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Masaharu Takigawa *Editor*

CCN Proteins

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Edited by

Masaharu Takigawa

Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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Editor
Masaharu Takigawa
Advanced Research Center for Oral and Craniofacial Sciences
Okayama University Dental School/Graduate School of Medicine
Dentistry and Pharmaceutical Sciences
Okayama, Japan

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Preface

CCN is an acronym that stands for *C*yr61 (Cysteine-rich 61)/CCN1, *C*TGF (connective tissue growth factor)/CCN2, and *N*ov (nephroblastoma overexpressed)/CCN3, which are the three founder members of this family. This family now comprises six distinctive members with the addition of three more members, namely, WISP (Wnt-induced secreted protein) 1-3/CCN4-6. They are all cysteine-rich secreted proteins and composed of four distinct modules connected in tandem, i.e., IGF binding protein-like, von Willebrand type C, thrombospondin type 1 repeat, and C-terminal modules, except for CCN5, which lacks the CT module. They are known to play roles in fundamental biological processes by serving as multifunctional growth and differentiation regulators that interact physically with various cytokines, extracellular matrices, and cell membrane proteins in various microenvironments. Abnormal regulation of these proteins is also involved in various diseases such as fibrosis and malignancy.

This volume will be valuable for all those interested in CCN proteins and serve as a valuable manual for cutting-edge methodologies and practical tips to overcome any obstacles with experimentation pertaining to the chemistry, biology, physiology, pathology, and pharmacology of CCN proteins in the context of basic, medical, and dental science's. We also believe that this comprehensive guide to methods and protocols for CCN research utilizing both basic and state-of-the-art techniques will be a valuable resource for a wide audience, ranging from the experienced CCN researchers looking for new approaches to junior graduate students taking their first steps into the field of CCN research.

Okayama, Japan ***Masaharu Takigawa***

Contents

Contributors

María José Acuña • *Facultad de Ciencias Biológicas, Departamento de Biología Celular y Molecular, Pontificia Universidad Católica de Chile, Santiago, Chile*

Afruja Ahad • *Department of Cell Biology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA; Department of Ophthalmology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA*

Eriko Aoyama • *Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*

Manish V. Bais • *Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, MA, USA*

Snigdha Banerjee • *Cancer Research Unit, VA Medical Center, Kansas City, MO, USA; Division of Hematology and Oncology, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA*

Sushanta K. Banerjee • *Cancer Research Unit, VA Medical Center, Kansas City, MO, USA; Division of Hematology and Oncology, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA; Department of Pathology, University of Kansas Medical Center, Kansas City, KS, USA; Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA*

Enrique Brandan • *Facultad de Ciencias Biológicas, Departamento de Biología Celular y Molecular, Pontificia Universidad Católica de Chile, Santiago, Chile*

David R. Brigstock • *The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA; Molecular, Cellular, and Developmental Biology Program, The Ohio State University, Columbus, OH, USA; Department of Surgery, Wexner Medical Center, The Ohio State University, Columbus, OH, USA*

Sarah Campbell • *Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI, USA*

Muriel Cario-Andre • *INSERM 1035, Bordeaux Cedex, France; University Bordeaux, Bordeaux Cedex, France; National Reference Center for Rare Skin Diseases, Bordeaux Hospital University, Bordeaux, France*

Cheng-Chi Chang • *Graduate Institute of Oral Biology, School of Dentistry, National Taiwan University, Taipei, Taiwan; Angiogenesis Research Center, National Taiwan University, Taipei, Taiwan*

Brahim Chaqour • *Department of Cell Biology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA; Department of Ophthalmology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA*

Li Chen • *The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA*

Lisa J. Crawford • *Haematology Research Unit, Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, UK*

Yilei Cui • *Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI, USA*
Archana De • *Cancer Research Unit, VA Medical Center, Kansas City, MO, USA*
Takanori Eguchi • *Department of Radiation Oncology, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, USA; Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; Department of Dental Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Menna Elaskandrany • *Department of Cell Biology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA; Department of Ophthalmology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA*
Gary J. Fisher • *Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI, USA*
Tomohiro Fukunaga • *Division of Orthodontics and Dentofacial Orthopedics, Tohoku University Graduate School of Dentistry, Sendai, Japan*
Takayuki Furumatsu • *Department of Orthopaedic Surgery, Okayama University Graduate School, Okayama, Japan*
Priyanka Ghosh • *Cancer Research Unit, VA Medical Center, Kansas City, MO, USA; Division of Hematology and Oncology, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA*
Hiroshi Hanagata • *R&D Department, Higeta Shoyu Co. Ltd., Kamisu, Ibaraki, Japan*
Takako Hattori • *Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Mitsuhiro Hoshijima • *Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Zhengshan Hu • *Department of Microbiology, Immunology, and Molecular Genetics, University of California Los Angeles, Los Angeles, CA, USA*
James Hutchenreuther • *Departments of Dentistry and Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada*
Alexandra E. Irvine • *Haematology Research Unit, Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, Northern Ireland, UK*
Shinsuke Itoh • *Department of Orthodontics and Dentofacial Orthopedics, Osaka University Graduate School of Dentistry, Osaka, Japan*
Danilo Janune • *Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Jie Jiang • *Department or Orthopaedic Surgery, School of Dentistry, University of California Los Angeles, Los Angeles, CA, USA*
Ken-ichi Katsube • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*

Yuko Katsuki • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*
Harumi Kawaki • *Department of Oral Biochemistry, Division of Oral Structure, Function, and Development, Asahi University School of Dentistry, Gifu, Japan*
Kazumi Kawata • *Department of Biochemistry and Molecular Dentistry, Okayama University Dental School and Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Ki-Hyun Kim • *Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA*
Seiji Kondo • *Faculty of Medicine, Department of Oral and Maxillofacial Surgery, Fukuoka University, Fukuoka, Japan*
Satoshi Kubota • *Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; Department of Membrane Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Min-Liang Kuo • *Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan*
Lester F. Lau • *Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA*
Andrew Leask • *Departments of Dentistry and Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada*
Sangmi Lee • *Department of Cell Biology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA; Department of Ophthalmology, SUNY Downstate Medical Center, College of Medicine and SUNY Eye Institute, State University of New York, Brooklyn, NY, USA*
Yue-Ju Li • *Angiogenesis Research Center, National Taiwan University, Taipei, Taiwan; Graduate Institute of Clinical Dentistry, School of Dentistry, National Taiwan University, Taipei, Taiwan*
Karen M. Lyons • *Department or Orthopaedic Surgery, University of California Los Angeles, Los Angeles, CA, USA; Department of Molecular, Cell, and Developmental Biology, University of California Los Angeles, Los Angeles, CA, USA*
Azusa Maeda • *Center for Promotion of Dental Education and International Collaboration, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama City, Okayama, Japan*
Gargi Maity • *Cancer Research Unit, VA Medical Center, Kansas City, MO, USA; Division of Hematology and Oncology, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA; Department of Pathology, University of Kansas Medical Center, Kansas City, KS, USA*
Yuki Matsushita • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*
Susan V. McLennan • *Greg Brown Diabetes and Endocrinology Research Laboratory, Sydney Medical School, Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia; Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia*

Danqing Min • *Greg Brown Diabetes and Endocrinology Research Laboratory, Sydney Medical School, Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia; Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia*
Tokutaro Minamizato • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*
Makoto Mizukami • *R&D Department, Higeta Shoyu Co. Ltd., Kamisu, Ibaraki, Japan*
Ricardo I. Monzon • *Department of Biological Science, Saint Xavier University, Chicago, IL, USA*
Kiyoshi Mori • *School of Phamaceutical Sciences, University of Shizuoka, Shizuoka, Japan*
Masashi Mukoyama • *Department of Nephrology, Kyoto University Graduate School of Medicine, Kyoto, Japan; Department of Nephrology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan*
Koichiro Muromachi • *Department of Pulp Biology and Endodontics, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Kanagawa, Japan*
Takashi Nishida • *Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Mitsuaki Ono • *Department of Molecular Biology and Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama City, Okayama, Japan*
Toshifumi Ozaki • *Department of Orthopaedic Surgery, Okayama University Graduate School, Okayama, Japan*
Kei Sakamoto • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*
Akira Sasaki • *Department of Oral and Maxillofacial Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Japan*
Debashree Saxena • *Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, MA, USA*
Tsuyoshi Shimo • *Department of Oral and Maxillofacial Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School, Okayama, Japan*
Hiroshi Sugiya • *Laboratory of Veterinary Biochemistry, Nihon University College of Bioresource Sciences, Fujisawa, Kanagawa, Japan*
Teruko Takano-Yamamoto • *Division of Orthodontics and Dentofacial Orthopedics, Tohoku University Graduate School of Dentistry, Sendai, Japan*
Nobuo Takeshita • *Division of Orthodontics and Dentofacial Orthopedics, Tohoku University Graduate School of Dentistry, Sendai, Japan*
Masaharu Takigawa • *Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Dental School/Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Nobuyuki Tani-Ishii • *Department of Pulp Biology and Endodontics, Graduate School of Dentistry, Kanagawa Dental University, Yokosuka, Kanagawa, Japan*
Katherine Thompson • *Departments of Dentistry and Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada*

Naohiro Toda • *Department of Nephrology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
Philip C. Trackman • *Department of Molecular and Cell Biology, Henry M. Goldman School of Dental Medicine, Boston University, Boston, MA, USA*
Stephen M. Twigg • *Greg Brown Diabetes and Endocrinology Research Laboratory, Sydney Medical School, Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia; Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, NSW, Australia*
Xiaoyu Wang • *Greg Brown Diabetes and Endocrinology Research Laboratory, Sydney Medical School, Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia*
Tomoichiro Yamaai • *Department of Oral Function and Anatomy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
Akira Yamaguchi • *Department of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan*
Hideki Yokoi • *Department of Nephrology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
Norie Yoshioka • *Department of Oral and Maxillofacial Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Japan*
Marian Young • *Molecular Biology of Bones and Teeth Section, Craniofacial and Skeletal Diseases Branch, National Institutes of Health, Bethesda, MD, USA*

Chapter 1

The CCN Proteins: An Overview

Masaharu Takigawa

Abstract

I introduce the general structures and functions of CCN proteins and possible molecular mechanisms regarding the unique biological actions of this new family of signaling regulators, which may be referred to as "signal conductors." Relevance to pathology is also briefly introduced. The information provided in this overview should be useful for readers of the following chapters.

Key words CCN family, Structure, Signaling, Signal conductor, Matricellular protein

1 Introduction

CCN is an acronym that stands for *C*yr61 (cysteine-rich 61)/βIG-M1/Cef10/CCN1, *C*TGF (connective tissue growth factor)/Hcs-24(hypertrophic chondrocyte-specific gene 24)/βIG-M2/Fisp-12/HBGF-0.8/CCN2, and *N*ov (nephroblastoma overexpressed)/CCN3, which are the three founder members of this family. This family now consists of six distinctive members with the addition of three more members, WISP (Wnt-induced secreted protein)-1/ELM-1/CCN4, rCOP-1/WISP2/CTGF-L/CCN5, and WISP-3/CCN6 (Fig. 1) [1–4]. The unifying nomenclature of CCN1–CCN6 for these proteins, which are numbered in the order of their discovery, was proposed in 2003 [5].

2 Structures of CCN Proteins and Genes

The CCN proteins are all cysteine-rich secreted proteins composed of four distinct modules connected in tandem, i.e., IGF-binding protein-like (IGFBP), von Willebrand type C (VWC), thrombospondin type 1 repeat (TSP-1), and C-terminal (CT) modules, except for CCN5, which lacks the CT module. The prototype of CCN protein contains 38 conserved cysteine residues; IGFBP, VWC, TSP-1, and CT modules contain 12, 10, 6, and 10 cysteine

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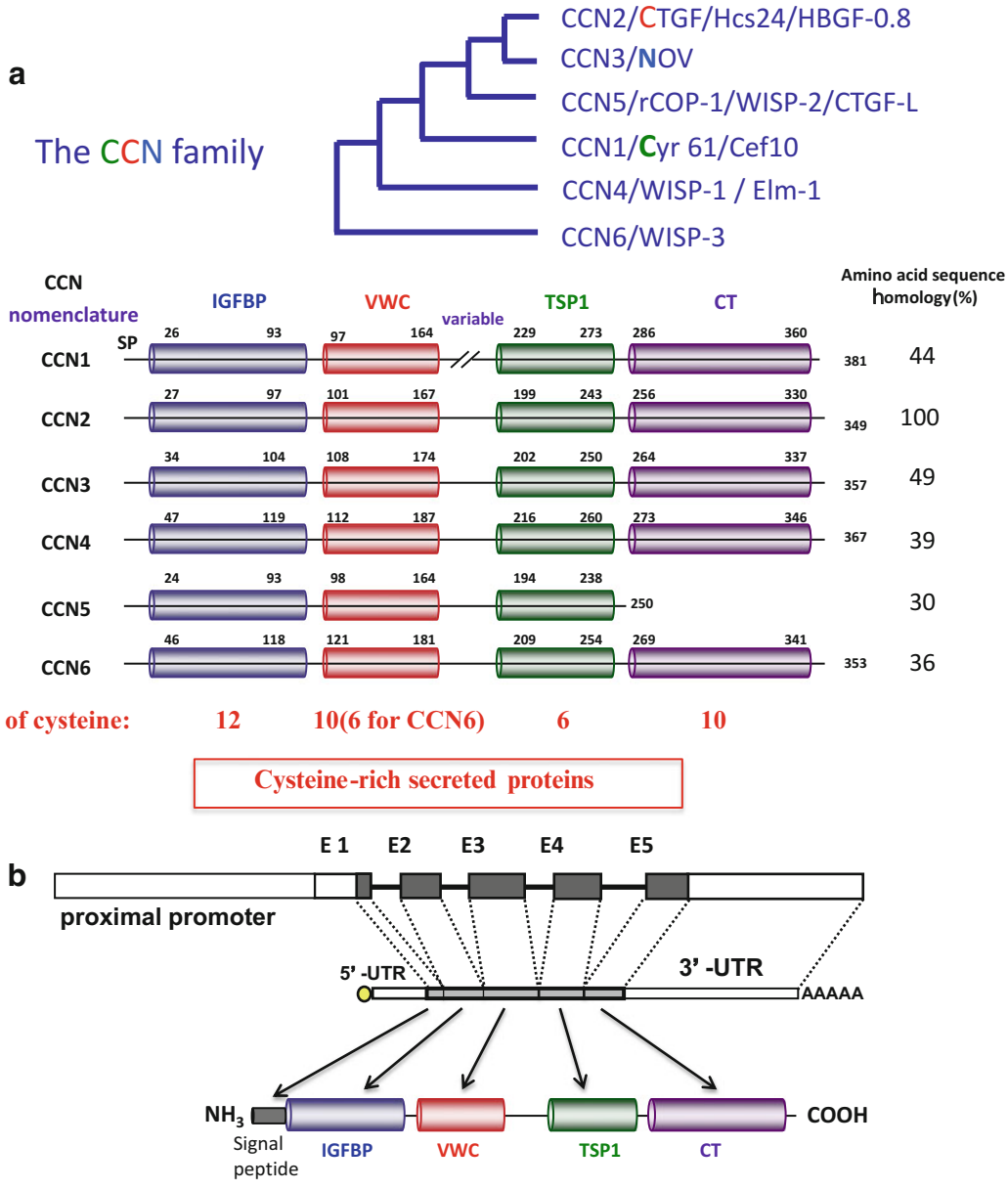

Fig. 1 The CCN family. (**a**) Dendrogram of CCN family proteins and their general structure. *IGFBP* IGF-binding protein-like module, *VWC* von Willebrand type C module, *TSP-1* thrombospondin type 1 repeat module, and *CT* C-terminal cystine module. *SP* signal peptide. (**b**) Structure of CCN family genes. *E1 to E 5* exon 1 to Exon 5. *UTR* untranslated region. As transcriptional regulatory proteins acting on proximal promoter, HIF1α, MMP3 Ets1, AP1, NF-kB, Smad, Sp1, SRF, TAZ, FoxO3a, and STAT have been reported. The mRNAs encoding the prototypic CCN protein are characterized by a long 3′-UTR. MicroRNAs such as miR-18a, miR-19b, miR-26a, miR-30, miR-133, miR-145, and miR-214 act on the long 3′-UTR as posttranscriptional regulatory RNAs. The long 3′-UTR is also a target of GAPDH and nucleophosmin (NPM) which are posttranscriptional regulatory proteins

residues, respectively, except that VWC module of CCN6 contains only six cysteine residues [1–4]. This characteristic modular structure with a number of cysteine residues is one of the causes of the difficulty in handling these proteins.

The prototypic CCN protein is encoded by five exons. Exon 1 encodes a signal peptide; and exon 2 to exon 5 encode IGFBP, VWC, TSP-1, and CT modules, respectively (Fig. 1b). The mRNAs encoding the prototypic CCN protein are characterized by a long 3′-UTR [6–8], which allows us to investigate the posttranscriptional regulation via 3′-UTR as a typical model [1, 6–8] (*see* Chapter 19).

3 Physiological Functions of CCN Proteins

The CCN proteins are known to regulate cell growth, proliferation, differentiation, adhesion, and migration and to play roles in fundamental biological processes such as development and growth, including chondrogenesis, endochondral ossification, osteogenesis, angiogenesis, nephrogenesis, wound healing, and tissue repair and regeneration by serving as multifunctional growth and differentiation regulators. Details of biological processes that CCN proteins are involved in are described in previous review articles [1–4, 6–13].

It has been suggested that the multiple functionalities of CCN proteins are produced by physically interacting with various growth factors and cytokines, extracellular matrices (ECMs), and cell membrane proteins in various microenvironments (Fig. 2) [7–10]. CCN proteins not only act through their own putative receptors but also modify actions of various growth factors and cytokines (Fig. 2). They also reserve those growth factors and cytokines in ECM, resulting in modification of their actions (Fig. 2). Importantly, modules contributing to these bindings are different depending on binding partners, suggesting that CCN proteins can bind several factors at the same time with their four hands (modules) (Fig. 3). Therefore, CCN proteins can participate in several extracellular signaling pathways described in Fig. 2 at the same time, and as a result, the sum of these actions would become the final outcome of biological action of CCN proteins (Fig. 2). Because microenvironment is different depending on the types of cells and tissues and differentiation stages of these cells, the diverse biological actions of CCN protein on various types of cells and tissues would depend on this difference in microenvironment (Fig. 2). This characteristic of full-length CCN proteins would contribute to their functions to promote harmonized tissue development and growth as well as regeneration [9].

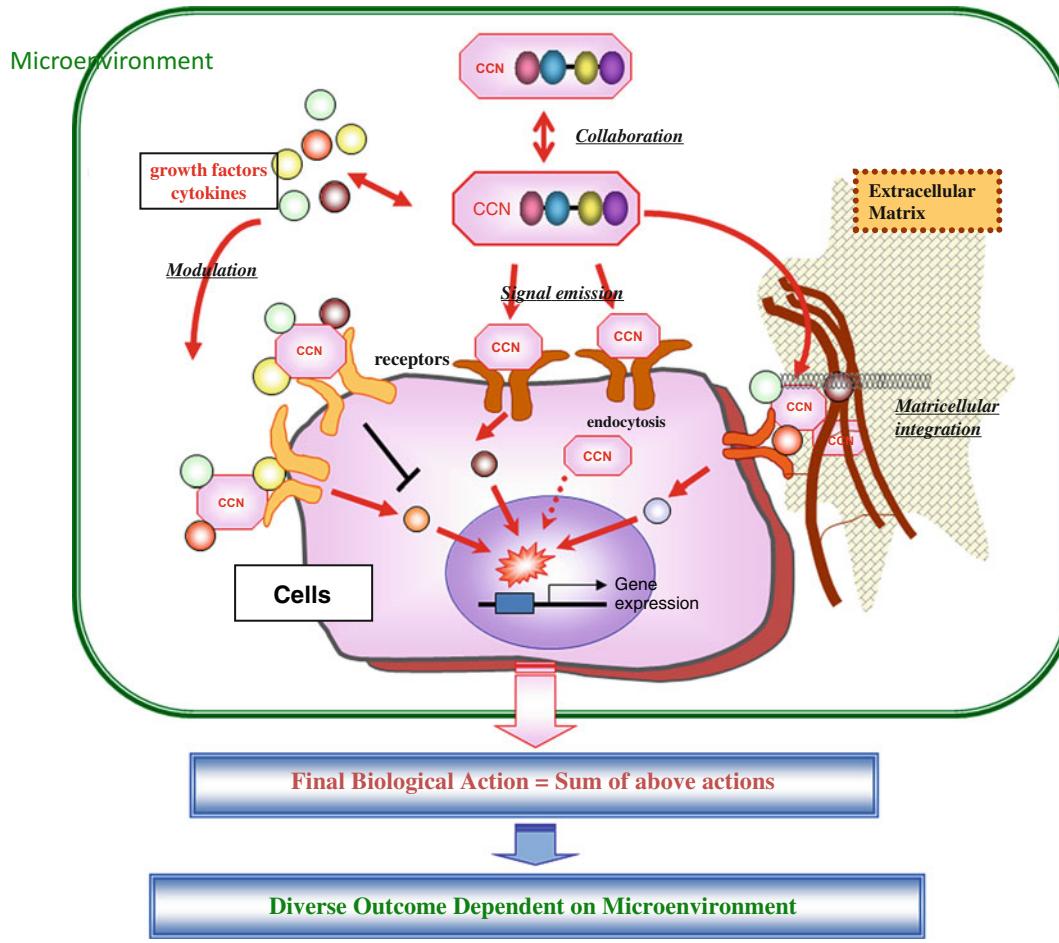

Fig. 2 CCN proteins modify various signaling pathways. A CCN protein such as CCN2 not only acts through its own receptors but also modifies actions of various growth factors and cytokines. The sum of these actions would become the final outcome of biological action of the CCN protein. In other words, various biological actions of a CCN protein on various types and different differentiated stage of cells would depend on different microenvironment of the cells. In addition, another CCN protein may modify the CCN actions by binding to the CCN protein

Since C-terminal module of CCN2 has been shown to have many activities such as cell adhesion, attachment, and proliferation [1–4] and to have many binding partners such as integrin alpha(v) beta(3), heparan sulfate proteoglycan, and fibronectin [7–12, 14, 15], there had been a hypothesis that this module was the active module of CCN protein while there had been another hypothesis that other modules also contribute to other biological functions [16]. Nowadays, it is believed that other three modules also have various biological activities and binding capacity to other molecules including CCN proteins [17, 18]. Detailed investigation of module-specific activities of CCN proteins as well as searching new binding partners and determination of their affinity and biological

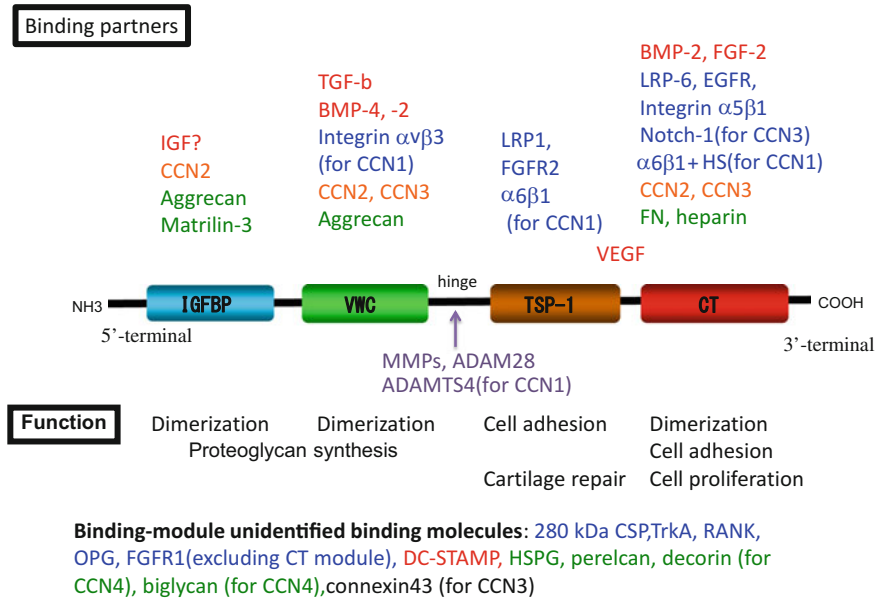

Fig. 3 Module-specific binding partners of CCN2 protein and function of each module. *Red*, *blue*, *orange*, and *green* letters indicate binding molecules of which categories are growth factors and cytokines, their receptors, CCN proteins, and ECMs and their degrading enzymes, respectively. The binding molecules have been reported to bind the module of CCN2 right under the binding partners. Module-specific functions are described under the modules, respectively. MMP and ADAM28 bind to hinge region between VWC and TSP-1 modules and cleave CCN2 protein. Binding-module unidentified binding molecules are listed up at the bottom. This figure mainly shows binding partners of CCN2 protein and its function, but the binding molecules with parentheses such as "Notch-1 (for CCN3)" mean binding partners for the CCN proteins in the parentheses, respectively

significance are one of the research directions of CCN proteins [17, 19–21]. Moreover, very recently the TSP-1 has been shown to be more active than full-length CCN2 in repairing articular cartilage in vivo [19]. This finding accelerates research toward possible clinical use of CCN fragments and engineered CCN proteins. This methods described in this book would be useful for testing them.

4 Pathological Roles of CCN Proteins

CCN proteins have been implicated in various diseases such as fibrosis in the skin (*see* Chapter 34), kidney (*see* Chapter 36), lung, liver (*see* Chapter 37), heart, blood vessels, gingiva etc. and, inflammation, muscle dystrophy (*see* Chapter 43), diabetes, retinopathy (*see* Chapter 46) and various types of malignancy (*see* Chapters 40 and 41). Details of pathological roles that the CCN proteins are involved in are described in previous review articles [1–4, 6–13]. Most of them can be attributed to abnormal

regulation of gene expression and activities of these proteins. For example, CCN2 has been shown to be involved in fibrosis of the various tissues described above. However, it is well known that CCN2 stimulates adhesion, migration, and proliferation of mesenchymal cells and their ECM production, which are normal cellular functions of CCN2 (Fig. 4). CCN2 is highly expressed during embryonic development and rapid growth of ECM-rich tissues such as cartilage and bone as well as during would healing which is believed to be reproduction of embryonic development (Fig. 4). When development and growth are completed or when wound defects are covered, if up-regulated expression of CCN2 is approximately terminated, these processes are called "physiological states" (Fig. 4). However, if the overexpression continues, it would cause fibrosis and scarring which are pathological states. In other words, pathological role is the reverse side of physiological role (Fig. 4). Therefore, methods analyzing physiological functions are applicable for better understanding pathological roles of CCN proteins, and methods analyzing pathological roles can be used for better understanding physiological roles of CCN proteins.

In contrast to CCN2, CCN1 and CCN3 have been shown to have anti-fibrotic effects, suggesting possible use of CCN1 and CCN3 proteins for anti-fibrosis therapy [8]. However, development of inhibitors for CCN2 such as CCN2-derivative antagonist against CCN2 is also a strategy for development of anti-fibrosis

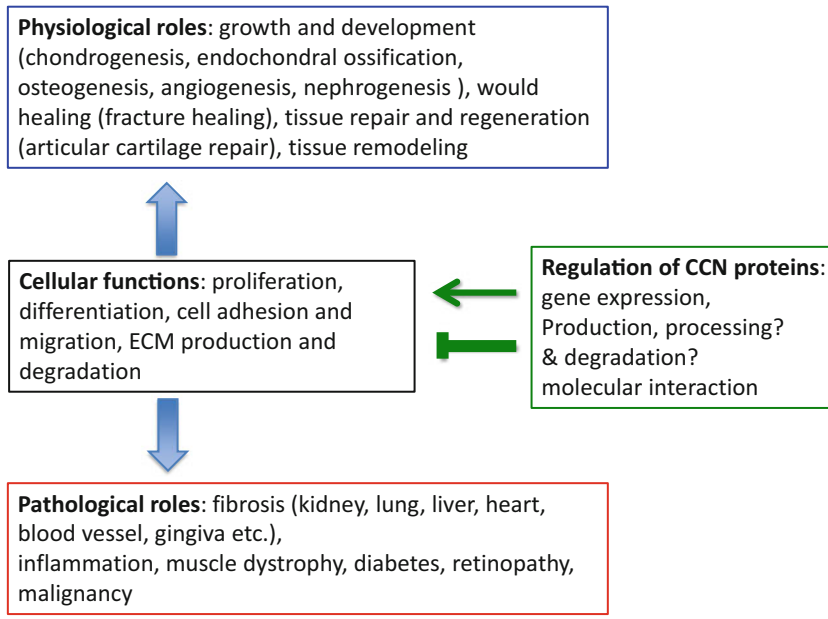

Fig. 4 Physiological and pathological roles of the CCN proteins and their cellular functions. Regulation of the CCN proteins modulates their cellular functions, causing physiological and pathological processes

therapy. In this case, methods analyzing physiological role of CCN2 protein could be useful. Therefore, in many cases, methods for analyzing physiological function can be applied for analyzing pathological roles and *vice versa*. Such a strategy is also applied for investigating physiological roles of other CCN proteins and pathological roles of all CCN proteins in other disorders including malignancy.

5 Concluding Comments

In this chapter, I have briefly introduced the general characteristics of CCN proteins and their physiological and pathological roles and merits of use of the following chapters for further investigation of the roles and translational research toward their therapeutic use. As already stressed in this introduction, because the CCN family members have unique structures as proteins and genes, they have been increasingly attracting the interest of chemists, biochemists, and molecular biologists. The biological roles of the CCN proteins as multifunctional proteins, which are new types of regulators for the control of cell proliferation and differentiation during normal development, growth, wound healing, and regeneration of various types of tissues, are likewise attracting the attention of biochemists, molecular biologists, and cell biologists. The involvement of CCN proteins in pathological processes such as fibrosis, renal, vascular, dental, muscle, and metabolic diseases and malignancy has also been attracting physician-scientists and dentist-scientists and pharmacologists.

This volume will be valuable for all those interested in CCN proteins, serving as a valuable manual for cutting-edge methodologies and practical tips to overcome any obstacles with experimentation pertaining to chemistry, biology, physiology, pathology, and pharmacology of CCN proteins. In addition, this volume provides comprehensive guides to methods and protocols for CCN research utilizing from fundamental to state-of-the-art techniques.

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Part I

Basic Methods and Protocols

Chapter 2

Gene Expression Analysis of CCN Proteins: Whole-Mount In Situ Hybridization of *Ccn2* in Developing Calcified Tissues

Tomoichiro Yamaai and Masaharu Takigawa

Abstract

A procedure for whole-mount in situ hybridization developed for detecting gene expression of *Ccn2* in developing calcified tissues of mouse embryos is presented. In this method, embryos are hybridized with Dig-labeled riboprobes, and the riboprobes are detected by use of the alkaline-phosphatase reaction in the presence of a 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT + BCIP) mixture. Obvious detection of positive signals for *Ccn2* in the cartilage of developing phalanges indicates that this method can be applied to gene expression analysis of other *Ccn* genes in developing calcified tissues.

Key words *Ccn2* gene expression, Whole-mount in situ hybridization, Dig-labeled riboprobe, Mouse embryo

1 Introduction

Whole-mount in situ hybridization itself is a well-known technique used to localize approximate gene expression sites of various genes; but somehow the procedure is more complicated than that of in situ hybridization on a slide glass, because each laboratory has its own technique. We introduce here the procedure used for whole-mount in situ hybridization in our laboratory, which was developed for detecting gene expression of *Ccn2* in developing calcified tissues. In this method, digoxygenin (Dig)-labeled riboprobes are used for hybridization, and the probes are detected by performing the alkaline-phosphatase reaction in the presence of a mixture of 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT + BCIP) in a refrigerator. Embryos up to E11.5 work well, whereas older embryos will only give signals at well-accessible positions such as limbs, tail, and central nervous system. Older embryos should be trimmed to get intense signals. Since CCN proteins have been suggested to be involved in embryonic

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development [1–3], the method described here can be applied to investigate the roles of other CCN proteins in embryonic development as well. The fundamental procedure is based on the method developed by Bober et al. [4] and referred to by Meyer et al. [5].

In situ hybridization techniques for the detection of CCNs in formalin-fixed, paraffin-embedded sections are detailed in Chapters 26 and 41.

2 Materials

1. 10× PBS(−) (Dulbecco's PBS without $CaCl_2$, 100 ml). Store at 4 °C.
2. Fixative: 2% paraformaldehyde in a Millonig's phosphate buffer (e.g., American MasterTech, *see* **Note 1**).
3. 20× SSC. Store at 4 °C.
4. TBST: 0.14 M NaCl, 25 mM KCl, 25 mM Tris–HCl pH 7.5, 0.5% Tween-20, 2 mM levamisole (*see* **Note 2**). Make 10× solution.
5. Dig-labeled sense and antisense riboprobes for CCN2 (*see* **Note 3**).
6. Embryonic powder: Grind E11–E14 mouse embryos after fixation with acetone and dry.
7. PBT: 1× PBS(−) with 0.1% Tween 20.
8. Prehybridization mixture (50 ml): 50% Ultrapure (deionized is better) formamide, 5× SSC, 0.04% heparin (made after 100 mg/ml DW) (instead of BSA), 0.5% tRNA (made after 11 mg/ml DW), + 1% salmon sperm DNA (made after 10 mg/ml), 0.1% Tween 20, add RDW to 50 ml. Store under −20 °C. Heat tRNA and ssDNA at 90 °C for 5 min to denature and then adjust pH with 1 M citrate or conc HCl to pH 4.5–5.0 (ca. 4–5 ml/50 ml mixture) before preparation of the mixture because riboprobes are unstable at alkaline pH.
9. Washing solutions:

   Solution I: 50% Formamide, 3× SSC, 0.1% Tween 20.

   Solution II: 0.5 M NaCl, 10 mM Tris–HCl pH 7.5, 0.1% Tween 20.

   Solution III: 50% Formamide, 1.5× SSC, 0.1% Tween 20.

   Solution IV: 0.5–1.0× SSC, 0.1% Tween 20.

   Concentration of SSC depends on an expected signal intensity. Use lower concentration of SSC carefully, because stringent washing might sometimes induce lower signal intensity.
10. Alkaline phosphatase (ALP) buffer: 100 mM NaCl, 100 mM Tris–HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20. Use within

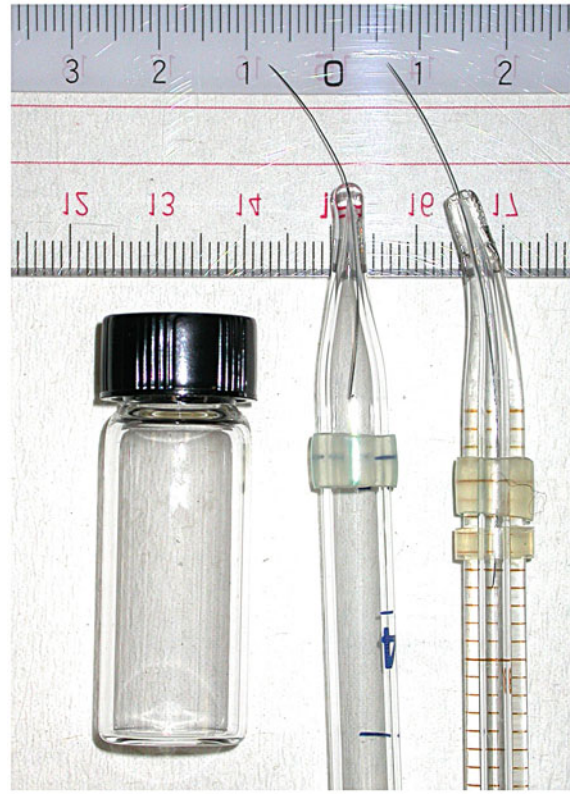

Fig. 1 Photograph shows sharp tungsten needles and a glass sample vial. Prepare adequate size glass vial for use, such as the one in the figure, and sharpen the needles by a finest sandpaper for convenient use

a week. 2 mM Levamisole is not necessary only in 4 °C. Tween 20 might block RNase activity in each sol'n during ISH.

11. Anti-Dig-ALP conjugate.
12. NBT + BCIP mixture (Boehringer Mannheim, Germany).
13. Glass sampling tubes (size: 1 ml) (Fig. 1).

3 Methods

The modified methods described here are based on the method by Bober et al. [4] and require at least 5 days to perform them.

3.1 Fixation and Dehydration

On the first day, fixation and dehydration are performed as follows:

1. Fix whole embryos at 4 °C by immersing embryos up to E12 in the fixative (*see* Subheading 2, **item 2**) at pH 7.3. However, embryos over E13 should be perfused with same fixative

through the left cardiac ventricle at 37 °C and then placed in a sample tube filled with the same fixative at 4 °C.

2. Irradiate the fixed embryos on ice in the same fixative mentioned above with six cycles of 5-s irradiation at 600 W and 15-s interval to cool down without irradiation by use of a kitchen microwave oven (*see* **Note 4**). This step is required for better fixation. Also, this microwave irradiation method is available not only for the fixation but also for dehydration up to 70% methanol or 70% Et-OH, immunoreactions, histochemical reactions, and washing (*see* **Note 5**).
3. Perform additional fixation after irradiation by keeping the samples in the same fixative for 30 min–3 h (depending on the sample size) at 4 °C on a shaker to obtain better morphology.
4. Wash samples 2 × 30 min with PBT (1× PBS(−) with 0.1% Tween 20).
5. Make punctures in samples (fixed embryos or organs) in PBT (or during 50–70% methanol) dehydration by a very sharp, thin needle, to obtain a better reaction (Fig. 1) (*see* **Note 6**).
6. Dehydrate samples by passing them through an ascending methanol series (50%, 70%, 80%, 90%, 95%, 100%) on ice for at least 15 min for each concentration of methanol (embryos can be stored in 100% methanol at −20 °C for 3 months) or overnight at 4 °C.

3.2 In Situ Hybridization

On the second day of the procedure, in situ hybridization should be performed as follows:

1. Generate Dig probe (*see* **Note 3**).
2. Transfer samples to room temperature (RT) from 4 °C.
3. Bleach samples with a solution of methanol:H_20_2 = 5:1 for 1 h at RT with shaking.
4. Rehydrate the samples by passing them through a descending methanol series (100%, 95%, 90%, 80%, 70%, 50%) on ice.
5. Rinse with PBT 3 × 5 min at RT.
6. Etch samples with proteinase K (1–20 μg/ml PBT, use DNase- and RNase-free grade only) for 10–30 min at RT (*see* **Note 7**).
7. Refix samples for 20 min at RT with freshly made 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde (GlA) in adequate buffer (dilute 25% GlA that was frozen at −20 °C:2% PFA = 1:125).
8. Rinse with PBT for 3 × 5 min at RT. If desired, acetylate samples twice for 20 min each time at RT to reduce the background (*see* **Note 8**). Rinse acetylated samples with PBT for 10 min at RT.

9. Add prehybridization mixture, wait for 5 min (floating samples should settle to the bottom of the tube within 5 min), and then incubate in fresh prehybridization mixture for 0.5 h at 50 °C with gentle rotation.
10. Hybridize samples with 7 μl (0.7 μg) of Dig-riboprobe/2 ml of hybridization mixture/bottle overnight at 55–70 °C with gentle rotation in an incubator. Temperature could depend on the size of the probe. Denature probes for 5–10 min at 80 °C before use.

3.3 Immunological Reaction

On the third day of the experiment, the immunological reaction should be performed as follows:

1. Wash samples 3 × 60 min with prewarmed solution I with shaking at the same temperature as used for the hybridization.
2. Wash samples with shaking with a mixture of solution I:solution II = 1:1 for 10 min at the same temperature used for the hybridization.
3. Wash with solution II, 3 × 10 min at RT with shaking (only for RNaseA treatment).
4. Treat with RNaseA/solution II (100 μg/2 ml solution II) for 30 min at 37 °C with shaking (*see* **Note 9**).
5. Rinse with shaking with solution III, 3 × 60 min at the same temperature as used for hybridization.
6. Rinse with solution IV for 15–30 min at RT (shaking is optional).
7. Wash samples with TBST 3 × 10 min at RT; in the meantime, heat inactivate a small amount of embryo powder (enough to stick to the tip of a Gilson's yellow tip) in 2 ml of TBST for 30 min at 56 °C.
8. Block embryos with heat-inactivated 1–3% normal goat serum in TBST for 1 h at RT; in the meantime spin down the embryo powder quickly, discard the supernatant (SNT), cool on ice, and add 1:2000–10,000 dilution of an anti-Dig-ALP conjugate in 1% heat-inactivated normal goat serum in 1× TBST; disperse powder well and incubate for 1 h at 4 °C.
9. Spin down the embryo powder for 5 min at 10,000×*g*, and then dilute the SNT 1:4 with 1% heat-inactivated normal goat serum in 1× TBST.
10. Remove the blocking solution and then add 2 μl of the diluted anti-Dig-ALP solution per tube, mix well, and incubate at 4 °C overnight with rotation.

3.4 Washing

On the fourth day of the experiment, washing is performed as follows:

1. Remove anti-Dig-ALP solution by washing with TBST 3 × 10 min at RT on a shaker.

2. Wash all day with TBST at RT, change TBST several times during this time, and continue to wash with it overnight at 4 °C with shaking.

3.5 Color Reaction

On the fifth day, start color reaction with the NBT + BCIP mixture in alkaline phosphatase (ALP) buffer at 4 °C (not at RT), for about 1 day–1week. This lower temperature blocks mammalian endogenous nonspecific ALP activity.

1. Wash with freshly made ALP buffer 2 × 60 min at RT with shaking.
2. Start the color reaction in glass vials, with 2 ml of ALP buffer, 1 mM Levamisol, and 1/10–1/20 concentration of NBT + BCIP mixture (for instance, 100 μl mixture/100 ml ALP buff.) in the dark at 4 °C for about 1 day–1week (*see* **Note 10**).
3. Place vials in the dark (cover with aluminum foil); staining might start in about 20 min after the addition of the color reaction mixture and may continue for 1 week. But generally the color reaction on whole-mount samples will be finished within 1 day. Monitor S/N (signal/noise) ratio under a microscope.
4. Stop the reaction when the background has increased, rinse with PBT + 1 mM EDTA, three times, and store at 4 °C (if the color reaction is not adequate, try **steps 1–3** again).
5. Refix with 2% PFA with 0.2% GIA in adequate buffer for 30 min at RT.
6. To take photographs, whole-mount samples are cleared in 70% glycerol and 30% 1× PBS (Figs. 2 and 3, and also *see* Refs. 6, 7). Store samples in PBT at 4 °C.

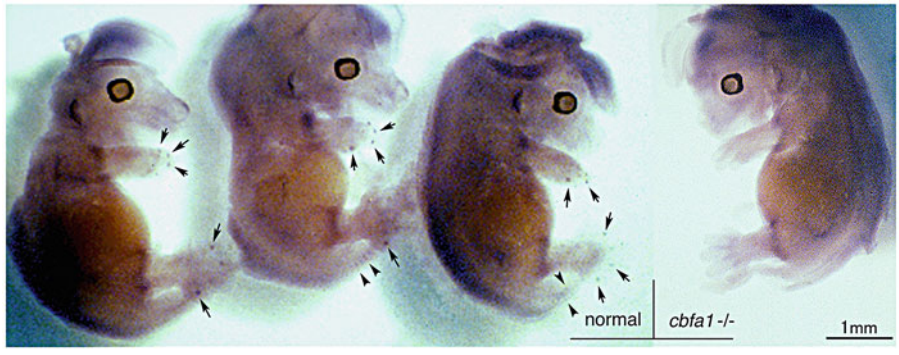

Fig. 2 Sample of gene expression in mouse embryos at E16.5. The gene expression of *Ccn2* is observed in the fingers (*arrows*) and tails (*arrowheads*) of wild-type embryos (*left 3*), but not in those of *cbfa1-null* mutants. Embryos are cut into half on the median line after perfusion for better fixation

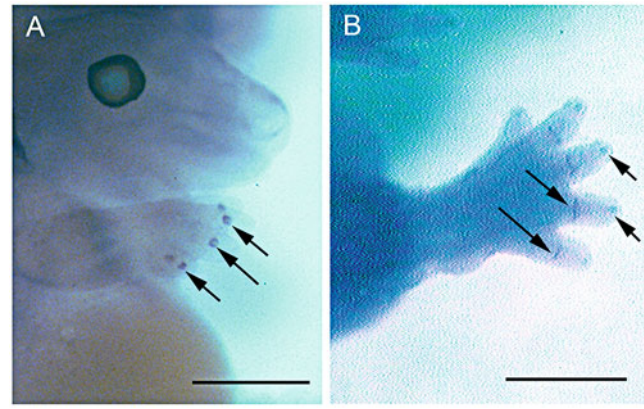

Fig. 3 Sample of gene expression (*Ccn2*) in the phalanges of wild-type mouse embryos at E15.5 (*left*) and E16.5 (*right*). *Arrows* indicate the sites of the gene expression of *Ccn2* observed in distal phalanxes indicated dark blue in E15.5 (**a**). In E16.5 embryo (**b**), the gene expression of *Ccn2* is also observed in interphalangeal joints, as indicated by the *arrows*. Bars in (**a**) and (**b**), 1 mm

4 Notes

1. Millonig's fixative is good for electron microscopy observation. The fixative composed of a higher concentration of the buffer (440 mOsm) maintains the true morphology of the embryos.
2. Levamisole is essential to decrease the background when performing ISH on an endogenous alkaline phosphatase-rich tissue or cell, as liver, kidney, testis, ovary, placenta, adrenal gland, small intestine, salivary glands, osteoblast, endothelial cell, lymphocyte, etc. in this system.
3. We prepared a noncoding region of mouse *Ccn2* as a probe for hybridization, which was amplified from cDNA by using the following primers: 5′-GGGTAAGGTCCGATTCCTACC-3′ (sense) and 5′-CTAGAAAGGTGCAAACATG-TAAC-3′ (antisense).

   The mouse CCN2 fragment inserted into the pGEM-T plasmid easy was cut by Pst I restriction enzyme for sense and by Nco I restriction enzyme for antisense template. Dig-labeled riboprobes are generated as follows by using a DIG RNA labeling kit (Roche, Mannheim, Germany; *see* Refs. 8, 9): Briefly, mix 10× transcription buffer (2 μl), Dig-NTP (2 μl), template (linearized) DNA (1 μl), RNA polymerase (2 μl), HPRI (RNase inhibitor, 1 μl), and sterilized DW (12 μl). Incubate the mixture for 1 h at 37 °C for T7 polymerase (sense) and at 36 °C for Sp6 polymerase (antisense). After that, add 1 μl of DNase I and incubate for 0.5 h at 37 °C before precipitating with 4 M LiCl and 100% ET-OH. Dissolve precipitate in a mixture

comprising 8 μl DEPC-treated DW, 1 μl HPRI, 1 μl 1 mM DTT, and 10 μl of formamide, and store at −70 °C. Use glass sampling tubes for ISH; never use plasticwares. Plasticwares may cause adsorption of probes.

4. Irradiation with microwaves gives better fixation.
5. Authors recommend to use methanol instead of ethanol during dehydration to obtain better signal contrast.
6. This procedure is very immportant to obtain fine signals.
7. Time of treatment and concentration of proteinase K are highly dependent on the position of the expected signal in the embryo; for example, for the *met*, *sf/hgf* signaling stream in the dermis of E10.5 embryos, 2 μg/ml PBT for 10 min is required, whereas in the case of the sympathetic system, 20 μg/ml PBT for 30 min is needed.
8. Prepare 10 ml of DW + 25 μl of HCl + 150 μl of triethanolamine + 25 μl of acetic anhydride (add and shake just before use).
9. This step is not necessary in many cases.
10. The color reaction should be performed at 4 °C with dilute reagent (1/10–1/20) to block endogenous mammalian (mouse) alkaline phosphatase activity.

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We want to thank Prof. Dr. Carmen Birchmeier (MDC, Berlin) for her kind suggestion regarding the process of a whole-mount in situ hybridization. This study was supported by grants (#JP24390415 and #JP15H05014 to M.T.) from the program Grants-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science.

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Chapter 3

Expression of CCN Genes and Proteins in Human Skin: Methods and Protocols

Yilei Cui, Sarah Campbell, and Gary J. Fisher

Abstract

CCN proteins are vital to a variety of biological processes that contribute to the normal development and function of skin. Quantitative real-time reverse transcriptase PCR (qPCR) quantifies mRNA levels of these key genes. Immunostaining localizes CCN proteins within skin tissue, and provides semiquantitative information regarding relative levels of the proteins. Reliable determinations of the expression levels of CCN genes and proteins are essential to uncovering their roles in skin physiology and pathology.

Key words CCN proteins, Gene expression, Real-time q-PCR, Immunostaining, Skin

1 Introduction

The CCN family of proteins contains six members, designated CCN1–CCN6 (previously named CYR61, CTGF, NOV, and WISP1-3, respectively). CCN proteins contribute to a range of biological processes such as cell growth, adhesion, migration, angiogenesis, and regulation of extracellular matrix production [1, 2]. They are essential to the development and function of multiple organs. In skin, CCN1 and CCN2 are the most studied CCN family members. CCN1 is reduced in the dermis of aged human skin and this reduction contributes to decreased production and increased degradation of the dermal extracellular matrix [3, 4]. CCN1 has also been shown to be a critical regulator of angiogenesis, wound healing in mouse skin [5, 6], and implicated as critical for epidermal development and homeostasis in mouse skin [7], and keratinocyte proliferation [8]. CCN2 is best characterized as a profibrotic factor that cooperates with transforming growth factor-β in a scleroderma and other fibrotic conditions [9–11]. With the exception of CCN6, all members of the CCN family have been reported to be expressed in human skin [12].

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2 Materials

1. All solutions are prepared with ultrapure water (18 MΩ, 25 °C) from a Millipore purification system or HPLC-grade water, and analytical/molecular grade reagents. Preparation and storage of all solutions are at room temperature (unless otherwise indicated).

2.1 Human Skin Samples

1. The protocols described below are conducted with full-thickness skin samples obtained from volunteers. Samples are taken with a 4 mm punch biopsy instrument, using local lidocaine anesthetics, by trained medical personnel. The procedure must be approved by an appropriate ethical board and all volunteers must provide written informed consent. Skin samples are either immediately snap frozen in liquid nitrogen, or immediately embedded in optimal cutting temperature compound (OCT, Tissue-Tek, Sakura, USA) and then snap frozen in liquid nitrogen. Skin samples are transferred to −80 °C for storage (*see* **Note 1**).
2. Twelve-well BioPulverizer (Biospec Products, Bartlesville, OK), which consists of stainless steel mortars and pestles. This instrument is used to pulverize skin samples prior to RNA extraction. Store the BioPulverizer in −80 °C freezer prior to use.

2.2 Isolation of Total RNA from Human Skin Samples

1. RNA isolation is performed by utilizing the RNeasy Mini Kit (Qiagen, Germantown, MD) (refer to kit handbook for further details).
2. RNeasy reagents used: RLT+ 2-mercaptoethanol (β-ME) (10 μl β-ME in 1 ml RLT; RLT can be used for 1 month after β-ME addition), RW1, RPE (*see* **Note 2**).
3. Standard reagents used: RNase-free H_2O, 70% ethanol.
4. Other reagents: DNase digestion kit (Qiagen), RNase inhibitors from reverse transcriptase kit (*see* **step 2**, Subheading 2.3).
5. Consumables: P1000, P250 sterile tips, 2.5 mm glass beads (e.g., Biospec Products Inc.), 1.7 ml sterile microfuge tubes, latex gloves.
6. Instruments: Leica Cryostat (accessories including brush, tweezers, and knife) or an equivalent, Vacuum flask, microfuge, vortex mixer, P1000, P200, and P20 pipettes.

2.3 Synthesis of cDNA from Human Skin Total RNA

1. Lab spectrophotometer (*see* **Note 3**).
2. Synthesis of cDNA from total skin RNA is accomplished using a commercial reverse transcriptase kit (e.g., TaqMan Reverse Transcription Kit, Applied Biosystems, Foster City, CA).
3. Thermal Cycler (e.g., Bio-Rad Laboratories, Model T100).
4. MicroAmp Optical 8-cap strip (e.g., Applied Biosystems).
5. MicroAmp RNase-free optical tubes for DEPC H_2O and mix tube.

2.4 Real-Time PCR Components

1. Real-time PCR instrument (e.g., ABI 7300 Applied Biosystems).
2. 96-Well PCR plate and plate cover.
3. TaqMan SYBR green PCR master mix (Invitrogen, Carlsbad, CA).
4. Forward and reverse primers for CCN1-6 and internal control 36B4 genes (*see* Table 1 for primer sequences and **Note 4**).

2.5 Immunostaining Components

1. Acetone (histological grade).
2. 2 % Paraformaldehyde (PFA): 20 ml Formalin, 100 ml 10× calcium- and magnesium-free phosphate-buffered saline (PBS).
3. 20× Super Sensitive Wash Buffer (sodium chloride, potassium phosphate dibasic anhydrous, Brij 35, Biogenex, Fremont, CA) diluted to 1× with water.
4. Link-Label detection system (e.g., Biogenex).
5. Microscope slides (Fisherbrand Superfrost Plus electrostatically treated glass slides, 25 mm×75 mm, Fisher Scientific, Pittsburgh, PA).
6. Water repelling pen (e.g., DAKO Pen, DAKO, Carpinteria, CA), to draw a barrier around the skin sections in order to retain reagents in contact with the tissue.
7. Permanent mounting medium (e.g., SuperMount, Biogenex) used to preserve samples on slides.
8. Research microscope (e.g., Carl Zeiss AG, Oberkochen, Germany) equipped with digital camera and software (such as manufactured by Spot Imaging, Sterling Heights, MI).
9. Primary antibodies directed against human CCN proteins (*see* **Note 5**).

Table 1
Primer sequences for qPCR of human CCN and 36B4 internal control mRNA

CCN gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
CCN1	TCAAAGACCTGTGGAACTGGTATC	CACAAATCCGGGTTTCTTTCA
CCN2	GTTTGGCCCAGACCCAACT	GGAACAGGCGCTCCACTCT
CCN3	CCGCTGTCAGCTGGATGT	CTCCAGGCACCTCAACTTTTCT
CCN4	AGAGGCATCCATGAACTTCAC	CAAACTCCACAGTACTTGGGTTGA
CCN5	ATGAGAGGCACACCGAAGAC	CTGGGTACGCACCTTTGAGA
CCN6	CATTATCATAATGGCCAAGTGTTTCA	CAATGGCCCCACTCACACA
36B4	ATGCAGCAGATCCGCATGTT	TTGCGCATCATGGTGTTCTT

Primer sequences are designed to have optimal annealing temperature of 60 °C

3 Methods

3.1 Isolation of Total RNA from Human Skin Samples

3.1.1 Preparation of Frozen Skin Samples

1. Frozen human skin samples need to be pulverized prior to RNA extraction. This process is simplified by use of a 12-well BioPulverizer.
2. Place the BioPulverizer in a shallow container and pour liquid nitrogen over the mortars and pestles. Then place one mortar on the lab bench.
3. Place frozen skin sample in the mortar.
4. Place pestle on top of the sample and pulverize by repeated blows with the hammer.
5. Dip scraper tool in liquid nitrogen and then scoop crushed tissue into labeled tubes containing RLT + β-ME RNA extraction buffer.
6. Vortex the tubes for 10 s and then store at −80 °C.
7. Repeat with remaining skin samples.
8. Further pulverization of the skin samples is performed by vortexing with 2.5 mm glass beads (Biospec Products Inc., Bartlesville, OK) immediately prior to RNA isolation.

3.1.2 Preparation of OCT-Embedded Human Skin Samples

For each skin sample that is embedded in OCT, collect 20 consecutive frozen sections (50 μm/section), using a cryostat set at −25 °C. Place all sections in one 1.7 ml RNase-free microfuge tube and place the tube in liquid nitrogen or on dry ice until starting isolation of RNA.

3.1.3 Total RNA Isolation

Total RNA is isolated using a commercial kit according to the manufacturer's protocol. Keep all reagents at 4 °C or on ice.

1. For frozen, pulverized skin, skip this step and directly go to **step 2**. For frozen OCT sections, OCT must be removed immediately prior to RNA isolation. This removal is accomplished by adding 300 μl RNase-free H_2O to one sample at a time, followed immediately by centrifugation in a microfuge at 3,300 × *g* for 5 s to dissolve the OCT and pellet the skin sections. Aspirate the supernatant with vacuum.
2. Label 1.7 ml sterile microfuge tubes that are needed for RNA isolation. Each sample requires four tubes that are used for (1) spin column washes, (2) holding 2.5 mm glass beads, (3) collecting the RNA eluted from the spin column, and (4) aliquot of isolated RNA for quantification.
3. Add 300 μl RLT + β-ME buffer, repeatedly draw up, and expel buffer to break up and suspend tissue pellet. Add eight 2.5 mm diameter sterile glass beads (described above), vortex for 30 s, and put on ice for a minimum of 2 min. Repeat with remaining samples. Centrifuge in microfuge at full speed for 2 min to pellet insoluble tissue material and glass beads.

4. Add 300 µl 70% ultrapure ethanol into separate microfuge tubes. It is convenient to use the same tubes that previously held the eight glass beads. Then add 300 µl of sample supernatant to the ethanol. Mix by inversion until sample is reasonably clear.
5. Load sample (approx. 600 µl) into a Mini Kit spin column, place column in collection tube, and centrifuge at 9,300 × *g* in a microfuge for 15 s. Remove liquid from collection tube and discard. Add 350 µl RW1 buffer to the spin column, place column in collection tube, and centrifuge at 9,300 × *g* in a microfuge for 15 s. Remove liquid from collection tube and discard.
6. Make DNase solution (70 µl DNase buffer, 10 µl DNase per sample) in one tube for the entire batch of samples. Add 80 µl DNase solution directly to column matrix. Incubate at room temperature for 15 min.
7. Add 350 µl RW1 a second time to spin column. Place column in collection tube and centrifuge at 9,300 × *g* for 15 s in a microfuge. Remove liquid from collection tube and discard.
8. Add 500 µl RPE buffer to spin column. Place column in collection tube and centrifuge at 9,300 × *g* in a microfuge for 2 min. Remove liquid from collection tube and discard.
9. Repeat **step 7**.
10. Centrifuge spin column at 9,300 × *g* in a microfuge for 1 min with cap open to dry and remove excess liquid. It is imperative that column matrix is as dry as possible to maximize elution of RNA.
11. Add 1 µl RNase inhibitor to a fresh sterile collection tube, and insert spin column into the tube, taking care to point the storage cap out from the center of the rotor.
12. To elute RNA from the spin column, add 50 µl RNase-free H_2O, and incubate at room temperature for 1 min. Centrifuge at 9,300 × *g* in a microfuge for 1 min. Mix eluate briefly by pipetting up and down before transferring 2 µl to a clean tube for RNA quantification.

3.2 Reverse Transcriptase (RT) Synthesis of cDNA

1. Components of the RT reaction mixture are listed in Table 2. Calculate how much of each RT reagent is needed based on the number of total RNA samples.
2. Pipette 30.75 µl RT mixture into each individual optical tube.
3. Based on the sample concentration of total RNA and the amount used per RT reaction (100–200 ng recommended), calculate the amount of DEPC H_2O that is needed for each reaction mixture. Add required volume of total RNA to individual labeled tubes. Final volume for each reaction is 50 µl.
4. After all RT reaction components are added, mix them briefly by vortexing. Centrifuge the tubes in a microfuge for 15 s before transferring them to the thermocycler.

Table 2
Components of reverse transcriptase reaction

$MgCl_2$	11 μl
dNTP	10 μl
10× buffer	5 μl
Random hexamers	2.5 μl
RNase inhibitor	1 μl
Reverse transcriptase	1.25 μl
Sample (100–200 ng RNA)	20 μl

RT-PCR cycling conditions: 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min, hold at 4 °C

5. The reaction is carried out for 45 min, as described in Table 2. cDNA is stable at 4 °C and can be used immediately or stored at −20 °C.

3.3 Real-Time Quantitative Polymerase Chain Reaction (q-PCR) Gene Expression Analysis

1. qPCR is performed using a dedicated real-time-PCR instrument. While details regarding instrument operation differ among different manufacturers' instruments and models, the principles of the procedure remain the same. The procedure described below is for the real-time PCR instrument such as the ABI 7300, which uses a 96-well plate sample format. Internal control gene 36B4 is run for every sample on the same 96-well plate as the sample (*see* **Note 6**).
2. Use at least 4 μl of cDNA sample for every PCR reaction. Run all samples in duplicate. Use a 10 + 2 method for determining volume of reaction mixture needed; add two additional reactions for every ten reactions (remember that ten wells will run five samples in duplicate).
3. The reaction components placed into each well of a 96-well plate are listed in Table 3. Standard reaction volume for each well is 20 μl. An automated liquid-handling station is preferred to reduce pipetting variability and save time.
4. It is helpful to create a map of each 96-well plate indicating the location, identity, and gene primers used for each sample (*see* **Note 7**). It is often convenient to arrange samples by gene. When performing measurements of multiple genes for the same samples on different plates, it is recommended to place the samples in the same location in each plate, to avoid instrument variability and to simplify data analysis.
5. A standard run takes approximately 2 h (for running conditions *see* Table 3). If a dissociation step is included, then the run will take approximately 3 h (*see* **Note 7**).

Table 3
Components of qPCR reaction

Reagent for samples to be run	Volume in one reaction
SYBR Green Master Mix (2×)	10 µl
Forward primer 1 µM (10×)	2 µl
Reverse primer 1 µM (10×)	2 µl
DEPC H_2O	4 µl
cDNA	2 µl
Volume total	20 µl

q-PCR cycling conditions: (1) 50 °C for 2 min (once), (2) 95 °C for 10 min (once), (3) 95 °C for 15 s, (4) 60 °C for 1 min (repeat **steps 3** and **4** for 40 cycles)

6. Check well setting before starting a run (*see* **Note** 7).
7. Save and start run. Create a folder on each instrument to house your run and data files. (Data should be automatically archived on the computer.)
8. Collect data when run is complete. Cycle thresholds (Ct) should be set consistently (0.2 is standard, needs may vary, *see* **Note** 7). Export results into a .csv file and process in Excel for data analysis.

3.4 Immunostaining

1. Clean cryostat chamber, instruments, red marking pen, exterior, and chucks with 70% isopropyl alcohol.
2. Maintain cryostat at −25 °C during sectioning.
3. Cut sections (7–10 µm) and place on slides. Slides with sections may be stored in a protective slide box at −80 °C for a few weeks prior to immunostaining.
4. Use water-repelling pen to draw a barrier around the skin sections.
5. Prepare peroxide block (to reduce background from endogenous reactive oxygen species) by 1:10 dilution of 3% stock solution of H_2O_2 in PBS.
6. Remove all slides from −80 °C freezer 15 min prior to staining. Do not let slides dry out during the fixation procedure.
7. Fixation of the skin section may alter exposure of the epitope that is recognized by the primary antibody (i.e., antibody to CCN protein of interest), and therefore may improve or hinder immunostaining. Optimal fixation conditions for each antibody must be empirically determined. Common fixations for skin section prepared from OCT-embedded samples are acetone, methanol, 2% paraformaldehyde, or formalin (*see* **Note 8**).

8. Wash slides with 1× Super Sensitive Wash Buffer for 5 min. Repeat for a total of three washes (*see* **Note 9**).
9. Peroxide block (dilute 1:10 in DI H_2O) for 10 min.
10. Wash (repeat **step 8**).
11. Protein block for 20 min.
12. Primary antibody incubation for 1 h.
13. Wash (same as **step 8**).
14. Link for 10 min, secondary antibody incubation (+ biotin).
15. Wash (repeat **step 8**).
16. Label for 10 min (streptavidin + peroxidase).
17. Wash (repeat **step 8**).
18. Substrate treatment for 3 min (try longer times).
19. Wash slides 2× with DI H_2O and then put slide holder into DI water to rinse.
20. Hematoxylin counterstain for 45 s (*see* **Note 10**).
21. Rinse and mount slides with permanent mounting medium (*see* **Note 11**).
22. Stained tissue sections are viewed with a high-resolution upright microscope coupled to a computer-controlled digital photography system.

4 Notes

1. Storing skin biopsies in −80 °C freezer is critical to prevent tissue from thawing. Human skin has very high levels of RNase activity that can rapidly degrade RNA. RNA degradation introduces variability, and may invalidate qPCR measurements, depending on the degree of degradation. During preparation and manipulation of RNA samples, use only RNase-free materials and reagents and changing gloves often helps protect RNA from contamination and degradation.
2. β-ME must be used processed in fume hood to prevent inhalation and avoid its pungent odor. β-ME can be toxic if ingested, inhaled, or absorbed through the skin.
3. 100–200 ng total RNA for each reverse transcriptase reaction yields optimal generation of cDNA. Calculation of the RNA concentration (small samples, 1–2 μl) can be quantified by measuring the absorbance (OD) at 260 nm using a general-purpose laboratory spectrophotometer, or a dedicated instrument such as Epoch (Biotek Instruments Inc., Winooski, VT). The concentration of RNA is calculated by multiplying the absorbance at 260 nm times 40, times any dilution factor

(OD 260 nm × 40 × dilution factor). Generally, the purity of RNA is assessed by the ratio of the absorbance at 260 and 280 nm. A ratio of >1.8 is considered high purity. A low ratio indicates contamination with protein. An Agilent 2100 Bioanalyzer is very useful for assessing RNA quality.

4. Optimal primer concentration is between 0.1 and 0.5 μM. Our lab uses 0.1 μM in q-PCR reactions. For example, to prepare 1 μM forward and 1 μM reverse primers (10×): simply add 10 μl of 100 μM stock forward primer or 10 μl of 100 μM stock reverse primer to 990 μl of HPLC water to make a 1 μM solution (stock primer concentration may vary). Briefly vortex each mixture in order to mix completely, and then centrifuge for 30 s at 9,300 × *g* in a microfuge.
5. There are no certified or universally accepted primary antibodies for immunostaining CCN proteins. Different laboratories have reported variable success with different antibodies from a variety of sources. Antibodies that our laboratory have found to be acceptable include CCN1, CCN2, CCN4, and CCN6 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, see Ref. 8, 13, 14); CCN3 rabbit polyclonal antibody K19M [15]; and CCN5 antibody described in [16].
6. A set of gene-specific forward and reverse primers hybridize to the cDNA sample and prime synthesis of a complementary DNA strand by TAQ polymerase. During successive cycles of denaturing, annealing, and synthesis, a doubling of primer-specific DNA (amplicon) occurs. A plot of the amount of amplicon versus cycle number results in a sigmoidal curve. The initial linear region of this plot is termed cycle threshold (Ct) and is proportional to the amount of target gene cDNA in the sample.
7. Real-time PCR instrument setup generally includes identifying SYBR green as the fluorogenic readout from the probe selection list. If a different fluorophore probe is used, then consult instrument technical documents. Passive reference should be set to ROX. Once run information is generated, a blank plate map will appear. Label plate according to experimental design. Under instrument tab, set reaction volume to the appropriate value (20 μl is the standard but experiment needs may vary). To verify primer specificity it is advisable to determine a temperature melting curve for the amplified PCR product. Specific amplification yields a single sharp peak at a temperature at which the two strands of the double-stranded PCR products dissociate. Multiple peaks indicate synthesis of multiple PCR products, which may be caused by lack of primer specificity. Use of alternative primers may elevate this situation to provide higher quality data. To collect melting curve data select an option for a dissociation step in the thermal cycling profile.

8. The slides need to be warmed at least 15 min at room temperature before fixation. Acetone or methanol fixation: Incubate slides in cold acetone or methanol for 10 min at 4 °C, and then allow slides to air-dry. Paraformaldehyde (2%) fixation: Incubate slides for 20 min at 4 °C. Formalin fixation: Incubate slides for 30 min at room temperature. Perform fixation in covered Coplin staining jars.
9. 1× PBS can be substituted for 1× Super Sensitive Wash Buffer. Multiple washings of slides may remove the lines made by the water-repelling pen. Replace the circles around the sections if necessary.
10. Monitor staining by eye to ensure that the slides do not become too dry, and hematoxylin counterstaining does not result in excessive pink background.
11. Dry six slides at a time and add seven drops of permanent mounting medium per slide. Spread with a pipette tip to ensure that the mounting medium completely covers each section. Allow slides to dry overnight loosely covered and protected from dust.

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Chapter 4

Analysis of Expression of CCN Family Genes in Skeletal Tissue-Derived Cells

Harumi Kawaki, Satoshi Kubota, and Masaharu Takigawa

Abstract

The quantitative reverse transcription polymerase chain reaction or real-time PCR has become a routine technique for the detection and comparison of amounts of specific mRNA transcripts, done by measuring amplified levels of specific cDNAs. In this chapter, we provide our real-time RT-PCR experimental procedure using SYBR Green I for the quantitative analysis of CCN family gene expression. Especially, we describe the extraction and purification steps for RNA derived from mesenchymal cells, such as chondrocytes and osteoblasts that produce a large amount of extracellular matrix in detail.

Key words Mesenchymal cells, Chondrocyte, Osteoblast, Extracellular matrix (ECM), Collagens, Proteoglycans, Matrix of cartilage, Matrix of bone, RNA purification, Real-time PCR, qRT-PCR

1 Introduction

CCN family members are known to be notable functional molecule in a variety of tissues and organs, as mentioned in other chapters [1–4]. Especially in bone formation, these members have been shown to be important factors, e.g., being closely involved in the differentiation of chondrocytes and osteoblasts [1–7]. Our previous studies showed that all CCN members are expressed in a certain stage during bone formation. We comparatively analyzed the gene expression patterns of the CCN family members in primary cultures of murine chondrocytes and osteoblasts by using PCR-based quantitative methods [5–7].

The quantitative reverse transcription polymerase chain reaction (qRT-PCR) or a real-time PCR is a major development in PCR technology for the detection and comparison of amounts of specific mRNA transcripts, often called "gene expression," done by measuring amplified levels of specific cDNAs [8, 9]. Compared with Northern blotting, an alternative method for the detection of specific RNAs, PCR-based methods are relatively simple and rapid, requiring only a moderate amount of RNA, and allowing for a certain level of

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degraded RNA. As long as the RNA is intact within the target region, this region of interest will be amplified by PCR [9, 10]. PCR-based methods for gene expression analysis consist of two parts. One is the process of preparation of RNA samples and synthesis of complementary DNA (cDNA) from the RNA samples by reverse transcription. The other is the process of amplification of specific cDNA by PCR and assessment of the amplicons. High-quality RNA samples are still required for PCR-based methods, as low-quality RNA may compromise the reliability of the experimental results [9, 10].

We have particularly paid attention to the mesenchymal tissues, especially skeletal tissues such as cartilage and bone. Cells derived from these tissues generally produce and deposit several extracellular matrix (ECM) components even in culture. Chondrocytes produce a large amount of cartilaginous matrix components, such as type II collagens and aggrecans. Osteoblasts are responsible for bone formation by producing bone matrix. These mesenchymal cells develop sheet-like ECM deposits approx. 2 weeks after being placed in culture. After this time point, the ECM produced by chondrocytes or osteoblasts eventually becomes mineralized. For this reason, successful gene expression analysis requires not only careful handling during all steps for RNA sample preparation but also some additional modifications made to the widely used protocol. In this chapter, we describe the real-time RT-PCR experimental procedure using SYBR Green I for the quantitative analysis of CCN family member gene expression. Especially, RNA extraction and purification steps are described in detail.

2 Materials

All chemicals are of regent grade. Use RNase- and DNase-free ultrapure water for all solutions.

2.1 Cell Culture and Sample Preparation

1. Cells of interest, e.g., primary murine chondrocytes or osteoblasts (*see* **Note 1**).
2. Culture medium (*see* **Note 2**).
3. Cell culture vessels (*see* **Note 3**).
4. Cell lysing buffer (e.g., Buffer RLT, a component of RNeasy Mini Kit, Qiagen) (*see* **Note 4**).
5. β-Mercaptoethanol (β-ME).
6. Cell scrapers.
7. 1.5 ml Microcentrifuge tubes.

2.2 RNA Purification and Cleanup

A number of conventional protocols are available for preparation of total RNA from tissues and culture cells. Commercial kits are also available from a variety of manufacturers for the preparation

and purification of total RNA or mRNA. This step can also be done conventionally by using NP-40-based lysing solution, phenol, and commercial Trizol (Invitrogen) containing phenol and guanidine isothiocyanate, or similar products. In the case of the RNA extraction and purification from ECM-rich mesenchymal cells, we perform this procedure by using RNeasy Mini Kit (Qiagen) (*see* **Notes 4–6**). This protocol is adapted from the supplier's instruction with the addition of optional procedures and our proper modifications.

1. RNA extraction kit (e.g., RNeasy Mini Kit, Qiagen) (*see* **Note 4**). The kit includes RNeasy spin columns, Buffer RLT, Buffer RW1, and Buffer RPE.
2. Homogenize tools, e.g., QIAshredder spin columns (Qiagen), homogenizer pestles, or 20-gauge needles and syringes (*see* **Notes 5**).
3. Ethanol.
4. DNase I (e.g., RNase-Free DNase Set (Qiagen); lyophilized RNase-free DNase I and Buffer RDD are supplied with the set) (*see* **Note 6**).
5. Spectrophotometer (*see* **Note 7**).
6. 1.2% Agarose gels and gel electrophoresis apparatus (optional) (*see* **Note 8**).

2.3 Reverse Transcription

1. Retroviral reverse transcriptase and RT reaction components (e.g., SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, containing RT enzyme and every other components required in the RT reactions) (*see* **Note 9**).
2. Thermal cycler or block incubator.
3. Micro tubes for the RT reactions.

2.4 PCR and Quantitative Analysis

1. cDNA dilution solution, e.g., RNase- and DNase-free water containing carrier RNA (*see* **Note 10**).
2. Target gene-specific PCR primer sets (Table 1).
3. A specific primer set for a housekeeping gene, e.g. *gapdh* (Table 1).
4. SYBR Green I PCR master mix.
5. Real-time PCR instrument and optical tubes, plates, or capillaries required the instrument.

3 Methods

In this protocol, we describe the procedure for total RNA extraction from ECM-rich cells by using RNeasy Mini Kit including the optional processes adapted in our lab.

Table 1
Primer sequences of mouse CCN family members and gapdh, internal control for real-time PCR

Gene	Primer sequence	Amplicon size (bps)
Ccn1	5′-ATGAAGACAGCATTAAGGACTC-3′ (S)	172
	5′-TGCAGAGGGTTGAAAAGAAC-3′ (AS)	
Ccn2	5′-CCACCCGAGTTACCAATGAC-3′ (S)	169
	5′-GTGCAGCCAGAAAGCTCA-3′ (AS)	
Ccn3	5′-TGAAGTCTCTGACTCCAGCATT-3′ (S)	230
	5′-TGGCTTTCAGGGATTTCTTG-3′ (AS)	
Ccn4	5′-TGAGAACTGCATAGCCTACAC-3′ (S)	192
	5′-TACACAGCCAGGCATTTC-3′ (AS)	
Ccn5	5′-GCTGTGATGACGGTGGTT-3′ (S)	194
	5′-GACAAGGGCAGAAAGTTGG-3′ (AS)	
Ccn6	5′-CTGCAAAGTCTGTGCCAAG-3′ (S)	151
	5′-GAACTCACATCCAACTGCC-3′ (AS)	
Gapdh	5′-ATCTTGGGCTACACTGAGGA-3′ (S)	122
	5′-CAGGAAATGAGCTTGACAAAGT-3′ (AS)	

S sense, *AS* antisense
Annealing temperature for all primer sets is 60 °C

3.1 Cell Culture and Sample Preparation

1. Harvest the cells of interest from duplicate culture vessels or more during the experimental period, routinely lasting 7–28 days in our lab. For the long-term culture experiments, the cells, e.g., chondrocytes or osteoblasts, are grown to confluence. At confluence (day 7), prepare initial RNA samples; and thereafter process additional cells for samples every 7 days according to the following procedure:
2. Prepare the Buffer RLT supplemented with 1% β-ME before use.
3. Aspirate the culture medium completely.
4. Add immediately 400–700 μl of Buffer RLT to the cell culture vessels to lyse the cells directly (*see* **Note 11**).
5. Collect cell lysates with a cell scraper immediately.
6. Pipette the cell lysates into microcentrifuge tubes.
7. Vortex the lysates for a few seconds to mix them.
8. Store the lysates at −80 °C until RNA purification can be done (*see* **Note 12**).

3.2 RNA Purification and Cleanup

Carry out **steps 4–25**, **28**, and **29** at room temperature. The centrifugation temperature (**steps 4–25**, **28**, and **29**) should be 20–25 °C.

1. Prepare the Buffer RPE by adding four volumes of ethanol just before the first use.
2. Thaw the cell lysate samples and spin them down.
3. Homogenize each lysate at 4 °C with a QIAshredder spin column, homogenizer pestle, or 20-gauge needle fitted to a syringe. In the case of homogenization using the QIAshredder spin column, pipet the lysate into the column placed in a 2 ml collection tube (supplied). Close the lid. Centrifuge for 2 min at 20,000 × *g* (*see* **Note 5**).
4. Add a volume of 70% ethanol approx. equal to that of the RLT buffer (usually 400–700 μl) to each lysate and mix well by pipetting (*see* **Note 13**).
5. Transfer 600 μl of the sample into an RNeasy spin column placed in a collection tube (supplied). Close the lid (*see* **Note 14**).
6. Centrifuge the tube for 15 s at 8000 × *g*.
7. Discard the flow-through. (Reuse the collection tubes through the following wash steps.)
8. Repeat **steps 4–6**, using the remainder of each sample.
9. To wash the column membrane, add 350 μl of wash buffer RW1 to each spin column. Close the lid.
10. Centrifuge the tubes with spin columns for 15 s at 8000 × *g*.
11. Discard the flow-through.
12. Prepare DNase I reaction mixture (*see* **Notes 4** and **6**). Add 10 μl of DNase I stock solution to 70 μl of Buffer RDD.
13. Add the DNase I reaction mix (80 μl per sample) directly to the RNeasy spin column membrane. Incubate for 15 min at room temperature (20–30 °C).
14. Repeat **steps 8–10**.
15. Wash the column membrane with wash buffer RPE. Add 500 μl of Buffer RPE to each spin column. Close the lid.
16. Centrifuge the tubes with spin columns for 15 s at 8000 × *g*.
17. Discard the flow-through.
18. Add 500 μl of Buffer RPE to each spin column. Close the lid.
19. Centrifuge the tubes with spin columns for 2 min at 8000 × *g*.
20. Discard the flow-through.
21. Place each spin column in a new tube (supplied in the kit). Centrifuge the tubes with spin columns for 1 min at 8000 × *g* (optional; *see* **Note 15**).
22. Place each spin column in a new tube (supplied).

23. Add 40 μl of RNase-free water (supplied) to the column membrane. Close the lid.
24. Incubate for 1 min.
25. To elute the RNA, centrifuge the tubes with spin columns for 1 min at 8000 × *g*.
26. Determine the RNA quality and concentration of each sample by using a spectrophotometric method. Quantify RNA yield based on the OD_{260} value (*see* **Note 7**).
27. Check the RNA quality by electrophoresis, loading 1 μg of each RNA sample onto a 1.2% agarose gel (optional; *see* **Note 8**).
28. RNA cleanup (optional; *see* **Note 16**): Add RNase-free water to the samples up to 100 μl. Add 350 μl of Buffer RLT to the samples and mix thoroughly by pipetting. Add 250 μl of 100% ethanol to the samples and mix thoroughly by pipetting. Transfer the samples into new RNeasy spin columns. Repeat **steps 6–25**. If DNase digestion steps are not needed, skip **steps 12–14**, and wash the column membrane with 700 μl of Buffer RW1 instead at **step 10**.
29. If a high RNA concentration is required, repeat **step 25** by using the eluate from **step 25**. Reuse the collection tube from **step 25**.

3.3 Reverse Transcription

Perform the RT reactions with 1–5 μg of each total RNA sample by one's choice of RT reaction system. A typical real-time PCR protocol with SYBR green I is described in Chapter 3 regarding human skin.

3.4 PCR and Quantitative Analysis

1. Prepare the template cDNA dilution solution.
2. Prepare serial dilutions of control samples with the dilution solution in **step 1** for establishing a standard curve (*see* **Note 10**).
3. Dilute each sample with the dilution buffer according to the instruction of one's selected real-time PCR system.
4. Prepare the PCR reaction mix.
5. Perform the real-time PCR reaction. A typical real-time PCR protocol with SYBR green I is described in Chapter 3 regarding human skin.

4 Notes

1. In this chapter, we describe especially high-quality RNA preparation from mesenchymal cells that produce a large amount of ECM under particular conditions, e.g., assessment of differentiation stage-dependent gene expressions of CCN family members. One can use this protocol for the preparation of total RNA from murine chondrogenic cell-line ATDC-5, osteoblastic MC3T3-E1, human osteoblastic SaOS-2, and human chondrocytic HCS-2/8 cells.

2. Use a medium optimized for the cells of interest. Classical media, such as Dulbecco, Eagle, Ham, and others, or specialty media, e.g., chondrogenic differentiation medium.
3. A 3.5 cm dish or a 6-well plate is recommended with careful consideration of its ease of handling and capacity for providing a sufficient amount of cell lysate for loading onto the RNA purification spin column. We usually perform cell culture-based assays in duplicate or triplicate.
4. We routinely use a commercial RNeasy Mini Kit (Qiagen) to prepare total RNA from chondrocytes and osteoblasts in primary culture for the following reasons: It is simple and rapid, giving high-quality RNA. RNA can be obtained in less than 1 h. If the quality of RNA is lower than that of one's expectation because of ECM-derived protein contamination, one can rapidly clean up the RNA by use of the spin column technique. In addition, any contaminating genomic DNA (gDNA) can be removed from the RNA by the on-column DNase digestion method during the RNA purification procedure. This RNA extraction kit can also be used to clean up RNA extracted from murine cartilaginous tissue [5].
5. Incomplete homogenization of cell lysates leads to reduced RNA yields and a clogged membrane of the spin column. One may well homogenize the cell lysates by passing the lysates through a 20-gauge needle fitted to an RNase-free syringe or using a homogenizer pestle. However, we recommend the spin-column method for the complete homogenization of cells with mineralized ECM. One can homogenize completely cell lysates containing calcium deposits in only 2 min by centrifugation using QIAshredder columns.
6. To eliminate the gDNA, optional DNase I Set for the on-column gDNA digestion method during RNA purification using RNeasy Kits is available. One can easily remove any contaminating gDNA from the RNA preparation by using this method.
7. The concentration of RNA should be determined by measuring its absorbance at 260 nm with a spectrophotometer. The ratio between the absorbance values at 260 and 280 nm should also be measured to estimate the purity of the RNA.
8. Agarose gel electrophoresis is one of the commonly used methods for analyzing RNA species. The quality of RNA is determined by the appearance of the large (28S) and small (18S) ribosomal RNAs as two sharp bands. For mammalian rRNA, generally, a 28S:18S ratio of 2:1 is representative of good-quality RNA. If gDNA is a contaminant in RNA samples, it can also be visualized in the agarose gel, as gDNA runs slower in the gel than RNA.
9. A number of reverse transcription kits that include reverse transcriptase and every other component needed for the RT

reaction are currently available (e.g., SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, or equivalent kits).

10. To avoid the adsorption of cDNA to the inside tube, we recommend to use a solution containing some carrier such as for the dilution of cDNA samples.
11. For complete cell lysis, increase the volume of Buffer RLT per dish along with the increase in the differentiation of the cells or increase in the number of days in culture.
12. If one conducts a time-course analysis, cell lysates should be stored at −80 °C until RNA purification is done.
13. Prepare 70% ethanol for RNA work in advance and store it at room temperature.
14. One can put a sample of up to 700 μl into a spin column. However, we transfer less than this amount because the cell lysate samples are slightly bubbly.
15. To ensure that no ethanol is carried over during the RNA elution procedure, perform the optional centrifugation to dry the membrane of spin columns.
16. If the A_{260}/A_{280} value of one's RNA sample is low, one can easily clean up such samples by using the RNA purification spin column.

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Chapter 5

Western Blotting Analysis of CCN Proteins in Calcified Tissues

Harumi Kawaki, Satoshi Kubota, and Masaharu Takigawa

Abstract

Western blotting is widely used for protein analysis. We routinely perform such analysis for evaluating the production levels of CCN family proteins in a variety of cells under various conditions. In this chapter, we describe our Western blotting protocol to estimate protein production profiles of CCN family members after having assessed the specificity of the antibodies against each CCN member protein to ensure no cross-reaction with other CCN member proteins.

Key words Amino acid homology, Conserved module, Member-specific antibody, Cross-reaction, Redundancy of CCN members

1 Introduction

CCN family proteins with a molecular weight of approximately 30–40 kDa have a high degree of amino acid sequence homology. The proteins in this family are composed of four conserved modules except for CCN5, which lacks the C-terminal (CT) module [1]. These members are critical signaling and regulatory molecules active in a variety of tissues and are involved in several cellular processes including adhesion, migration, proliferation, differentiation, and survival [1–6]. Consequently, the distribution of CCN family members overlaps, and these proteins function redundantly in certain types of cells and tissues. Hence, estimation of the production of a particular CCN protein without cross-reaction with other family members is required to elucidate the physiological roles of a given CCN protein in those cells or tissues.

Western blotting is a routine technique used in research to separate proteins based on their molecular weight through gel electrophoresis followed by the identification of specific proteins. The specificity of the interaction between antibody and antigen enables us to identify a target protein from a complex mixture of proteins. Many researchers including us employ this method to

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identify targeted proteins, ever since Renart [7] developed the protein blotting and detection method using antisera after the establishment of the protein separation technique using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli and his colleagues [8] and by Towbin [9]. However, it is difficult to distinguish a particular CCN protein from other CCN proteins, except for CCN5, by their molecular weights. In addition, due to the homology among CCN member proteins [1], the key for detection of a particular CCN member by Western blotting is the use of well-selected antibodies having high specificity for the target CCN member protein and not cross-reacting with any other members.

Thus, for the specific detection of the six CCN family proteins, we carefully evaluate the specificity of their respective antibodies against each CCN family member by performing Western blotting with control proteins before analyzing biological samples [2]. In this way, we could clarify the protein production patterns of CCN family members, which are differentially expressed in chondrocyte and osteoblast lineages [2, 4]. In this chapter, we give detailed information on the procedures for Western blotting analysis of human or mouse CCN proteins, in which chemiluminescent substrates are used for the discriminative detection of specific CCN family member proteins.

2 Materials

All chemicals are of reagent grade. Use ultrapure water for all solutions.

2.1 Sample Preparation

1. Phosphate-buffered saline (PBS).
2. Detergent-based cell lysis buffer (*see* **Note 1**). Add proteinase inhibitor cocktail to the cell lysis buffer according to the manufacturer's protocol and keep on ice before use (*see* **Notes 1** and **2**).
3. Proteinase inhibitors (e.g., phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin hemisulfate salt, and pepstatin A) (*see* **Note 2**).
4. 2× Concentrate Laemmli [8] sample buffer (*see* **Note 3**).
5. Reagents or kits for measurement of protein concentration (e.g., bicinchoninic acid (BCA) assay reagents) (*see* **Note 4**).
6. Spectrophotometer or microplate reader.

2.2 Electrophoresis

1. 10–15% Polyacrylamide gel: We commonly use shop-made pre-casting gels for "mini gel" system (*see* **Note 5**).
2. 20 mM Tris-buffered saline (TBS), pH 7.6.
3. Electrophoresis buffer: 25 mM Tris aminomethane, 192 mM glycine, 0.1% SDS.

4. Protein ladder maker (*see* **Note 6**).
5. Protein electrophoresis equipment.

2.3 Blotting

1. Blotting membrane, e.g., polyvinylidene difluoride (PVDF) membrane (*see* **Note 7**).
2. Blotting buffer: 25 mM Tris aminomethane, 192 mM glycine, 20 % methanol.
3. Several sheets of filter paper are needed.
4. Blotting unit for electro-transfer.

2.4 Immuno-detection and Image Analysis

1. Primary antibodies specific to the target CCN family member(s). Our selected six antibodies [2]: SC-13100 (lot. J2105) for CCN1 (Santa Cruz), ab6992-50 (lot. 314202) for CCN2 (Abcam), K-19M [10] for CCN3, SC-25441 (lot. B2806) for CCN4 (Santa Cruz), SC-25442 (lot. K0805) for CCN5 (Santa Cruz), and SC-25443 (lot. A2604) for CCN6 (Santa Cruz) (*see* **Note 8**).
2. Antibody dilution and blocking buffer: 5 % Skim milk in TBS-Tween 20 (TBS-T). A Tween 20 concentration of 0.1 % is suitable.
3. Wash buffer: TBS-T.
4. Horseradish peroxidase (HRP)-conjugated secondary antibodies specific to the primary antibodies.
5. Chemiluminescence detection reagents (e.g., Amersham™ ECL™ series, GE Healthcare and SuperSignal™ Chemiluminescent Substrate series, Thermo Scientific).
6. Shaker.
7. Washing trays.
8. Image analysis system: Charge-coupled device (CCD) camera-based imaging or X-ray film-based signal detection system.

2.5 Recombinant CCN Proteins

In this chapter, because we describe Western blotting analysis for human CCN proteins as typical examples, the following recombinant human CCN proteins that we commonly use as standards are listed up. If western blotting analysis for other species of CCN proteins is required, one should select proper combinations of sources of CCN proteins, their corresponding primary antibodies, and secondary antibodies.

1. Recombinant human CCN1 (rhCCN1) (PEPROTECH).
2. Recombinant human CCN2 (rhCCN2) (Bio Vender Laboratory).
3. Recombinant human CCN3 (rhCCN3) (PEPROTECH).
4. Recombinant human CCN4 (rhCCN4) (PEPROTECH).

5. Recombinant human CCN5 (rhCCN5) (PEPROTECH).
6. Recombinant human CCN6 (rhCCN6) (PEPROTECH).

All rhCCN proteins are to be prepared in 0.1% bovine serum albumin (BSA)-PBS at a concentration of 50 μg/ml as stock solutions.

3 Methods

Perform gel electrophoresis and transfer proteins to the membrane according to standard protocols. We employ the semidry transfer method, which is faster than wet transfer and expends less blotting buffer. This semidry transfer system works well for the detection of CCN proteins, although it may be less efficient for the transfer of larger proteins.

3.1 Sample Preparation

Prepare protein extract of interest (*see* **Note 9**). In this section, we describe our protocol to extract proteins from adherent cells. Cells grown in a monolayer in cell culture vessels can be lysed directly in the vessels. Carry out all procedures at 4 °C or on ice.

1. Wash cells in the tissue culture dish with cold PBS and gently rock it manually.
2. Discard the PBS. Add ice-cold lysis buffer with a protease inhibitor cocktail and collect cell lysates with a cell scraper immediately (*see* **Note 10**). Pipette the cell lysates into microcentrifuge tubes.
3. Homogenize the lysates (*see* **Note 11** and **Note 5** in Chapter 4). Centrifuge them at 13,000 × *g* for 5 min at 4 °C, and then transfer the supernatants into fresh tubes.
4. Determine the protein concentration of each sample spectrophotometrically.
5. Adjust the sample volume to the desired protein concentration to permit loading the same volume for each sample. In our lab, 20–30 μg of proteins are separated for the detection of CCN proteins.
6. Add an equal volume of 2× Laemmli sample buffer. Boil the protein samples at 95 °C for 5 min before loading them onto the gel. After boiling, spin down the samples to bring down the condensation on the inner surface of the tubes.
7. Store at −80 °C before use.

3.2 SDS-PAGE

1. Load the samples and protein markers onto the gel.
2. Run the gel at a constant voltage of 120 V for 60 min, or until the dye front runs off the bottom of the gel (*see* **Note 12**).

3.3 Electro-Transfer (Blotting)

1. Immerse a membrane in the transfer buffer before electrophoresis is completed.
2. After electrophoresis is completed, equilibrate the gel in the transfer buffer for 20 min to remove the SDS.
3. Transfer separated proteins to the membrane at a constant current of 144 mA for 45 min at room temperature.
4. On completion of transfer, place the protein-bound membrane into TBS to prevent the membrane from drying out.

3.4 Immunodetection and Image Analysis

1. Discard the TBS and add blocking buffer to block the nonspecific binding sites. Incubate the membrane at room temperature for 60 min with gentle shaking (*see* **Note 13**).
2. Prepare the primary antibody solution. Dilute the primary antibodies specific for each CCN family member by using antibody dilution buffer at the supplier's recommended concentration. Store them on ice before use.
3. Discard the blocking buffer, add the primary antibody solution to the membrane, and incubate the membrane at 4 °C overnight with gentle shaking (*see* **Note 14**).
4. Discard the primary antibody solution and wash the membrane three times with TBS-T for 5 min each time.
5. Make a secondary antibody solution by mixing antibodies and antibody dilution buffer.
6. Discard the TBS-T washing buffer and apply the secondary antibody solution to the membrane. Incubate the membrane at room temperature for 60 min with gentle shaking.
7. Discard the secondary antibody solution and wash the membrane three times with TBS-T for 5 min each time.
8. Prepare chemiluminescence reagents following the manufacturer's protocol.
9. Incubate the membrane in the solution of chemiluminescent reagents.
10. Visualize the signals (*see* **Note 15**).

4 Notes

1. Cell lysis with detergent is easier and milder than alternative physical disruption methods. Cell lysis solutions are detergent-based buffers and reagent sets (cell lysis buffer) that have been optimized for a variety of samples and applications. A number of cell lysis buffers with different detergents and buffer formulations are currently available. We commonly use a CelLytic™-M lysis buffer (Sigma) or a Cell Lysis Buffer (Cell Signaling Technology) for mammalian cells.

2. To prevent degradation of extracted proteins and to increase the yield of intact proteins, the addition of the proteinase inhibitors to the cell lysis buffer is recommended. Solutions, tablets, or powder forms of proteinase inhibitor cocktails of varied formulations, which cocktails contain inhibitors with a broad specificity for serine, cysteine, and acid phosphatases, as well as aminopeptidases are commercially available (e.g., Protease Inhibitor Cocktail P8340 as a 100× concentrate ready-to-use solution, Sigma Aldrich).
3. We commonly use a 2× Laemmli sample buffer (Bio Rad).
4. Pierce™ BCA Protein Assay Kit (Life Technologies) or an equivalent kit may be used. We commonly use the former.
5. For the detection of full-length CCN proteins, we commonly use 12.5 % gels. It has been reported that multiple functions of CCN proteins ought to be enabled by the differential use of four modules. To address this issue, we had raised and evaluated antibodies for independent detection of each single module of hCCN2. In that case, we use 15–25 % gradient gels and individual modules of hCCN2 produced by *Brevibacillus choshinensis* transformed with an expression plasmid for one of the modules [12].
6. We frequently use a broad-range recombinant ladder with multiple colors, which allows easy band referencing and orientation for protein transfer (e.g., Rainbow Markers, GE Healthcare).
7. Nitrocellulose and polyvinylidene difluoride (PVDF) membranes are popular. We commonly use Amersham™ Hybond P (GE Healthcare) PVDF membranes.
8. We selected the six antibodies listed in Subheading 2.4, none of which cross-react with the other members [2]. We confirmed this lack of cross-reactivity after preliminary Western blotting analysis using human recombinant CCN proteins listed in the next subsection (see Subheading 2.5). We also tested protein samples derived from human cell lines and murine cells in primary culture with these six antibodies. To assess the specificity of primary antibodies against specific CCN family member proteins without cross-reaction with the other CCN family members, we perform Western blotting analysis using rhCCN proteins. We offer a result obtained with anti-CCN2 antibody, ab6992-50 (lot. 314202) (Fig. 1). In this experiment, we used rhCCN2 secreted by HeLa cells transformed with hCCN2 overexpression plasmids [11] and bacterially derived rhCCN proteins described above. Afterwards, we elucidated changes in the production levels of all CCN family member proteins in CCN2-deleted murine chondrocytes [2]. We also demonstrated differentiation

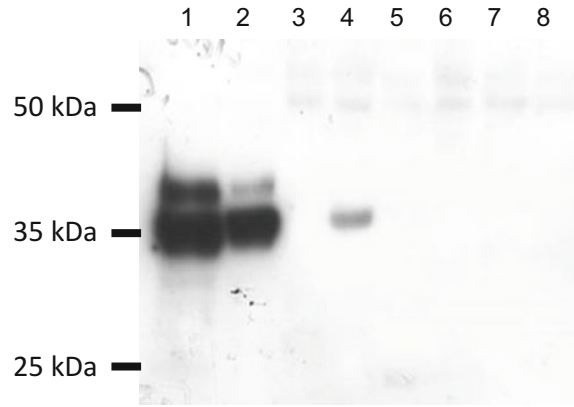

Fig. 1 Western blotting analysis for evaluation of the specificity of a CCN2 antibody. The anti-CCN2 antibody, ab6992-50 (lot. 314202, Abcam), was evaluated for its specificity by performing Western blotting using recombinant CCN member proteins. *Lanes 1* and *2*: 50 and 20 ng, respectively, of rhCCN2 secreted by HeLa cells transformed with an hCCN2 overexpression plasmid. *Lanes 3–8*: 5 ng of bacterially derived rhCCN1, rhCCN2, rhCCN3, rhCCN4, rhCCN5, and rhCCN6, respectively, with 10 ng of BSA as a protein carrier. Note that the ab6992-50 reacted well with rhCCN2 but was unreactive with any of the other CCN family members

stage-dependent CCN protein production in primary cultures of murine calvarial osteoblasts, with the recombinant proteins used as a positive control [4].

9. The gene expression of CCN family members has been suggested to be regulated at multiple steps, by multiple stimuli such as growth factors [1, 6, 13], hormones [1, 6, 14], and even mechanical stress loading [1, 15]. For the preparation of stimulated or unstimulated samples, we commonly use 3.5 cm dishes for cell culture and harvest the cells with 200–400 μl of lysis buffer. We have performed Western blotting analysis for the detection of cell-associated chicken CCN2 in protein samples obtained from smaller scale cultures, as well as for that of secreted CCN2 secreted into the culture medium of chicken calvarial osteocytes in culture [15].
10. Keep the culture dish on ice throughout the process of harvesting the cell lysate.
11. Certain types of cells, such as chondrocytes and osteoblasts, generally produce a large amount of extracellular matrix. In this case, the homogenization or sonication of cell lysates is needed. We frequently use QIAshredder spin columns (QIAGEN). The QIAshredder homogenizer is acceptable for small-scale lysates of less than 100 μl.
12. We perform SDS-PAGE in a cold room.

13. Blocking buffer should be freshly prepared and blocking agents should be completely dissolved. Casein Buffer (Vector Laboratories) [2] and 3–5 % BSA in TBS buffer [15] also work in our lab.
14. Setting up shakers or rotators in a cold chamber or cold room is recommended.
15. To enhance the intensity of positive signals, we occasionally use biotinylated secondary antibodies and avidin–biotin complexes (ABC system, Vectastain Elite ABC kit, Vector) following the manufacturer's protocol [2].

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Chapter 6

Immunohistochemical Analysis of CCN Proteins in Calcified Tissues

Harumi Kawaki, Satoshi Kubota, and Masaharu Takigawa

Abstract

Immunohistochemistry is a major technique to determine the distribution and localization of differentially produced proteins in the context of an intact tissue. It exploits one of the properties of antibodies, specific binding to an antigen, i.e., to the epitope of its target protein, in combination with a color-developing enzymatic reaction or tagged fluorophore. We have clarified the spatial and temporal expression patterns of CCN family proteins in several different types of animal tissues by using this immunohistochemical technique to support our corresponding data obtained in vitro. In this chapter, we provide our protocol for immunohistochemistry optimized for paraffin-embedded sections after having determined the optimal conditions for the use of antibodies against each member of the CCN family.

Key words Paraffin-embedded sections, Tissue morphology, Masked epitope, Antigen retrieving, Monitoring protein distribution

1 Introduction

Immunohistochemistry (IHC) is widely used to determine the localization pattern of a protein in a biological sample through the specific binding of an antibody to the protein. This technique is especially useful for monitoring the distribution and localization of biomarkers and differentially produced proteins in different parts of a biological tissue by specifically detecting a wide variety of antigens, despite its having less quantitative value than other immunoassays such as Western blotting (WB) and enzyme-linked immunosorbent assay (ELISA). Since Coons and his colleagues first used fluorescein isothiocyanate (FITC)-labeled antibodies to detect target proteins [1, 2], a number of different methods for visualizing antibody–antigen interaction have been reported. Detection systems utilizing enzyme labeling such as peroxidase [3] or alkaline phosphatase [4] conjugated systems are commonly used by many researchers.

For IHC experiments to be successful, there are two major considerations. First, obtaining the optimal antibodies for the

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target proteins is critical. For example, we tested dozens of antibodies including 6 antibodies selected for WB analysis [5]. Then, as a result of preliminary experiments, we finally adopted these same six antibodies for use in immunohistochemical analysis. Second, adequate epitope retrieval is important. One of the most common forms of IHC involves the use of tissues fixed with paraformaldehyde (PFA) and embedded in paraffin, since paraffin-embedded sections allow superior retention of tissue and cell morphology. However, most PFA-fixed tissues require an antigen retrieval step before proceeding to IHC, because this fixation process can cause protein cross-linking that masks the epitope recognized by the antibody. We tested our selected antibodies against each CCN member protein and determined the optimal antigen-retrieval conditions for IHC.

After optimization of the general immunohistochemical protocol, we clarified the spatial and temporal protein distribution patterns of CCN family members in several different types of animal tissues, such as bone and cartilage tissues, by using this immunohistochemical technique to support our in vitro data [5–10]. In this chapter, we describe in detail our IHC protocol, optimized for paraffin-embedded samples, for detecting proteins of the CCN family.

IHC protocol optimized for frozen sections is described in Chapter 3.

2 Materials

All chemicals are of reagent grade. Use ultrapure water for all solutions.

2.1 Sample Preparation

1. Microtome.
2. Water bath.
3. Flattening table for paraffin-embedded sections.
4. Charged glass slides (*see* **Note 1**).

2.2 Deparaffivnization/Rehydration

1. Xylene.
2. Graded ethanol (100%, 90%, 80%, 70%, and 50%).
3. Methanol.
4. 3% hydrogen peroxide (H_2O_2) in methanol as a blocking solution for endogenous peroxidase (*see* **Note 2**).
5. Glass staining jars.
6. Slotted stainless steel slide rack fitting into the glass staining jar.

2.3 Antigen Retrieval

1. Antigen retrieving buffer (e.g., Target Retrieval Solution, pH 6.1, DAKO) (*see* **Note 3**).
2. Microwavable vertical staining jars (*see* **Note 4**).

3. 20 mM Tris-buffered saline (TBS), pH 7.4.
4. Microwave oven.

2.4 Immunoreaction

1. Primary antibodies. Specific to the target CCN member(s). Our selected six antibodies that do not cross-react with the other members; SC-13100 (lot. J2105) for CCN1 (Santa Cruz), ab6992-50 (lot. 314202) for CCN2 (Abcam), K-19M [6] for CCN3, SC-25441 (lot. B2806) for CCN4 (Santa Cruz), SC-25442 (lot. K0805) for CCN5 (Santa Cruz), and SC-25443 (lot. A2604) for CCN6 (Santa Cruz) (*see* Chapter 5). Dilutions of the primary antibodies are listed in Table 1. We employed higher concentrations of primary antibodies than those in WB experiment.
2. Blocking solution and antibody diluent. As a blocking reagent, select suitable one for immunodetection system (*see* **Note 5**).
3. 20 mM TBS, pH 7.4.
4. Wash buffer. TBS containing 0.1 % Tween 20 (TBS-T).
5. Humid chambers.
6. Shaker.

2.5 Immunostaining Components

1. Horseradish peroxidase (HRP) conjugate immunohistochemical detection system (*see* **Note 6**).
2. Substrate for detection system such as 3,3′-diaminobenzidine (DAB) for HRP (*see* **Note 7**).
3. Counterstaining solution. Methyl green or hematoxylin (optional).

2.6 Dehydration and Mounting

1. Graded ethanol (70 %, 80 %, 90 %, and 100 %).
2. Xylene.
3. Coverslips.
4. Suitable mounting media.

Table 1
Antibodies diluent conditions

Target	Antibody	Dilution
CCN1	SC-13100	1:100
CCN2	ab6992-50	1:200
CCN3	K-19M	1:500
CCN4	SC-25441	1:100
CCN5	SC-25442	1:100
CCN6	SC-25443	1:100

3 Methods

The main steps of this protocol are sample preparation, deparaffinization/rehydration, antigen retrieval, immunoreaction, immunostaining, and dehydration/mounting. Do not allow slides to dry at any time until the samples have been mounted with coverslips.

3.1 Sample Preparation

1. Prepare paraffin-embedded tissues of interest and store them until sectioning (*see* **Note 8**).
2. Use a microtome to cut the embedded samples into 5-μm-thick sections and then float them in a 45 °C water bath containing distilled water.
3. Mount the sections on charged glass slides and allow them to dry at 42 °C overnight (*see* **Note 9**).

3.2 Deparaffinization/Rehydration

Perform this procedure according to Table 2.

1. To remove the paraffin, place the slides sequentially into three staining jars of xylene for 5 min each time (*see* **Note 10**).

Table 2
Deparaffinization and dehydration procedure including the blocking reaction for endogenous peroxidase

Solution	Repetitions	Incubation time (min)	
Xylene		5	
Xylene	Three changes	5	Deparaffinization
Xylene		5	
100% ethanol		5	
100% ethanol	Three changes	5	To rinse off the xylene and start rehydration
100% ethanol		5	
100% methanol	One time	5	
3% H_2O_2–methanol	One time	10	Blocking endogenous peroxidase activity
90% ethanol	One time	5	
80% ethanol	One time	5	
70% ethanol	One time	5	
50% ethanol	One time	5	
TBS		5	
TBS	Three changes	5	To rinse off the ethanol
TBS		5	

2. For rehydration, place the slides sequentially into three jars of 100% ethanol for 5 min each time and then into one jar of 100% methanol for 5 min.
3. Quench endogenous peroxidase activity by placing and keeping the slides in 3% H_2O_2–methanol for 10 min.
4. Place the slides into a graded ethanol series for 5 min for each concentration. Start from 90% and proceed downwards to 50% ethanol.
5. To complete the rehydration process, wash the slides three times in TBS buffer for 5 min each time.

3.3 Antigen Retrieval

PFA fixation forms protein cross-links that mask the epitope sites of target proteins in specimens, thereby creating weak or false-negative staining for immunohistochemical detection of certain proteins. We tested which antigen retrieval methods were most suitable for antibodies against CCN family proteins and adopted the heat-induced method using Target Retrieval Solution and a microwave (*see* **Note 3**).

1. Add 50 ml of 1× concentrated Target Retrieval Solution in a microwavable vertical staining jar.
2. Insert the sample slides into slots of the jar (*see* **Note 11**).
3. Heat the sections twice by microwaving until the buffer starts to boil, usually requiring 1 min each time (500-W microwave oven) or twice for 30 s each time (700-W microwave oven) (*see* **Note 12**).
4. Cool the slides at room temperature for 30 min.
5. Wash the slides in TBS buffer twice for 5 min each time.

Typical comparative results for antigen retrieval for detecting CCN3 are shown in Fig. 1.

3.4 Immunoreaction

All incubation steps should be carried out in a humid chamber to avoid drying of the specimens.

1. Block each section with 100–200 µl of blocking solution for 1 h at room temperature in the humid chamber.
2. Aspirate off the blocking buffer.
3. Apply 100 µl of primary antibody diluted in blocking solution onto each section.
4. As a negative control, if necessary, apply 100 µl of normal rabbit IgG in blocking solution onto another slide of the same sample. The same concentration of IgG as that of the primary antibody should be used.
5. Incubate the slide at 4 °C overnight in the humid chamber.
6. Wash the slides three times with TBS-T wash buffer for 5 min each time, with gentle shaking.

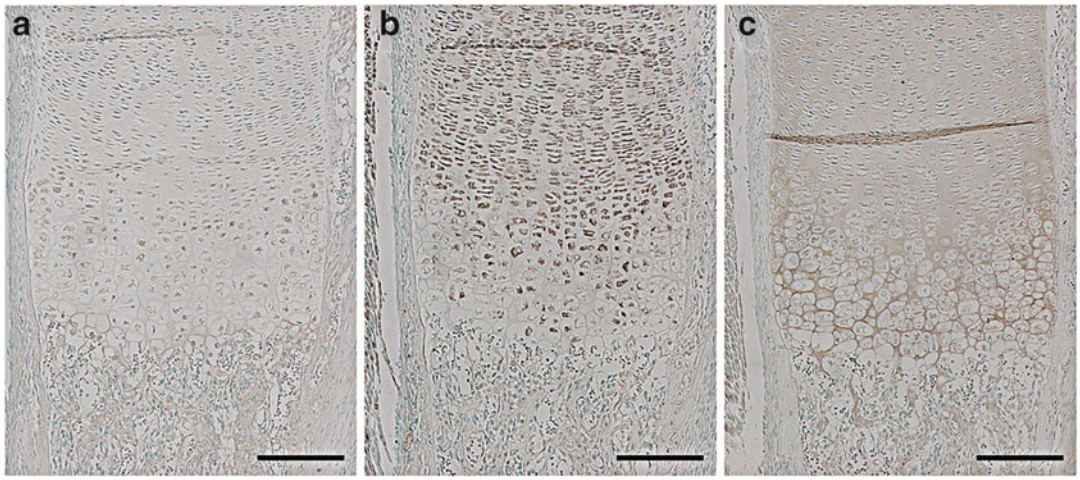

Fig. 1 Antigen retrieval comparison. Embryonic day 19 murine tibial sections were stained with anti-CCN3 rabbit antibody. Results of no retrieval reaction (**a**), microwave heat-induced retrieval with Target retrieval solution (DAKO) (**b**), and enzymatic digestion with pepsin for epitope unmasking (**c**) are shown. A clear increase in signal intensity is seen in the section after unmasking the antigen by the microwave heat-induced method with Target Retrieval Solution. Scale bars: 100 μm

7. Apply one drop of immunodetection reagent onto each section.
8. Incubate the slide for 30 min at room temperature in the humid chamber.
9. Wash the slide three times with TBS-T for 5 min each time, with gentle shaking.
10. Wash the slide with TBS for 5 min before starting the DAB chromogen reaction.

3.5 Immunostaining

This protocol is for chromogenic staining with DAB. See Subheading 3.7 for immunofluorescence staining.

1. Prepare DAB reagent or suitable substrate according to the manufacturer's instructions immediately before use.
2. Apply 100 μl of DAB or suitable substrate solution to each section and monitor its staining carefully.
3. As soon as the brown reaction product develops, immerse the slide in deionized water.
4. Counterstain the section with methyl green or hematoxylin for 20 s, if necessary.
5. Wash the slide in tap water until the water becomes clear.

3.6 Dehydration and Mounting

1. Dehydrate slides according to Table 3.
2. When all the necessary wash steps have been completed, mount the sections with coverslips using suitable mounting media, being careful to avoid the introduction of air bubbles.

Table 3
Rehydration procedure

Solution	Repetitions	Incubation time (min)
70% ethanol	One time	5
80% ethanol	One time	5
90% ethanol	One time	5
100% ethanol		5
100% ethanol	Three changes	5
100% ethanol		5
Xylene		5
Xylene	Three changes	5
Xylene		5

3. Examine the sections under a microscope and capture images as required.

3.7 Immunofluorescence Staining

In addition to the DAB chromogenic immunohistochemical analysis one can also analyze CCN member proteins in a bone fragment retaining its three-dimensional organization by using the immunofluorescence technique [10] described briefly below.

1. Prepare post-natal day 3 murine calvarial bone fragments trimmed into pieces approximately 3 mm × 3 mm.
2. Fix these fragments with 4% PFA-PBS for 30 min at 4 °C.
3. Render the fragments permeable by incubating them in 0.5% Triton X-100 in PBS for 15 min at room temperature.
4. Wash and block the fragments.
5. Reactions of primary antibodies are carried out as described above for the detection of CCN family members.
6. Use secondary antibodies conjugated with a suitable fluorescent dye as secondary antibodies (*see* **Note 13**).
7. Additionally, stain the fragments with phalloidin conjugated to a fluorescent dye and a fluorescent nuclear counterstain for morphological observation.
8. After fluorescence labeling, immediately observe the samples with a confocal laser scanning microscope.

4 Notes

1. Charged glass slides stabilize the adherence of sections to the slides. Using charged slides such as silanized ones, effectively preventing possible loss that can be expected during the microwave treatment.
2. Depending upon the type of antibodies used, a series of blocking steps is needed to minimize the nonspecific reactions of the antibody or endogenous enzymes. Some types of cells and tissues contain endogenous peroxidase. In this protocol, the use of the HRP-conjugate immunodetection system may result in high, nonspecific background staining. For blocking this endogenous peroxidase activity, we use 3% H_2O_2–methanol. Some cell-surface proteins are sensitive to 3% H_2O_2–methanol quenching. However, this is not the case for CCN family member proteins.
3. There are currently several antigen retrieval methods available. We tested other antigen retrieval buffer such as ethylenediaminetetraacetic acid (EDTA)-based alkaline buffer and sodium citrate buffer. Enzymatic digestion with pepsin was also tested. A representative result of immunostaining with anti CCN3 rabbit antibody is shown in Fig. 1. Weak signals were detected with EDTA-based or sodium citrate buffer retrieval (data not shown) or without retrieval (Fig. 1a). An incorrect method of antigen retrieval causes high background signals (Fig. 1c). Signal intensity is increased in sections after the microwave heat-induced reaction with Target Retrieval Solution (Fig. 1b).
4. Vertical staining jars such as a Coplin jar hold slides vertically for easy manipulation. A microwavable one is necessary. We commonly use microwavable jars that are made of poly-methylpentene (PMP).
5. We commonly use a commercially available blocking reagent suitable for immunodetection systems. We also use 10% normal serum from the same species that produced the secondary antibody.
6. We routinely use commercially available high-sensitivity and ready-to-use polymer-based immunodetection reagents. In this chapter, we use a Histofine® Simple Stain reagent kit (Nichirei Biosciences Inc. Tokyo, Japan), which contains reagents for human, mouse, and rat tissues. These reagents are labeled polymers prepared by combining amino acid polymers with peroxidase and secondary antibody minimized to its Fab' fragment to confer high sensitivity. SignalStain® Boost IHC Detection Reagents (CST) are also polymer-based reagents.
7. We commonly perform immunohistochemical experiments with the HRP-DAB chromogenic system. DAB is a widely

used chromogen for IHC staining. DAB produces a brown precipitate that is insoluble in alcohol. Slides developed with DAB can be dehydrated, cleared, and permanently mounted.

8. We prepare tissue samples fixed with freshly made 4% PFA-PBS.
9. If the slide is dried at a lower temperature, the adhesive strength of the tissue section becomes diminished. After the slide is completely dry, store it at 4 °C prior to staining. It is better practice that sections be freshly cut before use.
10. Incomplete deparaffinization cause spotty staining, background staining, or a reduced immunoreaction.
11. All slots of the jar must be filled. Insert blank slides into any empty slots to ensure even heating of the slides.
12. Since equipment differs, the heating protocol must be optimized for the make and model of microwave used in one's laboratory whose optimization is performed by making a trial run using blank slides.
13. We commonly use Alexa Fluor® dye conjugate secondary antibodies (Life Technologies), because the Alexa Fluor® series covers the visible spectrum and extends into the infrared. Also, a number of species-specific secondary antibodies are prepared by the supplier.

Acknowledgements

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Chapter 7

Analysis of CCN Expression by Immunofluorescence on Skin Cells, Skin, and Reconstructed Epidermis

Muriel Cario-Andre

Abstract

During a long time, immunofluorescence has been neglected to benefit of molecular biology especially genetics, transcriptomics, and proteomics analyses. These techniques give good results on cell culture but for organs that are made of numerous cells with several compartments, various states of differentiation as in epidermis, immunohistochemistry is always relevant. Double (triple) staining by immunofluorescence allows positive cells identification in complex cell structure (for example, pericytes and endothelial cells in vessels) and subcellular localizations. In order to, due to improvement of antibodies avoiding especially species cross-reactions, microscopy and specific softwares, quality of staining, and acquired images have been upgraded. Consequently, this technique permits, as molecular biology analyses, quantification of the level of expression as intensity of fluorescence can be measured in each cells and each compartments (nuclear, cytoplasmic). In order to immunofluorescence on cells and tissue needs few materials and gives at the same times qualitative and quantitative results and must be used more widely especially when a mutation was associated to a disease.

Key words Immunofluorescence, Paraffin-embedded sections, Triple staining, Quantification

1 Introduction

Immunodetection of protein on fixed cells (immunocytochemistry) and tissue (immunohistochemistry) has been used since a long time [1, 2]. According to Brandtzaeg, immunostaining for cell markers represents a way to "talk with cells," because it allows not only the histological origin of the cell to be identified but also indicates its function in vivo, when duly investigated with the correct antibodies [3, 4]. Use of immunohistochemical (IHC) markers by anatomopathologists to help medical doctors in establishing diagnosis especially in cancer characterization [5, 6] have participating in the improvement of immunostaining techniques. Antibodies which were previously developed to be used on frozen section where now largely developed on paraffin-embedded section. Immunohistochemistry on frozen tissue allows direct and rapid

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DOI 10.1007/978-1-4939-6430-7_7,

detection of antigen but tissues and sections may be stored at −80 °C. Immunohistochemistry on paraffin-embedded sections is more time consuming (dehydration of tissue prior to embedding and rehydration prior to detection of antigen). The critical step is antigen retrieval since fixation induced often masking even damaging of antigen [4, 7, 8]. Nevertheless, paraffin-embedded tissue are stored and cut at room temperature which is more convenient. Study of Manne et al. demonstrated that markers p53 and Bcl-2 are detected without loss of staining on paraffin-blocked store from 13 years and 6 years, respectively [9]. The choice of embedding in paraffin or OCT (frozen) depends of antigen. Some antigens do not support heating and need to be analyzed on frozen section (fresh or fixed cryopreserved). For a long time, it was admitted that immunofluorescence can only be made on frozen sections but this is not the case, immunofluorescence can be performed either in frozen or paraffin-embedded tissue and allows colocalization and quantification of expression by measure of intensity of fluorescence.

Background due to autofluorescence was a limitation but improvement of staining techniques, antibodies sensitivity (monoclonal vs. polyclonal) and antibodies specificity especially secondary antibodies have permitted to circumvent this problem. In order to, multiple fluorescence finds renewed interest with confocal microscopy allowing choice of excitation and emission wavelengths and the development of a broad range of fluorochrome conjugated antibodies ranging from 442 to 665 nm for emission wavelengths, i.e., 18 different antibodies for Alexa fluor® dyes. Multiple immunofluorescences can be made at the same time for various antigens until 3 plus Dapi under classic epifluorescent microscopy. However, for epifluorescence, excitation and emission wavelengths of the filters and fluorochromes have to be carefully chosen to avoid overlapping which may results in false positive results.

In order to fluorescence has a great interest associated with trangenesis. Indeed use of genetically modified cells expressing in addition to the gene of interest, a fluorescent protein (green fluorescent protein (GFP), red fluorescent protein (DsRed, dTomato), yellow fluorescent protein (YFP)) permits to detect without staining the autofluorescent cells of interest and with antibody staining the actors implicated in the pathway modified by the gene of interest. If all cells do not express the transgene this technique allows detecting local changes. It was especially interesting in organs as skin that have cells with various states of differentiation since gene of interest can be expressed differentially according to layers. In our laboratory, we have developed a model of reconstructed epidermis that can be produced with normal, pathological, or genetically modified cells such as CCN3 silenced melanocytes [10–12]. In vitiligo using double immunofluorescence we could detect that melanocyte in perilesional skin sometimes loose the expression of CCN3 and in vitro that silencing of CCN3 in melanocyte is

deleterious and induced their death in monolayer culture or their detachment in epidermal reconstructs as observed by Fukunaga-Kalabis [12, 13].

2 Materials

For human epidermal reconstruction, all manipulations are made in a biosafety level 2 cell culture laboratory, all solutions are sterilized by heating or 0.22 μm filtration.

2.1 Epidermal Reconstruction

1. Trypsin 0.25%, EDTA 0.1% in Hank's Balanced Salt Solution (HBSS) (*see* **Note 1**).
2. Fetal bovine serum (FBS) 10% diluted in HBSS.
3. Keratinocyte medium: modified MCDB153 medium (as compared to classical commercial MCDB 153, keratinocyte medium has two times more L-arginine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-valine and choline chlorine and less NaCl 0.104 M, Hepes 2.29×10^{-2} M and $NaHCO_3$ 1.19×10^{-2} M) supplemented with insulin 5 μg/ml, bovine pituitary extract 70 μg/ml, hydrocortisone 1.4 μM, Epidermal Growth Factor 10 ng/ml, and penicillin streptomycin 1% or KGM2.
4. Melanocyte medium: commercial MCDB153 medium supplemented with insulin 20 μg/ml, bovine pituitary extract 140 μg/ml, hydrocortisone 1.75 μM, 3% FBS, and penicillin streptomycin 1% or melanocyte medium M2 supplemented with 1% penicillin streptomycin (*see* **Note 2**).
5. Epidermal reconstruction medium: 500 ml Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 167 ml MCDB153 supplemented for keratinocytes, 50 ml FBS, 6 ml penicillin streptomycin or DMEM/F12 supplemented with 50 ml FBS, 6 ml penicillin streptomycin. 0.5 μg/ml hydrocortisone, 5 μg/ml insulin and 10 ng/ml EGF
6. Melanocytes and keratinocytes are isolated from skin sample (*see* Subheading 3.2). Alternatively, one can purchase commercially available melanocytes (e.g., human skin-derived melanocytes, PromoCell, and ATCC) and keratinocytes (e.g., human skin-derived keratinocytes, PromoCell, and ATCC).

2.2 Immunofluorescence on Cells

1. Lab-Tek® chamber slide or cell culture wells.
2. Formaldehyde 3.7%: formaldehyde 37% diluted in Phosphate buffered saline (PBS) pH 7.2–7.4.
3. Permeabilizing solution: PBS-0.2%Triton X-100.
4. Blocking solution: Fetal bovine serum (FBS) 2.5–10% diluted in PBS.

5. Primary antibody: We recommend the following anti-CCN3 antibody (N20 sc-18677 SantaCruz biotechnology) but other commercial antibodies exist.
6. Dapi (4′,6-diamidino-2-phenylindole) (stock solution 2 mg/ml) diluted at 1/10,000 in PBS

2.3 Immunofluorescence on Tissue Sections

1. Superfrost + slide.
2. Rinsing solution: PBS or TBS supplemented with tween (0.01–0.1%) (*see* **Note 3**) according to manufacturer's instruction if any.
3. Once the buffer have been selected (PBS or TBS), use it as saline buffer for all the steps.
4. Solution for antigen retrieval (*see* **Note 4**) according to manufacturer's instruction.
5. Blocking solution: Fetal bovine serum (FBS) 2.5–10% diluted in saline buffer.
6. Antibody diluent: Saline buffer without tween.
7. Primary antibodies: We recommend the following mix of antibodies Rabbit anti-CCN3 (abcam, ab137677, 1/300°) and mouse anti-melanA (Dako, M7196, 1/100°) in PBS overnight at 4 °C but other antibodies exist.
8. Secondary antibodies: We recommend the following mix Donkey anti-rabbit Alexa Fluor® 555 and chicken anti-mouse Alexa® 488 but antibodies from other species or coupled with other fluorochromes can be used.
9. Dapi (4′,6-diamidino-2-phenylindole) (stock solution 2 mg/ml) diluted at 1/10,000 in PBS.
10. Fluorescent mounting medium with DAPI or not (*see* **Note 5**).

2.4 Immunofluorescence Analysis

Imaging software: NIS element BR (Nikon) or an equivalent ImageJ.

3 Methods

3.1 Deepidermized Dead Dermis (DDD) Preparation

1. Remove grease from skin sample.
2. Put skin in a container filled with HBSS.
3. Incubate the closed container in prewarmed water bath at 54 °C for 20 min.
4. Peel off epidermis.
5. Rinse shortly in 70 °C ethanol.
6. Rinse in HBSS.
7. Put in a 50 ml tube filled with HBSS supplemented with 1% penicillin streptomycin and 1% amphotericin.

8. Make two Freeze-Thaw cycles.
9. Store at −20 °C.

3.2 Culture of Melanocytes and Keratinocytes

1. Degrease, thin the samples and cut into fragments with a maximum size of 0.5 × 0. 5 cm.
2. Incubate in trypsin 0.25% EDTA 0.1% for 3 h at 37 °C or overnight at 4 °C.
3. Neutralize trypsin with same volume of FBS.
4. Remove the epidermis and scrape the basal layer not too hard to avoid fibroblasts in HBSS.
5. Filter the obtained suspension (40 µm filter strainer) and count cell.
6. Centrifuge at 250 × *g* for 5 min.
7. Suspend the pellet in appropriate medium (keratinocyte or melanocyte medium).
8. Seed cells at a density of 100,000 per cm^2.
9. Change medium three times a week (*see* **Note 6**) after rinsing with HBSS.
10. After about a week, the primo-culture will be at 70–80% confluence and cells may be amplified or used for reconstruction.

3.3 Epidermal Reconstruction (Fig. 1)

1. Thaw DDD.
2. Place one DDD epidermal side up in each well of a six wells plate (*see* **Note 7**).
3. Put an incubation chamber (stainless ring) on each DDD and push with a curved clamp.
4. Add 0.5 ml of reconstruction medium in each well to keep moisture.
5. Keep at 4 °C (preparation the day before) or 37 °C (the day of use).
6. Rinse keratinocytes and melanocytes culture (passages 1–3) with HBSS to improve trypsin action when culture reach 70–80% confluence (*see* **Note 8**).
7. Incubate melanocytes or keratinocytes in the trypsin working solution (0.025% trypsin, 0.01% EDTA) less than 1 min for melanocytes around 5 min for keratinocytes. Follow detachment under microscope (*see* **Notes 9–11**).
8. Neutralize trypsin with equal volume of FBS 10% (3 ml for 75 cm^2).
9. Collect the solution containing cells (note the volume) and before centrifugation take 50 µl to count cells.
10. Centrifuge the solution at 250 × *g* at room temperature 5 min.

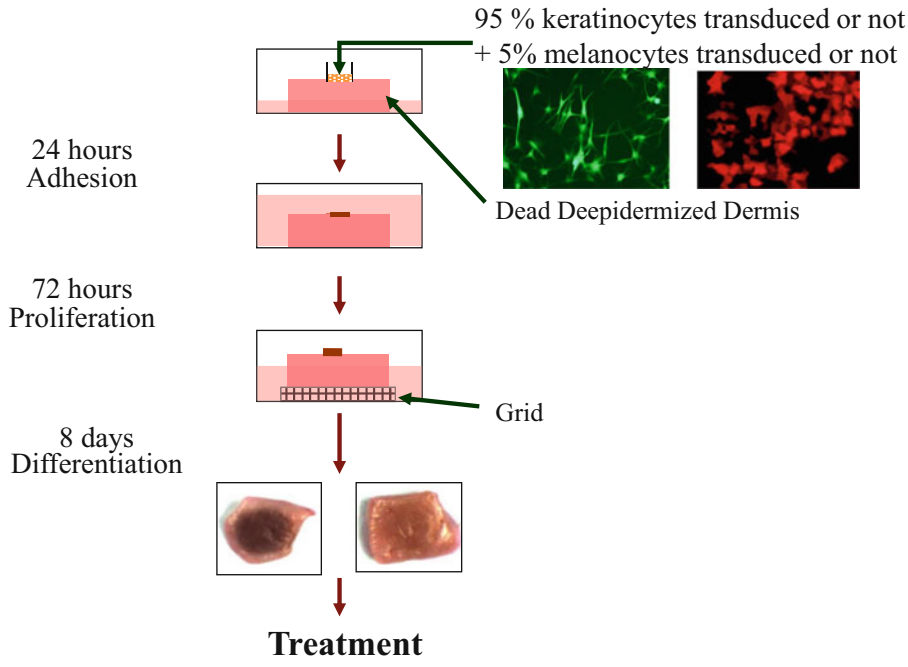


Fig. 1 Schematic representation of epidermal skin reconstruction

11. Dilute 50 µl of solution containing cells in 50 µl trypan blue and count non-blue cells.
12. Suspend the cell pellet in corresponding medium in order to have 190,000 keratinocytes in 100 µl and 10,000 melanocytes in 50 µl.
13. Mix melanocytes and keratinocytes at a ratio of 1:20 (5%).
14. Seed in each incubation chamber the mixed solution at 4×10^5 cells per cm^2, i.e., 150 µl for 0.5 cm^2 (Day 0).
15. 6–24 h after remove incubation chamber and submerge with reconstruction medium (Day 0 or 1).
16. 72 h later change medium (reconstruction medium) and shift to the air–liquid interface (*see* **Note** 7) (Day 3 or 4).
17. Change the medium (reconstruction medium) every 2–3 days.
18. Stop culture day 11 or 12 to day 18–19 by immersing in 4% formaldehyde during 3–24 h.

3.4 Immuno-fluorescence on Cells

1. Culture cells in Lab-Tek® chamber slide or cell culture wells (96 wells plate) for 6–24 h.
2. Rinse cells three times with cold PBS 7.2–7.4.
3. Fix cells with formaldehyde 3.7% for 10 min at 4 °C.
4. Rinse cells three times with cold PBS 7.2–7.4.

5. Permeabilize cells with 0.2% triton X-100 in PBS (*see* **Note 12**).
6. Wash three times with PBS.
7. Incubate with FBS 2.5–10% 20 min at 4 °C (*see* **Note 13**).
8. Incubate with primary antibody diluted according to manufacturer in PBS 1 h at room temperature to overnight 4 °C in a moisture chamber.
9. Collect diluted antibody and keep this dilution at the antibody storage temperature (*see* **Note 14**).
10. Wash with PBS three times.
11. Incubate with secondary fluorescent antibody raised against the host species used to generate the primary antibody diluted in PBS according to manufacturer, 1 h at room temperature in the dark.
12. Collect diluted antibody and keep this dilution at the antibody storage temperature (*see* **Note 14**).
13. Wash with PBS three times.
14. Incubate with Dapi 10 min (*see* **Note 5**).
15. Wash with PBS three times.
16. Mount with fluorescent mounting medium.
17. Observe the cells under an immunofluorescence microscope (Fig. 2) (*see* **Notes 15** and **16**).

3.5 Double Immunofluorescence on Tissue Sections

1. Cut sections of 4 μm (*see* **Notes 17–20**).
2. Put ribbon in a water bath (50–55 °C) and catch section with superfrost + microscopic slide. Or put two consecutive sections on a hot drop of water on a superfrost + microscopic slide.
3. Remove excess of water to avoid tissue dislocation. Let's dry until paraffin was melted.
4. Deparaffinization of paraffin section.
   Incubate 20 min sections at 60 °C then dewax by three successive baths of xylene.
5. Rehydration of paraffin section.
   Incubate in baths of decreasing Alcohol concentrations (2 × 100°, 1 × 95°, 1 × 70°,1 × 50°) then rinse one time with H_2Od and two times with PBS or TBS (according to buffer recommended for antibody).
6. Antigen retrieval (*see* **Notes 4** and **21**).
   Incubate sections in prewarm (70–98 °C) adequate antigen retrieval solution (microwave, water bath, or pressure cooker), heat at the adequate temperature and adequate time (classically 20 min at 97 °C) and finally let's cool to 70 °C.
7. Wash three times with PBS or TBS 0 to 0.1% tween (*see* **Note 3**).
8. Incubate with FBS 2.5–10% 30 min at room temperature (*see* **Note 13**).

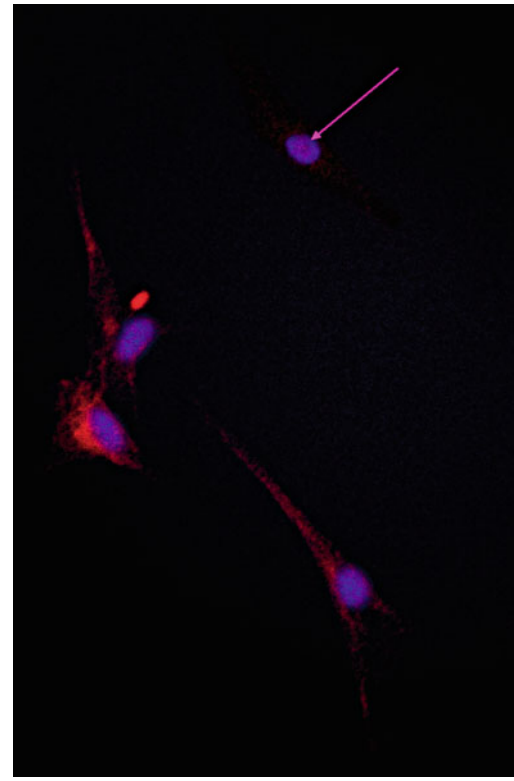


Fig. 2 Expression of CCN3 (*red*) in culture of melanocytes. Counterstaining of nuclei with dapi (*blue*). CCN3 was expressed in cytoplasm and sometimes also in nucleus which appeared in *pink* (*pink arrow*)

Incubate with the two primary antibodies diluted (according to manufacturer's instruction) in PBS or TBS overnight 4 °C or 1 h at room temperature (*see* **Note 22**).

9. Wash with PBS or TBS three times.
10. Incubate with the two different secondary fluorescent antibodies raised against the host species used to generate the primary antibodies diluted in PBS or TBS 1 h at room temperature in the dark (*see* **Note 23**).
11. Incubate with Dapi 10 min at room temperature (*see* **Note 5**).
12. Wash with PBS or TBS three times.
13. Mount with fluorescent mounting medium.
14. Observe the tissues under an immunofluorescence microscope (Fig. 3) (*see* **Notes 15** and **16**).

3.6 Analysis

1. Take pictures of interesting areas for each spectra (*see* **Note 24**).

Pictures may be taken beginning by the highest wavelength to finish by Dapi, a bright field picture can also be made (for example, to estimate the degree of pigmentation (melanin) in skin).

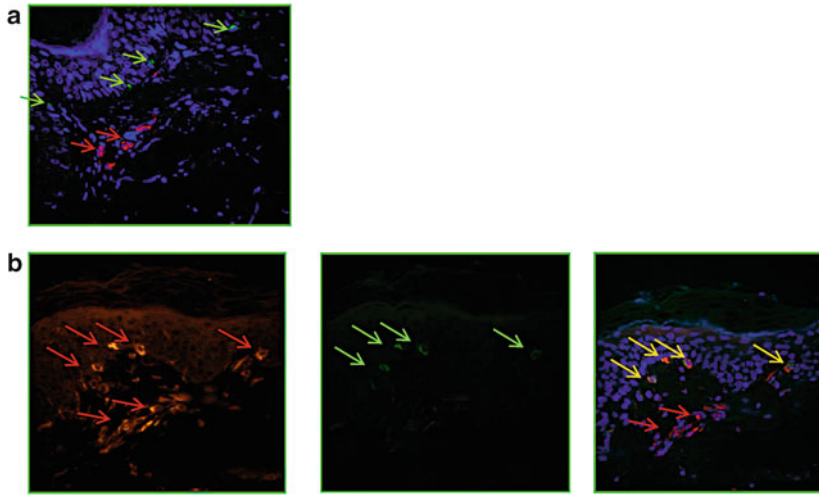
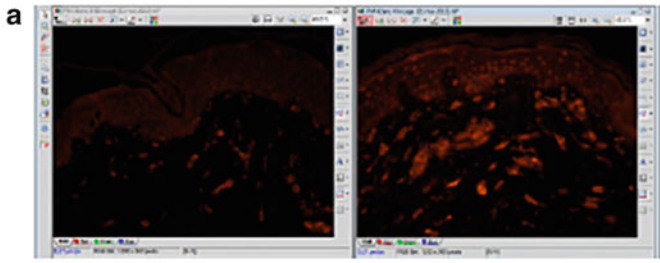
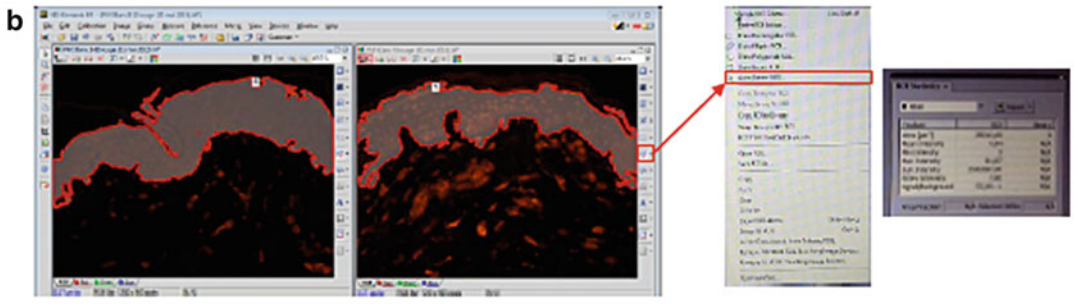


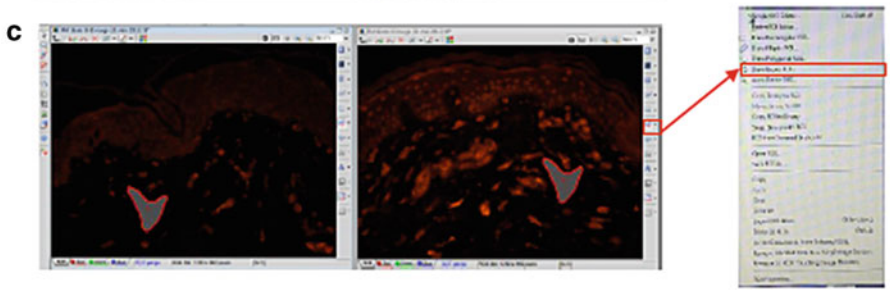
Fig. 3 Triple staining of perilesional skin of two non segmental vitiligo patients (**a**, **b**). CCN3 was detected using anti-CCN3 followed with alexa 555 anti-rabbit antibody and appeared in *red. Red arrows* point out cells highly expressing CCN3. Melanocytes were detected using anti-melanA followed with alexa 488 anti-mouse antibody and appeared in *green* (*green arrows*). Nuclei were counterstained with Dapi and appeared in *blue*. For patient A only the merged picture of the triple staining was shown whereas for patient B, CCN3, MelanA, and merged picture were presented. CCN3 was not expressed in melanocytes of patient A (*green arrow*) but since some dermal cells highly expressed CCN3 (*red arrows*) it was not a false negative staining. On the contrary, melanocytes of patient B expressed CCN3 (*yellow arrows*). Patient B expressed same level of CCN3 in melanocytes and dermal cells. Dermal cells are not melanA positive (no green fluorescence) confirming specific high expression of CCN3 in melanocytes and not false positive expression due to spectra overlapping

Determine the exposure time and the gain for each wavelength. Take pictures for all the sections to be analyzed (Fig. 4a).

2. Merge the pictures to see if there is colocalization. If the two antibodies are in FITC channel (green) and Cyanine 3 channel (red), colocalization appears in yellow. However, a same cell can express the two antibodies in two different locations (red nucleus surrounding by a green cytoplasm) (Fig. 3).
3. To quantify fluorescence (*see* **Note 25**), define the ROI (region of interest) you want to quantify manually or automatically according to your software (Fig. 4b).
4. If you have background staining duplicate the ROI to obtain the value of the background in an area of same size or define manually the ROI (ROI can be copy in a file and paste in another file to quantify the same area in NIS-Elements BR imaging software) (Fig. 4c) (*see* **Note 26**).
5. Transfer the results of ROI statistics especially mean intensity, minimal intensity, maximal intensity, sum intensity, in your statistic files (export to excel is automatic in NIS-Elements BR) (Fig. 4d).







d

FileName	PM 38 years old II (31 may 2013)		PM 42 years old VI (31 may 2013)	
	epidermis	Background	epidermis	Background
ROI Area	23247.71	1133.72	18253.31	1133.72
ROI Mean Intensity	12.49	1.56	17.57	3.34
ROI Min Intensity	0.66	0	3.66	1
ROI Max Intensity	55	7	94.66	9
ROI Sum Intensity	3989068	24386.33	4405285.66	52001.66
ROI StDev Intensity	3.36	0.89	6.64	0.92
ROI Signal/Background	15.5	6	11	7

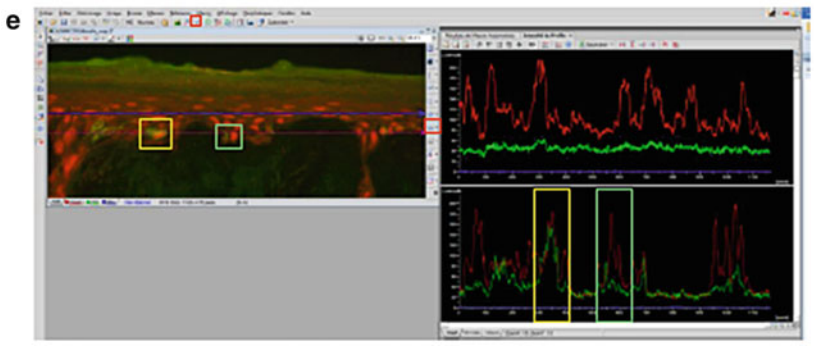


Fig. 4 Analyzes of fluorescence using NisElement Br (Nikon). (**a**) Two representative pictures of CCN3 staining. Staining realized same days with same settings for two skins of phototypes III (*left*) and VI (*right*). (**b**) Automatic detection of ROIs (region of interest) in epidermis. (**c**) Manual detection of background and copy of the same

6. If background is high, remove mean background from your mean intensity and compare mean intensity of your representative area.
7. Intensity profile along an axis may also be obtain to quantify expression of a protein in a same layer (for example, melanocytes and keratinocytes of the basal layer) and confirm colocalization (Fig. 4e).

4 Notes

1. Make sure to be at 37 °C no more to avoid inactivation of trypsin.
2. Melanin produced by high phototype melanocytes hampers cell growth. Thus for high phototype melanocyte M2 medium is more adapted or MCDB153 has to be supplemented with PTU (phenyl thiourea) 300 μM, an inhibitor of tyrosinase. We commonly use MCDB153 without PTU for phototype I–IV melanocytes and M2 medium for phototype V and VI.
3. Tween 20 is a detergent, according to antibody it can be added in PBS or TBS at various concentration 0.01–0.1 %. Some antibody doesn't need tween. Not enough tween can be associated with no staining but too many tween can induce background. For first use a concentration of 0.05 % is generally recommended. Tween allows also a good spreading of the solution on the slide (important for automated staining). There is no real difference between PBS or TBS in the quality of staining. Other detergent such as triton X100 can also be used.
4. Antigen retrieval is a critical step and depends of antibody. Antibody can be retrieved by enzymatic digestion (proteolytic enzyme induced epitope retrieval (PIER): trypsin or pepsin (1 mg/ml) proteinase K (20 μg/ml), 10–20 min room temperature or 37 °C) or heat induced epitope retrieval (HIER) can be performed at pH 6, 7, 8, 9 at different temperatures 97–120°, with high pressure or not and during various time 10–45 min. Read carefully the data sheet to find the retrieval technique. Most antibodies are retrieved with citrate buffer pH 6, 20 min at 97–98 °C.

   pH 6 Buffer: 10 mM Sodium Citrate Buffer.
   pH 8 Buffer: 1 mM EDTA.
   pH 9 Buffer: 1 mM EDTA Solution, 0.05 % Tween 20.

Fig. 4 (continued) area in the second picture. (**d**) Statistics of epidermal ROI. In phototype VI epidermis the mean of fluorescence intensity is higher than in phototype II epidermis. (**e**) Profile intensity of CCN3 (*red*) and melanA marker of melanocytes (*green*). *Yellow* and *green rectangles* point out location and relative intensity of CCN3 and MelanA in a melanocyte (*yellow*) and a keratinocyte (*green*). These two cells expressed same level of CCN3

Commercial solutions are available from antibodies distributors. Some solutions also allow at the same time paraffin removal rehydration and antigen retrieval but for instance any of them give high quality results.

5. Fluorescent mounting medium with DAPI is very efficient to stain nucleus but is expensive.
6. If no melanocytes were seen after 2–3 days in culture, the scratching was not strong enough.
7. A grid can be placed under each DDD before placing the incubation chamber (make sure that the height of this assembly allows closing the plate and that this assembly doesn't float in submersion phase) or the grid can be inserted at the air/liquid interface.
8. If culture of keratinocytes reaches complete confluence, cells will be in contact inhibition and not in proliferative state and thus could not provide a correct reconstruction.
9. If culture are not pure, you must make a short trypsination to detach melanocytes (30 s–1 min); and then a second trypsination to collect keratinocytes.
   Process as follow: first, add Trypsin 0.025 % EDTA 0.01 % and follow melanocytes detachment, second collect the solution containing melanocytes and neutralize with same volume of FBS 10 %, third add a second times Trypsin 0.025 % EDTA –0.01 % in each flask to detach keratinocytes, fourth neutralize trypsin with equal volume of FBS 10 % in each flask and finally collect the solution containing keratinocytes.
10. If melanocytes detach too quickly and if it is difficult to purified culture, use a less concentrated solution of trypsin-EDTA as 0.0125 % trypsin, 0.005 % EDTA.
11. If keratinocytes detach too slowly more than 5 min at 37 °C, collect detached cells and add new trypsin. The number of active unit/mg may vary according to batch, use a more concentrated solution of trypsin-EDTA but to have around 19 BAEE unit/ml.
12. Permeabilization step can be avoid if the protein is membranous and that antibody target extracellular domain.
13. Manufacturer recommends using serum from species of the secondary antibody but FBS is efficient for most staining. Incubation with FBS 2.5–10 % avoids aspecific fixation, the percentage has to be adjust according to the background. Start with 2.5 %. Otherwise commercial blocking solutions are available.
14. Some diluted antibodies can be used up to three times.
15. Be sure that objectives are fluor objectives.

16. Intensity of fluorescence depends also of the lamp, if a decrease in fluorescence is observed perhaps the lamp has been used more than the expected time generally 250 h.
17. Always use a negative control to detect if there is background. This is important for skin because some antibodies induced an aspecific staining of stratum corneum. In order to autofluorescence is observed for red blood cells. Negative control is process as samples but instead of incubating section with primary antibody, incubating only with PBS or TBS.
18. To detect a new antibody always use a positive control: tissue already know to greatly express the protein of interest.
19. If the protein of interest is weakly expressed in your tissue, always use positive and negative controls to be sure of your staining.
20. If the protein is greatly expressed in your tissue, you can only use negative control.
21. To perform double staining you may be sure that the two epitopes can be unmasked with the same technique.
22. To perform double staining you may be sure that the two antibodies are not made in the same species.
23. To perform double staining you may use two secondary antibodies with different excitation and emission non overlapping spectra which are raised in different species to avoid cross reaction (for example, first antibodies raised in mouse and rabbit second antibodies: chicken anti-mouse 488 (green fluorescence) and donkey anti-rabbit 568 (red–orange–fluorescence) double staining may be yellow. A simple staining of each antigen on different sections is recommended to detect a possible overlapping of the fluorescence and thus false positive staining if one of the antigen is more expressed and have a great level of fluorescence.
24. To compare immunofluorescence intensity of different sections, pictures may be taken for each wavelength the same day with same settings (i.e., time of exposure, gain, noise reduction, size), same optics. Pictures may be taken quickly and beginning by the highest wavelength which has the lowest energy to avoid quenching of fluorescence. However, settings may be modified between wavelengths. In general exposure time is lowest for DAPI. Ensure that the exposure chosen is not too highest to avoid saturation of some pixels.
25. For quantification, original file does not have saturated pixels.
26. To compare several pictures, ROIs may be representative of the staining. Indeed, the most part of the time each section is different and it is not possible to take area of the same size. For this reason mean intensity of fluorescence has been chosen for statistic analyzes.

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Chapter 8

Production of Recombinant CCN2 Protein in *Escherichia coli*

Eriko Aoyama, Takako Hattori, Satoshi Kubota, and Masaharu Takigawa

Abstract

Recombinant proteins are important tools for understanding molecular functions in vitro. Recent progress in the generation of recombinant proteins is amazing. However, when we plan to produce them, we should choose the best method according to the nature and the use of the target recombinant protein. Degradation and mis-folding are major problems in producing active recombinant CCN2. The method shown in this chapter describes the appropriate conditions under which we can produce CCN2 and its truncated fragments in *Escherichia coli*.

Key words *Escherichia coli* (*E. coli*), Histidine$_6$-tag, FLAG-tag, Imidazole, FLAG peptide, Ni-NTA agarose gel

1 Introduction

Recombinant protein is an essential tool to analyze the chemical properties of proteins or the biological effect of exogenous proteins in vitro. Though many recombinant proteins are commercially available to date, we occasionally need to produce them by ourselves, if they are not affordable or cannot be obtained commercially. To get chemically active proteins, it is important to design them appropriately and choose the most suitable protein-producing system and purification tools.

First, the production system for recombinant proteins is grouped into the "cell-free" system or "in cell" system. The cell-free protein synthesis system consists of extracts of cells, such as wheat germ or rabbit reticulocytes, required factors, and salts [1, 2]. This system is relatively easy to handle and can make even toxic proteins that would be difficult to express in living cells. However, the production efficiency of this system is sometimes low and the procedure costly. The other system, "in cell," is further grouped into the one based on the use of cultivated cells or transgenic animals such as silkworms, eggs, milk, and so on. Although transgenic

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animals can produce large amount of proteins, a gene transfer technique to generate these animals and equipment for the maintenance of the transgenic animals are required [3]. For these reasons, animal-based tools are usually used in industrial production rather than in the laboratory. The cultivated cell system is a convenient tool for producing recombinant proteins in the laboratory. Mammalian, insect, bacillus, and other bacterial cells are used as tools for producing recombinant proteins because of the availability of materials and technical accessibility. For example, the advantages of using *Escherichia coli* (*E. coli*) are its growth speed and availability of many kinds of convenient tools. However, this system also has certain disadvantages in that the proteins produced in the cells do not undergo posttranslational modification, such as glycosylation, and sometimes are produced as insoluble forms in inclusion bodies.

In terms of the design of the expression form of the protein, such as secreted or non-secreted form, the secreted form has many advantages such as that the produced protein is not damaged by detergents present in lysis buffer and can already be activated by necessary cleavage occurring naturally. However, we need a large amount of culture supernatants to collect sufficient proteins, because the concentration of protein in the medium is usually rather low. Other disadvantages of producing a protein as its secreted form are degradation by enzymes in outer culture media, loss by adsorption on the culture container, and so on. Additionally, fusion of peptide tags to recombinant proteins is also a notable option in protein designing. Tags are not only markers, but some of them also enhance the solubility of proteins [4, 5]. At the same time, they add to the molecular weight of the product; and some of them may add an extra property.

To purify the protein from cell lysates, we need to select the proper lysis buffer and purification tools. Lysis buffer normally includes detergent, denaturing agents, chelating agents, and some buffering salts. These contents of the lysis buffer need to be determined depending on the purification tools used and the molecular nature of the protein. Protein purification resins are usually used based on specific affinity to each protein. A continuous column is used for a sample of large volume, whereas a batch column is used for a sample of small volume.

Based on the above considerations, the appropriate method fitting the purpose of use and the nature of the protein needs to be chosen. For example, CCN2 is sensitive to degradation by some kinds of enzymes or repetitive freezing/thawing. Many cysteine residues in CCN2 also increase the difficulty of proper protein refolding. Thus, we selected the *E. coli* expression system and lysis buffer without strong denaturing agent, such as guanidine or urea, to avoid the denaturation of the protein by these agents. We examined the conditions for culturing *E. coli* and the composition of the

lysis buffer to overcome the insolubilization of CCN2 protein produced in *E. coli*. Thus, we designed recombinant proteins with two types of tags (His_6 and FLAG), which are useful for two-step purification and help their solubilization. We were successful in producing and purifying ten types of recombinant CCN2-truncated fragments. Some of these fragments showed binding activities toward other proteins such as FGF1, FGF2, or aggrecan [6–8]. Recombinant CCN2 protein and its truncated fragments produced in *E. coli* are useful tools for the analysis of protein-protein interaction, and below we present the design and protocol for the generation of full-length CCN2 and its truncated fragments in *E. coli* and their purification from these cells.

2 Materials

Prepare all buffers with distilled water. For stock solutions stored at 4 °C, sterilize the solutions without protease inhibitors or peptides. Protease inhibitors and peptides need to be added before use. Chemicals used are of analytical grade.

2.1 Design of Expression Vectors and Cultivation of E. coli Including Expression Vectors

1. Equipment for PCR.
2. Restriction enzymes.
3. Equipment for DNA ligation.
4. Expression vector pT7-flag-1™ (Sigma-Aldrich, St. Louis, MO).
5. *E. coli* Rosetta™ 2(DE3)pLysS (Novagen, Madison, WI).
6. LB media with ampicillin and chloramphenicol.
7. Isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2 Lysing Cell Bodies and Purification of Proteins

1. Lysis buffer: 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A.
2. Nickel affinity gel (e.g., Ni-NTA Agarose, Qiagen, Chatsworth, CA).
3. Chromatography column.
4. Elution buffer: 20 mM Tris–HCl, pH 8.0, 10 mM KCl, 0.5 mM PMSF, 1 μM pepstatin A.
5. Imidazole/elution buffers: 20, 50, 150, 500, 1000 mM imidazole in elution buffer.
6. Anti-FLAG affinity gel (e.g., ANTI-FLAG® M2 Affinity Gel Freezer-Safe, Sigma, St. Louis, MO).
7. Tris-buffered saline (TBS): 50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 0.5 mM PMSF, 1 μM pepstatin A.

8. FLAG peptide (e.g., FLAG® PEPTIDE, Sigma, St. Louis, MO).
9. Equipment for SDS-PAGE.
10. Coomassie brilliant blue.
11. Anti-FLAG Ab (e.g., Monoclonal ANTI-FLAG® M2 Antibody, Sigma, St. Louis, MO).
12. Anti-His Ab (e.g., 6×-His Epitope Tag Antibody (3D5), Invitrogen, Carlsbad, CA).

3 Methods

3.1 Design of Expression Vectors and Cultivation of *E. coli* Bearing Expression Vectors

1. Design primers for each derivative of CCN2 cDNA (Fig. 1a–c).
2. Produce each PCR fragment with the above primers.
3. Clone PCR fragments into pT7-flag-1 vector at EcoRI/BglII sites.
4. Transform *E. coli* Rosetta™ 2(DE3)pLysS cells with pT7-flag-1/hCCN2.
5. Culture the transformed *E. coli* at 25 °C for 4 h in 1 L of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (*see* **Note 1**).
6. Add 0.05 mM IPTG (*see* **Note 2**) when the O.D. at 600 nm reaches between 0.4 and 0.6.
7. Culture the cells for another 18 h and then harvest them by centrifugation (approx. 5,000 × *g* for 20 min) and freeze the pellet (*see* **Note 3**).

3.2 Purification of Recombinant Proteins from Crude Lysates

1. Thaw the frozen pellet with 5 ml of lysis buffer without Triton X-100 and mix well (*see* **Notes 4** and **5**).
2. Add 45 ml of lysis buffer with Triton X-100 into the bacterial suspension and mix well on ice.
3. Sonicate the suspension for 20 s on ice and centrifuge (approx. 5,000 × *g* for 30 min) at 4 °C to harvest the supernatant.
4. Mix the supernatant with Ni-NTA agarose gel pre-equilibrated with lysis buffer.
5. After rotating gently for 1 h at 4 °C, wash the gel first with lysis buffer and subsequently with elution buffer without imidazole five times with each.
6. Wash the gel with 20 mM imidazole elution buffer once (*see* **Note 6**).
7. Load the adsorbed gel onto a Poly-Prep® chromatography column.

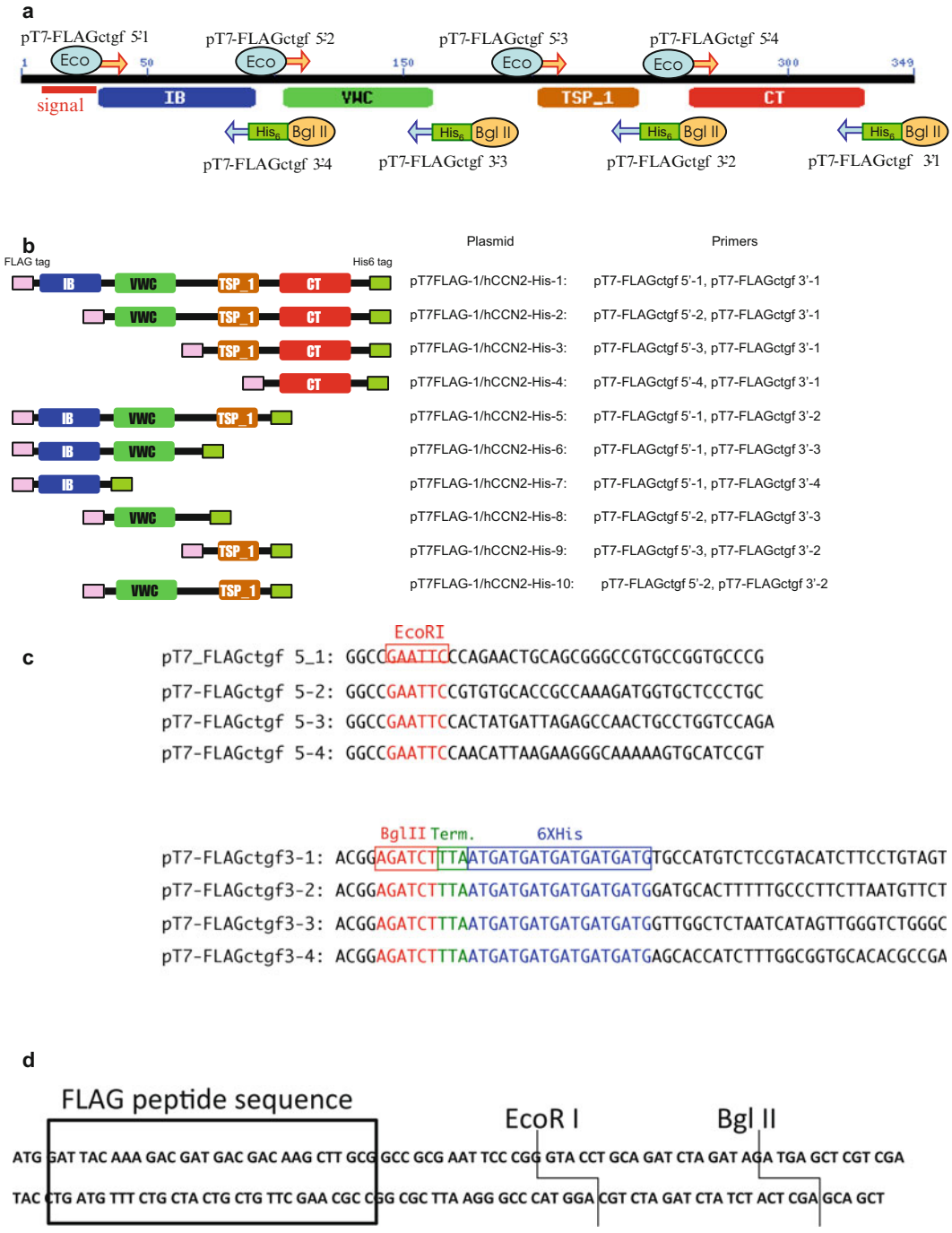

Fig. 1 Designs of full-length and truncated recombinant CCN2 proteins and primer sets for the amplification of cDNA for each protein. (**a**) Schematic representation of human *CCN2* cDNA with its four domains and the position of primers used for amplification of the full-length and truncated *CCN2* fragments. *Orange arrows* indicate forward primers containing *EcoRI* sites at 5′ ends, and *blue arrows*, reverse primers containing His_6-tags and following *BglII* sites. (**b**) Diagrams of all recombinant proteins and *E. coli* expression plasmids with each primers set used for amplification. (**c**) Sequences of primers. Forward primers have *EcoRI* recognition sites (indicated in *red*), and reverse primers, *BglII* recognition sites (indicated in *red*). Stop codon (indicated in *green*) and codons for six histidine residues (indicated in *blue*) are also indicated. (**d**) The sequence of multiple-cloning site in pT7flag-1 vector. FLAG peptide cDNA is followed by *EcoRI* and *BglII* site sequences

8. Elute the protein from Ni-NTA agarose gel with 10 ml of each of 50, 150, 500, and 1000 mM imidazole/elution buffer and collect the eluent as 1 ml samples into tubes.
9. Load small aliquots of each sample onto an SDS-PAGE gel and stain the gel with Coomassie brilliant blue to find which sample includes the recombinant protein (Fig. 2a–c; *see* **Note** 7).
10. Mix the samples including the recombinant protein with 0.4 ml of ANTI-FLAG® M2 Affinity Gel Freezer-Safe equilibrated with TBS.
11. Incubate the gel suspension for 2 h at 4 °C with gentle rotation.
12. Wash the gel with TBS once.
13. Add 0.2 ml of elution buffer containing 400 μg/ml of FLAG peptide to the adsorbed agarose gel and incubate for 20 min at 4 °C with gentle rotation.

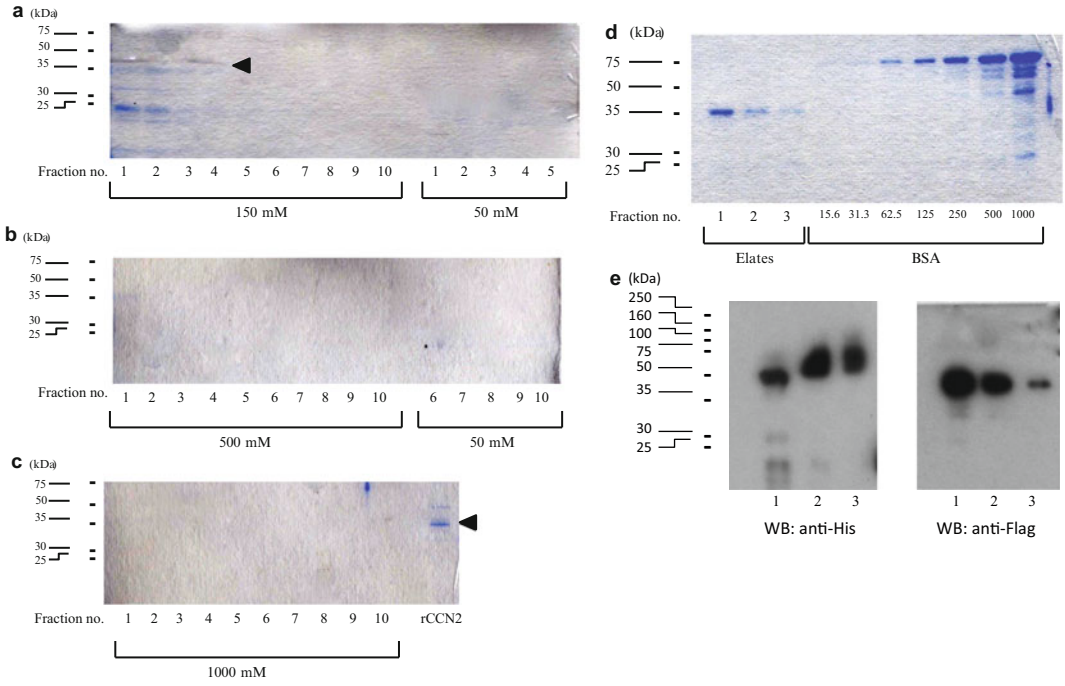

Fig. 2 (**a**–**c**) All samples eluted from the Ni-NTA column were loaded onto an SDS-PAGE gel, which was then stained with Coomassie brilliant blue after electrophoresis. Imidazole concentrations of eluates are indicated at the bottom. Fraction numbers are named in the order of elution. The bands indicated by *arrowheads* in 150 mM Fr.1, 2, and 3 represent the recombinant protein (full-length CCN2 in this case). (**d**) Small aliquots of three eluate fractions from the anti-FLAG agarose gel were loaded onto an SDS-PAGE gel, and following electrophoresis the gels were stained with Coomassie brilliant blue. (**e**) Portions of three eluate samples from the anti-FLAG agarose gel were loaded onto an SDS-PAGE gel, and CCN2 was detected by Western blotting with anti-Flag and anti-His antibodies

14. Centrifuge to collect supernatant. Repeat **steps 13** and **14** three times.
15. Load *a part of three supernatant samples* to the SDS-PAGE gel and check the purity and quantity of the recombinant protein by Coomassie brilliant blue staining (Fig. 2d) and Western blotting analysis with anti-FLAG Ab and anti-His Ab (to check for possible deletions in the recombinant proteins; Fig. 2e). The single bands in Fr.1, Fr.2, and Fr.3 in Fig. 2d indicate that these protein solutions were without appreciable contaminants. The fact that bands are detected with both anti-FLAG Ab and anti-His Ab suggests that the protein does not have any deletions at either end.
16. Compare the band density of each recombinant protein with that of standard BSA to calculate the protein concentration of each sample. In the case shown in Fig. 2, the concentration of protein in each fraction is 235, 99, and 58 μg/ml (*see* **Note 8**).
17. Add one-twentieth volume of 2% BSA/PBS to the recombinant protein solution (final concentration: 0.1% BSA/PBS). Store the protein solution as aliquots with adequate volume in a siliconized tube at −80 °C (*see* **Note 9**).

4 Notes

1. Culture the cells slowly at 25 °C to avoid accumulation of insoluble proteins in inclusion bodies. In the case of a low-molecular-weight protein (under 15 kDa), it can be produced as a soluble protein even if the cells are cultured at 37 °C.
2. IPTG is usually used at 0.1–1 mM. A low concentration of IPTG such as 0.05 mM is also useful to avoid accumulation of the proteins in inclusion bodies.
3. Freezing and thawing is a mild way to break the cell walls. However, do not repeat this step, because some damage to the protein itself may occur.
4. After this step, all work should be performed on ice or in a cold room, and a swing-out rotor should be used for centrifugation.
5. The cells should be premixed with buffer without Triton-X 100. Otherwise, a huge volume of DNA from cells broken by Triton-X 100 results in high viscosity, which disturbs even mixing with buffer.
6. The 20 mM imidazole solution washes out proteins nonspecifically bound to the gel.
7. To optimize the dose of imidazole for elution, a part of every sample is separated by SDS-PAGE and subjected to Coomassie

brilliant blue staining. Once the optimal dose has been determined, this process can be skipped. Figure 2b and c shows that 150 mM imidazole solution is suitable for the elution of CCN2 protein.

8. Conventional assays for protein quantification (e.g., Bradford or Lowry) are not useful for the quantification of the purified protein with this protocol, because these solutions include a vast amount of FLAG peptides. If you dialyze these protein solutions, you can then use them in these assays. However, dialysis sometimes causes deposition or precipitation and consequently loss of the protein.
9. CCN2 protein easily binds to walls of most tubes and containers. To avoid loss of protein, siliconization is useful.

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Chapter 9

Production of Recombinant CCN Proteins by *Brevibacillus choshinensis*

Hiroshi Hanagata and Makoto Mizukami

Abstract

Brevibacillus choshinensis is an excellent host for the production of secretory proteins. This host has also been applied successfully to efficient production of CCN proteins. Described herein are methods of constructing plasmids for CCN protein production (IGFBP-, VWC-, TSP-, and CT-domain) with *Brevibacillus* as a host, cultivation methods for protein production, and methods of purification for domain proteins using his-tag.

Key words *Brevibacillus choshinensis*, BIC system, IMAC

1 Introduction

A number of protein expression systems are currently used as production systems for foreign proteins. Individual protein expression systems have distinctive characteristics. For production of structurally complex proteins such as antibodies, mammalian system, *Escherichia coli* which are the most commonly used protein expression systems, and many other expression systems, such as yeast, insect cell, and cell-free systems, are available and selectively used depending on properties of a target protein.

Although *E. coli* is the most extensively used bacterial expression system and superior in high production of proteins, the target protein expressed intracellularly often aggregates and forms insoluble inclusion bodies. Since inclusion bodies are inactive, the protein is required to be converted to the active form through denaturation solubilization followed by refolding. This process is cumbersome and not always successful. Furthermore, since *E. coli* is a Gram-negative bacterium, endotoxins are produced and necessitate an additional process to remove them, which can lead to a lower yield of the protein. If a desired protein can be produced and secreted extracellularly using a Gram-positive bacterium intrinsically incapable of producing endotoxins, the foregoing disadvantages

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may be overcome, and an increased productivity, acquisition of the protein in correct high-order structures, and simplification of separation and purification procedure can be expected. From this perspective, Takagi et al. screened bacterial strains for a high capacity of secretory protein production, protease activity deficiency, and ease of culture and genetic manipulations, and found *Brevibacillus choshinensis* HPD31 (formerly *Bacillus brevis*) [1]. As further improvement, inactivation of the spore-forming ability and disruption of two weak protease genes were accomplished thereafter. Thus, a highly versatile protein production host, *B. choshinensis* HPD31-SP3 strain [2, 3], was established. Main benefits of the *Brevibacillus* expression system are listed below.

- Foreign proteins are produced and secreted efficiently.
- Proteins produced are virtually free from degradation by proteases in cultures.
- Endotoxins are not produced.
- Active proteins maintaining correct conformations can be produced.
- Culturing, sterilization, and genetic manipulations are straightforward.
- All required components are commercially available as an all-inclusive kit, and thus anyone can carry out expression experiments.

Taking advantage of these features, the system has been employed in successful secretory production of more than 300 proteins thus far, and has achieved high production particularly of secretory proteins. More recently, a very simple gene cloning technique has been developed and is being applied to construct high-expression plasmids.

CCN-family proteins have many intramolecular S-S bonds, and have been expected to be produced in the correct conformation with *Brevibacillus*. We introduce here a method of culture production and Ni-chelate column purification of each domain in the active form.

2 Materials

Culture media, reagents, pipette tips, etc. for genetic engineering experiments and microbial culture should be autoclaved before use.

2.1 Construction of Strains Expressing CCN Proteins

1. Vector pBIC3 DNA: For expression of IGFBP, VWC, and TSP domains (Takara Bio Inc.).
2. Vector pNCMO2 DNA: For expression of CT domain (Takara Bio Inc.).

3. Primer:

   IGFBP-forward: 5′-agttccgcattcgctcagaactgcagcgggccgtg-3′.

   IGFBP-reverse: 5′-catcctgttaagcttaatgatgatggtggtggtgaccatctttggcggtgcac-3′.

   VWC-forward: 5′-agttccgcattcgctgcatgcctggtccagaccac-3′.

   VWC-reverse: 5′-catcctgttaagcttaatgatgatggtggtggtggttggctctaatcatagtt-3′.

   TSP-forward: 5′-agttccgcattcgctgcaccctgcatcttcggtgg-3′.

   TSP-reverse: 5′-catcctgttaagcttaatgatgatggtggtggtgctttttgcccttcttaatg-3′.

   CT-forward: 5′-tgctcccatggctttcgctgcatgcatccgtactcccaaaatc-3′.

   CT-reverse: 5′-gccgaagcttaatggtgatggtgatggtgatggtggagctttgccatgtctccgtacatc-3′.

4. Template DNA: Gene synthesis or cDNA comprising respective genes.
5. *Brevibacillus* Competent Cells (Takara Bio): Including Competent Cell, MT medium, Solution A, and Solution B.
6. MTNm plate: 1% Phytone peptone (Becton & Dickinson), 0.5% yeast extract (Becton & Dickinson), 0.2% Ehrlich Bonito Extract (Kyokuto Pharmaceutical), 1% glucose, 20 mM $MgCl_2$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 10 mg/L $MnSO_4{\cdot}4H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 50 μg/ml neomycin, 1.5% agar, pH 7.0.
7. Neomycin stock solution (×1000): 50 mg/ml Neomycin (SIGMA). Store at −20 °C.
8. PCR enzymes (*see* **Note 1**).
9. Restriction enzymes: NcoI EcoRV, HindIII.
10. Agarose gel: 1.5% Agarose.
11. DNA recovery kit.
12. Ligation kit.
13. Plasmid extraction kit.
14. Molecular weight markers for DNA.
15. Incubator.
16. Thermostatic shaker.
17. Thermal cycler for PCR.

2.2 Culture Production

1. Selection medium (MTNm plate): *See* Subheading 2.1.
2. 2SYNm medium for production: 4% Phytone peptone (Becton & Dickinson), 0.5% yeast extract (Becton & Dickinson), 2% glucose (separately sterilized), $FeSO_4{\cdot}7H_2O$, 10 mg/L $MnSO_4{\cdot}4H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 50 μg/ml neomycin (prepared using 1/1000 volume of neomycin stock solution), pH 7.2.

3. TMNm medium for production: 1% Phytone peptone (Becton & Dickinson), 0.5% yeast extract (Becton & Dickinson), 0.2% Ehrlich Bonito Extract (Kyokuto Pharmaceutical), 1% glucose, 10 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $MnSO_4 \cdot 4H_2O$, 1 mg/L $ZnSO_4 \cdot 7H_2O$, 50 μg/ml neomycin (prepared using 1/1000 volume of neomycin stock solution), pH 7.0.

2.3 Purification

1. Immobilized metal ion absorption chromatography (IMAC) media: Use chelating Sepharose FF (GE Healthcare) for IGFBP, VWC, and TSP domains; and Ni-NTA (Life Technologies) for CT domain.
2. Native purification buffer (Buffer A): 50 mM Sodium phosphate, 500 mM NaCl, pH 7.4.
3. Native elution buffer (Buffer B): 50 mM Sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4.
4. Denaturing binding buffer: 8 M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 8.0.
5. Denaturing wash buffer: 8 M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.3.
6. Denaturing elution buffer: 8 M Urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4.5.
7. Dialysis buffer for IGFBP domain: 50 mM Sodium phosphate, pH 7.0.
8. Dialysis buffer for VWC and TSP domain: 50 mM Sodium acetate, pH 4.0.
9. Dialysis buffer for CT domain: Phosphate-buffered saline (PBS), 0.5 M arginine, pH 7.4.
10. Dialysis tubing: Use seamless cellulose tubing (Wako) or an equivalent product.
11. Centrifugal filter units: Use Centricon Plus-70 (Merck Millipore) for ultrafiltration; and Centriprep YM-10 or an equivalent product for concentration.

3 Methods

3.1 Construction of Expression Strains

3.1.1 Construction of IGFBP, VWC, and TSP Expression Vectors (Fig. 1)

1. Carry out PCR using the template DNA and forward and reverse primers for each domain.
2. Mix 1 μl (100 ng) of pBIC DNA and the insert at an approximate molar ratio of 1:2, add sterile water to the mixture to obtain a final volume of 5 μl, and then carry out transformation with Brevibacillus Competent Cells (product code, HB116).
3. Allow Solution A, Solution B, and MT medium to melt in advance.

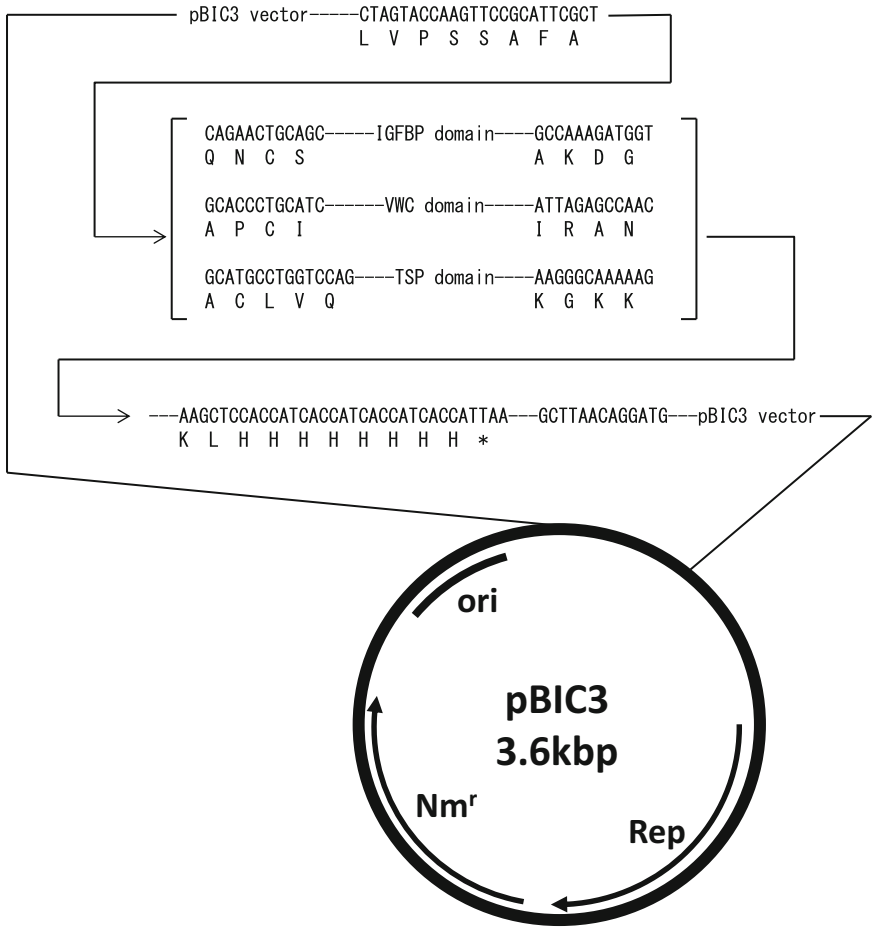

Fig. 1 Expression plasmid map for IGFBP, VWC, and TSP domain production based on pBIC3 vector. Rep: replication protein, Nm^r: neomycin-resistant protein, ori: replication origin from pUB110 plasmid

4. Allow *Brevibacillus* Competent Cells to thaw rapidly (~30 s) in 37 °C warm water.
5. Collect bacterial cells with a microcentrifuge (13,000 × *g*, 1–2 min), and remove the supernatant completely with a micropipette.
6. (At room temperature from this step onward) Mix the DNA solution prepared in 5 μl and 50 μl of Solution A.
7. Add the entire DNA mixture to the tube of 5, and suspend the bacterial pellets completely by vortex (*see* **Note 2**).
8. Let stand for 5 min.
9. Add 150 μl of Solution B (PEG solution), and mix by vortex to give a homogeneous solution (5–10 s).
10. Collect bacterial cells with a microcentrifuge (3000 × *g*, 5 min) and remove the supernatant.

11. Briefly centrifuge (5000 rpm, ~30 s) again and remove the supernatant completely.
12. Add 1 ml of MT medium and suspend cells completely with a micropipette.
13. Shake at 120 rpm for 1 h at 37 °C.
14. Spread an aliquot of the suspension onto MTNm plate and incubate at 37 °C overnight.
15. Pick 6–10 random colonies on the MTNm plate, and inoculate 2 ml TMNm medium with them (*see* **Note 3**).
16. Culture at 37 °C for 15–18 h, and then collect bacterial cells (*see* **Note 4**). Extract the plasmid using a commercial plasmid extraction kit. The DNA yield is generally 1–2 μg.
17. Carry out restriction enzyme digestion (EcoRV, HindIII) using an appropriate quantity of DNA (*see* **Note 5**).
18. Verify the presence of the insert by agarose gel electrophoresis.
19. Once the insert is verified to be present, determine the sequence. The following forward and reverse primer sequences can be used to confirm the sequence.

    Forward sequencing primer: 5′-CGCGATATCAGGATT CGG-3′.

    Reverse sequencing primer: 5′-CAATGTAATTGTTCCCT ACCTGC-3′.

3.1.2 Construction of CT Expression Plasmid (Fig. 2, See **Note 6***)*

1. Carry out PCR using forward and reverse primers for CT domain.
2. Treat the insert and 1.0 μg of pNCMO2 DNA with two types of restriction enzymes (NcoI, HindIII).
3. Subject each reaction mixture to agarose gel electrophoresis to collect/purify the desired fragment.
4. Allow 100 ng each of purified DNA to react with the ligation reagent (DNA Ligation Kit Ver. 1).
5. Transform *Brevibacillus* Competent Cell using 5 μl of the reaction mixture.
6. Verify the presence of the insert in the same manner as described in **steps 3–18** in Subheading 3.1.1.
7. Once the insert is verified to be present, determine the sequence. The following forward and reverse primer sequences can be used to confirm the sequence.

    Forward sequencing primer: 5′-CGCTTGCAGGATTCGG-3′.

    Reverse sequencing primer: 5′-CAATGTAATTGTTCCCTA CCTGC-3′.

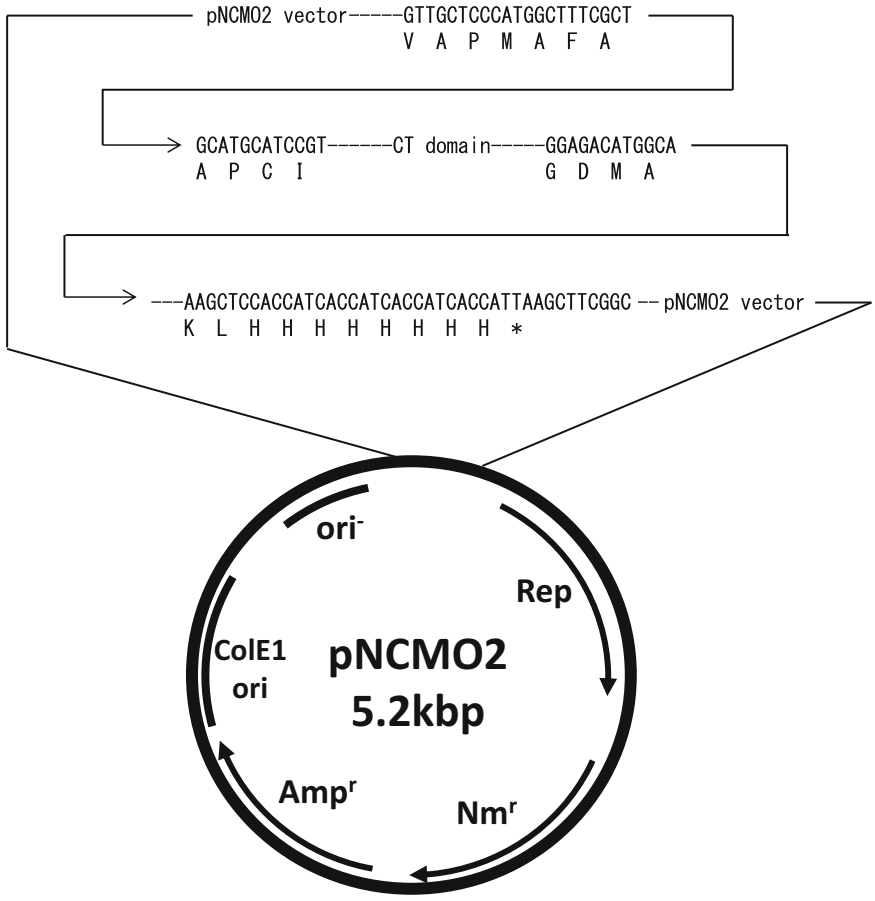

Fig. 2 Expression plasmid map for CT domain production based on pNCMO2 vector. Rep: replication protein, Nm^r: neomycin-resistant protein, Amp^r: ampicillin-resistant protein, ColE1 ori: replication origin from pUC plasmid, ori: replication origin from pUB110 plasmid

3.2 Culture Production

1. Transfer the sequence-verified expression vector again into *Brevibacillus* Competent Cells. Using 1–5 μl of the resulting plasmid DNA, conduct **steps 3–14** in Subheading 3.1.1.
2. Pre-culture: Inoculate 2SYNm medium (IGFBP, VWC, TSP) or TMNm medium (CT) in a test tube or flask with a colony taken from the MTNm plate, and incubate at 30 °C overnight.
3. Inoculate a necessary volume of the medium with 1% volume of the pre-culture fluid, and culture with shaking at 30 °C for 48 h. As in the pre-culture, use 2SYNm medium for IGFBP, VWC, and TSP or TMNm medium for CT.
4. After completion of culturing, centrifuge the culture fluid and collect the supernatant.

3.3 Purification

3.3.1 IGFBP, VWC, and TSP Domains

1. Dialyze against ≥100-fold volume of Buffer A at 4 °C. For example, repeat 24-h dialysis with ≥10-fold volume of the buffer two times.
2. Collect the dialysate.
3. Load the dialysate to Ni-bound resin. Use the flow rate and loading amount recommended in the manufacturer's instruction, as they differ depending on the column size.
4. Wash with 2CV (Column Volume) of Buffer A.
5. Elute with a linear gradient with 0.5 M imidazole. The gradient condition shall be 0% → 100% in 10CV, and the fraction size shall be ~1/5CV (thus ~50 elution fractions).
6. Analyze the fractions by SDS-PAGE, and collect the fractions containing the target protein.
7. Dialyze against ≥10^4-fold volume of 50 mM phosphate buffer, pH 7.0 (IGFBP) or 50 mM acetate buffer, pH 4.0 (VWC and TSP) (*see* **Note 7**). For example, repeat 24-h dialysis with ≥100-fold volume of the buffer two times.
8. Collect the filtrate with a centrifugal ultrafiltration membrane (Centricon Plus 100K).
9. Concentrate with a centrifugal ultrafiltration membrane (e.g., Centriprep YM-10) as needed.

3.3.2 CT Domain

1. Dissolve urea in the supernatant to give a final concentration of 8 M, and adjust the pH of the resulting solution to 8.0 with 1 N NaOH or 1 N HCl (*see* **Note 8**).
2. Equilibrate a column with 5CV of denaturing binding buffer.
3. Load the sample solution containing 8 M urea to the column. The loading volume shall be up to fivefold of the resin volume.
4. Wash with 2CV of denaturing binding buffer.
5. Wash with 2CV of denaturing wash buffer.
6. Elute with 3CV of the denaturing elution buffer, and collect fractions in a volume of 1/5CV.
7. Analyze the fractions by SDS-PAGE, and collect the fractions containing the target protein.
8. Collect the filtrate with a centrifugal ultrafiltration membrane (Centricon Plus 100K).
9. Dilute the external dialysis solution tenfold with dialysis buffer for CT domain, and continue dialysis.
10. Repeat **step 9** three times to decrease the urea concentration to 1/1000 (0.008 M).
11. Concentrate with a centrifugal ultrafiltration membrane (e.g., Centriprep YM-10) as needed.

4 Notes

1. Use highly accurate high-fidelity PCR enzymes for PCR.
2. Use vortex to achieve full suspension. The transformation efficiency will be substantially reduced if pellets are left.
3. If small and large colonies are formed, choose both colonies. The size difference may reflect the growth difference due to production of the target protein.
4. Aeration agitation is essential for *Brevibacillus* to grow. If a test tube with a screw-type cap is used, loosen the cap during culturing to facilitate aeration. Moreover, adjust the inclination of the test tube such that the medium is shaken well.
5. When EcoR V and Hind III are used, the fragment generated will be in a size of 200–220 bp plus a gene of interest.
6. This conventional construction method is now obsolete. Much easier method, "BIC method," is currently recommended instead.
7. If VWC and TSP domains are dialyzed against the same buffer as used for IGFBP, i.e., 50 mM sodium phosphate, pH 7.0, a precipitate will be formed in a considerable quantity, and the yield of the target protein will be reduced.
8. CT domain cannot be purified under a native condition due to aggregation with contaminating proteins and is thus required to be solubilized using urea.

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Chapter 10

Production of Recombinant CCN2 Protein by Mammalian Cells

Takashi Nishida, Satoshi Kubota, and Masaharu Takigawa

Abstract

Recombinant CCN2 protein (rCCN2) is available from many companies; however, most of them are produced in *E. coli*. To investigate true functions of rCCN2, glycosylated protein with proper folding needs to be used. Therefore, we use rCCN2 produced by mammalian cells. Conditioned medium (CM) of HeLa cells stably transfected with a *CCN2* expression vector are collected, and the recombinant *CCN2* protein produced and secreted into the CM is purified by two-step chromatography, first with a heparin affinity column and then with an anti-CCN2 affinity column prepared with a monoclonal antibody against CCN2. The purified rCCN2 shows the bands of 36–38 kDa with sliver staining after gel electrophoresis, which can be confirmed by Western blotting. This chapter describes these methods in detail.

Key words Recombinant CCN2 protein (rCCN2), Heparin affinity chromatography, Anti-*CCN2* affinity chromatography, Stable transfection, HeLa cells, *CCN2* expression vector, ASF serum-free medium 104

1 Introduction

CCN family proteins were named by using the first letter of each of the initially identified members, i.e., *C*ysteine-rich 61 (Cyr61; Ref. 1), *C*onnective tissue growth factor (CTGF), and *N*ephroblastoma overexpressed (Nov); and their structure is characterized by four distinct modules; insulin-like growth factor binding protein-like (IGFBP), von Willebrand factor type C (VWC), thrombospondin type 1 repeat (TSP1), and a carboxyl terminal cystein-knot (CT; Refs. 1, 2). At present, the CCN family now comprises six members with the addition of Wnt-inducible secreted proteins (WISP) 1, 2, and 3. So now the family consists of six distinct proteins with similar structures except for WISP2, which lacks the CT module [3]. In 2003, a unified nomenclature was proposed by the International CCN Society, by which each member protein was renamed numerically in the order of its discovery [4].

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DOI 10.1007/978-1-4939-6430-7_10,

CTGF is the second member of the CCN family of proteins (CCN2), and It has been reported to be involved in a number of biological processes during development, including chondrogenesis [5, 6], osteogenesis [7], and angiogenesis [8], as well as in tissue remodeling such as bone fracture repair [9] and fibrosis [10]. In particular, we have studied the physiological roles of CCN2 in the biology of bone and cartilage cells [11]. To investigate the effects of CCN2 on these cells, we prepared recombinant CCN2 (rCCN2) protein produced by mammalian cells by using our original methods [12]. Currently, rCCN2 is commercially available from many companies. Most of these recombinant proteins are produced in *Escherichia coli* and are homogeneous, nonglycosylated proteins. However, it is known that native CCN2 has possible glycosylation sites at asparagine residues 28 and 225 that cause heterogeneity in molecular weight [13]. Therefore, using rCCN2 produced in mammalian cells is important for investigating the true functions in mammals.

In this article, we explain how to produce rCCN2 in mammalian cells and to purify it by using our proprietary chromatographic techniques.

2 Materials

All solutions were prepared by using ultrapure water (prepared by purifying deionized H_2O to attain a resistance of 18 MΩ cm at 25 °C) and were sterilized by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on a liquid cycle.

2.1 Stable Transfection of HeLa Cells with a CCN2 Expression Plasmid by Electroporation

1. Dulbecco's modified Eagle's medium (DMEM): Weigh 9.5 g of DMEM powder autoclavable and add ultrapure water to a volume of 1-L (*see* **Note 1**). Mix and sterilize by autoclaving. After cooling down room temperature, add 2 mM glutamine, 0.1% $NaHCO_3$ (*see* **Note 2**) and 10% fetal bovine serum (FBS).
2. Selection medium: To prepare a G418 sulfate stock solution at a concentration of 200 mg/mL, weigh titrated 200 mg of G418 sulfate powder and add purified water to a volume of 1-mL. Sterilize the solution by filtration, and store it at −20 °C. Section medium is DMEM containing G418 sulfate solution at a final concentration of 200 μg/mL (*see* **Note 3**).
3. Collection medium: Serum-free medium without animal components (e.g., ASF medium 104, Ajinomoto Co.) or an equivalent (*see* **Note 4**).
4. Trypsin-EDTA: Weigh 0.25 g of trypsin powder and add phosphate-buffered saline (PBS) to a volume of 100 mL. Then, add 0.5 M EDTA (pH 8.0) solution at a final concentration of

0.5 mM and stir it overnight (*see* **Note 5**). Sterilize the solution by filtration, and store at 4 °C.

5. Vector: Purify pcDNA3.1(−) *CCN2* plasmid by plasmid purification kits. In this plasmid, whole coding region of human CCN2 cDNA amplified by polymerase chain reaction (PCR) is cloned into pcDNA3.1(−) plasmid (*see* **Notes 6** and **7**).
6. Cells: HeLa cells are available from American Type Culture Collection (ATCC).
7. Electroporation device and cuvettes (*see* **Note 8**).

2.2 Primary Purification of Serum-Free Media Conditioned with HeLa Cells Transfected with CCN2 Expression Vector by Heparin Affinity Chromatography

1. Washing buffer: PBS containing 0.2 M NaCl. Weigh 11.7 g of NaCl and add PBS to a volume of 1-L.
2. Elution buffer: PBS containing 0.75 M NaCl. Weigh 22.0 g of NaCl and add PBS to a volume of 500 mL.
3. Heparin affinity chromatography (e.g., HiTrap heparin column, GE Healthcare) or an equivalent (*see* **Notes 9** and **10**).

2.3 Secondary Purification of the Heparin Column Elutes by Affinity Chromatography Using an Anti-CCN2 Antibody

1. Anti-CCN2 monoclonal antibody (*see* **Note 11**).
2. Washing buffer: PBS.
3. Elution buffer: 0.1 M glycine buffer pH 2.5. Weigh 0.75 g of glycine and add water to a volume of 100 mL. Mix and adjust pH to 2.5 with HCl.
4. Neutralization buffer: 0.75 M Tris–HCl pH 8.0. Weigh 9.1 g of Tris base and add water to a volume of 100 mL. Mix and adjust pH to 8.0 with HCl (*see* **Note 12**).
5. Coupling buffer for anti-CCN2 antibody to column: 0.2 M $NaHCO_3$ and 0.5 M NaCl pH 8.3. Weigh 16.8 g of $NaHCO_3$ and 29.2 g of NaCl. Add water to a volume of 1-L. Mix and adjust pH to 8.3 with HCl.
6. Affinity column with an anti-CCN2 antibody: To be prepared (*see* Subheading 3.4). Anti-CCN2 monoclonal antibody is coupled with HiTrap NHS-activated column (*see* **Note 13**).
7. UV/Vis spectrophotometer (e.g., DU730, Beckman Coulter) or an equivalent.

2.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blotting, Silver Staining, and Protein Assay

1. Loading buffer (2×): 0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue (BPB).
2. SDS-PAGE running buffer: 0.025 M Tris–HCl (pH 8.3), 0.192 M glycine, 0.1% SDS.
3. Tris-bufferd saline (TBS; 10×): Weigh 24.2 g of Tris base and 80 g of NaCl. Add water to a volume of 1-L. Mix and adjust pH to 7.6 with HCl (*see* **Note 14**).

4. TBST: TBS containing 0.1% Tween-20.
5. Blocking solution: 5% skim milk in TBS.
6. Silver staining kit (e.g., 2D-silver staining kit, Daiichipure Chemicals Co.) or an equivalent (*see* **Note 15**).
7. Polyvinylidene difluoride (PVDF) membrane.
8. Primary antibody; Anti-CCN2 polyclonal antibody (Abcam, ab6992; *see* **Note 16**).
9. Protein assay kit (e.g., Micro BCA protein assay kit, Pierce) or an equivalent (*see* **Note 17**).

3 Methods

3.1 Stable Transfection of HeLa Cells with CCN2 Expression Plasmid by Use of Electroporation

1. Culture HeLa cells until they reach a subconfluence state (*see* **Note 18**). Use trypsin-EDTA to release adherent cells, and centrifuge them at 500 × *g* for 5 min at room temperature.
2. Resuspend the cell pellet in DMEM containing 10% FBS and measure the cell number by using a hemocytometer.
3. Collect the cells by centrifugation at 500 × *g* for 5 min again, and resuspend them in PBS at a cell density of 4.0×10^7 cells/mL.
4. Transfer 500 μL aliquots of the cell suspension into electroporation cuvettes on ice. Add 10 μg of plasmid DNA in a volume of up to 50 μL to each cuvette. Gently mix the cells and DNA by pipetting the solution (*see* **Note 19**).
5. Set the parameters on the electroporation device. A typical capacitance value is 960 μF, and voltages range is 300 V for 31 ms (*see* **Note 20**).
6. Discharge electricity from the condenser to the cuvette. Immediately transfer the electroporated cells to a culture dish and incubate them for 48 h in DMEM containing 10% FBS at 37 °C under 5% CO_2 in air (*see* **Note 21**).
7. To isolate stable transfectants, transfer the cells to the selection medium. Change the selection medium every 2–4 days for 2–3 weeks to remove the cellular debris and dead cells and to allow the resistant cells to grow into colonies.
8. When colonies are formed, aspirate the medium and place a sterile cloning ring with a touch of grease around its bottom edge on the plate to surround an individual clone. Add 100 μL of trypsin-EDTA to the cloning ring and immediately remove it. After incubation for 2 min at 37 °C, add 200 μL of selection medium and pipet the medium to pick up colonies. Transfer the colonies picked from the plate to a 96-well multiwell plate for expansion in selection medium (*see* **Notes 22** and **23**).

3.2 Preparation of Conditioned Medium (CM) from Stable HeLa Transfectants with CCN2 Expression Vector

1. Inoculate HeLa cells stably transfected with *CCN2* expression plasmid into 100-mm-diameter culture dishes containing the selection medium and culture them until they reach subconfluence (*see* **Note 18**).
2. When the stable transfectants reach subconfluence, replace the selection medium with collection medium and culture these cells for 3 days.
3. Collect and pool the conditioned media (CM) and filter it through a 0.8-μm filter to remove cells and cellular debris (*see* **Note 24**). Store the CM at −20 °C until over 1 L of it has been collected.

3.3 Use of Heparin Affinity Chromatography for Purification of CM from Cultures of Stable HeLa Transfectants Prepared with CCN2 Expression Vector

1. Start a peristaltic pump beforehand at the flow rate to 1 mL/min and then connect the heparin affinity column to the pump (*see* **Notes 25** and **26**).
2. Run an approximately tenfold column volume of distilled water through the column, and then equilibrate the column with a tenfold column volume of PBS.
3. Apply the CM at flow rate of 1 mL/min (*see* **Note 27**).
4. After the sample has been applied to the column, wash the column with PBS at a flow rate of 1 mL/min for 2 h. Then, change the buffer from PBS to the Washing buffer and apply it at 1 mL/min for 1 h (*see* **Note 28**).
5. To elute the heparin binding proteins from the column, switch the buffer to PBS containing 0.75 M NaCl and continue pumping in the same direction at a flow rate of 1 mL/min.
6. Collect the eluted fractions and measure their optical absorbance at the wavelength of 280 nm (*see* Fig. 1a).
7. To clean the column for reuse, wash it with both PBS and subsequently with distilled water at a flow rate of 1 mL/min for 2 h. Then fill the column with 20% ethanol before storage (*see* **Notes 29** and **30**).

3.4 Coupling of Anti-CCN2 Antibody to HiTrap NHS-Activated HP 1-mL Column

1. Connect HiTrap 1-mL column to a syringe and wash out the isopropanol with 1 mM HCl (*see* **Note 31**).
2. Wash with six column volumes of 1 mM HCl manually at a flow rate of 1 mL/min.
3. Immediately inject 1 mL of anti-CCN2 monoclonal antibody (5 mg) in coupling buffer into the column.
4. Seal the column, and then let it stand for 30 min at room temperature.
5. To deactivate any excess active groups that have not coupled to the ligand, wash the column with 6 column volumes of a solution of 0.5 M ethanolamine and 0.5 M NaCl (pH 8.3),

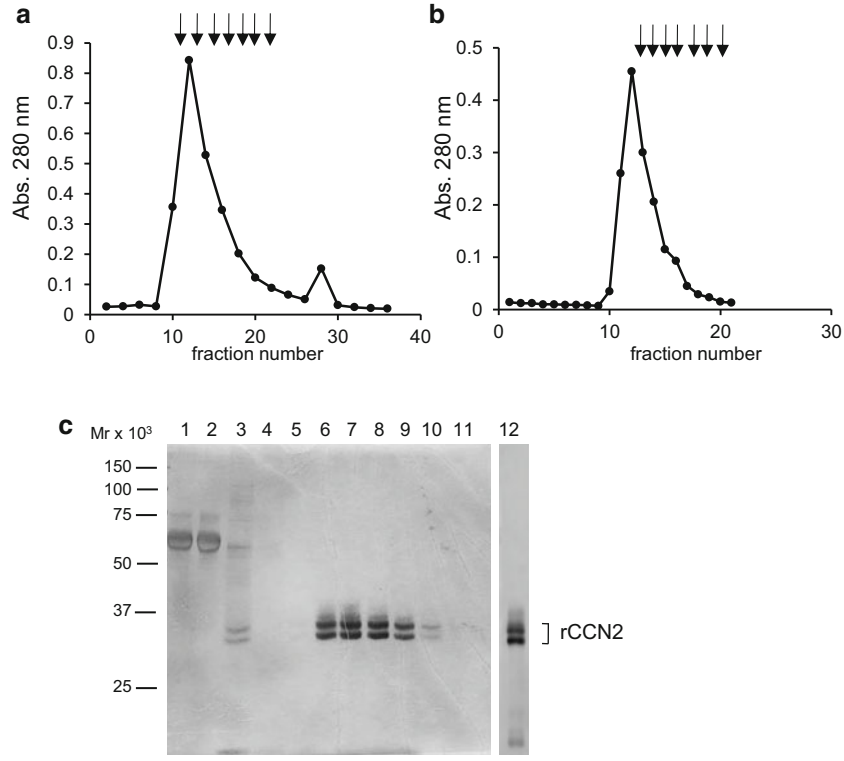

Fig. 1 Purification of rCCN2 from conditioned medium of HeLa cells stably transfected with a *CCN2* expression vector. (**a**) Elution profile of heparin affinity chromatography. The peak fractions are collected and pooled (*arrows*). Then, these samples are applied onto an anti-CCN2 antibody affinity column. (**b**) Elution profile of anti-CCN2 antibody affinity chromatography. The peak fractions are collected (*arrows*) and analyzed by silver staining and Western blotting. (**c**) Detection of purified rCCN2 by silver staining and Western blotting. *Lane 1*, conditioned medium of HeLa cells transfected with *CCN2* expression vector; *lane 2*, conditioned medium passed through the heparin affinity column; *lane 3*, pooled fractions eluted from heparin affinity column; *lane 4*, flow-through fractions of the anti-CCN2 antibody affinity column chromatography; and *lanes 5–11*, corresponding elution fractions of indicated *arrows* in "**b**". *Lane 12* shows the results of Western blotting of the *lane 7*

and then again with the same volume of a solution of 0.1 M acetate and 0.5 M NaCl (pH 4.0). Then, wash it once more with six column volumes of a solution of 0.5 M ethanolamine and 0.5 M NaCl (pH 8.3).

6. Leave the column for 30 min at room temperature.
7. Inject six column volumes of a solution of 0.1 M acetate and 0.5 M NaCl (pH 4.0), followed by the same volume of a solution of 0.5 M ethanolamine and 0.5 M NaCl (pH 8.3). Then, inject the same volume of the former solution into the column.
8. Finally inject 2 mL of 0.05 M Na_2HPO_4 (pH 7.0) to equilibrate the column.

3.5 Final Purification of the Recombinant CCN2 by Affinity Chromatography Using an Anti-CCN2 Antibody

1. Start the pump at a flow rate to 0.5 mL/min and then connect the outlet tubing to the affinity column conjugated with anti-CCN2 monoclonal antibody (*see* **Note 26**).
2. Run approximately a tenfold column volume of distilled water through the column, and then equilibrate the column with a tenfold column volume of PBS.
3. Apply the pooled fractions eluted from the heparin affinity column with application at a flow rate of 0.5 mL/min.
4. When the sample has been loaded, wash the column with a tenfold column volume of PBS.
5. To elute the target protein, switch the buffer to 0.1 M glycine buffer (pH 2.5) and collect the eluted fractions at a flow rate of 0.3 mL/min. Immediately neutralize the collected fractions with 0.75 M Tris–HCl pH 8.0 (*see* **Note 32**).
6. Measure the optical absorbance at the wavelength of 280 nm by using a spectrophotometer to detect the target protein (*see* Fig. 1b).
7. To clean the column for reuse, wash it with a tenfold column volume of PBS and subsequently with the same volume of distilled water at a flow rate of 0.5 mL/min. Fill the affinity column with 20% ethanol before storage (*see* **Notes 29** and **30**).

3.6 Detection and Quantitation of the Purified Recombinant CCN2 Protein

1. Mix 5 μL of the sample from the fractions and the same volumes of Treatment buffer (2×) and heat the mixture at 95 °C for 5 min before electrophoresis.
2. Electrophorese at a constant current of 30 mA until the BPB dye in the Treatment buffer reaches the bottom of the polyacrylamide gel.
3. Following electrophoresis, transfer the protein onto a PVDF membrane by using a semidry blotting apparatus for Western blotting or rinse the gel with 50% methanol and 10% acetic acid solution for silver staining.
4. For Western blotting, block the PVDF membrane with Blocking solution for 1 h at room temperature and then incubate it overnight at 4 °C with anti-CCN2 polyclonal antibody (*see* **Note 16**) diluted at 1:1000 in TBS. Thereafter, rinse the membrane with TBST three times (*see* **Note 33**).
5. Add horseradish peroxidase-coupled anti-rabbit IgG in Blocking solution and incubate the membrane for 1 h. Rinse with three cycles of TBST followed by TBS.
6. Detect the bands with the chemiluminescence substrate (*see* Fig. 1c).
7. For silver staining, use a sliver staining kit according to the manufacturer's instructions (*see* Fig. 1c; *see* **Note 34**).

8. Determine the concentration of the purified CCN2 protein by using a protein assay kit, including bovine serum albumin (BSA) standards, according to the manufacturer's instructions (*see* **Note 14**).
9. Examine the activity of rCCN2 by performing cell biological assays with the desired mammalian cells, such as chondrocytes [5, 14–16], osteoblasts [7, 17], osteoclasts [18, 19], vascular endothelial cells [8, 20] or myoblasts [21].

4 Notes

1. Keep the powder of DMEM at room temperature before using it. Otherwise, the powder absorbs humidity and the DMEM becomes degraded.
2. Because the pH of the medium is one of the important factors for cell growth in culture, the most suitable pH condition of the medium is determined based on the color of the medium rather than on the absolute weight of $NaHCO_3$ used to adjust the pH.
3. G418 stock solution is stored at −20 °C, and is added to DMEM immediately before using.
4. We have used ASF medium 104 (Ajinomoto Co.) with good success [5, 7, 8, 15–18, 20, 21], although other commercial producers are also available. This medium maintains high activity of the cells. Therefore, it can be used as an assured medium for protein production followed by purification.
5. The addition of EDTA to the trypsin solution enhances dissociation of the Ca^{2+} bridges that support the binding between the cells and culture dish. Therefore, as the time of treatment with trypsin-EDTA is shorter than that of trypsin only, the damage to the cells is less.
6. We use the pcDNA3.1(−) expression vector because this vector has the human cytomegalovirus (CMV) immediate-early promoter that provides stable and transient expression at a high level in a wide range of mammalian cells.
7. Design appropriate restriction sites in sense and antisense primers. When a target gene is generated by PCR, the restriction enzymes are incorporated in the target gene. Then, the target gene can be inserted into a vector easily.
8. We use the Gene Pulser (Bio-Rad Laboratories Inc., Hercules, CA, USA), although other commercial devices may also be used.
9. Because CCN2 binds strongly to heparin, we perform heparin affinity chromatography as a first step of purification.
10. More than 1-L of CM is loaded onto the column for purification as the first material. Therefore, a 5-mL column is necessary.

11. We use monoclonal antibody produced by hybridomas of BALB/c mice immunized with a partially purified CCN2 protein.
12. When the glycine buffer and the neutralization buffer are kept at 4 °C for long-term storage, debris is produced easily in these buffers. To avoid this, make tenfold concentrations of these buffers and sterilize them by autoclaving. Immediately before use, these buffers are diluted with distilled water.
13. When we coupled antibody to an affinity column in preliminary experiments, the coupling efficiency of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) was lower than that of the HiTrap NHS-activated column. Therefore, we recommend use of the HiTrap NHS-activated column.
14. The pH of Tris buffer is dependent on the temperature. If adjustment of pH is performed at room temperature, it should be stored at room temperature.
15. We use the 2D-sliver staining kit (Daiichipure Chemicals Co.), because this kit has an easy protocol and gives good success (Fig. 1c). However, other commercial kits are also available.
16. Because we have used this antibody with good success (Fig. 1c), we recommend this antibody. However, other commercial sources exist.
17. We use a micro BCA protein assay kit to reduce the amount of sample for measurement. However, other commercial kits may also be used.
18. When the cells have grown to 70% confluence, collect them from the culture dish. Do not allow growth beyond 70% confluence.
19. Be careful not to introduce air bubbles into the mixture of the cells and plasmids during the mixing step.
20. We have used the electroporation protocol recommended by Bio-Rad Laboratories.
21. Because several ions in the cells are released from the pores in the cell membrane made by electroporation, do not keep the cells on ice for long-term storage.
22. Pick up big colonies formed independently.
23. Although autoclaving is applicable to sterilize the cloning ring, it should be dried very well. Otherwise, the trypsin solution may leak from under the ring because a greased wet ring does not stick well to the culture dish.
24. Centrifuge the CM at 750 × *g* for 5 min to remove dead cells and big cellular debris before filtration.
25. All purification steps are performed in a cold room.

26. Connect the outlet tubing from the pump to the column once the distilled water appears "drop by drop" at the end of the tubing to avoid introducing air into the column.
27. Apply the sample to the heparin column overnight, and be cautious to avoid drying of the column.
28. Wash the column with over a tenfold column volume of PBS followed by the same volume of Washing buffer.
29. After confirming that the optical absorbance at a wavelength of 280 nm has returned to base line during the elution step, switch to PBS.
30. Wash the column with ten column volumes of 20% ethanol at a flow rate of 1 mL/min. Store the column in 20% ethanol at 4 °C.
31. Do not wash out the isopropanol until the coupling reaction is ready to be performed.
32. Using a pH test paper, confirm immediately that collected fractions have been neutralized.
33. The amount of antibodies and incubation time used to perform Western blotting may vary according to the experimental design and may need to be optimized.
34. Even if Stopper is added to the staining solution, the gel color gradually continues to develop. Therefore, wash the gel with water immediately and dry it up by using a gel-dryer.

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Chapter 11

In Vitro Transfection with and Expression of CCN Family of Genes

Danilo Janune and Masaharu Takigawa

Abstract

The ability to engineer cells to express a protein of interest in an inducible manner and stably for a long period is a valuable tool in molecular biology and also one that holds promise for regenerative medicine in the future. CCN proteins have been suggested to be involved in tissue regeneration. In this chapter, we describe an in vitro method for stable and inducible expression of CCN protein in a chondroprogenitor cell line and in chondrocytes in primary culture that does not involve the use of any viral vector.

Key words PiggyBac, Transposon, Stable, Expression, CCN

1 Introduction

The most common methods to deliver naked DNA, plasmid or not, to mammalian cells are lipofection, calcium precipitation, and electroporation. Primary chondrocytes are cells that, in order to maintain their phenotype and polygonal morphology in vitro, must be cultured at high densities. The production of abundant extracellular matrix is another inherent remarkable characteristic of primary chondrocytes that, combined to the high density in which they are cultured, greatly decreases the efficiency of transfection of these cells by lipofection or calcium precipitation methods. Electroporation, on the other hand, is carried out with the cells in suspension (no extracellular matrix) and in a very small volume, which increases the chances of the DNA molecules entering the cell. Therefore, electroporation is, in our experience, the method with the highest efficiency when delivering foreign DNA to primary chondrocytes, provided that suitable reagents and machines are used.

Regardless of the method used for delivery of naked DNA to a mammalian cell, the rate of integration of this DNA element into the host genome is very low; and the integration site also greatly

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affects the activity of the transgene [1, 2], with the transgene expression lasting only as long as the episomal form of the DNA element lasts. Therefore, in order to achieve stable, long-term transgene expression with conventional DNA elements, time-consuming drug selection and cloning steps are necessary. The use of recombinant viral vectors based on the retroviridae family of viruses, such as retrovirus or lentivirus, is an easier option to achieve stable transgene expression. This method, however, involves the use of special cabinets and facilities, and there is also the extra step of the virus production. When inducible long-term transgene expression is needed, the drawbacks pointed earlier are doubled; as at least two DNA elements need to be integrated into the host genome when a bipartite inducible expression system is used.

The piggyBac transposon (PBT) is a transposable DNA element, originally isolated from the cabbage-looper moth, that recombines specifically at the TTAA sites of DNA [3, 4]. The native PBT consists of two inverted terminal repeats flanking an ORF that encodes the transposase, the enzyme that mediates the transposition. PBT versions used in biotechnology have the terminal repeats flanking the genetic elements of interest and the transposase expressed from another construct without the flanking repeats. PBT, combined with the transient expression of its transposase, can efficiently insert constructs as large as 9 kb [5] into the host genome. This high efficiency in inserting DNA elements into the host genome makes PBT the ideal tool for a bipartite inducible expression system and enable the creation of a bulk cell culture with robust, inducible and long-term transgene expression by selecting the cells with a drug directed to only one of the inserted elements in the bipartite system [6]. We describe here how to use PBT to regulate and stably express the red fluorescent protein tdTomato in the chondroprogenitor cell line ATDC5 and the full-length human CCN3, as well as tdTomato, in articular chondrocytes in primary culture. As a matter of fact, the PBT system can be used to rapidly create bulk cultures expressing any CCN family member, as well as their separate modules, alone or combined, and their truncated forms.

2 Materials

1. Chondroprogenitor cell line ATDC5.
2. Primary articular chondrocytes from 4-weeks old Sprague-Dawley rats.
3. A mixture of HAM-F12 and DMEM 1:1 culture medium for ATDC5 cells.
4. α-MEM for primary articular chondrocytes (*see* **Note 1**).
5. Collagenase: 1.5 mg/mL in the cells' respective complete culture media.

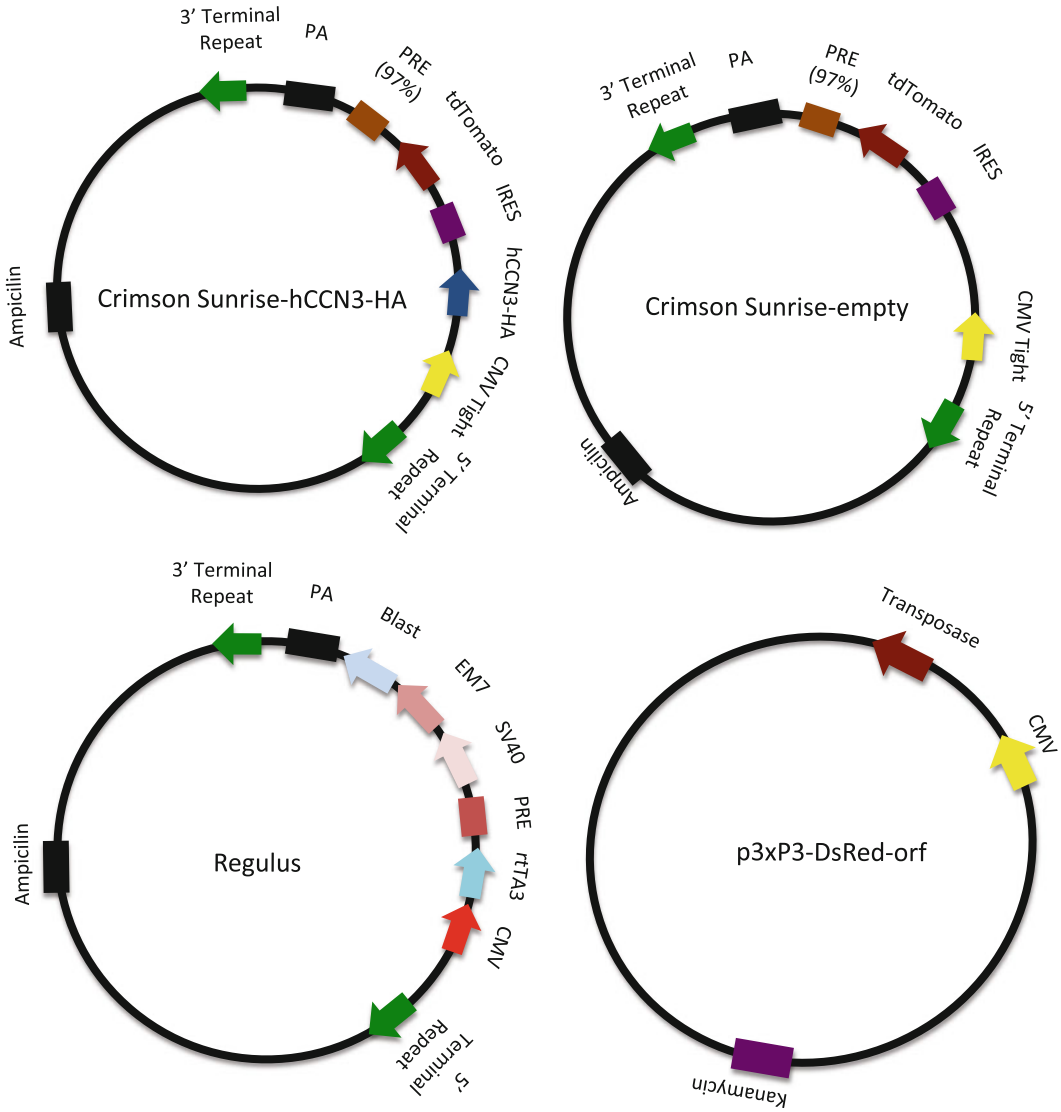

Fig. 1 Scheme illustrating the four plasmids employed and the approximate location of the relevant elements

6. Nucleofector® II machine (Amaxa Biosystems).
7. Amaxa® Human Chondrocyte Nucleofector® (Amaxa Biosystems).
8. Plasmids: Crimson Sunrise-hCCN3-HA, Crimson Sunrise-empty, Regulus, p3xP3-DsRed-orf (*see* Fig. 1), all dissolved in ultra pure water (*see* **Note 2**).

3 Methods

1. Isolate chondrocytes from articular cartilage and plate them at $5 \times 10^4/cm^2$ in 60-mm culture dishes (*see* **Note 3**).

2. After about 4 days in culture, the articular chondrocytes reach confluence and are then prepared for electroporation (*see* **Note 4**).
3. Before completing the preparation of the articular chondrocytes for electroporation, Human Chondrocyte Nucleofector® solution is prepared, with the plasmids in it, at room temperature (*see* **Note 5**).
4. Once the articular chondrocytes are ready for electroporation, pellet 10^6 of them, discard the supernatant, immediately add the Nucleofector®/supplement solution with the plasmids to the cell pellet, and proceed to the electroporation (*see* **Note 6**).
5. After electroporation, immediately add 500 μL of complete culture medium to the cells in a cuvette and, with the kit-provided plastic pipette, gently remove the cells from the cuvette (*see* **Note 7**).
6. Place the cells in one well of a six-well plate and make the final complete culture medium volume up to 2 mL (*see* **Note 8**).
7. For the electroporation of ATDC5 cells, the procedure is exactly the same once the cells have reached confluence.
8. Four days after electroporation, start the drug selection with blasticidin in order to establish a bulk culture (*see* **Note 9**).

4 Notes

1. Both media are supplemented with penicillin (57 μg/mL)–streptomycin (100 μg/mL). The culture medium for ATDC5 cells is supplemented with 5% fetal bovine serum (FBS); and that for the articular chondrocytes, with 10% FBS. For the drug selection, blasticidin is added to the medium to a final concentration of 5 μg/mL. For the activation of the transgenes, doxycycline is added to the medium to the final concentration indicated in the figures.
2. In principle, any plasmid construct with the piggyBac terminal repeats and any plasmid construct expressing the piggyBac transposase can be used. Crimson Sunrise-hCCN3-HA, Crimson Sunrise-Empty, and Regulus were assembled at our laboratory by putting together elements of the following plasmids: human CCN3 pLEGFP-N1 [7], pXL-BacII [3, 4] (provided by Dr. Malcolm J. Fraser at the University of Notre Dame, IN, USA), LeGO-iT2 [8], pLenti CMV rtTA3 Blast, and pLenti CMVtight Hygro DEST (Tet-on Advanced Vectors by Eric Campeau, unpublished). The last three plasmids were obtained from the plasmid repository Addgene, MA, USA. p3xP3-DsRed-orf [3, 4] (also provided by Dr. Malcolm J. Fraser) is used here without any modification. Crimson Sunrise-hCCN3-HA (or Crimson Sunrise-Empty) is the PBT

plasmid with the CMVtight and, downstream to it, the ORF of the full-length human CCN3 fused to an HA tag and the ORF of the red fluorescent protein tdTomato with an IRES element between them (Fig. 1). CMVtight is the promoter that contains the binding domain for the third generation transactivator (rtTA3). Regulus is the PBT plasmid that expresses the resistance gene against blasticidin and the rtTA3 that binds to the CMVtight promoter in the presence of doxycycline, which initiates transcription. p3xP3-DsRed-orf is the plasmid that encodes the transposase enzyme, the enzyme responsible for the integration of the PBT elements into the cell genome.

3. Cartilage is dissected from the distal femoral epiphysis and digested for 5 h with collagenase (1.5 mg/mL) in complete medium. Cells are plated just after digestion. Roughly, the two femurs of one rat will yield enough cells for one 60-mm culture dish.
4. In order to remove the abundant extracellular matrix surrounding the cells, collagenase digestion (1.5 mg/mL in complete culture medium) is performed for 3 h. Some cells will remain attached to the culture dish, probably through integrin-mediated adhesion. Do not worry about them. Usually, the collagenase digestion alone will provide the 10^6 cells necessary for electroporation. If you need the cells attached to the dish to be recovered, routine trypsin digestion is adequate.
5. We use 81.8 μL of the Human Chondrocyte Nucleofector® solution with 18.2 μL of its supplement. The plasmid amount is 4 μg of the Crimson Sunrise-hCCN3-HA (or Crimson Sunrise-empty), 0.5 μg of the Regulus, and 0.5 μg of the p3xP3-DsRed-orf. It is important to keep the original plasmid concentrations at no less than 800 μg/μL in order to not dilute the final Nucleofector®/supplement/plasmids solution too much.
6. Carefully resuspend the cells in the Nucleofector®/supplement/plasmids solution without creating bubbles, and transfer 100 μL of it into the cuvettes provided with the kit. When performing the electroporation itself, the fabricant protocol says that either the electroporation program U-024 or U-028 is fine. In our empirical experience, the program U-028 gives a better survival rate.
7. Avoid vigorous pipetting in all steps immediately after electroporation, as it seems to decrease cell survival.
8. It is advisable to change the medium of the cells no longer than 14 h after electroporation. Whenever we changed the medium at a time longer than that, we noticed a decrease in cell survival.
9. Drug selection with blasticidin (5 μg/mL) is directed against the Regulus element of the bipartite system. For articular chondrocytes in primary culture, the drug selection lasts 5 days,

whereas it requires 14 days for ATDC5 cells. Theoretically, the longer the selection period, the more robust transgene expression is achieved; however, 14 days' drug selection for the articular chondrocytes causes them to lose their polygonal shape, a sign of their dedifferentiation. Nevertheless, 5 days' drug selection still works well for them, as can be seen in Fig. 2b.

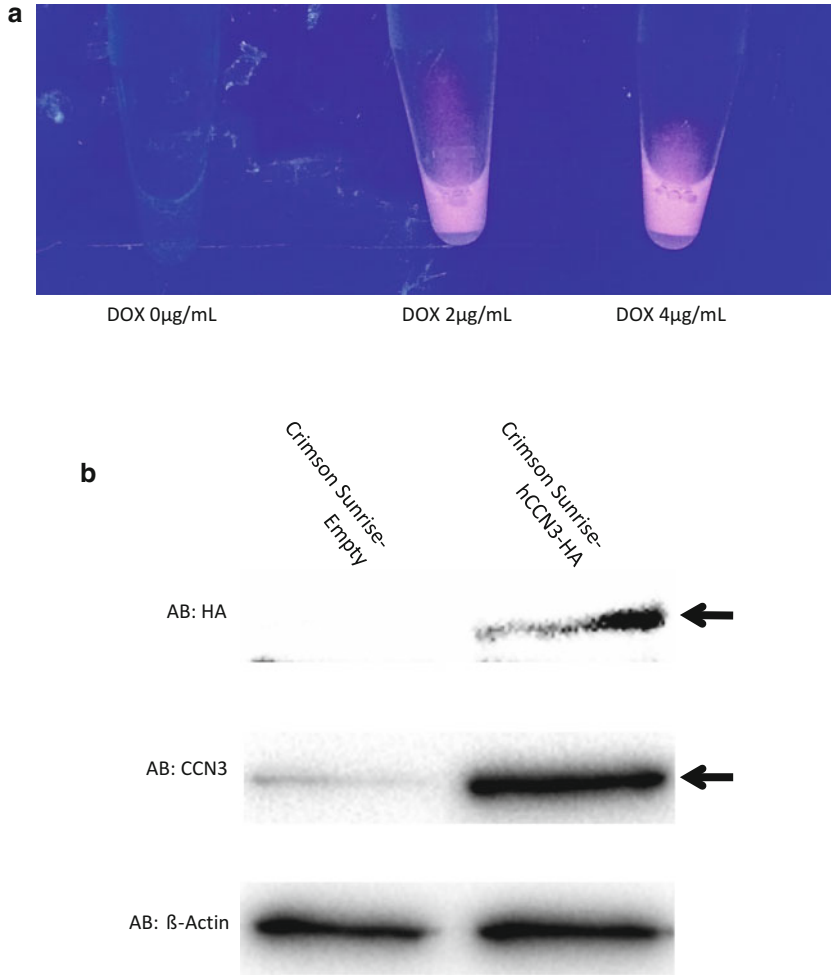

Fig. 2 Representative figures showing the expression levels of the transgenes reached by the methods described in this chapter. (**a**) Cell lysate of ATDC5 cells that were transduced with the Crimson Sunrise-empty plasmid, visualized with an ultraviolet transilluminator. After 14 days' drug selection, the cells were replated at 10^4 cells/cm^2, cultured to confluence, and treated with doxycycline at the indicated concentrations for 10 days prior to the lysate collection. Note that the resulting bulk culture after 14 days of drug selection expresses the transgene so intensely that the tdTomato fluorescence can be detected with a conventional ultraviolet transilluminator. (**b**) Western blot of the cell lysate of articular chondrocytes (in primary culture) that were transduced with Crimson Sunrise-hCCN3-HA. After 5 days' drug selection, the cells were replated at 5×10^4 cells/cm^2, cultured to confluence, and treated with 4 μg/mL of doxycycline for 14 days. In this case the drug selection period must be shorter (5 days), as explained in **Note 9**; but still robust recombinant human CCN3 expression is obtained. Total protein per lane is 3 μg. The allows indicate hCCN3-HA protein

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Chapter 12

Preparation of Module-Specific Antibodies Against CCN Family Members

Satoshi Kubota and Masaharu Takigawa

Abstract

Specific antibodies against biomolecules are conventional, but robust tools for the structural and functional analysis of target molecules. Since CCN family proteins are composed of four distinct modules that together determine the functionalities as full-length molecules depending upon extracellular microenvironment, specific antibody against independent modules are quite useful in CCN family research. Three distinct strategies are considerable for raising antibodies specific to four modules: IGFBP, VWC, TSP1, and CT modules. In the first strategy, full-length CCN family proteins are used to immunize mice to obtain a number of hybridoma clones producing different monoclonal antibodies, which are to be characterized to locate the epitopes in particular modules. Second methodology is a straightforward one, in which each modular protein fragment or synthetic peptide is prepared and is used for the immunization of animals independently. Finally, DNA immunization technology is recently known to be useful in developing module-specific antibodies against CCN family proteins as well. Preparation of antibodies is a quite classical and established technique, and thus nowadays is managed mostly by professional and commercial facilities. Therefore in this chapter, essentials of each strategy are introduced, rather than experimental details in each process.

Key words CCN family, CTGF, Monoclonal antibodies, Modules, DNA immunization

1 Introduction

Antibodies can be utilized not only for specific detection of proteins and other molecules with higher ordered structures, but also for the purification and functional characterization of these biomolecules. As described in other chapters, antibody affinity column chromatography enables us to prepare highly pure recombinant proteins of our interest. Blocking/neutralizing antibodies is widely used to ascertain particular aspects of molecular function. Conversely, antibodies are occasionally utilized for the stimulation of cell surface receptor molecules as agonists. As such, in order to execute these highly specified missions, antibodies are sometimes required to recognize and bind to particular substructures, such as domains and modules, in the protein. Here, scientific significance

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and utility of module-specific antibodies against CCN family members is emphasized.

All but one CCN family protein members consist of conserved tetramodular structure with IGFBT, VWC, TSP1, and CT modules [1, 2]. These modules bind to their proper cofactors, and the net functionality of the full-length CCN family protein is determined under the collaboration of these four modules [2, 3]. Therefore, blocking the function of a particular module by a module-specific antibody would provide us precious scientific information in clarifying the molecular function of the CCN family protein [4, 5]. Moreover, another reason why module-specific antibodies are needed for CCN2 research can be pointed out. According to a number of previous studies, several CCN family members are present in our body, not only in the form of full-length protein, but also as modular fragments processed from the full-length [2, 6–10]. These truncated forms of CCN family proteins can be produced either by alternative splicing at a posttranscriptional stage, or by posttranslational processing by proteinases. At present, molecular function of a significant part of these CCN family fragments remain to be clarified. Obviously, module-specific antibodies are powerful tools to explore this issue.

For the preparation of module-specific antibodies against the CCN family proteins, several different approaches may be employed, each of which can be meritorious or inappropriate, depending upon the specific aim and design of experiments. Nowadays, development of antibodies specific against particular antigens is mostly conducted as professional services. Thus, instead of repeating the general protocols for established methods to prepare antibodies, essential comparison of the methodologies that are actually applied for raising CCN family module-specific antibodies is described herein.

2 Materials

2.1 Monoclonal Antibodies for General Use (with a Full-Length Immunogen)

1. Recombinant CCN family proteins for immunization and epitope mapping: Purified human recombinant CCN proteins produced by eukaryotic cells can be used to raise high-quality antibodies even against particular modules in CCN family proteins. For the preparation protocol, *see* Chapter 10. Bacterially produced CCN family individual modules are usually utilized for module-wise epitope mapping, as well as for immunizing animals. Experimental procedures to prepare these modular proteins are detailed in Chapters 8 and 9. Glutathione *S*-transferase (GST)-fusion technique is also useful for the production of these proteins [11].

2. Animals: For regular and genetic immunization to obtain monoclonal antibodies, BALB/c female mice are used.
3. Materials used in Western blotting analysis: Reagents and apparatus required for Western blotting analysis is described in Chapter 5.

2.2 Polyclonal Antibodies for General Use (with Synthetic Peptide Immunogens)

1. Synthetic peptides as immunogens: Synthetic oligopeptides may be chemically synthesized to raise antibodies directly targeting a particular module of a CCN family protein (*see* **Note 1**). Nowadays, custom synthetic peptides are commercially available. Although highly purified peptides (>90%) are desired, peptides for immunization may not be necessarily pure, and thus those with >70% purity can be used. Conjugating keyhole limpet hemocyanin (KLH) to the peptide as a carrier protein is recommended, since it remarkably enhances the immunogenicity of small molecules, such as peptides (*see* **Note 2**).
2. Animals: New Zealand White rabbits are recommended for obtaining antisera against CCN family proteins.
3. Materials for ELISA: *See* Chapter 16.

2.3 Antibodies Reacting to Modules in the Full-Lengths with Native Conformation

1. Expression plasmids for DNA immunization and epitope mapping: Theoretically, any mammalian constitutive expression vector for CCN family proteins is applicable. In order to drive the genes of immunogens, cytomegalovirus (CMV) and human β-actin promoters have been used. For DNA immunization, it is recommended to add glycosylphosphatidylinositol (GPI) anchor sequence following the coding region of the CCN family genes [13]. Addition of a peptide recognition tag, such as FLAG tag, between the signal peptide and the N-terminal module (recommended) is also necessary. Similar molecular constructs expressing each modular protein fragments need to be prepared mainly for epitope mapping.
2. Materials used in transient DNA transfection: For transient DNA transfection, either cationic liposome-mediated, DEAE-dextran, calcium phosphate, or electroporation method can be employed, since long-term cell culture may not follow it.
3. Cells for epitope mapping: Mammalian cell lines that are widely used in transient DNA transfection can be utilized. Usually, Chinese hamster ovary (CHO) cell line and human embryonic kidney (HEK) 293 cell line are used for this purpose and have yielded satisfactory results [13]. These cell lines can be maintained in regular Dulbecco's modification of Eagle's minimum essential medium (D-MEM), 10% fetal bovine serum (FBS).
4. *Microprojectile coated with DNA*: Gold particles of 0.6–1.6 μm in diameter (Bio-Rad), those of 2–5 μm in diameter (Alfa, Ward Hill, MA), or tungsten particles (Sylvania) around 4 μm in

diameter are used as microprojectile. For DNA coating, 2.5 M $CaCl_2$ and 1 M free base spermidine are required. Ethanol (70% and absolute) is also used for the wash and storage.

5. Particle bombardment for DNA immunization: A particle bombardment is necessary to introduce microprojectile coated with plasmid DNA in vivo [12, 14]. For example, Helios Gene Gun (Bio-Rad) is commercially available.
6. *Animals*: *See* Subheading 1, **item 2**.
7. Materials used in flow cytometry: Dissociation Buffer (Invitrogen, Carlsbad, CA) is recommended to collect CHO and HEK293 cells from adherent cell culture. EDTA (1 mM) in Dulbecco's phosphate buffered saline can substitute it. Immunostaining and flow-cytometric analysis are performed in PBS, 0.5% bovine serum albumin. An antibody conjugated with a fluorescent dye against the peptide tag fused to the CCN family protein, for example, anti-FLAG Cy5 conjugate, is also required.

3 Methods

3.1 Monoclonal Antibodies for General Use (with Full-Length Immunogens) (Fig. 1a)

Any monoclonal antibody recognizes a specific structure in the antigen. Therefore, unless it is created to recognize a trans-modular three-dimensional structure, most of monoclonal antibodies raised against a full length CCN family protein are module specific. In this way, one may expect several module-specific antibodies by a module-wise epitope mapping of monoclonal antibodies raised against a full-length CCN family protein (*see* **Note 3**). Obviously, it is more time-consuming to immunize animals with single modules in order to obtain antibodies against four modules independently than with the single full-length. In addition, larger proteins with more complex structure show higher antigenicity than the small and simple ones. Collectively, such a conventional method followed by module-specific epitope mapping is recommended to obtain module-specific antibodies for general use including Western blotting, immunoprecipitation analysis, ELISA, immunohistochemistry, and affinity chromatography. This method is well established and commercial service is available from a number of companies, except for module-specific epitope mapping. Below is

Fig. 1 (continued) all the antigens for both immunization and screening are used in a form displayed on the cell surface. This methodology is particularly suitable in the preparation of antibodies for diagnostic and therapeutic purpose, as well as for functional assays. (**d**) Module-wise DNA immunization to obtain antibodies that recognize modular fragments with no reactivity to the full length. In this experimental system, antibodies against fragment-specific conformation can be produced and selected. Discriminative detection of modular fragments with such antibodies can be valuable from medical point of view, particularly in the case of CCN2

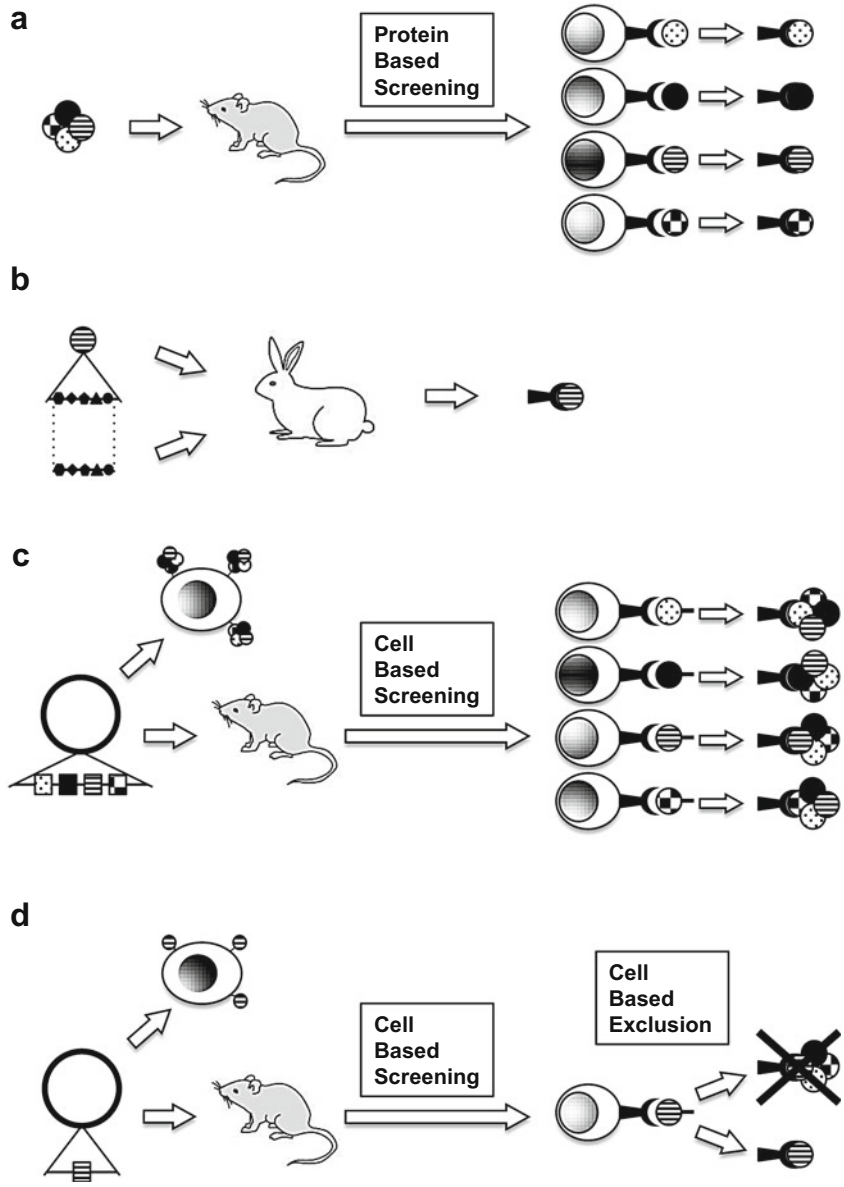

Fig. 1 Distinct strategies to be employed for the preparation of module specific antibodies under distinct objectives. *Spheres* with different patterns represent four modules in a CCN family protein, to which module specific antibodies bind. Four modular complexes denote full length CCN family proteins. *Elliptic objects* with nuclei indicate cells: The larger ones indicate those used for the verification of membrane-anchored display of CCN family proteins and modules, whereas smaller one with surface antibodies represent hybridomas. (**a**) Regular protein-based immunization to obtain module-specific anti-CCN family antibodies for general experimental use, in which screening is performed by solid phase ELISA and Western blotting. Different antibodies recognizing different modules can be usually obtained by immunizing mice with a single full-length CCN family protein. (**b**) Single modular immunization to obtain specific antibodies against a particular module. For the modules with low immunogenicity in the context of full-length protein, this strategy is useful. Synthetic peptides illustrated below the module can be used, occasionally as haptens. (**c**) DNA-immunization strategy to obtain module-specific antibodies that recognize native conformation in the context of full-length CCN family proteins. In this method,

a brief summary of a method that successfully raised a monoclonal antibody against a VWC module in human CCN2 [15].

1. Immunization of mice: The recombinant CCN family protein produced by HeLa cells and purified through heparin affinity column chromatography (*see* Chapter 10) is used as an immunogen. Every 7 days, female Balb/c mice are injected with the antigen on foot pad, followed by booster injection of Freund's adjuvant after 2 days. A few days after the last booster injection, sera are obtained from mice, and the presence of anti-CCN family protein antibodies is examined by ELISA.
2. Preparation of hybridomas: Cells containing immunized lymphocytes were obtained from popliteal lymph nodes. Hybridomas were produced by fusing these cells with mouse myeloma cells with PEG 1500.
3. Primary screening of hybridomas: Screening of hybridoma should be performed with the full-length CCN family protein via a conventional ELISA system. It is not recommended to use independent modular proteins at this stage.
4. Module-wise selection and epitope mapping: Western blotting analysis as described in Chapter 5 is employed. Recombinant single modular proteins prepared following the methods shown in Chapters 8 and 9 are independently loaded onto a SDS-polyacrylamide gel. These test-antigens are not necessarily highly pure (*see* **Note 4**) [16]. If an antibody failed to detect any of the four modules, native PAGE with each module and the full-length should be considered. Large-scale production of antibodies will follow the establishment and selection of hybridomas after epitope mapping, following a standard procedure.

3.2 Polyclonal Antibodies for General Use (with Synthetic Peptide Immunogens) (Fig. 1b)

Although module-specific antibodies can be obtained in most cases by the abovementioned technique by immunizing mice with a single full-length protein, one may find possible difficulties in raising antibodies against particular modules by this method (*see* **Note 5**). Owing to the structural hindrance, such a module may be folded inside the protein at the native condition, restricting the accessibility of immune cells. Even in this case, by immunizing animals with a synthetic peptide involved in the module, antibodies recognizing corresponding modules of CCN family proteins can be produced. Recombinant proteins of CCN modular fragments may be used as well, while preparation of synthetic peptides is generally easier and requires less time. However, it should be noted that antibodies raised against a synthetic peptide occasionally display higher background and nonspecific signals. Commercial antiserum production service is commonly available mostly with rabbits. Only a brief overview is described here.

1. Immunization of animals: Each rabbit is immunized subcutaneously by >0.2 mg of a synthetic peptide of around 20 amino acid residues with equal volume of Freund's complete adjuvant. Six cycles of immunization with an interval of 2 weeks is preferred, although it may be performed every week. It is important to bleed from the rabbits before immunization (for control). In case that immunization of rabbits is not efficient, guinea pigs can be used in place, which may yield different results. More than two animals should be used for the immunization by an antigen, since reactivity to a specific antigen may significantly differ among individual animals.
2. Bleeding: In case of rabbits, bleeding is performed from auricular marginal vein except for the last one. Test bleeding is started after the second immunization at a small scale (3–30 ml) per rabbit. Sera are immediately prepared from the blood samples and are subjected to titration. For final exsanguination, bleeding is carried out from the heart. Usually, >120 ml of blood can be obtained from a single rabbit.
3. Titration: Every time after the test bleeding, the titer of the serum is evaluated by solid-phase ELISA with the immunogen (*see* **Note 6**). This process is critical, especially at a later stage of immunization; for it provides the information to decide whether additional immunization is necessary, or not. Since the animals are immunized by antigens specific to modules, epitope mapping may be unnecessary.

3.3 Antibodies Reacting to Modules in the Full-Lengths with Native Conformation (Fig. 1c)

Protein function is determined principally by the three-dimensional conformation rather than the primary peptide sequence itself. In many cases, functional domains are constructed by assembling different amino acid residues that are distant in terms of primary peptide sequence through folding process. Therefore, antibodies that recognize such tertiary structure have better chance to modify the function of target proteins than the ones recognizing short linear peptide sequences. Such antibodies are quite useful for specified experimental or therapeutic aims. The most typical example is the utility of neutralizing antibodies. Among biological researches, these antibodies are used to confirm whether a particular biological event is mediated by the protein of interest, or not [5, 13]. More generally, humanized neutralizing antibodies [17] are widely used in actual clinics for molecular targeted therapeutics. In order to develop the antibodies targeting functional domains in tertiary structure, correctly folded native proteins have to be used as immunogens. However, preparation of purified CCN family proteins with correct conformation is difficult, since these proteins are easy to aggregate during purification process (*see* **Note 7**). Another problem for us is that little is known about the native tertiary structure of CCN family proteins. To overcome this difficulty, instead of administrating

recombinant CCN family proteins exogenously, one may think about producing them inside of animals by DNA immunization. Also, please note that an antibody recognizing tertiary structure of a protein usually does not react to denatured target antigen [13]. In other words, antibodies raised by this method may not be usable in Western blotting analysis (*see* **Note 8**).

1. Confirmation of cell surface display of the antigen by transient transfection of the expression plasmid: For CHO or HEK293 cells, most of conventional transfection methods are applicable, such as classical calcium-phosphate co-precipitate, DEAE-dextran, cationic liposome-mediated transfection and electroporation. All of these methods are established and protocols are available with corresponding experimental kits. Particle bombardment for DNA immunization may be also utilized (*see* next item). Forty-eight hours after transfection, the cells are recollected in 1 mM EDTA or Dissociation Buffer. After a wash with PBS, the cells are blocked for Fc-mediated nonspecific binding and are fluorescently stained with an antibody against the recognition tag fused at the N-terminus of the CCN family protein, and are analyzed by flow cytometry (*see* **Note 9**).
2. DNA immunization of mice and preparation of hybridoma cell lines: The gold microprojectile coated with the plasmid DNA is directly injected intradermally with a particle bombardment biweekly (*see* **Note 10**). DNA immunization is performed three times in total. Production of anti-CCN family antibodies in the sera can be monitored by flow cytometry with the cells described in the last item. Three days after the last immunization, mice are processed for the collection of B-lymphocytes. Hybridoma cell lines are prepared following an established standard procedure.
3. Primary screening of hybridomas: In this method, screening of hybridoma is performed with a cell-based ELISA system (*see* **Note 11**). The cells displaying native CCN family proteins are seeded and cultured until 80–90% confluence in 96 well multiwell plates, instead of coating recombinant CCN proteins. Although basic experimental procedure is based on the conventional ELISA, growth medium is used for all of the wash steps to keep the viability of the cells. Also, all of the handling processes should be gently performed.
4. Cell-based module-wise epitope mapping: After establishing hybridoma clones producing monoclonal CCN family proteins, module-wise epitope mapping is carried out by flow cytometry with the cells expressing membrane-anchored CCN family modular fragments. Reactivity to full-length CCN family proteins is subsequently confirmed by the same cell-based strategy. Western blotting analysis may be employed to determine if the antibody recognize a linear epitope and can be used in this method.

3.4 Antibodies Reacting to Fragment-Specific Conformational Epitopes (Fig. 1d)

In certain macromolecules, fragmentation of intact protein occasionally forms new structures (neo epitopes) that can be recognized by immune system. Antibodies recognizing these structures can distinguish protein fragments from the intact ones without size information. This type of antibody is particularly useful in CCN family research and for the development of CCN family-mediated diagnostics. Indeed, we can analyze the histological distribution of CCN4 subfragments in tissue sections and screen samples from patients for CCN2 N-terminal fragments associated with fibrotic disorders [10]. Preparation strategy is essentially the same as that used in last subsection. Negative selection process to exclude the ones with reactivity to the full length replaces the epitope mapping process in the last subsection.

1. Confirmation of cell surface display of the module by transient transfection: Under the same experimental methods described in Subheading 3.3, **step 1**, cell surface expression of the module of interest by DNA transfection is confirmed with a plasmid constructed to express the tagged module in a membrane-anchored manner.
2. DNA immunization of mice and preparation of hybridoma cell lines: Intradermal DNA immunization with the module expression plasmid and hybridoma preparation are performed as described in Subheading 3.3, **step 2**.
3. Primary screening of hybridoma cell lines: As described in Subheading 3.3, **step 3**, screening of hybridoma is performed with the cell-based ELISA system with those expressing the modular CCN family fragment.
4. Cell-based negative selection of clones reacting to the full length: Among the hybridoma cell lines producing antibodies against membrane-anchored modular fragments on the cell, clones producing the ones that bind to the full length CCN family proteins are excluded by cell-based experimental systems. If a number of hybridoma cell lines are obtained, primary selection may be based on the cell-based ELISA system. If not, and as a secondary selection, flow cytometry with the cells expressing full length CCN family proteins are employed. As a result of negative selection, several antibodies reacting to both the modular fragment and full-length CCN family protein are excluded from the group of desired antibodies. However, it is strongly recommended to keep and utilize them for alternative objectives.

4 Notes

1. Peptides for immunization can be designed by a commercial service, but it is important to know what kind of peptides are suitable as antigens. To obtain an antibody that react to the

peptide in the context of corresponding protein, it has to be exposed to surface in solution. In general, peptides with a significant number of hydrophilic amino acid residues, and those located amino termini (excluding signal peptides for secretion) or carboxy termini are selected. If tertiary structures of CCN family members were clarified, it is relatively easy to deal with this issue, but limited information is available to date [18]. A number of computer software programs to predict the secondary structure of proteins are available and can be utilized for this purpose [16].

2. We prepared antisera against the TSP1 module of CCN2 with a synthetic peptide of an amino acid sequence, H_2N-Arg-Pro-Cys-Glu-Ala-Asp-Leu-Glu-Glu-Asn-Ile-Lys-Lys-Gly-Lys-Cys-Ile-Arg-Thr-Cys-COOH. Several rabbits were immunized by this peptide with or without conjugating carrier KLH. Antisera that could yield data of publication quality were obtained only from the animals immunized by the KLH-conjugate.
3. Under such a conventional method, we could obtain four module-specific monoclonal antibodies against human CCN2 by immunizing mice with a partially purified full length human CCN2. One antibody was raised against the IGFBP and CT modules, respectively, whereas two different ones were found to react to the VWC module [16]. A few antibodies supposed to recognize tertiary structure of CCN2 were also found.
4. As antigens for module-wise epitope mapping, crude culture supernatants from *Brevibacillus choshinensis* producing single modular fragments of CCN family proteins are quite useful [16]. As described elsewhere, CCN family single modules are abundantly secreted out from these transformed bacteria. Usually, produced modular proteins can be recognized simply by SDS-PAGE and silver staining of crude culture supernatants as extra bands in the gel.
5. By immunizing mice with a full-length recombinant CCN2 from mammalian cells, no monoclonal antibody recognizing TSP1 was obtained [16]. This result was consistent with the in silico prediction of antigenic peptide sequences in human CCN2. Instead, we prepared antisera specific to TSP1, as described in **item 2** of this section.
6. It occasionally happens that even if an antiserum shows extremely high reactivity to the peptide used for immunization, it does not perform well for Western blotting. In those cases, high levels of nonspecific signals are overwhelming specific ones. Interestingly, individual rabbits immunized against the same oligopeptide produce antisera with variable titers and nonspecific signal levels. Selecting a best animal for each antigen is critical.

7. In fact, one should take particular care of the pH of the solvent used CCN2 purification. In this process, acidic conditions are preferred; otherwise, aggregate may be formed, which represent the structural instability of CCN2. It is suspected that CT module is mainly responsible for this structural instability, since a single modular recombinant protein of CT was found to easily form precipitate during purification procedure.
8. Following this DNA immunization strategy with a full-length CCN2 expression plasmid, specific monoclonal antibodies were raised against all of the four modules of human CCN2. Although all of the these antibodies efficiently recognized their target modules displayed on the cell surface, none of them did not recognize CCN2 under a denatured condition by Western blotting.
9. General procedure for flow cytometry can be found in a number of literatures [19]. No enzymatic treatment is allowed in the recollection process of the cells. Particularly, when 1 mM EDTA in PBS is used to detach the cells from the tissue culture dishes, careful pipetting is required for obtaining single cell suspension.
10. Strategy and methodology of this method are described in original literature [12–14].
11. Additional information is available in an original article published in 2004 [20].

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Chapter 13

ELISA of CCN Family Proteins in Body Fluids Including Serum and Plasma

Satoshi Kubota, Harumi Kawaki, and Masaharu Takigawa

Abstract

Enzyme-linked immunosorbent assay (ELISA) is the most popular methodology for absolute quantification of particular proteins in liquid samples. Especially for CCN family members that are associated with human diseases, utility of ELISA for those proteins in clinics as well as research laboratories is emphasized. However, in order to obtain accurate and stable results in ELISA, particular care should be taken in controlling the quality and quantity of standard CCN family proteins, which bind to various materials and can be unstable in a purified form. Recently, diagnostic value of the CCN family protein fragments in body fluids has been indicated in several diseases. Therefore, module-specific detection system for the CCN family members is desired as a promising tool in clinics. It should be also noted that modular fragments of CCN family members can be more stable than the full-length in purified forms, whose quality may be easier to control than that of the full-length ones.

Key words CCN family, ELISA, Platelet, Fibrosis

1 Introduction

Before twenty-first century, antibody-mediated recognition was almost the only method for the discriminative detection of macromolecules in liquid samples. Although biosamples are usually small in amount, containing a number of different molecules and sometimes involving limited concentrations of target proteins, immunodetection enables us to detect, or even quantify them therein. Particularly, enzyme-linked immunosorbent assay (ELISA) has been widely used in clinics as well as research laboratories, since absolute quantification of proteins can be relatively easily performed, so far as the system is well controlled and established. In comparison with Western blotting analysis, more accurate quantification is possible through a simpler experimental procedure, which does not critically depend on operator's skill. Recent advance in biotechnology yielded more accurate and comprehensive strategy for absolute quantification of proteome, which is represented

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by mass spectrometry with associated techniques. Nevertheless, such methodologies usually require special equipment and facility that may not be affordable for independent research groups and clinics. In addition, only well-trained operator can run such equipment. As such, ELISA still remains to be a major method to quantify a protein of interest in biosamples.

Diagnostic utility of CCN family members in diseases is best represented by CCN2 in fibrosis and other related human disorders [1–4]. According to recent reports, blood CCN2 level correlates the severity of chronic heart failure [5], scleroderma [6], liver cirrhosis [7], pulmonary fibrosis [8], biliary atresia [9], and kidney fibrosis [10]. Urine CCN2 level is also proposed as a biomarker of diabetic nephropathy [11]. Of note, accumulation of amino-terminal CCN2 fragment in blood and vitreous was found in scleroderma and proliferative diabetic retinopathy patients, respectively [6, 12]. Also in the case of CCN4, involvement of modular variants in gastrointestinal malignancies was reported [13]. These findings suggest a novel utility of module-specific ELISA system in medicine.

In this way, a number of research groups including those in commercial companies, have been trying to establish ELISA systems for the quantification of full-length CCN2 and its modular fragments. However, relatively less CCN family ELISA kits are commercially available than other popular growth factors. This is probably because of the difficulties associated with the molecular property of CCN family proteins. Although an established ELISA system is available, one should be careful in handling the components of the kit, particularly the standard protein, taking this point into account. Module-specific ELISA kit is currently not commercially available. Using module-specific antibody against CCN family members, an ELISA system that quantify a modular fragment excluding the full-length can be constructed, as described below.

2 Materials

Currently, a significant number of ELISA kits (*see* **Note 1**) and ELISA development kits (*see* **Note 2**) for the CCN family members are commercially available. Nevertheless, since no kit to specifically quantify a modular fragment of a CCN family member, it is necessary to construct the entire ELISA system for this objective. Below are typical examples of the materials needed.

1. Multiwell plates for coating primary antibodies: Ninety-six flat-bottom wells for ELISA are widely available from a variety of manufacturers (*see* **Note 3**).
2. Antibodies: Two antibodies recognizing different epitope on a CCN family protein are required for the development of a

sandwich ELISA system to warrant target specificity. One of them should recognize a tertiary structure of the CCN family protein rather than a linear epitope (*see* **Note 4**). Either horseradish peroxidase or alkaline phosphatase or biotin is going to be conjugated one of these antibodies.

3. Blocking buffer: Dulbecco's phosphate buffered saline (PBS), 3% bovine serum albumin (BSA).
4. Antibody diluent: PBS, 1% BSA.
5. Protein standard: A purified recombinant CCN family protein or modular fragment in 1% BSA is prepared at the highest concentration as a standard and aliquots into adequate numbers of small tubes at a volume of 100 μl each. It is critical to determine the accurate concentration of the standard CCN family protein. All of the aliquots are recommended to be stored at −80 °C and are for single use. Do not refreeze and reuse them after the first use (*see* **Note 5**).
6. Fluorometric (or colorimetric) substrate.

   In our system, 4-methylumbelliferyl-β-d-galactoside (UDG) is used as a fluorometric substrate in combination with β-galactosidase as the conjugated enzyme: 100 mM UDG, 10 mM Na/K phosphate buffer, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% BSA. Additionally as a colorimetric substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) is most widely utilized with horseradish peroxidase.
7. Stop solution: For terminating the enzymatic reaction by β-galactosidase, we use 1 M Na_2CO_3. In case that horseradish peroxidase is utilized, 1 M H_2SO_4 works as perfect stop solution.
8. Wash buffer and sample diluent, PBS-T: PBS, 0.05% Tween 20.
9. Streptavidin–enzyme conjugate: In order to amplify the signal from the secondary antibody, the biotin–avidin system is frequently utilized also in sandwich ELISA systems. In our hands, streptavidin–β-galactosidase conjugate yields stable results: 0.01% streptavidin–β-galactosidase conjugate, 20 mM HEPES, 0.5 M NaCl, 0.1% BSA.
10. Micro plate reader: Depending upon the substrate for signal detection, a micro plate reader for fluorometric or colorimetric signal quantification is necessary. The former one measures fluorescence emitted from the sample, whereas the latter quantifies optical absorbance through the sample. Since methodologies of signal detection are totally different between the two, it is not easy to find a single machine that can quantify both types of signals. A machine measuring both fluorescence and luminescence (for luciferase assay) is commonly available (e.g., Thermo Fisher Scientific, Waltham, MA, USA).

11. Multichannel pipettes (Optional): In order to accelerate the sample dispensing, this apparatus is strongly recommended. This device enables us quick and easy liquid handling, and thus possible experimental errors and variations in results can be minimized with it (*see* **Note 6**). Since 96-well plates are generally used for regular ELISA, 8-channel pipettes are particularly useful. This type of pipette is available widely from a number of relevant companies (e.g., Gilson, Middletown, WI, USA).
12. Multiwell plate washer (Optional): Especially in case of clinical ELISA, an automated programmable plate washer is sometimes used to economize the time and effort of laboratory technicians. Even in a research laboratory, if ELISA is performed daily, such a machine is recommended; for it also minimizes variations in results caused by wash processes. Many suppliers handle such plate washers (e.g., Thermo Fisher Scientific).

3 Methods

3.1 Development of a Modular Fragment-Specific ELISA System

Since a conventional ELISA kit is commercially available for each CCN family member, it is not noteworthy here to describe a general method to develop a conventional ELISA system. However, modular fragment-specific ELISA system is not commercially available. In order to quantify a modular fragment of a CCN family protein, excluding the full-length protein, two distinct strategies are considerable. The first one is to establish an ELISA system, in which the modular fragment is selectively recognized. In the other strategy, two ELISA systems are needed; one recognizes both modular fragment and the full length, while the other does the full length only. By subtracting the value obtained by the latter system from that by the former one, amount of the modular fragment can be computed. In this section, a methodology aiming to develop an ELISA system that enables the first strategy is introduced [14].

1. Selection of antibodies for modular fragment detection: Specific recognition of an antigen completely depends upon the selection of antibodies used in an ELISA system. Therefore, this step is the most critical in constructing an ELISA to selectively quantify a modular fragment of a CCN family protein. For this objective, most feasible primary antibody is the one that modulate the function of a full-length CCN family protein upon binding to the module of the interest. Functional modulation of a protein is usually caused by its structural alteration. Therefore, such an antibody is expected to change the tertiary structure of the full-length protein, which may result in the interference with the recognition by subsequent secondary

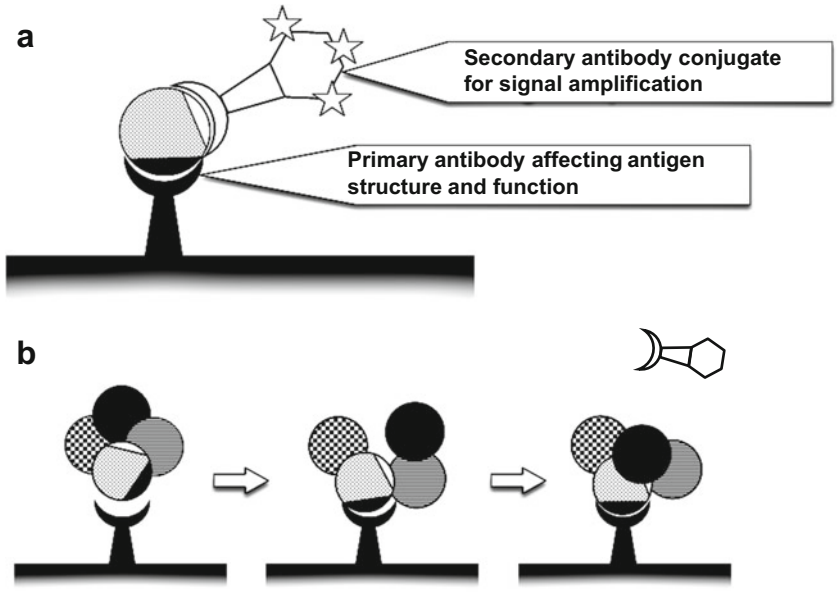

Fig. 1 An ELISA system that selectively quantify a modular fragment without recognizing the corresponding full-length CCN family protein. (**a**) Schematic representation of the detection of the target single module of a CCN family member by the sandwich ELISA system and antibodies employed therein. Primary and secondary antibodies are represented by solid *black* and *white* objects, respectively. A *sphere* represents the single module as the antigen, in which epitopes for primary and secondary antibodies are indicated by solid *black* and *white* areas, respectively. A *hexagon* conjugated to the primary antibody denotes biotin, whereas avidin-conjugated enzyme molecules are represented as *stars*. (**b**) A model explaining the mechanism of the specific detection of the single modular fragment. Immunocomplex formation between the primary antibody and the full-length CCN family member interferes with subsequent interaction between the antigen and secondary antibody. Structural alteration by primary antibody–antigen interaction leads to the structural hindrance of the secondary epitope, resulting in the inaccessibility for the secondary antibody

antibody (Fig. 1). Neutralizing antibodies and antibodies that rather enhance the biological activity of antigens may fall into this category [15–17] (*see* **Note 7**). If a secondary antibody recognizes a distinct epitope that is hindered by the structural modulation induced by primary antibody, we can successfully construct an ELISA system for modular fragment specific quantification.

2. Conjugation of the secondary antibody: To maximize the sensitivity, chemical conjugation of biotin to the primary antibody is recommended. More simply, horseradish peroxidase can be also covalently conjugated directly to the antibody. In most cases, conjugation is carried out via amine coupling method. Experimental kits are commercially available from a variety of suppliers, and conjugation should be performed following the manufacturer's instruction.

3.2 Sampling for the Quantification of CCN2 in Body Fluids

Considering the application to clinical diagnostics, ELISA is the most powerful tool for the evaluation of biomarkers. Until today, a number of clinical studies indicated the significant correlation between CCN2 levels in body fluids and related human diseases, most of which are fibrotic disorders of particular tissues and organs. These reports indicate possible utility of CCN2 as disease markers. In contrast, few reports can be found concerning the other CCN family members in this point of view. Therefore in this subsection, utility of CCN2 ELISA systems and sampling tips in diagnostics is summarized, based on the current relevant knowledge.

3.2.1 Plasma and Sera

It is widely recognized that blood CCN2 level has significant correlation to the severity of chronic heart failure, pulmonary fibrosis, biliary atresia, chronic viral hepatitis, and allograft renal fibrosis [3, 7–10], suggesting its potential as a biomarker of these human disorders. However, it is important to note that platelets contain abundant CCN2 in their granules and release CCN2 upon activation, which means a major part of serum CCN2 may originate from normal platelets [18]. If elevation of CCN2 level caused by such diseases is drastic enough to overwhelm the platelet-derived background, serum CCN2 level can be a biomarker of relevant diseases. Otherwise, useful results are hardly expected. One should also note that platelet numbers and CCN2 content per a platelet might vary among individuals. Therefore, plasma is more reliable for this objective. However, particular care should be taken to avoid blood coagulation during bleeding process and centrifugation (*see* **Note 8**).

Another possible problem in clinical research is blood CCN2 levels can be affected by particular medication. A typical example is observed in patients treated by corticosteroids; for these ligands strongly induce the CCN2 gene expression at a transcriptional level [19] (*see* **Note 9**).

3.2.2 Urine

Urine is one of the body fluids that can be obtained by minimal intervention and thus is a feasible sample for medical screening. Previous studies suggest the utility of urine CCN2 as a biomarker for kidney fibrosis caused by diabetes mellitus [20]. Since diabetes mellitus is a common and serious problem threatening human life, diagnostic application of CCN2 ELISA in this field is highly expected.

3.2.3 Dermal Interstitial Fluid

Etiological relationship between CCN2 and scleroderma is now clearly recognized [15]. Moreover, recent advance in medical technology enabled us to obtain dermal interstitial fluid without using needles [21]. Since significant correlation between the CCN2 level in dermal interstitial fluid and severity of scleroderma is observed [6], a CCN2 ELISA system can be utilized to monitor the status of scleroderma in affected individuals.

3.2.4 Vitreous

Compared to the body fluids exemplified above, vitreous is not easy to obtain, which may delimit its utility in diagnosis. However, it is noteworthy that an N-terminal modular fragment of CCN2 is released herein upon proliferative diabetic retinopathy [12]. An ELISA system that can discriminatively quantify these fragments may greatly help to analyze the pathogenic role of the modular fragments and the mechanism of CCN2 fragmentation therein.

3.3 Custom ELISA

1. To coat an ELISA plate with the primary antibody, 50 μl of primary antibody at a concentration of 20 μg/ml in PBS is added to each well of 96-well ELISA plate. Coating is performed at room temperature (RT) for 1 h.
2. After a wash with PBS, block unbound surface of the well with 200 μl of 3% BSA in PBS for 2 h at RT.
3. Prepare serial dilution of the standard protein. During the coating and blocking process, dilute the standard protein serially twice per each step up to 16-fold of the original concentration (*see* **Note 10**). Since 50 μl is required for each well, and all the measurement should be performed in duplicate, >100 μl (120 μl recommended) of each standard is necessary.
4. After three washes with PBS-T and thorough removal of the wash buffer remnant (*see* **Note 11**), add 50 μl of plasma or other body fluid to each well in duplicate.
5. Continue incubation for 1 h at RT.
6. After three washes with PBS-T, add 50 μl of secondary antibody conjugate (usually, at a concentration of 1 μg/ml) to each well.
7. Incubate the plate further at RT for 1 h.
8. After final three washes with PBS-T, discard wash buffer thoroughly.
9. Add 50 μl of diluted streptavidin β-d-galactosidase for signal amplification for 30 min at RT.
10. Initiate enzymatic reaction by adding 50 μl of 100 mM UDG to each well for fluorometric detection. For colorimetric detection via peroxidase reaction, TMB is used instead (*see* **Note 12**).
11. Terminate reaction by the addition of 50 μl of a respective stop solution (*see* **Note 13**). Measure fluorescence at a wavelength of 460 nm with excitation at 355 nm. Color development after stop reaction by TMB is measured at a wavelength of 450 nm.

4 Notes

1. Although relatively few companies are supplying ELISA kits for CCN family members, nowadays, one can obtain an ELISA kit for any of the six CCN family members. Particularly, a number

of human CCN1 and CCN2 ELISA kits are currently available (R &D Systems, Minneapolis, MN, USA; Assay Biotechnology, Sunnyvale, CA, USA). In contrast, one, or only a few CCN6 ELISA kits are supplied commercially (Antibodies-online.com, Atlanta, GA, USA). For the other family members, the availability of CCN4 ELISA kits follows that of the kits for CCN1/2. One may have less choice in selecting a better kit for CCN3/5 quantification (Antibodies-online.com). However, a module or fragment-specific ELISA system for any CCN family member is still unavailable.

2. Several suppliers provide a set of antibodies and protein standard to construct an ELISA system following the attached protocol, which is occasionally designated ELISA development kit. Usually, such a kit does not include most of the items required for ELISA, except for the most critical components; primary antibody, secondary antibody conjugated with an enzyme and protein standard. If it is designed to amplify the signal via biotin–streptavidin system, avidin–enzyme conjugate is supplied, together with a biotin-conjugated secondary antibody (Peprotech, Rocky Hill, NJ, USA). In case that a number of samples are tested, it is generally economical to employ this type of kit than the regular ELISA kit.
3. We routinely use ELISA plates from Greiner (Kremsmünster, Austria).
4. When we constructed an ELISA system for the measurement of full-length human CCN2, a monoclonal antibody recognizing the tertiary structure was used as the primary antibody coated on the surface of 96-well plates [9]. While this antibody gave low background interactions with other proteins, it failed to recognize murine CCN2. As such, this ELISA system could be used only for human samples. High specificity in detection is usually accompanied by limited utility of an experimental system. As a secondary antibody, another monoclonal anti-CCN2 antibody that recognized a linear epitope in the VWC module was used as a form of an HRP conjugate, which could be utilized in Western blotting analysis as well.
5. In the CCN2 ELISA system described above, we underwent a difficulty in maintaining the quality of standard proteins. Even in the presence of BSA as a carrier protein, purified CCN2 was found unstable. When aliquots of standard CCN2 in BSA was stored at −20 °C, ELISA signals developed by the same set of aliquots rapidly decreased over time. Interestingly, such a "decayed" CCN2 gave signals comparable to a "fresh" one in Western blotting under denaturing condition, indicating that denaturation rather than degradation occurred during storage. If an antibody recognizing a linear epitope is employed, this

problem may be solved. However in this case, less accuracy and sensitivity are expected.

6. The multichannel pipettes are very helpful, but particular care should be taken for its use. First, we need to make sure that all of the channels are capable of dispensing the same amount of liquid. Second, even if the apparatus is working properly, careless loading of pipette tips may severely affect the outcome. Usually, eight tips are simultaneously loaded from a pipette stand onto the pipette by a single action. Ideally, all of the tips are stood along a straight line on a stand, receiving the same force upon tip loading. Also, the operator has to try to give a force evenly to all of the channels upon tip loading. Otherwise, some of the tips can be loosely loaded, causing inaccuracy and even dislocation during operation. Proper loading of tips can be confirmed by fingers, if necessary.
7. In our ELISA system to selectively quantify the VWC single modular fragment of CCN2, a monoclonal antibody recognizing a linear epitope in VWC was employed as the primary antibody. Of note, this antibody was shown to repress the bone metastasis of breast cancer cells, in which CCN2 plays a critical role, in an animal model [17]. In another study, this antibody was also found to enhance the effect of CCN2 on chondrocytic cells [16]. These findings together suggest that this antibody modify the tertiary structure of CCN2 upon binding, which results in the neutralization of a certain molecular function, while enhancing another functionality of CCN2. As such, an antibody inducing structural modification on the antigen is feasible for this objective.
8. We evaluated the CCN2 level in blood samples from patients with chronic heart failure and normal healthy donors with our CCN2 ELISA system. At an initial stage, blood draw was performed without particular care, and plasma was prepared for the ELISA assay [5]. However the result showed highly elevated level of CCN2 even in a few samples from healthy donors. Suspecting the contamination of platelet-derived CCN2 therein, the research was repeated with newly drawn blood samples from the same patients under the strict indication to avoid possible coagulation. As a result, elevated CCN2 levels were not observed in control samples, which provided significant difference from those in patient samples.
9. Since corticosteroids are strong CCN2 inducers and sometimes used in the therapeutics of rheumatoid arthritis and systemic sclerosis, therapeutic background of the patients needs to be extensively examined prior to their enrollment in corresponding studies. Not only the patients with these clinical complications, but also patients with chronic disorders that do

not require steroid therapy may have been administrated with corticosteroids for the treatment of another condition. Systemic view of patients is critically required for the research, as well as in clinics.

10. The range of concentration of the standard protein depends on the titer of the antibody used in the ELISA system and its signal developing strategy. For the measurement of a CCN family member from human samples, an ELISA system that can quantify several nanograms of the protein is desired. If desired sensitivity is not achieved with a secondary antibody simply conjugated with an enzyme, signal amplification with the biotin–avidin system should be considered.
11. Thorough removal of wash buffer at the last wash is quite critical. If an automated plate washer is applied, no particular care is necessary. When wash is performed manually, a majority of wash buffer can be removed simultaneously from all of the wells by simply shaking it off directly to a sink or container. No further action is necessary except for the final wash. After the final wash, the wash buffer remnant may be removed by a multichannel pipette from tilted multiwell plates without leaving a drop. Another simple, but rough method for removing the buffer remnant is to beat a few sheets of paper towels on a bench with the multiwell plate held upside down. This makes significant noise, which can bother others who are working nearby.
12. Signal development is an enzymatic process with shorter incubation period (usually 10 min or around) than that for immunocomplex formation. Therefore, it is very important to minimize the variation in incubation period among the wells. Especially, when a multichannel pipette is unavailable, one should take particular care of this point, as it may take more than 1.5 min to fill all of the wells. The order of the wells, to which the substrate is serially dispensed, should be recorded. Time spent for liquid dispense may not be different among the wells. Thereafter, when the reaction is stopped, the order of dispensing the stop solution has to follow the one for substrate addition, spending the same time period as that for substrate.
13. In general, time period for enzymatic reaction is optimized to be around 10 min at RT. However, this period ought to be flexible; for RT is not a strictly defined temperature. For example, in the USA, room temperature is always around 20 °C, whereas it can be 8–28 °C in Japan, depending upon the season, which affects the optimal incubation time for this step. Therefore, one needs to monitor the development of the signals in the wells during the incubation to decide when the stop solution should be added. If a colorimetric substrate is used, signal development can be monitored, simply by taking a look

at the wells for the standard samples. However, if fluorometric substrate is used, one has to repeat the measurement with a microplate reader, until the signals from the standard samples reach optimal levels. Please note that in case of TMB, signals develops in blue and turn to yellow upon the addition of the stop solution.

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Chapter 14

Analysis of Signaling Pathways Activated by CCN Proteins

Harumi Kawaki, Satoshi Kubota, and Masaharu Takigawa

Abstract

CCN family proteins activate multiple intracellular phosphorylated kinase cascades to yield the multiple physiological functions of a variety of target cells. In this chapter, we describe our protocol examining the effects of these proteins on signal transduction pathways, especially mitogen-activated protein kinase cascades, activated by CCN member proteins, which examinations have been carried out mainly by using Western blotting methodologies.

Key words ERK1/2 (p44/42 MAPK), p38 MAPK, JNK, MAPK pathway, PI3K-PKB/Akt pathway, CCN signaling, Western blotting

1 Introduction

As mentioned in the previous chapters, CCN family proteins are known to be involved in various physiological events such as normal tissue growth and differentiation, tumor growth, tissue regeneration, and wound healing [1–3]. CCN member proteins exert differential effects such as promotion of cell proliferation or differentiation, chemotaxis, migration, adhesion, and/or extracellular matrix formation on a variety of target cells in various tissues and organs [1–3]. We have demonstrated the differential effects of CCN family proteins, especially CCN2 among other CCN members [1–8]. Furthermore, we reported that two classical mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK)1/2 and p38 MAPK, are triggered by CCN2, to promote the proliferation and differentiation, respectively, of chondrocytes [8], whereas the c-Jun N-terminal kinase (JNK) pathway is used for the CCN2 signal transduction toward both proliferation and differentiation of chondrocytes [8]. Moreover, these MAPK pathways are critically involved in the CCN family member-mediated modification of osteoblast activities and endothelial cell activities [9–11]. MAPK pathways modulate fundamental cellular processes such as cell growth, proliferation, differentiation, migration, and apoptosis [12, 13]. In addition, the

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involvement of the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB)/Akt pathway or protein kinase C (PKC) has been indicated to promote chondrocyte differentiation [8].

In this chapter, we describe our protocol for the detection of phosphorylated MAPKs by Western blotting, under the conditions altered by recombinant human CCN (rhCCN) member proteins, small interfering RNAs or inhibitors of MAPK pathways.

2 Materials

2.1 Activation of Signaling Pathways by rCCN Family Proteins

1. CCN1 (e.g., rhCCN1, PEPROTECH).
2. CCN2 (e.g., rhCCN2, BioVendor Laboratory) (*see* **Note 1**).
3. CCN3 (e.g., rhCCN3, PEPROTECH).
4. CCN4 (e.g., rhCCN4, PEPROTECH).
5. CCN5 (e.g., rhCCN5, PEPROTECH).
6. CCN6 (e.g., rhCCN6, PEPROTECH). All rhCCN protein stock solutions are to be prepared by following the protocol in Chapter 5.
7. Cells of interest (e.g., chondrocytes, osteoblasts).

2.2 Reduction of Signaling Pathways by CCN Family Gene Silencing

1. Small interfering RNAs (siRNAs). We used Ambion® Silencer® select pre-designed siRNAs directed against mouse Ccn1 (S68131), Ccn2 (S201352), Ccn3 (S70714), Ccn4 (S76050), Ccn5 (S76054), Ccn6 (S233359), and nonsilencing oligonucleotide (4390843) (Thermo Fisher Scientific) (*see* **Note 2**) as a control (*see* **Note 3**).
2. Transfection reagents: RNAiFect transfection reagent (Qiagen), Lipofectamine™ RNAiMAX transfection Reagent (Thermo Fisher Scientific), or equivalent (*see* **Note 4**).
3. Cells of interest (e.g., chondrocytes, osteoblasts).

2.3 Inhibition of MAPKs Signaling

1. U0126, a highly selective inhibitor of MEK 1 and MEK 2, as an inhibitor of ERK1/2 pathway (*see* **Note 5**).
2. SB203580, a selective inhibitor of p38 MAPK.
3. SP600125 as an inhibitor of JNK signaling. It confers a dose-dependent inhibition of the phosphorylation of c-Jun. All inhibitors are to be prepared in dimethyl sulfoxide (DMSO) at a concentration of 50 mM as stock solutions.
4. Cells of interest.

2.4 Western Blotting for Detection of Phosphorylated MAPKs

Add following cell lysis buffer and antibodies to the materials described in Chapter 5.

1. Cell lysis buffer for the detection of phosphorylated kinases: 25 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, 1 mM

EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 5 mM NaF, 10 mM sodium pyrophosphate, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM phenylmethyl sulfonyl fluoride, pH 8.0 (*see* **Note 6**).

2. Primary antibodies specific to the target kinases (*see* **Note 7**).
3. Horseradish peroxidase (HRP)-conjugated secondary antibodies specific to the primary antibodies.

3 Methods

3.1 Activation of Signaling Pathways by rCCN Family Proteins

1. Seed cells at a density of 5.0×10^3–2.0×10^4/cm^2 into cell culture dishes or plates. A 3.5-cm-diameter dish, 6-well plate, or 12-well plate is adequate in size.
2. Preincubate cells for 24 h in a growth medium (D-MEM or other medium suitable for the cells of interest) containing 10% fetal bovine serum (FBS).
3. Replace the culture medium with fresh growth medium without serum or with a reduced amount of it, and incubate the cells further for 24 h to exclude the effects of various factors present in FBS.
4. Add 50 ng/ml of rCCN protein to each cell culture vessel.
5. At various time points between 0 and 60 min after the addition of the desired rCCN protein, prepare cell lysates (*see* Subheading 3.4 and Chapter 5).

3.2 Reduction in Activity of Signaling Pathways by Silencing CCN Family Genes

1. Seed cells at a density of 2.0×10^4–4.0×10^4/cm^2 into cell culture dishes or plates. A 3.5-cm diameter dish, 6-well plate, or 12-well plate is of adequate size.
2. Transfect the cells with siRNA for the desired CCN gene by following the manufacturer's protocol for use of the transfection reagent (*see* **Note 4**).
3. After a 24–48 h incubation, prepare cell lysates (*see* Subheading 3.4 and Chapter 5).

3.3 Inhibition MAPKs of Signaling

1. Seed cells at a density of 5.0×10^3–2.0×10^4/cm^2 into cell culture dishes or plates. A 3.5-cm diameter dish, 6-well plate, or 12-well plate is adequate in size.
2. Preincubate the cells in a growth medium (D-MEM or other medium suitable for the cells of interest) containing 10% FBS.
3. Replace the culture medium with fresh growth medium without serum or containing a reduced amount of it, and incubate the cells further for 24 h to exclude the effects of various interfering factors present in FBS.
4. Add the MAPK inhibitor (10–50 μM) or an equal volume of the solvent, DMSO, before the addition of 50 ng/ml of rCCN protein to each cell culture.

5. At adequate time points after the addition of the desired rCCN family protein, prepare the cell lysate (*see* Subheading 3.4 and Chapter 5).

3.4 Western Blotting for Detection of Phosphorylated MAPKs

1. Prepare a protein extract of the rCCN-treated cells on ice. Stringently carry out all procedures at 4 °C or on ice.
2. Follow the previously described Western blot protocol (*see* Chapter 5).

4 Notes

1. Among the CCN family members, we especially focused on CCN2. Therefore, we established stable HeLa cells transformed by human CCN2 overexpression plasmids and also evaluated the physiological activities of the rhCCN2 secreted by these HeLa cells [1–5].
2. At least these siRNAs work for murine costal chondrocytes or calvarial osteoblasts [6, 9].
3. As a negative control for Silencer® select pre-designed siRNAs, Silencer® select negative controls #1, #2, and #3, which three sequences were designed based on three levels of GC content, are currently supplied by Thermo Fisher Scientific.
4. A number of transfection reagents are currently available. Other types of transfection systems such as electroporation and virus vector systems are also available. Therefore, we can now select the most suitable reagent or method from various options for efficient transfection. Transfection of cells such as chondrocytes or osteoblasts in primary culture is not easy. It is occasionally necessary to enzymatically digest the extracellular matrix with 0.1% of collagenase A and 0.1 U chondroitinase prior to transfection of chondrocytes with siRNAs [6].
5. In our previous studies, we used PD98059 as a MEK1/2 inhibitor. At present, U0126 is recommended based on its specificity for MEK1/2.
6. Equivalent lysis buffers are available from various manufacturers. We frequently use commercially available Cell Lysis Buffer (Cell Signaling Technology) containing Protease Inhibitor Cocktail (Sigma) and Phosphatase Inhibitor Cocktails 2 and 3 (Sigma) [9, 14].
7. To detect phosphorylated MAPKs, we use rabbit monoclonal antibodies against ERK1/2, phospho-ERK1/2, p38 MAPK, phospho-p38 MAPK, JNK, and phospho-JNK (Cell Signaling Technology; refs. [9, 14]).

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Chapter 15

Protocols for Screening for Binding Partners of CCN Proteins: Yeast Two-Hybrid System

Mitsuhiro Hoshijima, Takako Hattori, and Masaharu Takigawa

Abstract

Yeast two-hybrid screening is a powerful method to identify proteins that interact with a protein of interest. CCN2 consists of four domains, and identification of new proteins that bind to individual domains of CCN2 may reveal a variety of CCN2 functions. To identify CCN2-interactive proteins that regulate CCN2 activity, we carried out GAL4-based yeast two-hybrid screening with a cDNA library derived from a chondrocytic cell line, HCS-2/8, with CCN2 cDNA used as a bait. In this chapter, we describe our methods for screening for CCN2 binding partners by this system in detail. This protocol may be applied to other CCN proteins as well.

Key words Bait protein, CCN2 interactive proteins, cDNA library, Prey protein, Reporter gene, Yeast two-hybrid system

1 Introduction

The yeast two-hybrid (Y2H) technique has been proven to be a useful approach to screen for interactive partners of molecules [1]. In order to identify targets of CCN2 that may be involved in the regulatory functions of CCN2 in chondrocytes [2], we carried out a GAL4-based yeast two-hybrid screening to identify CCN2 interactive proteins with a cDNA library derived from a chondrocytic cell line, HCS-2/8 [3]. This technique is based on two domains of the yeast GAL4 protein; the DNA-binding domain (DNA-BD) and the transcriptional activation domain (AD) [4]. The activation of the reporter genes occur when the DNA-BD and the transactivation domain are coupled with two interacting proteins. In the Y2H system, the DNA-BD is fused with the gene of the bait protein, in our case CCN2; and the potential target proteins (prey proteins) are expressed in the yeast cells as proteins fused with the AD. Only when the DNA-BD with the CCN2 bait finds an interacting prey protein, the complex will activate the reporter gene (*see* Fig. 1)

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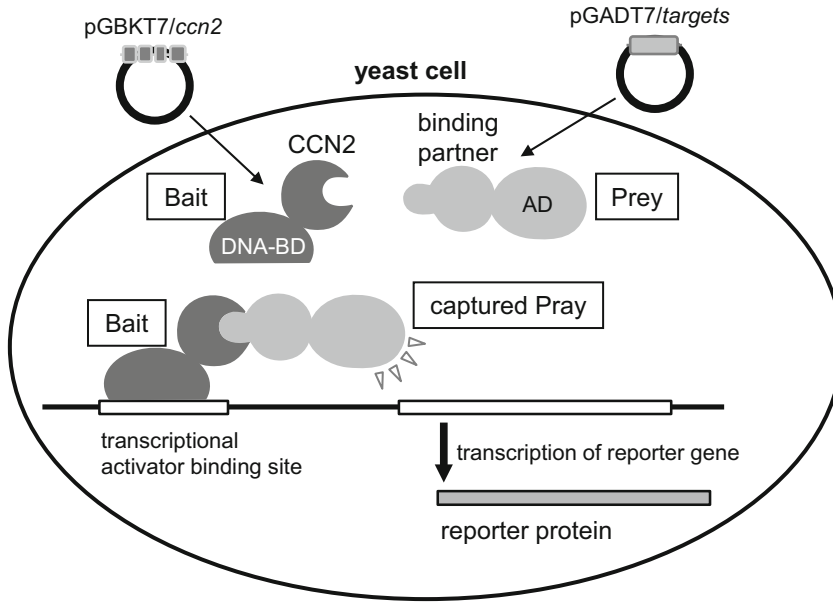


Fig. 1 Schematic representation of the yeast two hybrid system. In the AH109 yeast strain containing pGBKT7 DNA-BD and pGADT7 AD vector, each selection marker gene, *TRP1* and *LEU2*, respectively, is activated. The CCN2 bait protein fused to the GAL4 DNA-binding domain (BD) is used to screen for interacting proteins out of a library of potential target proteins fused to the GAL4 transcriptional activation domain (AD). Activation of the reporter genes such as *ADE2* and *HIS3* occur only in the yeast cells that contain interactive proteins coupling DNA-BD and AD and activating the GAL4 promoter

CCN2 cDNA was fused to the GAL4 DNA-BD as bait, and cDNAs from a chondrocyte library were fused to the cDNA of GAL4 AD and expressed as fusion protein in yeast cells. Interaction between the CCN2 and a library protein leading to transcriptional activation of GAL4 responsive promoter was monitored by using 4 independent reporter genes (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*; *see* Fig. 1). Only transformants viable on synthetically defined medium lacking tryptophan, leucine, histidine, and adenine, respectively, were grown and collected. In our study, we screened 5×10^4 individual clones, and 68 positive clones were identified. Plasmid DNA from these yeast clone candidates was prepared, and an *Escherichia coli* strain was re-transformed with them for further cloning and sequencing. This technique can readily identify CCN2 binding proteins in chondrocytes or other cell types when appropriate cDNA libraries are generated [5, 6]. These methods are applicable for not only CCN2 but also other CCN proteins [7].

2 Materials

Prepare all solutions using Milli-Q water which purified by filtration and deionization (typically 18.2 MΩ cm at 25 °C). Prepare and store all reagents at room temperature unless specified.

2.1 Preparation of Total RNA and the cDNA Library from HCS-2/8 Cells

1. pGADT7 AD vector (Clonteck).
2. *Eco*RI-*Eco*RI Adapter (STRATAGENE).

2.2 Construction of pGBKT7-ccn2

1. *Pfu* DNA Polymerase.
2. 10× *Pfu* PCR Buffer.
3. dNTP Mixture 2.5 mM each.
4. Restriction enzymes: *Eco*RI, *Bam*HI and *Nde*I with NEBuffer 2 (NEW ENGLAND BioLabs).
5. QIAquick Gel Extraction Kit (QIAGEN).
6. pGBKT7 DNA-BD vector (Clonteck).
7. DNA Ligation Kit <Mighty Mix> (TAKARA).
8. *E. coli* DH5α Competent Cells.
9. LB medium: 1 % Bacto tryptone, 0.5 % Bacto yeast extract, and 1 % NaCl.
10. LB plate: 1 % Bacto tryptone, 0.5 % Bacto yeast extract, 1 % NaCl, and 2 % agar (*see* **Notes 1** and **2**).
11. 20 mg/mL kanamycin (1000× stock solution): Filter-sterilize and store in aliquots at −20 °C.
12. GenElute™ Plasmid Miniprep Kit (Sigma).
13. TE buffer: 1 mM EDTA, 10 mM Tris–HCl, pH 8.0.
14. BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).
15. ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

2.3 Yeast Transformation and Selection

1. Yeast strain (*Saccharomyces cerevisiae*): AH109.
2. YPD medium: 10 g Bacto yeast extract, 20 g Bacto peptone, 20 g glucose, and distilled water to bring to 1000 mL (*see* **Notes 1** and **2**).
3. YPD plate: 10 g Bacto yeast extract, 20 g Bacto peptone, 20 g glucose, 20 g agar, and distilled water to bring to 1000 mL (*see* **Notes 1** and **2**).
4. 10× LiAc (pH 7.5): 1 M lithium acetate.
5. 50 % PEG 3350: Filter-sterilize.
6. 10× TE buffer: 10 mM EDTA, 0.1 M Tris–HCl, pH 7.5.

7. 10 mg/mL Herring Testes Carrier DNA, Denatured for K1606 (Becton, Dickinson and Company).
8. SD plate (–Leu/–Trp): 6.7 g yeast nitrogen base without amino acids and ammonium sulfate, 0.64 g –Leu/–Trp DO supplement, 20 g glucose, 20 g agar, and distilled water to bring to 1000 mL (*see* **Notes 1** and **2**).
9. SD plate (–His/–Leu/–Trp): 6.7 g yeast nitrogen base without amino acids and ammonium sulfate, 0.6 g –Ade/–His/–Leu/–Trp DO supplement, 20 mg ADENIN Hemisulfate salt, 20 g glucose, 20 g agar, and distilled water to bring to 1000 mL (*see* **Notes 1** and **2**).
10. SD plate (–Ade/–His/–Leu/–Trp): 6.7 g yeast nitrogen base without amino acids and Ammonium Sulfate, 0.6 g –Ade/–His/–Leu/–Trp DO supplement, 20 g glucose, 20 g agar, and distilled water to bring to 1000 mL (*see* **Notes 1** and **2**).

2.4 Isolate Plasmids from Yeast

1. SD medium (–Ade/–His/–Leu/–Trp): 6.7 g yeast nitrogen base without amino acids and ammonium sulfate (Sigma), 0.6 g –Ade/–His/–Leu/–Trp DO Supplement, 20 g glucose and distilled water to bring to 1000 mL.
2. Sterilized glass beads (425–600 μm).
3. Lysis solution: 2% Triton X-100, 1% SDS, 0.1 M NaCl, 0.01 M EDTA, and 0.01 M Tris–HCl, pH 8.0.
4. PCI: phenol–chloroform–isoamyl alcohol solution (25:24:1).
5. 5 M potassium acetate.
6. 80% ethanol solution in water.

2.5 Characterization of Interacting Clones

1. 50 mg/mL ampicillin (1000× stock solution): Filter-sterilize and store in aliquots at –20 °C.
2. Primer for sequencing: T7 Promoter/forward (5′-taatacgact cactataggg-3′).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of Total RNA and the cDNA Library from HCS-2/8 Cells

The cDNA used for the cDNA library and the bait plasmid was obtained by reverse transcriptase (RT)-PCR from the RNA isolated from HCS-2/8 cells. The cDNA library was constructed in the *Eco*RI site of the pGADT7 vector by using the *Eco*RI-*Eco*RI Adapter (*see* **Note 3**).

3.2 Construction of pGBKT7-ccn2

1. Mix 1–3 μL of the first-strand cDNA mixture obtained in Subheading 3.1, 5 μL of 10× *Pfu* PCR Buffer, 4 μL of 2.5 mM dNTP, a 1 μM concentration of each primer, and 1 μL of *Pfu* DNA polymerase in a volume of 50 μL.
2. Carry out PCR under three-step cycling conditions (*see* Table 1).
3. Purify the PCR products by ethanol precipitation and digest them with *Eco*RI and *Bam*HI (*see* **Note 4**).
4. Separate the PCR products obtained in Subheading 3.2, **step 1** by agarose gel electrophoresis. View the gel with a UV transilluminator and excise the DNA band by using a scalpel. Cut the slice into small pieces and transfer the gel pieces to a sterilized tube.
5. Extract and purify the PCR product from the gel by using a QIAquick Gel Extraction Kit.
6. Ligate the *Eco*RI–*Bam*HI fragment into a pGBKT7 vector, which was digested with *Eco*RI and *Bam*HI, by mixing 50 ng pGBKT7 vector, 10–100 ng PCR fragment, and 5 μL 2× Ligation Mixligase in a 10 μL volume. Incubate the ligation reaction mixture at 16 °C for 1 h (*see* **Note 4**).
7. Place 50 μL aliquots of *E. coli* DH5α competent cells into sterilized tubes (*see* **Note 5**).
8. Add 5 μL of ligation reaction mixture to *E. coli* competent cells. Mix gently and incubate the cells on ice for 30 min.
9. Heat the cells for 45 s in a 42 °C water bath, and then place them on ice for 1–2 min.
10. Spread the cells onto LB plates containing kanamycin and incubate the plates at 37 °C overnight.
11. Pick up single colonies from the plate, inoculate them into 2.5 mL of LB medium containing kanamycin, and incubate them overnight at 37 °C with shaking.

Table 1

PCR primers and amplification conditions used to amplify full-length *ccn2*

Primer sequences (5'–3')	
CCN2 (27–349 AA)-F	atccgaattccagaactgcagcgggccgtgccggtgcccg
CCN2 (27–349 AA)-R	atacggatccctcatgccatgtctccgtacatcttcctgt
PCR conditions	
1× 94 °C: 2 min	
30× 94 °C: 30 s, 55 °C: 30 s, 72 °C: 90 s	
1× 72 °C: 10 min	
Hold at 4 °C	

12. Isolate plasmid DNA by using a GenElute™ Plasmid Miniprep Kit according to the manufacturer's instruction.
13. Verify insertion in the plasmids by digestion with *Eco*RI and *Bam*HI followed by agarose gel electrophoresis.
14. Characterize several clones by sequencing analysis using BigDye Terminator and an ABI PRISM™ 310 Genetic Analyzer.

3.3 Yeast Transformation and Selection

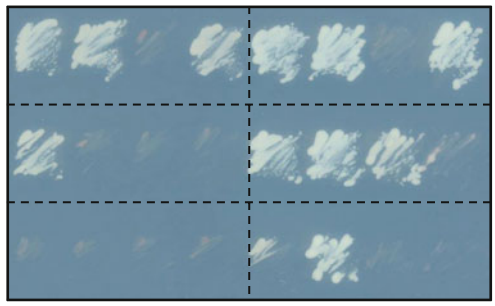
1. Streak fresh onto yeast host strain AH109 YPD plate to obtain single colonies. Incubate at 30 °C until colonies grow to 1–2 mm in diameter, usually taking about 3 days.
2. Inoculate a single colony into 75 mL of YPD and culture overnight at 30 °C with shaking at 200 rpm.
3. Inoculate 10 out of 75 mL overnight yeast culture into 300 mL YPD in 2 L flasks, and culture at 30 °C with vigorous shaking with monitoring OD_{600}. Continue culturing for about 6 h until the OD_{600} reaches between 0.45 and 0.6.
4. Harvest the cells by centrifugation at 1,000 × *g* for 10 min and remove the supernatant.
5. Resuspend the pellets in 90 mL of sterile water.
6. Collect the cells by centrifugation at 1,000 × *g* for 10 min and remove the supernatant.
7. Wash the cells again by adding 20 mL of sterile distilled cold water, centrifuge them at 1000 × *g* for 10 min, and remove the supernatant.
8. Resuspend the cells in 4.5 mL of sterile 0.1 M LiAc to make yeast competent cells.
9. Add 7.2 mL of 50% PEG 3350, 1.08 mL of 10× LiAc, and 0.75 mL of Herring Testes Carrier DNA (*see* **Note 6**) to 4.5 mL of yeast competent cell solution obtained in Subheading 3.3, **step 8** on ice. Mix well by vortexing.
10. For each reaction, add 1.56 mL of plasmid DNA solution (*see* **Note 7**) to the solution obtained in Subheading 3.3, **step 9** on ice and vortex vigorously (*see* **Note 8**).
11. Incubate at room temperature for 50 min.
12. Heat the tubes for 25 min in a 42 °C water bath and then rapidly chill them on ice for 1–2 min.
13. Centrifuge the cells for 15 s at 4,500 × *g* at room temperature and carefully remove the supernatant.
14. Resuspend the cells in 4.5 mL of sterile water.
15. Spread 0.1 mL of the transformed cells onto a 100-mm SD plate (–Leu/–Trp). Spread 4.4 mL of transformed cells onto ten 150-mm SD plates (–His/–Leu/–Trp; *see* **Note 9**). Yeast cells containing both pGBKT7 (the bait fusion vector) and pGADT7 (the cDNA library fusion vector) grow on the SD

plate (–Leu/–Trp). Yeast transformants in which interaction between the bait protein and proteins encoded by a cDNA library has occurred are detected as colonies of positive transformants (*see* **Note 10**) on the SD plates (–His/–Leu/–Trp).

16. Select colonies that grow well on the SD plates (–His/–Leu/–Trp). Streak these colonies on fresh SD plates (–Ade/–His/–Leu/–Trp) and incubate the plates for 2–4 days at 30 °C (*see* **Note 11**, Fig. 2).
17. Seal the plates with Parafilm and store at 4 °C.

3.4 Isolate Plasmids from Yeast

1. Suspend each positive clone in 2 mL of SD medium (–Ade/–His/–Leu/–Trp) and cultivate it overnight at 30 °C.
2. Transfer 1.5 mL of the culture to a sterilized centrifuge tube, spin down the cells at 3,000 × *g* for 5 min at room temperature and remove supernatant.
3. Resuspend the cell pellet in 100 μL of TE buffer and vortex vigorously.
4. Mix 0.2 mL of lysis solution, 0.2 mL of PIC, and 0.3 g of sterilized glass beads and vortex for 2 min.
5. Centrifuge the mixture for 5 min at 20,000 × *g*.
6. Transfer the aqueous phase into a new 1.5-mL tube.
7. Add 0.1 volume of 5 M potassium acetate and 2 volumes of 100% ethanol. Vortex well and incubate for 2 min at room temperature.
8. Centrifuge for 5 min at 20,000 × *g* and remove the supernatant.
9. Rinse the pellet with 80% ethanol and centrifuge it for 2 min at 20,000 × *g*.
10. Remove the supernatant and dry up.
11. Resuspend the pellet in 50 μL of sterile water (*see* **Note 12**).



1	2	3	4	5	6	7	8
9	10	11	12	13	14	15	16
17	18	19	20	21	22	23	24

An example of master plate

Fig. 2 Changing stringency conditions for screening of interactive clones. The first screening can be carried out on lower stringency medium, SD plates (–His/–Leu/–Trp). In secondary screening, positive clones can be further selected by using SD plates (–Ade/–His/–Leu/–Trp), which have higher stringency. These plates are incubated for 2–4 days at 30 °C and can be used as master plates for subsequent experiments

3.5 Characterization of Interacting Clones

1. Transform DH5α competent cells with 5 μL of each plasmid isolated from yeast cells (*see* **Note 13**).
2. Select transformants by growing them overnight at 37 °C on LB plates containing ampicillin (*see* **Note 12**).
3. Incubate 2.5 mL of cultures in LB medium containing ampicillin and isolate the plasmid DNA by using a GenElute™ Plasmid Miniprep Kit.
4. Verify whether each positive interacting clone is by digestion with appropriate restriction enzymes followed by agarose gel electrophoresis (*see* **Note 4**).
5. Further characterize one clone from each group by sequencing analysis using a BigDye Terminator and an ABI PRISM 310 Genetic Analyzer.
6. Compare the sequence of each clone against available databases (*see* **Note 14**).

4 Notes

1. For agar plates, 20 g agar is added to 1 L of medium and autoclaved. Each plate contains 20–25 mL per 100-mm or 50 mL of medium per 150-mm Petri dish.
2. Sterilize the medium by autoclaving for 15 min at 121 °C.
3. A total of 1×10^6 independent cDNA clones with an average insert size of –2.4 kbp (range: 2.0–4.0 kbp) is obtained.
4. CCN2 (27–349 AA)-F and CCN2 (27–349 AA)-R, which are the primers for cloning full-length CCN2 (27–349 amino acid: except signal peptide) contain *Eco*RI and *Bam*HI site, respectively. Therefore, the PCR product derived from the two primers is digested with *Eco*RI and *Bam*HI and ligated into the pGBKT7 vector that was digested with the same restriction enzymes.
5. Competent cells removed from a –80 °C freezer should be thawed on ice and gently mixed before being aliquoted.
6. Herring Testes Carrier DNA solution, prepared at 10 mg/mL, is diluted to a one-fifth and boiled for 20 min at 95 °C.
7. This 1.56 mL of plasmid DNA solution includes 15 μg of the pGBKT7-*ccn2* and 15 μg of the cDNA library which was constructed with pGADT7 vector.
8. In the case of small-scale transformation of yeast cells, resuspend the cell pellet in 4.5 mL of 0.1 M LiAc and put 150-μL aliquots into sterile 1.5-mL tubes. Then add 240 μL of 50% PEG 3350, 36 μL of 10× LiAc, and 25 μL of Herring Testes Carrier DNA to the competent yeast cells and vortex well. Add 52 μL of DNA solution containing a total of 2 μg of plasmid

DNA and vortex vigorously. The reaction can be multiplied according to the scale performed. We have successfully done up to 30-fold multiplied experiments.

9. Choose the selection medium depending on the reporter genes contained in the yeast expression vector. The pGBKT7 vector contains a *TRP1* gene for the yeast strain to show resistance in the absence of tryptophan in the medium. The pGADT7 vector, cloned cDNA library, contains a *LEU2* gene, which gives resistance to growth in medium lacking leucine. AH109, the yeast strain, has four reporter genes (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*) with the GAL4 promoter. These genes are expressed when the GAL4 DNA-BD fused CCN2 and the GAL4 AD fused cDNA library proteins interact. Expression of the *ADE2* and *HIS3* genes gives resistance to growth in adenine- and histidine-lacking medium, respectively. Expression of the *MEL1* gene turns colonies to blue color in the presence of X-gal as a substrate, and the *AUR1-C* gene gives resistance to growth in the presence of the antibiotic Aureobasidin A [8].
10. The number of clones screened is calculated from the colonies on SD plate (–Leu/–Trp) by multiplying with the total volume.
11. Select colonies that grow well on the SD plates (–Ade/–His/–Leu/–Trp) and incubate them for 2–4 days at 30 °C. These plates can be used as master plates for subsequent experiments. Wrap the plates in Parafilm and store at 4 °C.
12. This DNA preparation will contain a mixture of bait and library plasmids. The library plasmids, pGADT7 vectors, have ampicillin resistance and can be selected after the *E. coli* has been transformed. Similarly, the bait plasmid, pGBKT7 vector, is resistant to kanamycin.
13. *E. coli* DH5α competent cells are transformed with the DNA by the traditional heat-shock method. Transformation with electroporation provides higher transformation efficiencies [9].
14. The protein–protein interaction should be reexamined by co-transformation of yeast with the bait plasmid followed by selection on a SD plate (–Ade/–His/–Leu/–Trp).

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Chapter 16

Protocols for Screening Peptide Motifs Binding to CCN Family Proteins

Satoshi Kubota, Harumi Kawaki, and Masaharu Takigawa

Abstract

Function of CCN family proteins is determined by the interactions with multiple cofactors that are present in microenvironment. Therefore, finding out these cofactors is critically important in understanding the molecular function of the CCN family members. For this objective, bacteriophage random peptide display library is a quite feasible tool. In this library, each filamentous bacteriophage is designed to display an oligopeptide of random 12–16 amino acid residues on its surface. Bacteriophage clones that possess the peptides that bind to a CCN family protein are selected through several cycles of a process designated biopanning or affinity selection. By determining the nucleotide sequence of the DNA that encodes the displayed peptide, oligopeptides that specifically bind to the CCN family member can be specified. Obtained peptide sequences can be utilized for designing peptide aptamers for the CCN family protein, or as a key sequence to find out new CCN family cofactor candidates in silico.

Key words CCN family, Bacteriophage, Phage display, M13, Aptamer

1 Introduction

Bacteriophage peptide display library screening system was developed to substitute antibodies as a molecular tool for specific recognition of proteins [1]. As stated in Chapter 12 of this book, antibodies have been used for the detection, purification, and functional characterization of the proteins of interest, utilizing its binding specificity to the antigen. However, raising antibodies, particularly monoclonal antibodies, against proteins can be costly, or wasting and time-consuming. Therefore, a different type of material that selectively binds to the target protein and can be prepared easily in vitro has been desired, which is also favorable from the viewpoint of animal ethics. In this way, bacteriophage display system was initially developed as a next-generation antibody manufacturing system. However, although a bacteriophage that selectively binds to the "antigen" can be utilized for the detection and purification of the protein per se, it may not substitute a conventional antibody, especially in medical usage.

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Namely, antibodies are now actually used as molecular targeting drugs in clinics, which is by no means applicable to bacteriophages. In this context, bacteriophage display library has been renovated and is currently utilized to determine the antigen binding sites against the targets, which will be eventually built in the backbone of human IgG. Such a bacteriophage display-mediated design and production of human antibodies in vitro is already established [2]. With this kind of system, neither animals nor hybridomas may be necessary. Contribution of this sophisticated system to the antibody-mediated therapeutics should be noted.

As a CCN family research [3] tool, a basic screening system of random peptide display library is sufficiently useful. Nevertheless, owing to the unique molecular characteristics of the CCN family proteins [3–5], results obtained by this system are not always simple. In general, using a regular protein as bait, several bacteriophage clones that display identical, or nearly identical peptide sequences, are eventually selected. On the other hand, displayed peptides that are found to bind to a CCN family protein can be highly diverse [6]. In a sense, this is an expected outcome consistent with the molecular properties of the CCN family proteins interacting with a number of different biomolecules. As such, analysis, interpretation, and application of the obtained data are as critical as the experimental process itself in this research strategy.

2 Materials

To date, a number of different experimental kits for the screening of bacteriophage display peptide library are available from several commercial resources. Such a kit is usually composed of a bacteriophage display peptide library, recommended host strain, specific primers for sequencing the DNA segment encoding the displayed peptides. Positive controls, typically streptavidin and biotins, may be included together. Since we use a M13 phage-based phage display system (New England Biolabs, Ipswich, MA, USA) to extract peptides that bind to each module of human CCN2, strategies in finding out CCN family protein-binding peptide motifs based on this system is described here. Other experimental kits with other types of bacteriophage, such as T7 bacteriophage, are also available (Merck, Darmstadt, Germany).

2.1 Bacteriophage Titration

Prior to the in vitro screening process called biopanning [7], titration of the phage library is absolutely necessary. After each biopanning process, bacteriophage pools that bound to the target CCN family proteins or their associated molecules have to be amplified and partially purified. Therefore, host bacteria and basic materials used for the propagation of corresponding bacteriophage vector need to be prepared in advance.

1. Host bacteria: An *E coli*. strain, ER2738 is used. The genotype of this strain is (F' *proA+B+ lacIqΔ(lacZ) M15 zzf::Tn10(TetR)/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5 [rk– mk– McrBC–]*) (*see* **Note 1**). However, other F^+ strains, such as XL-1 blue and DH5αF′, can be used for a library with M13 coliphage background.
2. LB medium: 10 g Bacto tryptone, 5 g yeast extract, 5 g NaCl per liter. However, regular broth that is feasible for the growth of host *E. coli* can be utilized.
3. IPTG/Xgal Solution (25 ml): 1.25 g of isopropyl-β-d-thiogalactoside (IPTG) Mix 1.25 g and 1 g of 5-bromo-4-chloro-3-indolyl-β-d-galactoside (Xgal) are dissolved in 25 ml dimethyl formamide (DMF).
4. LB/IPTG/Xgal Plates: Simply add 15 g of Bacto agar to 1 l LB medium (Subheading 2.1, **item 2**) and sterilize by autoclave. After cooling it down to 60 °C, add 1 ml of IPTG/Xgal and mix them well. Immediately after the mixing, pour it into 10 cm sterile petri dishes.
5. Top Agar: 10 g of Bacto tryptone, 5 g of yeast extract, 5 g of NaCl, and 7 g of Bacto agar are mixed and filled up with distilled water to 1 l. After autoclaving, dispense it into 50 ml aliquots for storage at room temperature (RT). This has to be melted by microwaving prior to each use.
6. Tetracycline suspension**:** Tetracycline is stored at −20 °C in a mixture of equal volume of water and ethanol at 20 mg/ml (*see* **Note 2**).
7. LB/Tet Plates: Simply prepare LB-agar (*see* subheading 1, **item 4** in this section), add 1 ml tetracycline suspension and pour into petri dishes. These plates should be stored in the dark.
8. PEG/NaCl: 20% (w/v) polyethylene glycol-8000 in 2.5 M NaCl. During autoclaving, solution may separate, which can be mixed back after cooling down to 50 °C. This solution is necessary for the purification of M13 phages by precipitation.

2.2 Biopanning

Biopanning is a word specifically representing the affinity selection techniques for bacteriophage library. This process can be performed either in solid or liquid phase. In the former case, binding reaction is performed on the plastic surface, whereas it occurs in solution in the latter. If biopanning is carried out in a solution, target CCN family proteins has to be tagged by biotin, and bound phages are captured onto streptavidin conjugated to beads or plastic surface. In short, solid-phase biopanning process is relatively simpler than the liquid phase. For the screening of binding peptides to a CCN family protein, we recommend the simpler methodology, since CCN family proteins are all highly interactive and positive results is expected even with this option.

1. CCN family proteins or modules**:** For the preparation of a recombinant CCN family protein, or its modular fragment, protocols are given in Chapters 8–10.
2. Blocking Buffer: 0.1 M $NaHCO_3$, pH 8.6, 5 mg/ml bovine serum albumin (BSA). In order to avoid possible decay, sodium azide may be added.
3. Tris-buffered saline (TBS) wash buffer: 50 mM Tris–HCl; pH 7.5, 150 mM NaCl. Supplementation with 0.1% Tween 20 (TBST) can be effective to reduce nonspecific interaction and thus is used for washes.
4. Elution buffer: 0.2 M glycine-HCl pH 2.2, 1 mg/ml BSA. If this buffer is used, subsequent neutralization with 1 M Tris–HCl, pH 9.1, is necessary. Also, 100 μg/ml of the target CCN family protein in TBS is feasible.
5. Biopanning Plate: Since CCN family proteins binds efficiently to plastic surface, most of plastic plates for cell culture can be employed. Typically, 24- or 12-well plates are adequate in size.

2.3 Bacteriophage amplification

See Subheading 1 of this section.

2.4 DNA Extraction and Sequencing

1. Specific primers for sequencing the region encoding displayed peptides: Usually, oligonucleotide primers designed to read the DNA sequence encoding random peptides are supplied as components of the experimental kit. If not, primers need to be designed, based on the nucleotide sequence of the phage genome. In the system we used, peptides were designed to be displayed at the N-terminus of a minor coat protein, pIII [8, 9].
2. Rapid DNA Extraction Buffer**:** 10 mM Tris–HCl (pH 8 .0), 1 mM EDTA, 4 M sodium iodide (NaI). Store at RT in the dark. Discard if color is evident.
3. PEG/NaCl: *See* subheading 2, **item 8** in this section.
4. Absolute and 70% ethanol.

2.5 ELISA

Direct interaction between target CCN proteins and phages pooled after biopanning can be confirmed by ELISA, in which a single antibody conjugate is used. For general materials required for ELISA, *see* relevant protocol or Chapter 13 in this issue.

1. CCN family proteins and modules: The same protein used for biopanning is utilized.
2. Specific antibodies against bacteriophage: Enzyme-conjugated antibodies against M13 phages (e.g., horseradish peroxidase (HRP)-labeled anti-M13 from GE Healthcare).

3 Methods

We utilize a M13 bacteriophage-based experimental kit from New England Biolabs (Ph.D.-12 Library) for the screening of CCN family protein binding dodecapeptides. An overview of this method and subsequent analysis are illustrated in Fig. 1. Below is a brief protocol based on the instruction from the manufacturer [10]. More detailed protocol can be found in a document from the supplier.

3.1 Titration of Bacteriophage Library

Although phage library can be stored at 4 °C for a few weeks, it is strongly recommended to determine the titer immediately before screening. Also note that infection should occur at a multiplicity of infection (MOI) at 1.0 (or even less). Otherwise, a single plaque may not necessarily represent a single bacteriophage clone.

1. Seed the host *E. coli* from a single colony into 5 ml of LB. Incubate at 37 °C with shaking for 4–8 h until reaching mid-log phase.
2. In parallel with the bacteria preparation, melt Top Agar in microwave and dispense 3 ml into bacteria culture tubes and let them stand at 45 °C. Also, pre-warm one LB/IPTG/Xgal plate per expected dilution at 37 °C (>1 h).
3. Make 10- to 10^3-fold serial dilutions of the bacteriophage library in 1 ml of LB. Original or amplified library may start from 10^8-fold dilution, whereas panning eluates may be diluted from 10- to 10^3-fold (*see* **Note 3**).
4. Dispense 200 μl of the growing host cell suspension (*see* **item 1** in this subheading) into 1.5 ml microfuge tubes, one for each phage dilution.
5. Infect the host cells by adding 10 μl of each phage dilution to the corresponding tube, mix briefly with a touch mixer and incubate at RT for 5 min.
6. Transfer the infected cells to culture tubes with predispensed and pre-warmed Top Agar. Mix briefly, and immediately pour culture onto pre-warmed LB/IPTG/Xgal plates (*see* **Note 4**). Spread top agar suspension evenly on the plate by gentle tilting. Stand and cool the plates surface-up for 5 min; then invert and incubate overnight at 37 °C.
7. Next morning, count plaques on plates with around 100 plaques. Compute phage titer in plaque forming units (pfu) per 10 μl of original phage suspension (*see* **Note 5**).

3.2 Solid Phase Biopanning

Here introduced is the simplest protocol for biopanning to find out CCN family protein-binding peptides displayed on phages. Although the methodology is relatively simple, it takes a week to carry out three cycles of panning process. We usually perform

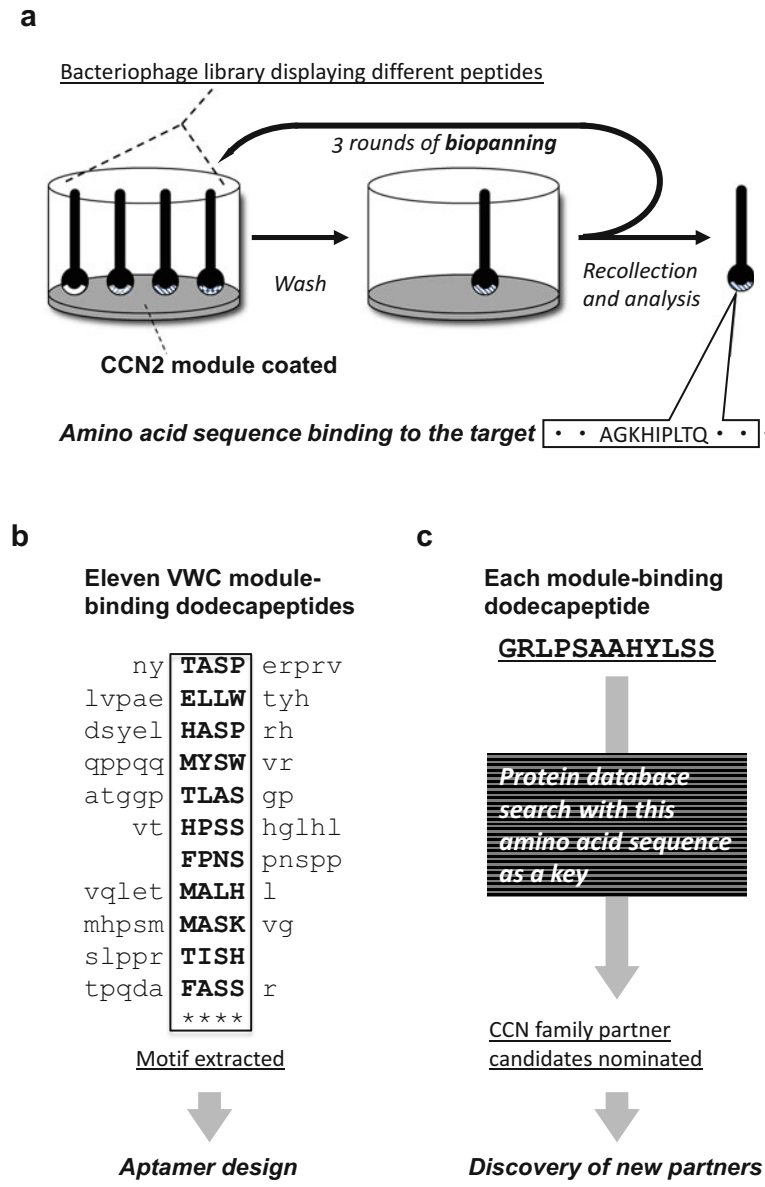

Fig. 1 Experimental workflow of the screening peptides that bind to CCN family proteins and their utility. (**a**) Typical selection process of bacteriophages that display dodecapeptides to a target CCN family protein. On the surface of the wells in a multiwell plate, a CCN family protein is coated. After the application of bacteriophage library displaying random dodecapeptides on the surface, unbound phages are washed out, and the bound ones are recollected and amplified. This process, called biopanning, is repeated three times. (**b**) Utility of obtained peptides sequences in designing CCN family specific protein aptamers. Note the remarkable diversity in the peptide sequences binding to the VWC module of CCN2 [6]. (**c**) Utility of an obtained peptide sequence in finding out a new CCN family partner

biopanning in duplicate, using 24-well plates, which can be changed to a larger scale, depending upon the availability of the target CCN family protein. However, in all cases, the number of input phage should remain the same (10^{11} virions).

1. Dilute a recombinant CCN family protein to 100 μg/ml in 0.1 M $NaHCO_3$, pH 8.6. If the protein is insoluble in this buffer, another buffer with similar ionic strength may substitute it.
2. Add 150 μl of the diluted target to each well and completely wet the well surface with it by horizontal shaking. Then, incubate the plate overnight at 4 °C with gentle shaking in a humidified container. The plate may be sealed into a plastic box with water-wet paper towels.
3. Next day, remove the coating solution from each well thoroughly and add the blocking buffer as much as possible. Incubate at 4 °C for >1 h.
4. During the incubation, start the culture of the host, ER2738, in 10 ml of LB + Tet medium (for titration), and in 20 ml of LB (for amplification) under vigorous shaking at 37 °C. The 20 ml culture should be performed in a 250-ml conical flask and carefully monitored to avoid overgrowth beyond early-log phase (OD 600 < 0.05).
5. Discard the blocking solution and wash the wells 6 times with TBST. Each time, remove TBST thoroughly by slapping the plate upside-down to a paper towel.
6. Prepare a 100-fold set of the original library (for example; 10^{11} phage for a library with 2×10^9 clones) with 100 μl of TBST. Dispense the phage suspension onto coated wells and incubate for 60 min at RT with gentle rocking.
7. Discard unbound phages and wash plates ten times with TBST as in Subheading 3.2, **step 5**. In order to avoid possible cross-contamination, using a clean paper towel each time is recommended.
8. Add 100 μl of the elution buffer for the collection of bound phages. Gently rock the elution mixture for 30 min at RT. Transfer the eluate into a 1.5 ml microcentrifuge tube and neutralize with 15 μl of 1 M Tris–HCl.
9. Determine the titer of the eluted phages with 1 μl of the eluate as described under the last subheading with the 10-ml culture prepared in **step 4** in this subheading.
10. Amplify the rest of the eluate in the 20-ml culture from Subheading 3.2, **step 4** by incubating with vigorous shaking for 4.5 h at 37 °C.
11. Transfer the cell suspension to a centrifuge tube and centrifuge it for 10 min at 12,000 × *g* at 4 °C. Take the supernatant to another centrifuge tube and repeat centrifugation. Transfer the

supernatant, leaving 10–20% of the bottom part, to another tube and add 1/6 volume of the PEG/NaCl. Keep this overnight at 4 °C.

12. Next day, recollect the phages by centrifugation at 12,000×*g* for 15 min at 4 °C. Discard the supernatant, spin again briefly and remove the supernatant with a micropipette. The phage pellet is usually visible in white.
13. Suspend the phages in 1 ml of TBS and transfer the suspension to a 1.5 ml microcentrifuge tube. Remove the residual bacteria by final spin down at the maximum rotation for 5 min at 4 °C.
14. Transfer the supernatant to another 1.5 ml tube and add 1/6 volume of PEG/NaCl and incubate it on ice for 60 min for re-precipitation. Recollect the phages by centrifugation at 17,000×*g* for 10 min at 4 °C. Remove the supernatant as described in **item 13** in Subheading 2.
15. Suspend the pellet in 200 μl of TBS (amplified eluate). Additional centrifugation for 1 min to ensure no remaining insoluble material is recommended.
16. Start the titration of the amplified eluate, following the protocol shown in the first subheading (*see* **Note 6**).
17. Prepare another coated 24-well plate for the second round of biopanning as described in **steps 1** and **2** in this subheading.
18. Determine the titer of the first round amplified eluate by counting plaques in pale blue. More than 10^{13} pfu/ml is expected.
19. Block the coated 24-well plate and perform a second round of panning and amplification, following the protocol given in steps **3–15** in this subheading (*see* **Note 7**). The same amount of bacteriophages should be used as that for the first biopanning. However, using phages with equivalent number to that of the clones in the original library is tolerable.
20. Begin the titration of the second round amplified eluate, and coat another 24-well plate for the third round of panning, as described above.
21. Perform a third round of panning by repeating steps described as **items 3–8** in this subheading, with the second round amplified eluate at an equivalent input titer.
22. Determine the titer of the original third round eluate, following **item 9** in this subheading. Plaques from this titration step are used for subsequent DNA sequencing and ELISA. Do not continue the incubation at 37 °C beyond 18 h; otherwise, deletion may occur. Plaques should be picked up within 3 days of storage period at 4 °C. The remaining eluate is stable for a week.
23. For DNA sequencing or ELISA, prepare a 10-ml overnight culture of host strain from a colony and amplify single plaques as described below.

3.3 Amplification of Phage Clones

In order to confirm the specific binding of the selected peptides to the target CCN family protein and to determine their deduced amino acid sequences, each clone needs to be amplified up to a sufficient amount, following this protocol.

1. After an overnight culture, dilute the host cell to 100-fold with LB. Aliquot 1 ml of diluted culture for each phage clone into culture tubes. At least ten clones from the third panning are necessary (*see* **Note 8**).
2. With a sterile yellow pipette tip (2–200 μl), stab a blue plaque from the plate from Subheading 3.2, **step 22** (*see* **Note 9**), and add it to the diluted culture.
3. Shake the tubes at 37 °C for 4.5–5 h (do not exceed).
4. Transfer the infected bacterial suspension to 1.5 ml microcentrifuge tubes, and centrifuge at a top speeed for 30 s. Take the supernatant into another tube and centrifuge again briefly. Carefully take the supernatant to another tube, leaving 10–21 % of the bottom part. This is the amplified phage stock (*see* **Note 10**). For long- term storage, add equal volume of sterile glycerol and store at −20 °C.

3.4 Determination of Peptide Sequence by DNA Sequencing

Amino acid sequence of the peptides displayed on bacteriophages can be deduced by determining the DNA sequences of the corresponding region in the phage genome (*see* **Note 11**). Since M13 bacteriophage is released from infected bacteria without bacteriolysis, phage DNA for regular DNA sequencing can be purified relatively easily.

Upon DNA sequencing, one should note that this type of phage genome is in the form of single strand DNA, and thus sequence can be determined unidirectionally. Since we use a library, in which codon usage is restricted and amber codon is suppressed to encode glutamine, a special reduced genetic code table supplied by New England Biolabs was used to deduce amino acid sequences, instead of a universal one.

1. At the final step of the plaque amplification procedure (*see* Subheading 3 in this section), take 500 μl of the phage-containing supernatant to another 1.5 ml tube.
2. Add 200 μl of PEG/NaCl solution, invert it to mix, and keep it for 20 min at RT.
3. Centrifuge at 17,000 × *g* for 10 min at 4 °C, and discard the supernatant. After another brief centrifugation, carefully remove remaining supernatant with a micropipette.
4. Suspend the pellet with 100 μl of Rapid DNA Extraction Buffer by vigorous tapping. Then, add 250 μl of absolute ethanol and incubate for 15 min at RT.
5. Centrifuge the tubes at 17,000 × *g* for 10 min at 4 °C, decant the supernatant, and wash the pellet with 500 μl of 70 % ethanol.

Centrifuge again for 5 min, discard the supernatant, and dry the pellet under vacuum for 2–3 min.

6. Dissolve the DNA pellet in 25 μl of pure water, or a conventional buffer (*see* **Note 12**).
7. Estimate the DNA concentration by spectrophotometry or agarose gel electrophoresis. Expected concentration is around 100 ng/μl, which would be sufficient for DNA sequencing.

3.5 Confirmation of Specific Binding to the CCN Family Protein by ELISA

To avoid fishing up nonspecific bacteriophages, this process may precede the DNA sequencing described above. Unlike the ELISA methods to quantify CCN family proteins and their modular fragments, which are introduced in another chapter, immunoreaction occurs only once between the bound phage and enzymatically labeled anti-phage antibody. However, basic methodology is almost the same as the one shown in the relevant chapter.

1. Perform the plaque amplification procedure (*see* Subheading 3) to obtain amplified phage stock.
2. Prepare 20 ml of the host culture for infection (*see* **items 4** and **10** in subheading 2; OD600 0.01–0.05) and add 5 μl of the phage stock to further amplify the phage clone. Follow the process described in **items 4–14** in the Subheading 2, and finally suspend the phage clone with 50 μl of TBS.
3. Determine the titer as described in the first subheading. Expected titer here is around 10^{14} pfu/ml.
4. Coat one row of 96-well ELISA plate wells for each clone with 50 μl of 100 μg/ml of the target CCN family protein in 0.1 M $NaHCO_3$, pH 8.6 for ELISA, following the same method used for biopanning. In addition, one uncoated row per clone is set up and simultaneously blocked to test the binding to BSA-coated plastic (*see* **Note 13**). Another uncoated microtiter plate should be also blocked for use in serial dilutions of phage.
5. In this blocked plate, make fourfold serial dilutions of the phage in 200 μl of TBST per well, starting with 10^{12} virions in the first well of a row until the 12th well.
6. Transfer half of each phage clone suspension to both coated and uncoated wells for each clone, and follow a regular ELISA process (*see* Chapter 13).

3.6 Analysis of Obtained Peptide Sequences In Silico

With this bacteriophage display system, a distinct amino acid sequence shared by most of the peptides binding to the target can be found in general cases, such as epitope mapping of an antibody that is used as a binding target. Nevertheless, because of its molecular nature, a CCN family protein binds to a variety of different peptide sequences. Therefore, remarkable variation is expected in the amino acid sequences that are shown to bind to the CCN

family protein. If this happens, it may be difficult to find a consensus sequence among those peptides at a glance, and thus analysis in silico is required. Indeed, we obtained more than ten peptides with remarkable sequence diversity as human CCN2 VWC module binding ones. As observed in Fig. 1b, no identical or highly similar sequences can be found among the 11 peptides. Therefore, we extracted a peptide motif from these sequences under the assistance of Gibbs Sampler algorithm [11]. A peptide aptamer that was designed based on this extracted motif actually bound to the VWC module of human CCN2, which was confirmed by surface plasmon resonance (SPR) analysis (*see* **Note 14**) [6].

The obtained peptide sequences are also useful from another point of view. Namely, each peptide can be used as a key peptide sequence to search a protein database for putative CCN family protein cofactors. A common database, such as a protein database provided by National Center for Biotechnology information (NCBI) or the Ensembl project, can be utilized (Fig. 1c). Following this strategy, we rediscovered receptor activator of NF-kB (RANK) as a new functional counterpart of CCN2 [12].

4 Notes

1. In this strain, amber codon is suppressed as a glutamine codon.
2. This stock is not a solution, but a suspension. Mix vigorously with a touch mixer before use.
3. Filter-embedded, aerosol-resistant pipette tips are recommended to prevent cross-contamination.
4. The phage vector used in this system, M13KE contains lac Z gene fragment for color selection. Since addition of unrelated peptides to the pIII gene product affects the infectivity and replication of the recombinant phage clones, it is critically important to discriminate library clones from possible contamination of environmental phages. This is the reason why IPTG/Xgal plates are used and only blue colonies are counted and expanded.
5. M13 bacteriophage is a temperate phage that does not cause host cell lysis, but restrain host growth. Therefore, each plaque appears vague in comparison with another phage causing cell lysis.
6. Early use of the eluate gives best results, though it may be stored a few weeks at 4 °C. If an equal volume of sterile glycerol is added, it can be stored at −80 ° C for a long time.
7. For second and third rounds of biopanning, concentration of the detergent in the wash buffer may be increased up to 0.5% Tween 20, which may increase the specificity and decrease the variety of binding peptides.

8. For a general target protein, ten clones are sufficient for the determination of a distinct binding sequence. However, in case that a CCN family protein is used as a target, analyzing more clones is recommended.
9. Plates should not be stored longer than 3 days and should not have too many (<100) plaques on it.
10. The amplified phage stock is relatively stable and can be stored at 4 °C for several weeks. Be sure to titer it again immediately before use.
11. Although this library was designed to display various dodecapeptides from random DNA sequences, the resultant peptide sequences are not necessarily random. This is partly because the genetic codes are not evenly assigned to all of the 20 amino acids. Additionally, bias in the distribution of the amino acid residues may result from the codon usage preferred by the host *E. coli* strain. It should be noted that, in this particular library, in spite of the expected frequency of 6.2%, proline residues are actually observed at a frequency of 12.2%. Contrarily, cysteine residues appear at a frequency of only 0.5%, although it is expected in 3.1% of total residues.
12. A typical example is TE buffer: 10 mM Tris–1 mM EDTA, pH 8.0.
13. Examination of background signal under this method is extremely important, especially if panning has been carried out in polystyrene multiwell plates.
14. Following this strategy, we designed, chemically synthesized, and evaluated the specific binding ability of three aptamers for the IGFBP, VWC, and CT modules of CCN2. Interestingly, in spite of the strongest binding ability, IGFBP aptamer revealed no biological effect, whereas VWC aptamer with the intermediate binding affinity showed a significant effect on chondrocyte proliferation.

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Chapter 17

Evaluation of Molecular Interaction between CCN2 Protein and Its Binding Partners by Surface Plasmon Resonance (SPR)

Eriko Aoyama and Masaharu Takigawa

Abstract

The surface plasmon resonance (SPR) biosensor is a useful tool to analyze numerically the interaction of certain molecules. The most important advantage of the SPR assay as compared with other protein–protein binding assays is that it can calculate the affinity between protein and its binding partner, for this affinity is necessary to determine the priority of interactions between proteins. Although CCN proteins have been shown to have various binding partners, the affinities of many of them have not yet been determined. Therefore, it is important to determine the unknown affinities of known binding partners and to find new binding partners whose affinities need to be determined. This chapter provides helpful tips to use the instrument for determination of the affinities of binding between CCN proteins and their binding partners.

Key words Surface plasmon resonance, Biacore, Binding affinity, Dissociation constant, Amine coupling, Resonance unit (RU)

1 Introduction

Surface plasmon resonance biosensor monitors the interaction between two molecules based on the SPR phenomenon (Fig. 1) [1]. SPR system is used to analyze and compare the affinities of protein and other factors such as proteins, peptides, or sometimes glycosides. This system has some advantages over other protein–protein interaction analysis methods such as the pull-down assay or solid-phase binding assay, either of which requires antibodies against the corresponding proteins. Furthermore, the latter method is costly. In addition, the variation; in antibody titers makes the comparison of results difficult. Moreover, these assays can reveal only whether proteins can bind to each other or not. In contrast, the SPR system can also determine kinetic parameters of interactions between these proteins.

Under natural circumstances, there are growth factors, cytokines, carbohydrate chains, or metal ions that can sometimes

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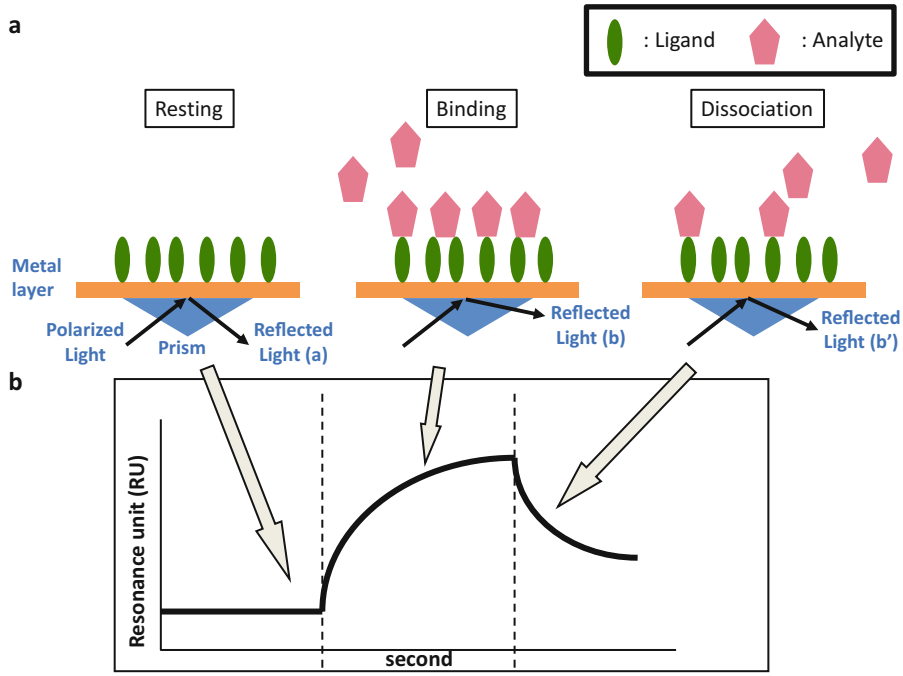

Fig. 1 Surface plasmon resonance (SPR) biosensor system. (**a**) The surface plasmon resonance (SPR) biosensor system consists of a prism with a thin gold surface on which the ligand molecules are immobilized. The analyte molecules flow over the ligand. The polarized light under a specified condition interacts with a surface plasmon of the metal layer and is absorbed. The phenomenon is called surface plasmon resonance (SPR). When the analyte binds to the ligand, the resonance condition changes. The SPR change alters the angle of reflected light from "a" to "b." Also, during the dissociation of the analyte, the angle is altered from "b" to "b.'" (**b**) The shift in angle is recorded in real time as a resonance unit (RU). The plot with time points on the *X*-axis and RU on the *Y*-axis known as a sensorgram. The association rate constant (k_a) and dissociation rate constant (k_d) are calculated from the sensorgram curve, and the dissociation constant (K_d) is given as k_d/k_a

influence the binding between targeted proteins; but the SPR system only analyzes protein–protein binding in the absence of other molecules. In other words, the results obtained from an SPR assay are based on only the characteristics of the targeted proteins themselves, such as structure, electric charge, and so on. However, many other factors are present under physiological conditions. In this respect, the pull-down assay using cell lysates might be closer to the natural condition; but this assay cannot provide kinetic parameters. Considering that kinetic parameters are the most important information concerning protein-protein binding, SPR is the best assay to prove the significance of the binding even though it has a minor disadvantage as described above. The results from an SPR assay in conjunction with those from other binding assays reflecting the natural situation would reveal the significance of the binding of proteins depending on their surroundings. Therefore, the SPR assay becomes more and more important when we investigate protein-protein interactions.

CCN2 consists of four modules, and each module has many binding partners, such as growth factors [2–5], extracellular matrices [6, 7], and cell components [7, 8]. CCN2 is also known to regulate these factors via direct binding to them. Thus it is necessary to investigate the affinities between CCN2 or its modules and binding factors to clarify which factor or which module is the most important in these binding situations.

By SPR analysis, we earlier showed (1) the affinities between CCN protein and other proteins, (2) the affinities between CCN protein and peptides, and (3) the effect of calcium ions on the affinity of CCN2 and its binding protein [3–6, 9]. Thus, SPR analysis has already contributed to identifying a number of interactions between CCN2 protein and other molecules and is expected to continue to make such a contribution. Moreover, SPR analysis can be applied to other CCN proteins such as CCN1, which is known to interact with integrins [10]; CCN3, which is known to bind to CCN2 [11] and Notch-1 [12]; and CCN4, which is known to bind to decorin and biglycan [13]. This chapter describes how to choose the equipment and methods to evaluate molecular interaction between CCN family protein and its binding partners by SPR.

2 Materials

All solutions for assay should be sterilized and kept in 4 °C. Chemicals are analytical grade.

1. Biacore® X (GE Healthcare UK Ltd., Buckinghamshire, England) (*see* **Note 1**).
2. Sensor chip (GE Healthcare UK Ltd.) (*see* **Note 2**).
3. Acetate 4.0 (GE Healthcare UK Ltd.) (*see* **Note 3**).
4. HBS-EP (GE Healthcare UK Ltd.): 10 mM Hepes-HCl, 150 mM NaCl, 0.3 mM EDTA, 0.005% surfactant P20 [pH 7.5] (*see* **Note 4**).
5. HBS-P (GE Healthcare UK Ltd.): 10 mM Hepes-HCl, 150 mM NaCl, 0.005% surfactant P20 [pH 7.5] (*see* **Note 5**).
6. Amine Coupling Kit (GE Healthcare UK Ltd.).
7. Glycine-HCl (pH 1.5) (GE Healthcare UK Ltd.).

3 Methods

There are two types of methods to analyze the affinity in the Biacore system. One is “multi-cycle kinetics” (Fig. 2), which can analyze the interaction between two sites or molecules, although it requires an analyte-removing procedure called “regeneration” after each reaction. Another is “single-cycle kinetics” (Fig. 3),

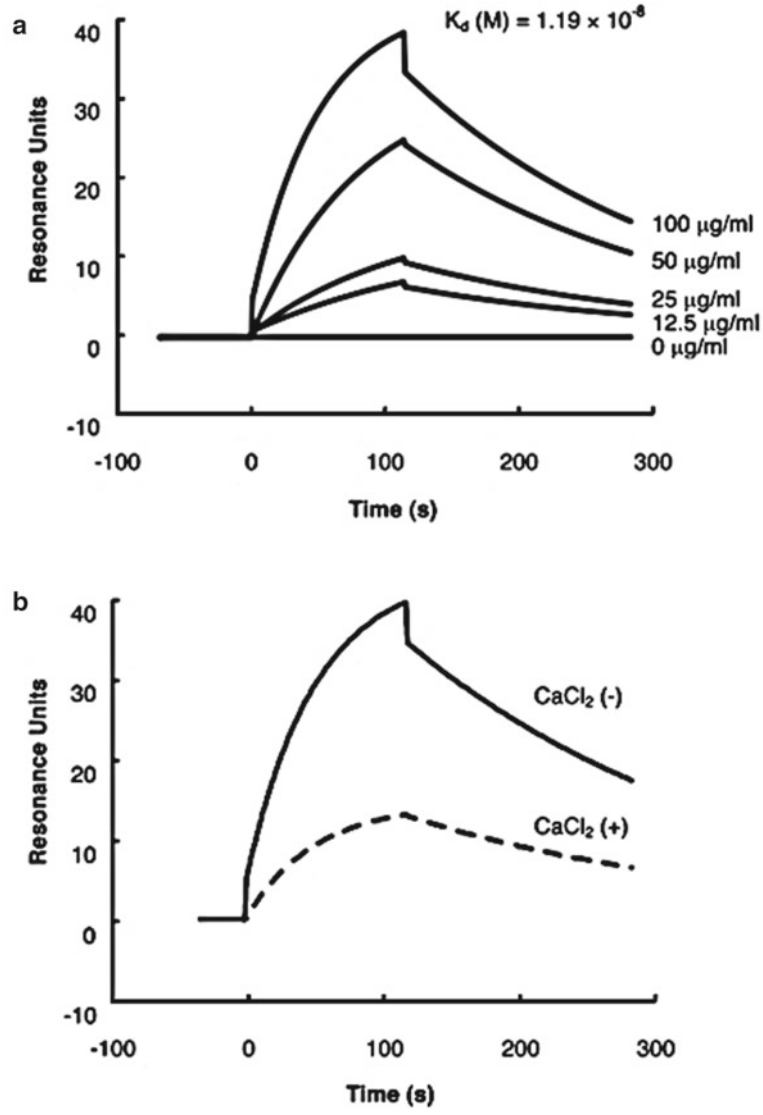

Fig. 2 Examples of sensorgrams with multi-cycle kinetics. (**a**) This sensorgram shows that the indicated concentrations of the aggrecan G3 domain bind dose dependently to CCN2 adsorbed on the sensor chip. The dissociation constant (K_d value) was calculated by analyzing these sensorgrams with Langmuir 1:1 binding. (**b**) This sensorgram presents the responses of the aggrecan G3 domain against CCN2 in the presence or absence of calcium ions (Reproduced from Aoyama et al. [6] by permission of the publisher)

which does not require this "regeneration" procedure after each reaction of analyte with ligand (An immobilized molecule is called the "ligand" in the Biacore assay and the molecule in flow buffer is called the "analyte."). However, it can analyze only 1:1 binding.

Because CCN2 is a strongly adhesive protein that binds to the Biacore sensor chip nonspecifically, it is impossible to remove

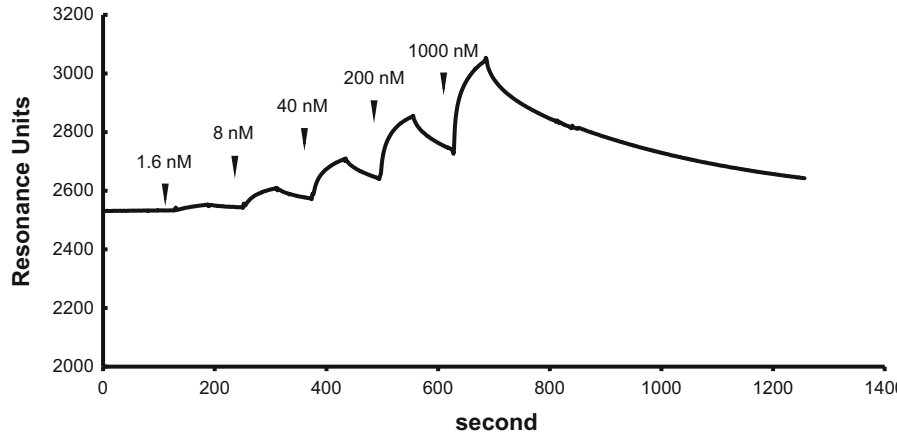

Fig. 3 An example of a sensorgram with single-cycle kinetics. The sensorgram shows that the indicated concentrations of fibroblast growth factor receptor 2 bind dose dependently to CCN2 adsorbed on the sensor chip. The dissociation constant (K_d value) was calculated by analyzing these sensorgrams with Langmuir 1:1 binding (Reproduced from Aoyama et al. [3] by permission of the publisher

CCN2 completely after the binding reaction if it is used as the analyte. Therefore, instructions described below show the methods using CCN2 as a ligand protein. In the case that CCN2 is used as an analyte, the C1 chip and running buffer including 0.005% surfactant P20 are suitable for the analysis.

3.1 The Operation for Single-Cycle Kinetics

1. Insert a sensor chip into the Biacore® X and perform the "Prime" procedure.
2. Treat flow cell 1 (reference cell) on the sensor chip with Acetate 4.0 and Amine Coupling Kit according to the standard "Amine Coupling" procedures.
3. Dilute recombinant CCN2 at concentrations of 10–25 μg/ml with Acetate 4.0 (*see* **Notes 6–8**).
4. Immobilize CCN2 protein on flow cell 2 by using a diluted CCN2 solution and Amine Coupling Kit.
5. Perform the "Priming" procedure three times to wash out nonspecifically bound CCN2.
6. Prepare five concentrations of analyte solution by diluting at the same ratio four times with running buffer (*see* **Note 9**).
7. Load diluted samples according to the guidance of the macro program for single-cycle kinetics.
8. Analyze the data with BIAevaluation software version 4.1 (GE Healthcare UK Ltd.) with the single-cycle kinetics support package (GE Healthcare UK Ltd.).

3.2 The Operation for Multi-cycle Kinetics

1. Immobilize the ligand protein and wash according to the instructions written in Subheading 3.1, **steps 1–5**.
2. Load the lowest concentration sample of five dilutions manually.
3. Remove analyte molecules binding to the ligand with glycine-HCl, pH 1.5 (*see* **Note 10**).
4. Repeat loading and removing from the lowest sample to the sample of highest concentration.
5. Analyze the data with BIAevaluation software version 4.1 (GE Healthcare UK Ltd.).

4 Notes

1. All experiments in this chapter are carried out with the most basic type of SPR instrument (Biacore® X). The methods described here are also useful for the assays with machines developed later.
2. Several types of sensor chip are purchased from the company. Usually the CM5 chip is the one of first choice, because it has a moderate capacity for protein immobilization. However, one should also prepare a C1 chip for the analysis for nonspecific binding of proteins, because nonspecific binding to the C1 chip is lower than that to the CM5 one.
3. Acetate 4.0 (acetate buffer, pH 4.0) is the most suitable for immobilization of recombinant CCN2. When another protein is used as a ligand, one should test which pH is best suited for the protein according to the Biacore BIAevaluation Software Handbook (GE Healthcare). All basic procedures are performed according to this handbook unless otherwise noted.
4. Running buffers (e.g., HBS-EP) need to be degassed by sonication with vacuum before use even if they are commercially available. Also, they should be filtered to remove debris when made by oneself.
5. HBS-P without EDTA is suitable for testing the influence of metal ions on the binding (Fig. 3b).
6. The protein as a ligand should be as pure as possible, because the sensorgram from contaminants immobilized on the chip can interfere with the analysis of the affinity between ligand and analyte.
7. The amount of immobilized protein should be adjusted depending on the assay. For just confirming the binding reaction, a sufficient mass of ligand should be immobilized by "Amine Coupling" procedures. For affinity analysis, the amount of immobilized ligand should be manually adjusted to

around 2000 resonance units (RU). When targeted analytes are those with low molecular weight such as peptides or are expected to have low affinity, it is recommended to immobilize as large a mass of ligand as practical, because the SPR response is proportional to the mass of the ligand.

8. For the CM5 chip, 10 μg/ml of CCN2 dilution is enough, whereas the C1 chip requires 25 μg/ml.
9. Ideally, the analyte concentration should be from ten times to one-tenth of the predicted Kd. However, if the K_d value cannot be estimated, the concentrations can be adjusted to the range of 10 nM to 1 μM. If the analyte is already dissolved in solvent/salts or cannot be dissolved in the running buffer, the amount of contaminating solvent/salts should be decreased as much as possible. If dilutions contain any solvent/salts except running buffer, prepare another series of five solutions by diluting the solvent/salts at the same ratio four times with running buffer, subject them to SPR in parallel, and subtract their sensorgrams from those of the corresponding analyte dilutions, respectively, because the solvent/salts sometimes interfere with obtaining an accurate sensorgram.
10. Glycine-HCl (pH 1.5) does not damage immobilized CCN2 so much. The damage can be confirmed by comparing the height of RU with that obtained previously at the same concentration of the same analyte.

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Chapter 18

Promoter Analyses of CCN Genes

Takanori Eguchi, Satoshi Kubota, and Masaharu Takigawa

Abstract

Promoter analysis is the most basics in the analysis of gene regulation. Luciferase gene is the most commonly used reporter gene in promoter analysis. Luciferase is an enzyme that is used when firefly and *Renilla reniformis* (sea pansy) emit light. The first experimental step in this reporter gene assay is to connect a particular DNA segment to a luciferase gene. The second step is to transfect the reporter construct into the cells. Thereafter, stable luciferase will be produced with the help of transcriptional machinery, mRNA transporters, and translational machinery in the cells. Luciferase assay measures the quantity of light that is emitted by luciferin–luciferase reaction. Consistent with the fact that CCN2 expression has been shown to be altered by a variety of stimuli, the CCN2 promoter region also haa been shown to be bound and regulated by multiple transcription factors such as Smad, MMP3, NF-κB, AP1, TCF/LEF, and Sox9.

Key words Promoter analysis, Transcription, Gene expression, Luciferin–luciferase reaction, Reporter gene assay, CCN2, CCN family

1 Introduction

Transcripts including messenger RNA (mRNA) are transcribed from their template DNA in genome in nuclei in eukaryotic cells before translated proteins are produced and have some roles in living bodies. Functional noncoding RNAs (ncRNAs) are also transcribed from genomic DNA through transcription. Gene expression in development is controlled in a spatiotemporal manner. CCN gene family members (CCNs) have crucial roles in development, wound healing, and tissue regeneration, so gene expression of CCNs should be promoted and repressed during defined periods at defined sites/cells during these processes [1–4]. Expressions of CCNs have been reported also in cancer and other diseases [1–4]. Thus this gene family is supposed to be controlled by oncogenes, tumor suppressor genes, genetic mutations, and epigenetic factors. Therefore promoter analyses of CCNs are important and useful in exploring basic developmental biology, pathology, and applied medical sciences of relevant fields. The authors have examined

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gene expression and transcriptional regulation of CCN2 [5–8]. In this chapter, we provide a high-throughput screening (HTS)-compatible protocol of promoter analyses, including analysis of CCN2 promoter that we published between 2001 and 2008 as an example and state-of-the-art in silico methods using databases and web tools.

2 Materials

2.1 In Silico Analysis

1. Personal computer.
2. Web browser and Internet connection.

2.2 Preparation of Plasmid Constructs

1. Reporter plasmids (pCCN2-luc and mutants) (*see* **Notes 1** and **2**).
2. Control plasmid (phRL-TK) (*see* **Note 3**).
3. (Option) Effector plasmid (pcDNA3-SMAD2) (*see* **Note 4**).
4. DH5α competent cells, LB, LB plates, ampicillin.
5. Plasmid purification kit (e.g., QIAGEN plasmid Midi kit) (*see* **Note 5**).
6. Spectrophotometer (*see* **Note 6**).
7. $T_{10}E_{0.1}$ buffer (*see* **Note 7**).

2.3 Cell Culture

1. Cells (*see* **Note 8**), medium.
2. Heat-inactivated FBS (*see* **Note 9**), penicillin/streptomycin (*see* **Note 10**).
3. CO_2 incubator, clean bench.

2.4 Transfection, Growth Factors and Inhibitors

1. Plasmids mentioned above.
2. Transfection reagent (e.g., FuGENE HD transfection reagent) (*see* **Note 11**).
3. Serum free medium (e.g., Opti-MEM I, GIBCO).
4. U-bottom plate (*see* **Note 12**).
5. Clean bench.
6. (Option) TGF-β, other growth factors or cytokines) or inhibitors.

2.5 Lysis and Luciferase Assay

1. Luciferase assay reagents (e.g., Bright-Glo luciferase assay system, Promega) (*see* **Note 13**).
2. Luminometer (*see* **Note 14**).
3. A 96-well white plate (*see* **Note 15**).

3 Methods

3.1 In Silico Promoter Analysis

1. Know the location of a promoter (*see* **Note 16**).
   Open UCSC genome browser (https://genome.ucsc.edu/) and search it with a keyword, e.g., CCN2. Change the size and range of the view (*see* **Note 17**). Taka a snapshot. Copy and paste onto a presentation file.
2. Download DNA sequence.
   Open Eukaryote Promoter database (EPD) (http://epd.vital-it.ch/). Search this with a keyword, e.g., CCN2. Input the range, e.g., –2000 and +500 (*see* **Note 16**) and then download the sequence.
3. Prediction of transcription factor (TF) binding sites.
   Open PROMO/TRANFAC (http://bit.ly/1LkHwon). Input the sequence obtained in EPD. TF binding sites and scores will be displayed (*see* **Note 16**). Copy and paste the search results onto a text editor or a presentation file.

3.2 Preparation of Constructs

1. Request plasmid constructs from researchers or purchase or prepare them by molecular cloning (*see* **Note 18**).
2. Subcloning of constructs (Optional) (*see* **Note 13**).
3. Transformation of competent *E. coli* cells with the plasmids.
4. Purify plasmids using a plasmid purification system (*see* **Note 4**).
5. Check the plasmids by cutting them with restriction enzymes and then check their sizes with agarose gel electrophoresis.
6. Determine the concentration and purity of the plasmid DNAs with a spectrophotometer as follows: Measure absorbances (optical density) A_{260} and A_{280} and estimate DNA purity via (A_{260}/A_{280}) value. This should be between 1.7 and 2.0 (*see* **Note 19**). Compute the DNA concentration (μg/ml) following this formula: (A_{260} reading – A_{320} reading) × dilution factor × 50 μg/ml. Dilute and adjust concentration of plasmids by adding $T_{10}E_{0.1}$ buffer to 100–1000 ng/μl for the 96-well transfection format (*see* **Note 20**). Store plasmid at –20 °C. Thaw before use.

3.3 Cell Culture

1. Seed cells in a 96-well plate in recommended medium (*see* **Note 21**).
2. Cultivate cells to be attached to dish and further to 60–70% confluency (*see* **Note 22**).

3.4 Transfection (×10 Transfections)

1. Prepare plasmids, FuGENE HD, Opti-MEM, and a U-bottom plate at RT in a clean bench.
2. Take 100 μL of Opti-MEM in a well of the U-bottom plate for ten transfections.
3. Add 8 μL of FuGENE HD into the Opti-MEM. Mix ten times by pipetting. Use 3–8 μL of FuGENE HD.

4. Dispense 10 × 10 μL of the FuGENE/Opti-MEM to ten wells of the U-bottom plate.
5. Add 0.4 μg of plasmid DNA into the Opti-MEM–FuGENE mixture. Reporter:Control:Effector = 5:1:5 (*see* **Notes 2** and **23**). Plasmids that are common among the transfections can be premixed within Opti-MEM after **step 2** or **3** before dispensing.
6. Incubate the mixture at RT for 15 min.
7. Change medium (*see* **Notes 9** and **10**). Add TGFβ, inhibitors, or other factors, if needed.
8. Add the plasmid–FuGENE–Opti-MEM mixture into cell culture medium. Gently swirl the cells.
9. Culture cells for 12–20 h. Cells should be 80–90% confluent. If they reach 100% confluence, lysis and reporter assay can be done here at day 1 after the transfection.
10. Change medium. Add TGFβ, inhibitors, or other factors, if needed. Culture cells overnight.

3.5 Lysis and Luciferase Assay

1. Dissolve Luciferin powder in 10 ml Bright-Glo luciferase assay buffer (lysis and reaction buffer). Dispense 1 ml × 10 tubes. Store at −20 °C. Avoid freeze–thaw cycles. Thaw before use.
2. Take out a 96-well tissue culture plate from an incubator. The amount of media can be around 90 μL due to evaporation.
3. Aspirate 50 μL of media with a multipipettor. Around 40 μL should remain.
4. Add 40 μL of Luciferase assay buffer (prepared at **step 1**) with a multipipettor.
5. Scratch the bottom of the wells with tips attached to the multipipettor (optional) (*see* **Note 24**).
6. Lyse the cells by incubation at RT for 10 min.
7. Pipet the cells every 3 min. This pipetting can promote lysis (optional).
8. Transfer 40 μL from each well to a 96-well white plate with a multipipettor (*see* **Note 25**).
9. Insert the plate into a luminometer and measure luminescence four times (*see* **Note 26**).
10. Save the data to USB memory. Calculate means and standard deviations in Excel. Generate graphs.

4 Notes

1. The authors and other research groups have prepared CCN promoter reporter plasmid constructs and mutants [5–12]. The authors are ready to provide a 802 bp CCN2 promoter

reporter plasmid (pCCN2-luc) [5], its derivative with mutated SMAD-binding element [7], that with mutated TGFβ response element [5], and that with mutated TRENDIC-putative MMP3 binding element [8]. A number of deletion mutants of the pCCN2-luc are also available. Plasmids published will generally be provided by the researcher(s) who cloned them originally. If you want to clone by yourself, please refer another guidebook, e.g., Molecular Cloning (*see also* **Note 17**).

2. Reporter assay, or reporter gene assay is a method to examine the regulation of promoters, 3′-untranslated regions (3′-UTRs) [13–18], and behavior of proteins as reporter-fusion proteins with artificial transfection of reporter gene constructs into cells. Luciferase, GFP-related fluorescent proteins, and LacZ have been frequently used as reporters that are not endogenously expressed in experimental animals or mammalian cells. The features of a reporter assay are as follows: it is (1) artificial, (2) easy and convenient, (3) has a wide range of applications including promoter analysis, drug screening, and in vivo imaging in transgenic animals to visualize where the regulatory sequence or the gene of interest is activated.
3. Researchers can notice and normalize the variation in experimental condition among the wells, e.g., transfection efficiency, number of cells, cell death (toxicity), somewhat by using Renilla luciferase expression control vector. The control vector can be omitted in the system of HTS. However, viability or toxicity should be examined in another assay in HTS. The authors recommend thymidine kinase (TK) promoter as control [5–8]. The authors tested also CMV, EF1α, and SV40 promoters; however, these promoters drive a control gene too strongly through usage of endogenous transcription factors too much and can disorganize regulation of other promoters.
4. One or two effectors can be tested here in the co-transfection experiment. pcDNA3 series are frequently used in driving cDNA expression from CMV promoter [7, 8].
5. Purity of the plasmids affects transfection efficiency and toxicity. LPS can possibly contaminate the plasmids during transformation and purification steps. LPS can trigger innate immune response in the experiments. To avoid LPS contamination, an LPS removal plasmid purification system is available from several manufacturers.
6. A spectrophotometer is an absorbance-based quantification machine. Fluorescence-based quantification of DNA is recently available as well.
7. Classical protocol of TE is 10 mM Tris and 1 mM EDTA. Because EDTA can inhibit the activities of some enzymes, the authors use Tris 10 mM, EDTA 0.1 mM.

8. The authors tested various types of cell lines in this protocol —HCS-2/8, COS7, HEK293, HeLa, 293T, HUVEC, and NIH3T3 [5–8]. Transfection efficiency and methods depend on the type of cells. Transfection reagents are good enough for cell lines such as 293 or HeLa cells with high transfection efficiency. In a difficult transfection case, such as the one with primary cells or slowly proliferating cells, electroporation can be adopted, e.g., Thermo Fisher Neon or Amaxa/LONZA Nucleofector systems (*see also* **Note 10**).
9. Serum depletion is not required for FuGENE transfection.
10. Removal of antibiotics is required for Lipofectamine series, but not for FuGENE.
11. FuGENE series (Roche/Promega) are less toxic than Lipofectamine series (Invitrogen) which though more toxic have higher transfection efficiency. As an experience, the authors previously examined secretion of HMGB1, a DNA binding protein upon DNA transfection. HMGB1 was known to be released from cells upon cellular damage, which is the so-called "danger signal." HMGB1 also has some role as damage-associated molecular pattern (DAMP). The authors applied Lipofectamine alone to RAW264.7 macrophage-like cells as a negative control. As a result, HMGB1 was detected at a high concentration in the cell culture supernatant. Thus Lipofectamine can damage cells, leading to release of HMGB1. It was suggested that Lipofectamine could not be useful in this particular experiment. However, FuGENE was useful here.
12. Low absorbance tube or plate is also useful.
13. The authors have used highly sensitive Bright-Glo system for high-throughput experiments and Dual-Glo system for controlled dual luciferase assay. Recently NanoLuc dual luciferase reporter assay system (Promega) has the highest sensitivity. Robotics is useful for HTS. Secretory luciferases are also recently available including *Cypridina* Luc (NEB) and Secreted *Metridia* Luciferase (Takara Clontech). Secretory luciferase can be helpful to establish HTS/monitoring, since it can be detectable and quantified within cell culture supernatant without cell lysis. GFP reporter is visible under fluorescent microscopy and quantitative with fluorescence-compatible cell counter LUNA FL.
14. This protocol is written for a 96-well format luminometer or a plate reader that can measure luminescence. Single tube type luminometer is useful for regular luc assay.
15. White plates have the tendency to enhance fluorescence, resulting in higher sensitivity and higher background, whereas black plates have the tedency to reduce fluorescence, resulting in lower background and lower sensitivity. Both are useful in this protocol. Clear plates are not useful in this system.

16. There are two definitions of a promoter. In a narrow sense, a promoter is a region required for minimum transcription and also known as a core promoter or proximal promoter, usually sized 50–100 bps in length. In a broad sense, a promoter is a region including a core promoter and regulatory sequences such as enhancers, usually a region around transcription start site (TSS) between −2000 and +500. This range has been in many cases examined and found functional; however, the other regions can have some role for gene regulation, for example through bending DNA. DNA binding type transcription factors can recognize *cis*-elements such as enhancers and silencers. Currently, TSS, promoter regions, and TF binding sites can be searched in the database by web tools as mentioned above. The ENCODE project revealed genome-wide promoter regions marked by histone H3 lysine 4 tri-methylated (H3K4me3) and genome-wide enhancer regions marked by H3K4me1 and H3K27ac [19]. These ENCODE data of promoters/enhancers can be viewed in UCSC genome browser. In order to investigate transcription events on genomic DNA, chromatin immunoprecipitation (ChIP)-seq, RNA-seq, and RT-qPCR are useful in combination with the reporter assay [8].
17. Only classical and established TFs will be displayed. These are landmarks in promoter analyses. However, other factors not shown in the prediction can frequently control transcription indeed [8]. Factors related to the TFs hit in this in silico analysis can be further tested in co-transfection experiments, with ChIP, ICC, and IHC as candidates of upstream transcriptional regulators [8]. In addition, transcription is not only controlled by DNA sequences as *cis*-elements and their *trans*-factors, DNA-binding TFs but also by non-DNA-binding type TFs including chromatin remodeling factors/complex, histone tail modifications by histone modification enzymes, DNA methylation and demethylation by DNA methyl transferases (DNMTs) and TETs, RNA polymerase II C-terminal repeat code (CTD code), insulators, DNA break and repair factors, and ncRNAs. For determination of further regulatory cofactors, co-immunoprecipitation, mass spectrometry, and next-generation sequencing are applicable.
18. There are four means to obtain a gene promoter—requesting a researcher, purchase, requesting a cloning protocol, or DIY. Currently Addgene has been established as a bank of cDNA clones and vectors. Researchers who prepared cDNA clones in plasmids or viral vectors can delegate administration and provision to Addgene. Users can purchase constructs at a low cost from Addgene. Some other companies and government agencies provide similar services. Researchers also can directly request a clone of interest from a researcher who originally prepared it.

19. If the A_{260}/A_{280} ratio is lower than 1.6, protein derived from *E. coli* can be contaminated. Repurification is required.
20. If the effects of some species of plasmid, e.g., various mutants, are going to be compared, transfection of same amount with same concentration is best for fair assay.
21. Six-, 12-, 24-, 48-well plates are also compatible [5, 6]. Larger scale requires more materials including plasmids and transfection reagents, but is easier for beginners to handle.
22. Usually between 4 and 48 h, depending on the type of the cells [5].
23. The ratio of the reporter, control, and effector plasmids is dependent on the context at hand. Another ratio can be tested.
24. This step can promote detachment and lysis of the cells.
25. The remaining lysate is valuable as a backup just in case.
26. Means and errors can be calculated from four times measurement. In addition, quadruplicate independent well experiments are recommended in the 96-well format, whereas triplicate is usually sufficient in 24-well plate format.

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Chapter 19

Analysis of Posttranscriptional Regulation of CCN Genes

Seiji Kondo, Satoshi Kubota, and Masaharu Takigawa

Abstract

Cells generally control the concentration of mRNA by transcriptional and posttranscriptional regulation, so the separate contributions of synthesis and degradation ("decay") cannot be discriminated by the quantification of mRNA. To elucidate the contribution of posttranscriptional regulation, all experimental procedures for the analysis of the total transcript level, transcriptional induction, and degradation of the target mRNA are performed either individually, or in combination. From our experience, measurement of the steady-state levels of the mRNA using quantitative real-time polymerase chain reaction is an essential first step in quantifying *ccn2* gene expression level. Subsequently, the effect of transcription rates should be assessed by reporter assays of the *ccn2* promoter and nuclear run-on assays. Finally, the stability of *ccn2* mRNAs is evaluated in the presence of a metabolic inhibitor actinomycin D, followed by mRNA degradation assays in vitro. Here, we describe the strategic methods used in a series of analyses to elucidate the possible involvement of the posttranscriptional regulatory mechanism of the *ccn2* gene and show how this approach can in theory be applied to elucidating the posttranscriptional regulation of other genes belonging to the CCN family.

Key words CCN, Gene expression, Posttranscriptional regulation, mRNA degradation, 3′-Untranslated region (3′-UTR)

1 Introduction

The expression level of a variety of human cytokine and chemokine genes is regulated at both the transcriptional and posttranscriptional levels. Transcriptional-level control is typically through changes in transcription rates, and posttranscriptional control is typically through changes in mRNA stability. The involvement of one mechanism versus the other depends on the cell-specific or tissue-specific behavior of the gene product; in addition, the type of regulation changes in response to the environment [1]. mRNA stability is a key aspect of the gene regulatory mechanism in the posttranscriptional processing of mRNA [2, 3]. Posttranscriptional processing of mRNA is an essential and well-controlled component of posttranscriptional control and often occurs in the cytoplasm by the cytoplasmic mRNA degradation ("decay") pathway.

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This mechanism allows a rapid increase or decrease in the concentration of a specific mRNA in response to the cell's requirements.

The CCN family is a group of genes encoding proteins with a novel structure and multiple functionalities and consists of *ccn1* (*cef-10/cyr61*), *ccn2* (*ctgf/Hcs24*), *ccn3* (*nov*), and several related genes such as *ccn4* (*elm-1/wisp-1*), *ccn5* (*ctgf-3/wisp-2/cop1*), and *ccn6* (*wisp-3*) [4, 5]. Control of the induction of the CCN2/connective tissue growth factor (CTGF) gene has been characterized most extensively in this family; activation occurs both through the induction of transcriptional activity [6, 7] and increased mRNA stability to enhance the intensity of gene expression in mammalian [8–11] and chick [12, 13] cells. We reported that the *ccn2* mRNA degradation pathways are integral components in the control of CCN2 gene expression. These degradation pathways are important determinants of the extent of gene expression and play key roles in maintaining cellular function [14–17]. Complete understanding of the contribution of the post-transcriptional regulatory mechanism in *ccn2* gene expression requires ruling out the effect of transcriptional induction from the overall induction of the gene, since gene expression reflects the combined outcome of transcript synthesis and degradation. Furthermore, the 3′-untranslated regions (UTRs) of all CCN members except CCN6 are relatively long and their posttranscriptional regulatory machinery can be characterized using an experimental strategy similar to that used for the characterization of *ccn2* gene expression [18]. The experimental procedure has three particularly important steps. First, the steady-state mRNA level, which is determined by the synthesis and decay rates of mRNA, is initially analyzed by quantitative real-time reverse transcription polymerase chain reaction (qPCR) to determine total *ccn2* mRNA expression. Second, the role of transcription is evaluated by transient transfection studies to measure the activity of the *ccn2* promoter. The initiation of the *ccn2* promoter in vivo cannot be determined solely by transient transfection with a promoter [15]; therefore, the effect of transcription rates is directly assessed using a nuclear transcript run-on assay. Finally, the *ccn2* mRNA decay rate in vitro is measured after the addition of a transcription inhibitor, actinomycin D, followed by an in vitro mRNA degradation assay mimicking cytoplasmic mRNA turnover by incubating the cytoplasmic extract with the artificial 5′-capped polyadenylated transcripts. Below we describe methods for measuring *ccn2* mRNA expression and for determining the contribution of the posttranscriptional regulatory mechanism on CCN gene expression in vitro.

2 Materials

The use of molecular biology grade water, polystyrene or polypropylene RNase/DNase/nucleic-acid-free tubes, and aerosol-barrier pipette tips is strongly recommended throughout. The following is

the exact protocol for each step. Degradation of RNA by RNases can be avoided by the use of powder-free gloves and carefulness in preparing the buffers.

2.1 Quantitative Real-Time PCR (qPCR) (*See* Note 1)

2.1.1 Cells, Growth Media

1. Cells (adherent culture cell lines, primary cells).
2. Complete Dulbecco's modified Eagle's medium (D-MEM) containing high glucose, with 10% fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS).
4. 35-mm tissue culture plate.

2.1.2 RNA Extraction

1. TRIzol and TRIzol LS (Life Technologies) (*see* **Note 2**).
2. 1 ml disposable syringe and 25 G disposable needle.
3. Proteinase K.
4. Chloroform.
5. Isopropyl alcohol.
6. 70% ethanol: add 70 ml of absolute ethanol to nuclease-free water to make up a volume of 100 ml.
7. TE buffer: 10 mM Tris, 1 mM EDTA.
8. TES buffer: 10 mM Tris, 1 mM EDTA, 0.2% SDS (*see* **Note 3**).
9. DNase I (e.g., RQ1 RNase-Free DNase, Promega).
10. Ribonuclease inhibitor (e.g., RNasin, Promega).
11. Phenol–chloroform–isoamyl alcohol (25:24:1, v/v, pH 8.0).
12. 1.5 ml polystyrene or polypropylene tubes.
13. 3 M sodium acetate, pH 5.2.
14. Glycogen solution (e.g., ethachinmate, Nippon Gene).
15. NanoDrop spectrophotometer (Thermo Scientific).

2.1.3 Reverse Transcription

1. cDNA synthesis kit (Bio-Rad iScript™ cDNA synthesis kit).
2. Reverse transcriptase (e.g., an avian myelosarcoma virus (AMV), Invitrogen; moloney murine leukemia virus (MMLV) reverse transcriptase, Bio-Rad).
3. RNase-free water.
4. Thin-walled PCR tubes (8-tube PCR tube strips) and optical adhesive covers.
5. Thermal cycler.

2.1.4 qPCR

1. Serially diluted standard cDNAs.
2. SYBR Green I fluorophores (e.g., FastStart DNA Master SYBR Green I, Roche Diagnostics; iQTM SYBR Green Supermix, Bio-Rad).
3. Specific primer sets (10 μM working stock solutions) (*see* **Note 5**).

4. Thin-walled PCR tubes (8-tube PCR tube strips) and optical adhesive covers.
5. 96-well PCR tube racks.
6. Real-time thermal cycler: (e.g., Light Cycler®; Roche Diagnostics, iCycler iQ® Bio-Rad).

2.2 Luciferase Assay

1. Cells (adherent culture cell lines, e.g., MDA-231 cells) [14].

2.2.1 DNA Constructs, Cells, Growth Media

2. Cell culture medium (Complete D-MEM containing high glucose), with 10% fetal bovine serum (FBS).
3. D-MEM without antibiotics.
4. PBS.
5. Reporter plasmid DNA (e.g., pMAMneo-LUC, Clontech; pGL3-control, Promega). Vector pTS589 encoding firefly luciferase gene under control of *ccn2* promoter (–820 to 0 bp from transcription initiation site).
6. Internal control plasmid (e.g., pRL-CMV, the cytomegalovirus (CMV) enhancer/promoter, and pRL-TK, the herpes simplex virus thymidine kinase (HSV-TK) promoter-driven *Renilla* luciferase gene involved).
7. 6-well or 35-mm tissue culture plates, 96-well tissue culture plates.
8. Polystyrene or polypropylene tubes.
9. Tissue culture incubator.

2.2.2 Transfection Solution

1. Serum-free transfection medium (Opti-MEM I, Invitrogen).
2. Cationic lipid reagent (e.g., Lipofectamine 2000®, Life Technologies; TransFast, Promega).

2.2.3 Luciferase Assay

1. Luciferase assay reagent (e.g.Dual-Luciferase® Reporter Assay System www.promega.co.jp Promega).
2. Luminometer (www.promega.co.jp Promega).

2.3 Nuclear Run-On Analysis

1. Cultured cell lines.
2. Cell culture medium (D-MEM) with serum.

2.3.1 Cells, Growth Media

3. PBS.
4. 10-cm tissue culture dishes.

2.3.2 Preparation of Nuclei

1. Cell scraper.
2. Nonidet P-40 lysis buffer: 10 mM HEPES (pH 7.3), 10 mM NaCl, 3 mM $MgCl_2$, 150 mM sucrose, and 0.5% Nonidet P-40.
3. Glycerol buffer: 50 mM Tris–HCl (pH 8.5), 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA.
4. 1.5 ml Polystyrene or polypropylene tubes.

2.3.3 Transcript Reaction

1. Transcription buffer: 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 200 mM KCl, 4 mM DTT, 0.5 mM each of rATP, rCTP, rGTP and 2.5 μM of rUTP, 200 mM sucrose, and 20% glycerol.
2. rATP, rCTP, rGTP, rUTP (Promega).
3. Biotin-16-rUTP (Roche).
4. Dithiothreitol (DTT).
5. Isogen LS (Nippon Gene).
6. Phenol–chloroform–isoamyl alcohol (25:24:1).
7. 70% ethanol.

2.3.4 RNA Pull Down

1. Streptavidin-agarose (Sigma).
2. 1× binding buffer: 5 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 1 M NaCl.

2.3.5 Elution

1. Elution buffer: 10 mM EDTA, 95% Formamide.
2. Ethanol.
3. TE buffer: 10 mM Tris, 1 mM EDTA.
4. Heat/chill block (NISSIN).

2.3.6 Reverse transcription (See Subheading 2.1)

Reverse transcriptase (e.g., an avian myelosarcoma virus (AMV), Invitrogen; moloney murine leukemia virus (MMLV) reverse transcriptase, Bio-Rad).

2.3.7 qPCR (See Subheading 2.1)

1. SYBR Green I fluorophores.
2. Primer sets.
3. Thin-walled PCR tubes (8-tube PCR tube strips) and optical adhesive covers.
4. 96-well PCR tube racks.
5. Real-time thermal cycler.

2.4 Measurement of Half-Life of ccn2 mRNA Levels

2.4.1 Cells, Growth Media, Reagents

1. Cultured cell lines (e.g., HCS-2/8 cells, established from a human chondrosarcoma) [6, 7].
2. Cell culture medium (Complete D-MEM containing high glucose), with serum.
3. Incubator.
4. PBS.
5. Actinomycin D.

2.4.2 RNA Extraction (See Subheading 2.1.2)

1. TRIzol and TRIzol LS (Life Technologies) (*see* **Note 2**).
2. Proteinase K.
3. TE buffer: 10 mM Tris, 1 mM EDTA.
4. TES buffer: 10 mM Tris, 1 mM EDTA, 0.2% SDS (*see* **Note 3**).

2.4.3 Reverse Transcription (See Subheading 2.1)

1. Reverse transcriptase (e.g., an avian myelosarcoma virus (AMV), Invitrogen; moloney murine leukemia virus (MMLV) reverse transcriptase, Bio-Rad).
2. cDNA synthesis kit (Bio-Rad iScript™ cDNA synthesis kit).

2.4.4 qPCR (See Subheading 2.1)

1. SYBR Green I fluorophores.
2. Primer sets.
3. Thin-walled PCR tubes (8-tube PCR tube strips) and optical adhesive covers.
4. 96-well PCR tube racks.
5. Real-time thermal cycler.

2.5 Analyzing 3′-UTR

2.5.1 DNA Constructs, Cells, Growth Media

1. Parental vector (e.g., pUC18, pcDNA3.1) for a double-step subcloning method.
2. Reporter plasmid DNA.
3. Internal control plasmid (e.g., pRL-CMV and pRL-TK).
4. Pfu DNA polymerase.
5. RT-PCR reagent (*see* Subheading 2.1).
6. DNA ligation kit Ver.2.1 (TaKaRa).
7. Unique restriction enzymes.
8. NEB buffer (New England Biolabs) for restriction enzymes.
9. SUPREC 01 (TaKaRa).
10. Low-melting temperature agarose (e.g., SeaPlague™ Agarose, Lonza).
11. Competent cells (e.g., *E. coli* JM109, *E. coli* DH5α, TaKaRa).
12. LB or SOC media, LB agar: 5 g NaCl, 5 g tryptone, 2.5 g yeast extract, 7.5 g agar, add dH_2O to 500 ml.
13. 10 cm polystyrene petri dish.
14. Cells (adherent culture cell lines; e.g., MDA-231 cells).
15. Cell culture medium (Complete D-MEM), with serum but no antibiotics.
16. PBS.
17. Incubator

2.5.2 Transfection Solution

1. Serum-free transfection medium (Opti-MEM I, Invitrogen).
2. Cationic lipid reagent (e.g., Lipofectamine 2000®, Life Technologies; TransFast, Promega).
3. 6-well or 35-mm tissue culture plates, 96-well tissue culture plates.
4. Polystyrene or polypropylene tubes.

2.5.3 Luciferase Assay

1. Luciferase assay reagent.
2. Luminometer.

2.6 In Vitro Cell-Free mRNA Decay Assay

2.6.1 Preparing the Template

1. Materials for molecular cloning of target (including specific sequence elements from 3′-UTR) cDNA fragment (e.g., buffer, enzymes, and apparatus for PCR methods, subcloning) (*see* **Note 20**).
2. pGEM4Zf plasmid (Promega).

2.6.2 Cytoplasmic Protein Purification

1. Cultured cell lines, medium, and incubator.
2. PBS.
3. Cell scraper.
4. Syringe with a 27G needle.
5. CelLytic NuCLEAR extraction kit (Sigma-Aldrich).
6. Hypotonic lysis buffer: 100 mM HEPES pH 7.9, 15 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, protease inhibitor cocktail.
7. Extraction buffer: 1.5 mM KCl, 15 mM $MgCl_2$, 100 mM, Tris–HCl, pH 7.4, 5 mM DTT.
8. Bovine serum albumin (BSA).
9. BCA protein assay kit (Pierce).
10. Densitometer (595-mm wavelength).

2.6.3 Preparation of Capped, Polyadenylated, and Radiolabeled Transcripts

1. Capped polyadenylated RNA preparation kit (e.g., mMessage mMachine® kit; Ambion by Life Technologies).
2. [α-^{32}P]UTP (18.5 MBq/ml; Amersham Biosciences).
3. Capped transcription mixture: 2× NTP/ARCA 5 μl, 10× T7 reaction buffer 1 μl, linearized template DNA (0.1–1 μg) 2 μl, [α-^{32}P]UTP 1 μl, enzyme mix 1 μl.
4. Polyadenylated mixture: mMessage reaction mix 11 μl, E-PAP 2 μl, 5× E-PAP buffer 10 μl, 25 mM $MnCl_2$ 5 μl, ATP solution 5 μl, H_2O 17 μl.
5. Scintillation counter.

2.6.4 IVDA Reaction

1. In vitro cell-free mRNA decay assay (IVDA) buffer: 10 mM Tris (pH 7.5), 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 10 mM creatine phosphate, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine.
2. ISOGEN-LS reagent (Nippon Gene).
3. 25:24:1 phenol–chloroform–isoamyl alcohol.
4. Ethanol.

2.6.5 Electrophoresis and Quantitation of the Remaining Undegraded Transcript

1. Loading dye.
2. A denaturing 6% polyacrylamide–7 M urea gel.
3. Nuclease-free solutions of 10× TBE (Life Technologies).
4. Filter paper.
5. X-ray film.
6. Molecular Dynamics PhosphorImager (GE Healthcare).
7. Computer software, Quantity One (PDI Inc.).

3 Methods

3.1 qPCR (See Note 1*)*

3.1.1 RNA Extraction

1. Wash cells once with 1 ml of ice-cold PBS. Discard PBS using a fluid aspiration system.
2. Add 500 µl of ice-cold PBS, scrape cells, transfer cells into 1.5 ml microcentrifuge tubes, and spin down $0.1–1 \times 10^5$ cells harvested from a 35-mm tissue culture plate.
3. Add 200 µl of TRIzol reagent (*see* **Note 2**).
4. Using a 1 ml disposable syringe, pass the cells lysate several times through a 25 G disposable needle.
5. Add 200 µl chloroform, vigorously vortex for 10 s, and incubate at room temperature on the bench for 2–3 min.
6. Centrifuge for 15 min at 13,000 × *g* in a microcentrifuge.
7. Recover the aqueous phase into a fresh 1.5 ml microcentrifuge tube, add 300 µl of isopropanol, mix, and centrifuge for 10 min at 13,000 × *g* at 4 °C.
8. Remove supernatant with a fluid aspiration system, and wash the pellets with 500 µl of 70% ethanol and air-dry.
9. Dissolve the pellet in 10–50 µl nucleic acid-free and nuclease-free water or TE buffer (*see* **Note 3**).

3.1.2 DNase I Digestion

1. Each reaction is set up as follows:

RNA	10 µl
10 × DNase buffer	5 µl
RNasin	0.5 µl
RQ1 DNase	2.0 µl
RNase-free H_2O	To 50 µl

2. Incubate samples at 37 °C for 30 min.

3.1.3 RNA Precipitation

1. Add 130 µl of RNase-free water.
2. Add equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) to each microcentrifuge tube containing the RNA

samples. Vortex and centrifuge for 5 min at 13,000 × *g*, and collect the aqueous layer into a new microcentrifuge tube.

3. Add equal volume of chloroform–isoamyl alcohol (24:1) to the aqueous layer. Vortex and centrifuge for 5 min at 13,000 × *g*, and collect again the aqueous layer into a new microcentrifuge tube.
4. Add 20 μl of 3 M sodium acetate, pH 5.2.
5. Add 2 μl of glycogen solution.
6. Precipitate the RNA by adding 500 μl of absolute ethanol.
7. Vortex vigorously and centrifuge for 15 min at 13,000 × *g* at 4 °C.
8. Remove supernatant using a fluid aspiration system, and wash pellet by 500 μl of 70 % ethanol and air-dry.
9. Dissolve the pellet in 10–50 μl nucleic acid and nuclease-free water or TE buffer.
10. Quantify RNA yield from the OD_{260} value (*see* **Note 4**), check by electrophoresis in an agarose gel.

3.1.4 Reverse Transcription

1. Generate a complementary DNA (cDNA) mixture as a template by reverse transcription using oligo (dT) and an AMV or MMLV reverse transcriptase. The procedure for reverse transcription of RNA is as follows:

Components	Final concentration	Volume per well
5× iScript reaction mix	1×	4 μl
iScript reverse transcriptase		1 μl
Template RNA	0.1–50 ng	μl
H_2O		To 20 μl
Total volume		20 μl

2. The reverse transcription mixture is placed in the thermal cycler and incubated for 30 min at 42 °C as follows:

   5 min at 25 °C.

   30 min at 42 °C.

   5 min at 85 °C.

   Hold at 4 °C.

3.1.5 Amplify by qPCR

1. PCR master mixture.
   qPCR requires fluorophores, SYBR Green I, a thermal cycler apparatus, and design/validation of qPCR primers (*see* **Note 5**). PCR master mixture is prepared to contain deoxyribonucleotides in a suitable buffer (the magnesium concentration is generally higher than that of the dNTPs and primers, and some optimization of the magnesium concentration may be

necessary for different templates). The PCR master mixture also contains a thermo-stable DNA polymerase. qPCR is started using a primer concentration of 0.2 μM, but the primer concentration should be increased if PCR efficiency is low. PCR master mixture is prepared with 2× iQTM SYBR Green Supermix containing $MgCl_2$, dNTP, and iTaq DNA polymerase (Bio-Rad) as follows:

Components	Final concentration	Volume per well
2× iQTM SYBR Green Supermix	1×	25 μl
primer 1 (10 μM)	0.2 μM primer	1 μl
primer 2 (10 μM)	0.2 μM primer	1 μl
Template cDNA	0.5–10 ng	1 μl
H_2O	N/A	To 50 μl
Total volume		50 μl

2. qPCR cycle.
   The qPCR cycle consists of template denaturation, primer annealing, and elongation. Each stage of the cycle must be optimized in terms of time and temperature for each template and primer pair combination. For the human *ccn2* gene, qPCR is conducted using the following parameters (*see* **Note 6**).
   One cycle 10 min 95 °C initial denaturation.
   45 cycles 15 s 95 °C denaturation.
   10 s 55 °C annealing.
   10 s 72 °C elongation.
3. Quantification of PCR results.
   Target nucleic acids can be quantified via either absolute quantification (number of copies of an mRNA transcript present in a sample (*see* **Note 7**)) or relative quantification (fold-difference in expression of a particular RNA transcript between experimental samples (*see* **Note 8**)).

3.2 Promoter Luciferase Assay (See Note 9, Also See Chapter 18)

3.2.1 Prepare the Plasmid DNA

The promoter subfragments are amplified by PCR using combinations of specific primers, which confers restriction enzymatic cutting sites at both ends, and connected to a firefly luciferase reporter gene at the corresponding unique restriction enzyme sites. Reporter parental plasmids at the multiple cloning sites located upstream of the firefly luciferase gene are available from various companies. The amount of reporter protein synthesized under a given condition is presumed to reflect the ability of the inserted sequence to promote transcription (*see* **Note 10**).

3.2.2 Cell Culture and Seeding

All culture incubations should be performed in a humidified 5% CO_2 atm at 37 °C. The number of cells used (70–90% confluent before transfection) should be appropriate for the size of the tissue culture well. Antibiotics should not be used at the time of plating and during transfection.

Example: Twenty hours prior to transfection, MDA-231cells (2×10^5) are seeded in a 35-mm dish and cultured in D-MEM containing 10% FBS.

3.2.3 DNA Transfection

1. On the day of transfection, DNA and transfection reagent are diluted into separate aliquots of the serum-free transfection medium, Opti-MEM I, and are then mixed together, leading to the formation of DNA–lipid complexes. The optimal ratio of nucleic acid (μg) and transfection reagent (ml) ranges from 1:0.5 to 1:5. The recommended cell density and amount of DNA and reagents can be determined from the transfection reagent product profile sheet (*see* **Note 11**) before beginning the transfection. An internal control plasmid containing the *Renilla* luciferase gene controlled by a constitutive promoter (e.g., pRL-CMV, pRL-TK), is needed for co-transfection in order to control transfection efficiency.
   Example: (*see* **Note 12**).
   For subconfluent cells in a 35-mm dish

pTS589	1.0 μg
pRL-TK	0.5 μg
Opti-MEM I	To 100 μl in 1.5 ml polypropylene tubes

Lipofectamine 2000®	10 μl
Opti-MEM I	To 100 μl in 1.5 ml polypropylene tubes

   Combine the diluted plasmid DNA and diluted transfection reagent, and allow to form the DNA–lipid complex (200 μl) for 15 min at room temperature. Then, add 800 μl of Opti-MEM I.
2. Before transfection, the cells are rinsed with 1 ml of PBS or Opti-MEM I, then the complexed transfection medium (1 ml) described above is added. The cells are incubated for several hours, then 1 ml of serum (+) medium is added (*see* **Note 13**).
3. Cells are incubated for an additional day to allow expression of the transgene (*see* **Note 14**).

3.2.4 Measurement of Luciferase Activities

The Dual-Luciferase Reporter Assay System is used for the sequential measurement of firefly and *Renilla* luciferase activities. Quantification of both luciferase activities and calculation of their relative ratios are carried out manually using a luminometer (*see* **Note 15**).

3.3 Nuclear Run-On Analysis (See Note 16)

3.3.1 Preparation of Nuclei Fractions

1. Wash cells grown in 10-cm tissue culture dishes twice with 5 ml of ice-cold PBS. Discard PBS using a fluid aspiration system.
2. Add 5 ml of ice-cold PBS, scrape cells, transfer cells into 15 ml centrifuge tubes, and spin down for 15 min at 500 × *g*, discard PBS.
3. Lyse the pellet of the cells in 4 ml of Nonidet P-40 lysis buffer, vortex for 10 s, and incubate on ice for 5 min.
4. Spin down for 5 min at 500 × *g*, discard the supernatant using a fluid aspiration system.
5. Resuspend the pellet in 200 μl of glycerol buffer, and store at −70 °C.

3.3.2 Transcription Reaction

1. Add 200 μl of transcription buffer to 200 μl of the nuclei along with 25 μl of Biotin-16-rUTP, and incubate for 30 min at 30 °C with shaking.
2. Aliquot 200 μl of reaction mixture to 1.5 ml tube.
3. Add 600 μl of TRIzol LS, vortex vigorously, and incubate for 5 min at room temperature.
4. Add 200 μl of chloroform, vigorously vortex for 10 s, and incubate at room temperature on the bench for 2–3 min.
5. Centrifuge for 15 min at 13,000 × *g* in microcentrifuge.
6. Recover the aqueous phase into a fresh 1.5 ml microcentrifuge tube, add 500 μl of isopropanol, mix and centrifuge for 10 min at 13,000 × *g* at 4 °C.
7. Remove supernatant with a fluid aspiration system, wash the pellets with 500 μl of 70% ethanol and air-dry.
8. Dissolve the biotin-labeled pellet in 50 μl nucleic acid and nuclease-free water or TE buffer.

3.3.3 RNA Pull Down

1. 20 μl of streptavidin-agarose per one sample is aliquoted into a tube.
2. Centrifuge at 5000 × *g* for 1 min and remove the supernatant.
3. The pellets are resuspended with 50 μl of 1× Binding buffer and centrifuged to remove the supernatant.

This step is repeated three times.

4. Reuspend the beads with 50 μl of 2× Binding buffer.
5. Add an equal volume of the isolated biotin-labeled RNA samples, incubate for 30 min at room temperature.
6. Centrifuge at 5000 × *g* for 30 s, and remove the supernatant..
7. Wash with 50 μl of 1× Binding buffer. Repeat the washing step three times.

3.3.4 Elution

1. The pellets are washed with 50 ml of elution buffer.
2. Heat at 65 °C for 10 min, and centrifuge at 10,000 × *g* for 1 min.
3. Recover the supernatant into a fresh 1.5 ml microcentrifuge tube.
4. Add 10 µl of 3 M sodium acetate, pH 5.2.
5. Add 1 µl of glycogen solution.
6. Precipitate the RNA by adding 250 µl of absolute ethanol.
7. Resolve in 10 µl of TE buffer.

3.3.5 Synthesis of cDNA Strands and qPCR

Synthesis of cDNA strands and the amplification of the DNA are conducted using the same procedure as described in the qPCR subsection above (see Subheading 3.1). qPCR is performed using the primer set of interest.

3.4 Measurement of the Half-Life of ccn2 mRNA Levels (See Note 17)

The mRNA half-life is analyzed with actinomycin D (*see* **Note 18**) in cultured cell lines. The medium is selected according to the cell type. An incubator calibrated to deliver a 5% CO_2 atm at 37 °C is used.

3.4.1 Cells, Growth Media

3.4.2 Actinomycin D Treatment

1. Add actinomycin D to the cells at the final concentration of 5–10 µg/ml.
2. Cells are harvested immediately (time zero) or after 30, 60, 120, 180, 360, or 600 min (*see* **Note 19**) of actinomycin D treatment.
3. Discard the supernatant and wash the attached cells gently with PBS buffer.
4. Total RNA is isolated with TRIzol at the indicated times.

3.4.3 RNA Preparation, Synthesis of cDNA Strands, and qPCR

RNA preparation, synthesis of cDNA strands, and the amplification of the DNA are conducted using the same procedure as described in the qPCR subsection above (*see* Subheading 3.1).

3.4.4 Quantification of PCR

1. The ΔΔCt (cycle threshold) method is used (*see* **Note 8**). The results are normalized to a housekeeping gene, *β-actin*. The Ct values for *β-actin* and *ccn2* are measured, and the relative transcript levels are calculated as $\chi = 2^{-\Delta\Delta Ct}$, where $\Delta C = \mathrm{Ct}_{\mathrm{ccn2}} - \mathrm{Ct}_{\dagger\ \mathrm{actin}}$ and $\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct}_{\mathrm{unknown}} - \Delta\mathrm{Ct}_{\mathrm{calibrator}}$. Consider the control group, to which the others will be compared, as the calibrator.
2. The *ccn2* mRNA half-life is determined by monitoring the subsequent rate of *ccn2* mRNA loss. The half-life is calculated from the simple parameter, $t_{1/2} = \mathrm{In}2/k$, where ($t_{1/2}$) is the half-life, the time required for 50% of the mRNA to decay if

the initial value is 100%, and (k) is the decay rate constant, $\Sigma_{i=1,n}[y(t_i) - \exp(-k \cdot t_i)]^2$. $y(t)$ is the mRNA concentration at time t.

3.5 Analysis of 3′-UTR (See Note 20)

3.5.1 Construction of Firefly Luciferase-CCN Chimeric Genes with Intact 3′-UTR

1. Prepare the parental vectors, designated pGL3L(+) and pGL3L(−). These vectors confer multiple cloning sites (MCS) immediately downstream of the luciferase gene in different orientations, allowing examination of the effect on gene expression in an orientation-dependent manner [8] (**Note 21**).
2. The cDNA of the entire 3′-UTR of the *ccn* gene is amplified by RT-PCR with Pfu DNA polymerase (**Note 22**). The primer comprises the linker sequence, that is, the unique restriction enzyme (such as *Xba*I and *Eco*RI)-recognition site overhangs. The amplicon is digested with the corresponding combination of unique restriction enzymes.

   Example:

Amplicon DNA	μl
10× NEB	5 μl
*Xba*I	2 μl
*Eco*RI	2 μl
H_2O	To 50 μl

3. Separate DNA fragment by agarose gel (SeaPlaque™ Agarose) electrophoresis.
4. Excise the band of the desired DNA out from the gel.
5. Insert the gel slice in the SUPREC 01 (TaKaRa).
6. Centrifuge at 15,000 × *g* for 10 min.
7. Add 200 μl of TE buffer to the gel slice and centrifuge at 15,000 × *g* for 10 min.
8. Discard the upper cartridge, and precipitate the DNA recovered in the filtrate by ethanol.
9. The cDNA is subcloned into a luciferase expression vector, pGL3L(+) or pGL3L(−), between the downstream end of the luciferase gene and the polyadenylation signal. T4 DNA ligation is performed using a molar ratio of 1:2 vector–insert.

   If 100 ng of a vector (3 kb) is ligated with insert of 0.5 kb in length:

$$\begin{aligned}\text{Insert}\,(\text{ng}) &= \frac{\text{vector}\,(\text{ng}) \times \text{insert size}\,(\text{kb})}{\text{vector size}\,(\text{kb})} \times \frac{\text{insert}}{\text{vector}} \\ &= \frac{10\text{ng} \times 0.5}{3} \times \frac{2}{1} \\ &= 33.3\text{ng}.\end{aligned}$$

Example:

Vector DNA (3 kb)	a μl (100 ng)
Insert DNA (0.5 kb)	b μl (33.3 ng)
Ligation I (TaKaRa)	a + b μl
Total	2(a + b) μl

For cohesive (sticky) ends, incubate at 16 °C overnight or at room temperature for 30 min. For blunt ends, incubate at 16 °C overnight.

10. Heat-inactivate at 65 °C for 10 min, chill on ice and transform 1 μl of the reaction into 50 μl competent cells.
11. Place the competent cell/DNA mixture on ice for 20 min.
12. For heat shock transformation, place tube into a 42 °C water bath for 45 s, and put the tube back on ice for 2 min.
13. Plate all of the transformants onto a 10 cm LB agar plate containing the appropriate antibiotic, and incubate plates at 37 °C overnight.
14. The resultant plasmids contain the entire 3′-UTR of *ccn* in different orientations at the end of the firefly luciferase gene.
15. Luciferase assay is used to examine the possible effects of the entire 3′-UTR on gene expression.

3.5.2 Deletion Analysis of 3′-UTR (See Note 23)

1. Sequence search of the entire 3′-UTR fragment: The entire nucleotide sequence alignment of the subcloned 3′-UTR fragment is analyzed, using a commercial software package, GENETYX ver.7 and to scan the cleavage sites of restriction enzymes over the entire 3′-UTR. If the intended target fragment of the 3′-UTR are obtained by one combination of enzyme sites involved in the MCS (*Xba*I, *Pst*I, *Eco*RI, *Afl* II, *Eco*RV, and *Nru*I), subclone it immediately into pGL3L(+) and/or pGL3L(−) at the corresponding enzyme sites to yield the 5′-direction or 3′-direction deletion mutant. If the cleavage sites of such enzymes are mismatched, a double-step subcloning method is used (**Note 24**).
2. If the upstream or d
ownstream deletion mutant of 3′-UTR is constructed by subcloning a PCR-derived fragment of the 3′-UTR using a PCR-based subcloning method, the target deletion fragment is acquired by PCR with specific primers comprising one restriction enzyme-recognition site overhang. The amplicon is digested by the corresponding combination of unique restriction enzymes, purified, and are introduced between the corresponding combination of enzyme cutting sites for pGL3L(+) and pGL3L(−) to yield the deletion mutant of interest.

3. Following the same experimental strategy as described in Subheading 3.5.1, the luciferase assay is used with these deletion mutants to search which portion of the entire 3′-UTR is the major determinant for the observed effects (**Note 25**).

3.6 In Vitro Cell-Free mRNA Decay Assay (IVDA)

3.6.1 Preparing the Template for the Degradation Assay (See Note 26)

1. Subclone the target cDNA fragment using restriction enzymes into the pGEM4Zf plasmid containing the T3/T7 promoters.
2. Linearize the pGEM4Zf with the intended target fragment by an appropriate restriction enzyme as a template to synthesize the artificial transcripts tested.
3. Transcribe the RNA probe, followed by phenol extraction and ethanol precipitation.

3.6.2 Capping, Polyadenylation, and Radiolabeling of the Transcripts (See Subheading 2.6.3)

1. Prepare the capped transcription mixtures (see Subheading 2.6.3. item 3) at room temperature and incubate at 37 °C for 60 min.
2. Add 1μl of DNase I and incubate for 15 min at 37 °C to destroy the DNA templates.
3. Prepare the polyadenylation mixture (see Subheading 2.6.3. item 4) at room temperature and incubate together at 37 °C for 45 min.
4. Remove the unincorporated NTPs by G-50 spin column chromatography, and aliquots are removed for scintillation counting.
5. Approximately 80,000 cpm of capped and polyadenylated RNAs are used for each decay reaction.

3.6.3 Cytoplasmic Protein Purification

1. Wash cells grown in 10-cm tissue culture dishes twice with 5 ml of cold PBS and collect in 1 ml of PBS using a cell scraper.
2. After centrifugation at 7000 × *g* for 10 min (4 °C), resuspend the packed cell volume (PCV) in 2 volumes of hypotonic lysis buffer and homogenize using a syringe with a narrow-gauge needle.
3. Centrifuge the homogenate at 10,000 × *g* for 20 min and collect the supernatant (cytoplasmic extract) carefully. Store at −70 °C.
4. Protein concentration is determined by optical densitometry using a BCA protein assay kit using BSA as a standard by densitometry.
5. To collect the nuclear extract, resuspend the pellet in 2/3 volumes of extraction buffer and centrifuge at 15,000 × *g* for 10 min to pellet the nuclei. Collect the supernatant (nuclear extract) carefully. The protein concentrations are determined using a BCA protein assay kit.

3.6.4 IVDA Reaction

1. The IVDA buffer is used to initiate mRNA degradation. Incubate the radiolabeled transcript (80,000 cpm) with 10 μg of cytoplasmic extract in a total volume of 70 μl (master mix) in IVDA buffer for the desired time at 30 °C.

2. At each time point, stop the reaction by transferring 10 μl aliquots from this master mix to 50 μl of ISOGEN-LS reagent and extract the RNA immediately with phenol–chloroform–isoamyl alcohol (25:24:1).
3. Precipitate the supernatant with isopropanol and wash the pellets once with 70% ethanol and air-dried.

3.6.5 Electrophoresis and Quantitation of the Remaining Undegraded Transcript

1. RNA samples are prepared in loading dye, boiled at 95 °C for 5 min, and then electrophoresed on a 6% polyacrylamide–7 M urea gel (*see* **Note 27**) at 150 V in 1× TBE (*see* **Note 28**).
2. After electrophoresis, the gels are fixed and dried on a filter paper.
3. The filter paper is exposed to an X-ray film and the intensity of the undegraded transcript at the different time points is analyzed using commercial software, Quantity One.

4 Notes

1. The steady-state *ccn2* mRNA level (i.e., the net outcome of *ccn2* gene expression) and the pattern of gene expression are measured within a certain time frame by qPCR and Northern blot analysis [14]. High specificity is one advantage of Northern blot analysis, allowing visualization of the position of the chain length of RNA on the membranes. Northern blot analysis is thus useful for detecting whether alternative splicing forms of the mRNA are present. Although nonisotopic (i.e., digoxigenin-labeled) northern blot analysis is now widely used, the qPCR protocol is relatively quick and more convenient than northern blot analysis. Therefore, gene expression analysis using qPCR is described here.
2. In this initial step, high-quality RNA extraction from the tested adherent cells is important for subsequent gene expression analysis. The use of molecular biology grade water, RNase/DNase/nucleic acid-free tubes, and aerosol-barrier pipet tips is strongly recommended in all procedures in this protocol. Established protocols for total cellular RNA from mammalian cells using acid-phenol extraction and guanidinium thiocyanate extraction [19] should be used. From our experience, a commercial product, Isogen or TRIzol reagent, gives best results [6, 12, 16].
3. During the extraction of RNA from some cells, such as human chondrocytes and embryonic chicken chondrocytes, the RNA can be degraded by extremely resilient proteins. This is a major problem. Enzymatic lysis steps are a key to isolating clean RNA. Proteinase K is often used in enzymatic lysis steps to degrade proteins in the sample, so the sample is treated with a

final concentration of 250 μg/ml of protease K on the final step of RNA preparation. Dissolve the pellet in 250 μl TES buffer at a final concentration of 250 μg/ml protease K for 16 h at 55–60 °C. Add 750 μl of TRIzol LS reagent and extract the clean RNA. The isolated RNA should then be treated with DNase I for 30 min at 37 °C to remove contaminating genomic DNA.

RNA	μl
10× DNase buffer	5 μl
RNasin	0.5 μl
RQ1 DNase	2 μl
H_2O	To 50 μl

4. High-quality (absorbance ratios (A_{260}/A_{280}) between 1.8 and 2.0) RNA is used in the gene expression analysis described in the next subsection.
5. RNA sequence information for the *ccn* of interest is searched from the appropriate resource (e.g., Genbank), and the locations of exon boundaries are determined by aligning the mRNA sequence with the *ccn* gene using the NCBI Entrez Gene Evidence Viewer (http://www.ncbi.nlm.nih.gov). After copying the sequence into the design program software, a primer set for which the primers anneal in different exons is selected and ordered. Generally, the primers should have 40–60% GC content and a melting temperature around 60 °C, and the PCR product should be 50–150 base pairs in length with a melting temperature between 85 and 95 °C. From our experience [15, 16], one pair of target templates, i.e.; *ccn2* or housekeeping gene, *β-actin* primers used were: for *human ccn2,* 5′-tgactgccccttcccgagaa-3′ and 5′-tcttccagtcggtaggcagctagg-3′; for *β-actin*, 5′-gatcattgctcctcctgagc-3′ and 5′-actcctgcttgctgatccac-3′. The validity of a new primer set using SYBR Green chemistry is tested as follows:

Components	Final concentration	Volume per well
2× SYBR Green mix	1×	5 μl
1:1 primer mix (1.25 μM each)	150 nM each primer	1.2 μl
Template cDNA	0.1–50 ng	μl
H_2O	N/A	To 10 μl

Following the instrument run, the dissociation (melting) curve is first checked. If a single peak is found, the primer set is valid and the PCR efficiency calculated from the slope of the

linear regression curve is checked. If the slope of the curve is -3.3 ± 0.1 with $R^2 = 0.99$, the primer set amplifies at 100% efficiency.

6. According to the instrument manual of a real-time thermal cycler (e.g., Light Cycler®; Roche Diagnostics, iCycler iQ® Bio-Rad), the running conditions should be changed for each primer set. Specifically, the optimal primer annealing temperature is dependent on the base composition (e.g., the proportion of A, T, G, and C nucleotides), primer concentration, and ionic reaction environment.
7. The most accurate standards are RNA molecules of known copy number or concentration. A proportion of the target RNA in the RNA sample is reverse transcribed and the resultant cDNA then serves as a template. A standard curve is generated using a dilution series of different concentrations of standard cDNA samples. The Ct (threshold cycle) values of the standard samples are determined, then the Ct value of the unknown sample is compared with the standard curve to determine the amount of target in the unknown sample.
8. In relative quantification, the ratio between the amount of target gene and a control gene (e.g., an endogenous reference gene) is determined. If the objective is measuring the fold-change in expression of a particular RNA transcript between experimental samples, the ΔΔCt (cycle threshold) method, which is direct comparison of Ct values, should be used. However, this method theoretically requires 100% amplification efficiencies of the primer sets. Preparation of a standard curve is only required in an initial experiment to determine the amplification efficiencies of the target and endogenous reference genes.
   (a) To calculate the amounts of target using Ct values, import raw data into Microsoft Excel.
   (b) For each of the three replicates of a sample, calculate the average (avg) cycle time (Ct) and then calculate the standard deviation (stdev).
   (c) Remove any outlier wells from the averaged Ct values (>0.3 stdev).
   (d) For each sample, normalize the gene of interest (GOI) (e.g., *ccn2*) Ct values to those of the reference gene (e.g., *β-actin*) for the same sample according to the equation: $\Delta Ct = {}_{avg}Ct_{GOI} - {}_{avg}Ct_{ref}$, that is,

$$\Delta Ct_{unknown} = {}_{avg}Ct_{GOI} - {}_{avg}Ct_{ref}$$

$$\Delta Ct_{calibrator} = {}_{avg}Ct_{GOI} - {}_{avg}Ct_{ref}$$

(e) After identifying the control group (calibrator) against which the others will be calibrated, ΔΔCt is determined for each sample according to the equation: $\Delta\Delta Ct = \Delta Ct_{unknown} - \Delta Ct_{calibrator}$

(f) Find the fold-change of expression (χ) for each sample relative to the calibrator according to the equation: $\chi = 2^{-\Delta\Delta Ct}$.

9. The luciferase assay is a widely used bioluminescent reaction catalyzed by luciferase and is used as a nonisotopic genetic reporter system. To determine the contribution of transcription to target mRNA expression, measuring the activity of a promoter is an easy way of determining if the promoter region can mediate transcriptional responses.
10. In our case, the resultant *ccn2* proximal promoter-driven firefly luciferase expression plasmid was constructed as pTS589 [6, 7]. When connecting a promoter to a firefly luciferase reporter gene, it is essential to ensure that there are no ATG codons between the transcriptional start site and the ATG of the reporter gene.
11. Lipofectamine® 2000 transfection reagent (Invitrogen by Life Technologies). Use of high-throughput 96-well plate transfection systems is economical. The following reagent amounts are recommended for minimizing the transfection scale: plating medium 100 μl, nucleic acid 0.2 μg, and transfection reagent 0.5 μl.
12. The cells are co-transfected with pTS589 and either pRL-TK or pRL-CMV using the cationic lipid-mediated transfection reagent, Lipofectamine® 2000.
13. The volume of the medium is increased or replaced with an antibiotic-free, serum (+) medium to avoid drying the cells and to bring the final cell concentration to that of a regular cell culture.
14. The post-transfection period before harvesting must first be optimized for each cell type.
15. The relative luminescence values for firefly luciferase vs. *Renilla* luciferase are calculated with a GloMax 96 microplate luminometer (Promega) by using high-throughput 96-well plate transfection systems to process multiple samples simultaneously.
16. Since the role of transcription cannot be elucidated directly by using transient transfection with a promoter [15], nuclear run-on assays should be carried out to determine possible involvement of the transcriptional regulatory mechanism.
17. As a first step toward determining the role of mRNA decay in *ccn2* gene expression, cells are simply cultured with the

transcription inhibitor actinomycin D and harvested at different times thereafter. The stability of *ccn2* mRNA is measured by qPCR.

18. Actinomycin D at final concentration of 5–10 μg/ml will arrest mRNA transcription [12, 13, 15].
19. Generally, the average mRNA half-life in mammalian cells is on the order of several hours, but in human cells it can be as long as 10 h [3].
20. mRNA stability determinants depend on *cis*-acting sequence elements usually present in the 3′-untranslated region (UTR) of the mRNAs and on sequence-specific trans-acting factors [8, 10, 11, 18]. Therefore, it is important to elucidate the 3′-UTR-mediated posttranscriptional gene regulation mechanism. A conventional reporter gene assay system is useful for this. The RNA sequence information and genomic context of each CCN family gene is searched using the NCBI Entrez Gene Evidence Viewer (http://www.ncbi.nlm.nih.gov).

Gene name	Gene ID	Length of the 3′-UTR (bp)
human *ccn1*	3491	925
human *ccn2*	1490	1085
human *ccn3*	4856	1406
human *ccn4*	8840	3877
human *ccn5*	8839	505
human *ccn6*	8838	52

21. The parental plasmid, pGL3-control (Promega), is modified to construct firefly luciferase-3′-UTR chimeric genes. A double-stranded synthetic oligonucleotide which confers multiple cloning sites (MCS; *Xba*I, *Pst*I, *Eco*RI, *Afl* II, *Eco*RV, and *Nru*I) is synthesized. This short DNA is built in at the unique *Xba*I site immediately downstream of the luciferase gene in pGL3-control. Consequently, two pGL3-control derivatives with the same MCSs in different orientations are obtained and designated pGL3L(+) and pGL3L(−).
22. The target fragment should be acquired by PCR using the high-fidelity DNA polymerase *Pfu* DNA polymerase [10], which exhibits 3′ to 5′ exonuclease proofreading activity. Consequently, *Pfu* DNA polymerase-generated PCR fragments have fewer errors than *Taq*-generated PCR inserts and are thus better suited to applications such as cloning and sequencing.
23. It is important to search for the minimal functional element of the 3′-UTR. Deletion analyses are experimentally useful for localizing the minimal functional element.

24. For example, in the construction of pGL3SS1 [10], a 0.8 kbp 3′-UTR fragment of the downstream deletion mutant excised from pGL3UTRS by *Kpn*I-*Eco*RI digestion was subcloned between the corresponding sites in pUC18. Then, the 3′-UTR fragment was recovered by *Sma*I-*Eco*RI digestion and fused with the luciferase gene at the *Eco*RV and *Eco*RI sites in pGL3L(−).
25. In addition to experiments using the deletion mutant of 3′-UTR, additional experiments should be conducted to estimate post-transcriptional regulation. A minimal functional element is located upstream of the promoter driving the luciferase gene and its effect can be evaluated [10, 18]. If no significant effect of the 3′-UTR at this location outside of the transcribed area is observed, the minimal element exerts its effect after transcription.
26. The generation of most mRNA involves nuclear processing events that result in the 5′ end being capped with a 7-methyl guanosine and a 3′ poly (A) tail, with the exception of metazoan histone mRNA [1]. mRNAs in the cytoplasm are degraded either by decapping-dependent 5′ → 3′ decay or 3′ → 5′ exosome-mediated mRNA decay via interactions of the cap-, and the poly(A)-binding protein. Furthermore, mRNA decay depends on both *cis*-acting sequence elements that are usually present in the 3′-untranslated region (UTR) of the mRNAs and sequence-specific trans-acting factors [1–3, 10–13, 15, 18]. Before IVDA, the functional properties of CCN 3′-UTR should be analyzed using the genetic reporter system. The functional element is identified by deletion analysis of the 3′-UTR (*see* Subheading 3.5.2) [10, 18]. The target cDNA fragment indicates that this functional element lies within the 3′-UTR. Therefore, incubation of the artificial 5′-capped polyadenylated transcripts, which include specific sequence elements from the 3′-UTR, with cytoplasmic extract mimics cytoplasmic mRNA turnover. In our case, a coding region of the firefly luciferase gene is flanked downstream by an 84-nucleotide fragment of the *ccn2* 3′-untranslated region (UTR). CEASAR [10, 11] is subcloned from pGL3 SA5 into pGEM4Zf(+) using unique *Xba* I-*Eco*RI sites. The resultant plasmid is named pGEM4Zf (+)-Luc-SA5. *Eco*RI-linearized pGEM4Zf(+)-Luc-SA5 is used as the template for synthesis of luciferase-SA5 transcripts [15].
27. Loading dye (2× TBE–urea sample buffer; 80% formamide, 1 mM Pipes, pH 6.8, 0.1 mM EDTA, xylene cyanol, bromophenol blue) and denaturing 6% polyacrylamide–7 M urea gels are purchased from Novex.
28. 1× TBE (90 mM Tris, 90 mM borate, 2 mM EDTA) is used for preparing gels and for the gel running buffer. Nuclease-free solutions of 10× TBE (0.9 M Tris, 0.9 M borate, 20 mM EDTA) is commercially available (Life Technologies).

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Chapter 20

Protein Imaging of CCN2 and CCN3 in Living Cells

Takako Hattori, Mitsuhiro Hoshijima, and Masaharu Takigawa

Abstract

Recent progress in molecular imaging technology has had a strong impact on improving our understanding of molecular translocation, receptor internalization, and interactions in living cells. The protocol in this chapter introduces an optimized technique for intracellular localization of CCN2 and CCN3 in live cells, one using GFP-tagged CCN2 and Halo-tagged CCN3.

Key words GFP, Halo, Molecular imaging, Protein interaction

1 Introduction

Molecular imaging of fluorescent probe-labeled proteins in cells, organs, and living animals has made a substantial contribution toward improving our understanding of molecular localization, translocation, and protein–protein interactions. There are many fluorescent proteins with different colors that are available not only for molecular imaging of proteins within cells, but also for cell tracing in the living animal. In that case, fluorescent proteins are expressed under cell-specific promoters, a powerful tool that allows cell migration. Halo-tag is also useful for molecular imaging and protein-interaction analysis carried out by using chemical fluorescent ligands and HaloLink resin.

CCN2, one of the members of the CCN family of proteins, is expressed in growth-plate cartilage during developmental stages from early embryonic to later stages of growth, and shows multiple cellular functions such as stimulation of cartilage-specific extracellular matrix (ECM) synthesis, as well as chondrocyte proliferation and maturation [1]. However, ectopic overexpression of it in soft tissues leads to various fibrotic events, because of its strong enhancing effects on ECM synthesis [2].

In search of additional extracellular or cell-surface targets for CCN2 that may be involved in the regulatory functions of CCN2 in chondrocytes, done by using the yeast two-hybrid screening assay,

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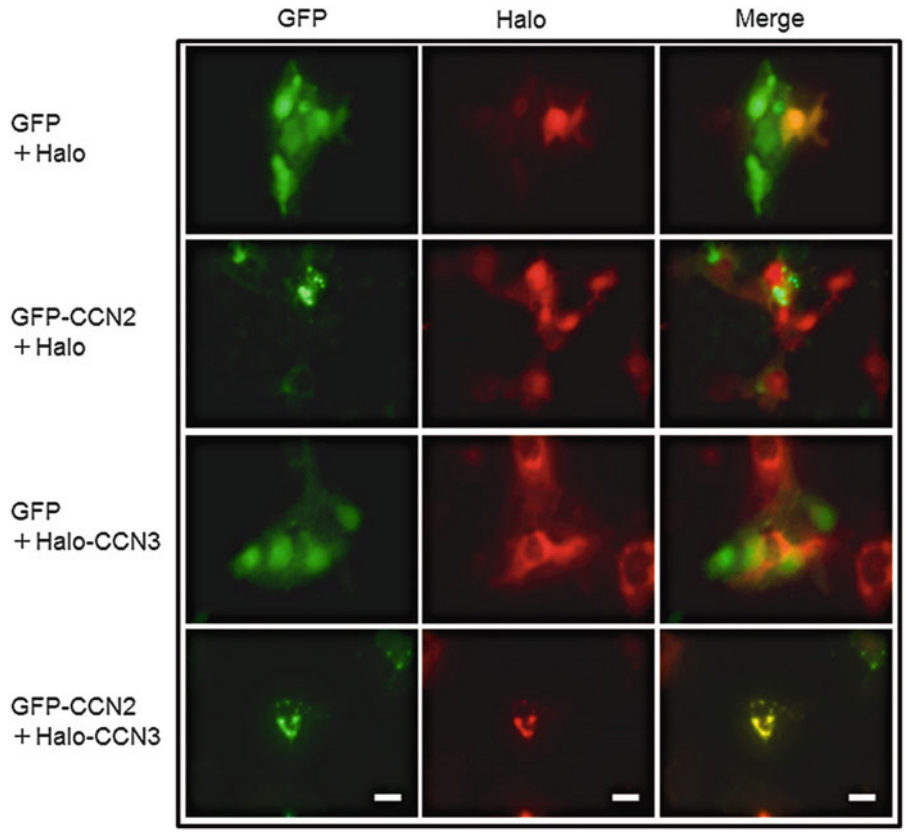

Fig. 1 Direct molecular imaging of CCN2 and CCN3 ectopically overexpressed in COS7 cells. GFP-tagged CCN2 and Halo-tagged CCN3 were overexpressed individually or together, and Halo-CCN3 detected by using TMB (*red*) ligands. Subcellular localization of CCN2 and CCN3 was overlapped

CCN2 and CCN3 (another CCN family member) were found as binding partners of CCN2, indicating that CCN2 forms homotypic dimers or heterotypic ones with CCN3 (Fig. 1) [3].

CCN3 is highly expressed in the central nervous system [4], blood vessels [5], and musculoskeletal system [6], as well as in pre- and early hypertrophic chondrocytes and osteoblasts [7]. CCN3 regulates CCN2 activity differently according to their molecular ratio [3].

Below we present protocols for generating DNA constructs for expression of pEGFP-N1/CCN2 and pFlag-CMV/CCN3-Halo compound proteins in COS7 cells for fluorescence imaging. CCN2 and CCN3 compound proteins carry a GFP and Halo-tag, respectively, at their C-terminus. Not only COS7 cells, but also cells from other sources including cells in primary culture, can be used for imaging proteins in them.

2 Materials

2.1 Generating a DNA Construct

1. pEGFP-N1 (Clonetech).
2. RNA isolation kit.
3. RNA from HCS-2/8 human chondrocytic cells.
4. primers for amplification of human *ccn2* cDNA; 5′-cttc-gaattcccatgaccgccagtatgggccccgtc-3′ and 5′-cggtggatcccg tgccatgtctccgtacatcttcctgta-3′.
5. EcoRI and BamHI.
6. pFLAG-CMV2 (Sigma).
7. pFN21AB3946 which expresses Sox9-Halo [8] protein as a template for halo-DNA.
8. primers for amplification of human *ccn3* cDNA: 5′-atccaagct-tatgcagagtgtgcagagcac-3′ and 5′-atacgaattcattttccctctggtagtct tc-3′.
9. Primers for amplification of halo: 5′-atacgaattcaatggcagaaatcg-gtactgg-3′ and 5′-atacggatccttagccggaaatctcgagcgtc-3′.
10. HindIII, EcoRI, BamHI.
11. Sequencing primers: pFLAG-F 5′-ctc cac ccc att gac gtc aat ggg ag-3′ and pFLAG-R 5′-ggt tcc caa tag acc ccg cag gcc ct-3′.

2.2 Fluorescence Imaging

1. COS7 cells.
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).
3. Fugene 6.
4. TMR, a fluorescent ligand.
5. 4% Formaldehyde/PBS.
6. Fluorescent microscopy (e.g., IX70, Olympus).
7. Axiovision software (Zeiss or other imaging software).

3 Methods

In the following protocol, we describe the preparation of pEGFP-N1/CCN2 and pFlag-CMV/*ccn3*-halo vectors (*see* Subheading 3.1) and the fluorescence imaging of GFP-CCN2 and CCN3-Halo proteins (*see* Subheading 3.2).

3.1 Preparation of pEGFP-N1/ CCN2

1. Isolate HCS-2/8 RNA by using any commercially available kit.
2. Amplify human *ccn2* cDNA by using the following primers: 5′-cttcgaattcccatgaccgccagtatgggccccgtc-3′ and 5′-cggtg-gatcccgtgccatgtctccgtacatcttcctgta-3′, which contain restriction sites at their 5′ ends. Any Taq DNA polymerase with high fidelity can be used for the amplification.

3. Treat 5′ and 3′ ends of the amplified *ccn2* fragments and pEGFP-N1 vector with EcoRI and BamHI, ligate them by using T4 DNA ligase, and use this vector to transform *E. coli*. Pick several clones and analyze their sequences with pFLAG-F and pFLAG-R primers. Select one clone with minimal misamplification with no codon changes.

3.2 Preparation of pFLAG-CMV/ccn3-Halo

1. Amplify human *ccn3* cDNA by using 5′-atccaagcttatgcagagtgtgcagagcac-3′ and 5′-atacgaattcattttccctctggtagtcttc-3′ as primers, which contain restriction sites at their 5′ ends, with any Taq DNA polymerase with high fidelity.
2. Amplify halo DNA by using primers 5′-atacgaattcaatggcagaaatcggtactgg-3′ and 5′-atacggatccttagccggaaatctcgagcgtc-3′ and pFN21AB3946, which expresses Sox9-Halo protein as a template.
3. Ligates the PCR fragments into the pFlag-CMV-2 vector at HindIII/EcoRI/BamHI sites.

3.3 Fluorescence Imaging of GFP-CCN2 and CCN3-Halo Proteins

1. Transfect COS7 cells with pEGFP-N1/CCN2 and pFlag-CMV/ccn3-halo vectors by using Fugene 6 and incubating the cells in DMEM/10%FBS for 24 h at 37 °C. As control experiments, pEGFP-N1 and pFlag-CMV/Halo vectors are used for transfection of COS7 cells (*see* **Note 1**).
2. Add the fluorescent ligand TMR, which recognizes the HaloTag, and incubate the cells for 15 min at 37 °C.
3. Wash the cells with DMEM.
4. Fix the cells with 4% formaldehyde/PBS for 15 min at room temperature if necessary.
5. Halo-tagged proteins (red) and GFP-tagged proteins (green) are directly monitored by fluorescence microscopy using an IX70 Microscope.
6. Analyze the images with Axiovision software.

4 Notes

1. As control experiments, perform transfection with combinations of pEGFP-N1 plus pFlag-CMV/Halo; pEGFP-N1/*ccn2* plus pFlag-CMV/Halo; pEGFP-N1/ccn2 plus pFlag-CMV/ccn3-Halo; and pEGFP-N1 plus pFlag-CMV/*ccn3*-Halo. GFP and Halo proteins show a broad intracellular localization, whereas GFP-CCN2 and CCN3-Halo are localized in small granular vesicles in the cells.

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Part II

Functional Analysis

Chapter 21

Cell Biological Assays for Measuring Chondrogenic Activities of CCN2 Protein

Takashi Nishida, Satoshi Kubota, and Masaharu Takigawa

Abstract

Growth-plate chondrocytes undergo proliferation, maturation, hypertrophic differentiation, and calcification; and these processes can be reproduced in vitro in a chondrocyte culture system. Using this system, we have shown that CCN family protein 2/connective tissue growth factor (CCN2/CTGF) promotes all stages of proliferation, maturation, hypertrophic differentiation, and calcification, thus suggesting that CCN2 is a multifunctional growth factor for chondrocytes and plays important roles in chondrocyte proliferation and differentiation. In this chapter, we describe how to evaluate CCN2 functions in these processes occurring in cultured chondrocytes. Evaluation strategies for cell proliferation include measuring DNA synthesis by [^{3}H]-thymidine incorporation, cellular metabolic activity, and cell number with a hemocytometer. Next, evaluation strategies to assess maturation are analysis of the gene expression of markers of mature chondrocytes, and examination of proteoglycan and collagen synthesis by using radioactive compounds. In addition, cytohistochemical detection of glycosaminoglycans (GAGs), such as chondroitin sulfate, by use of alcian blue and toluidine blue staining is useful to evaluate chondrocyte maturation. These methods can be also used for evaluation of physiological functions of CCN2 in permanent chondrocytes such as articular and auricular chondrocytes, which do not calcify under physiological conditions. Next, evaluation of hypertrophic differentiation is performed by detecting type X collagen, which is specific marker of hypertrophic chondrocytes, and by measuring alkaline phosphatase (ALP) activity. Finally, evaluation of calcification is performed by detecting matrix calcification by use of alizarin red staining and by examining the incorporation of ^{45}Ca into cartilaginous matrix. These methods would be useful for the evaluation not only of CCN2 but also of its derivatives and other CCN proteins.

Key words Recombinant CCN2 protein (rCCN2), Chondrocytes, Chondrocyte proliferation, Chondrocyte maturation, Proteoglycan, Collagen, Chondrocyte hypertrophy, Alkaline phosphatase, Chondrocyte calcification, Alizarin red staining

1 Introduction

Cartilage tissue is a specialized connective tissue without nerve and vascular systems, and these tissues consist of only chondrocytes embedded in an extracellular matrix (ECM; [1]). Cartilage ECM is mainly composed of type II collagen, chondroitin sulfate proteoglycan (aggrecan) and hyaluronate [1]. When chondrocytes

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become hypertrophic chondrocytes, they produce type X collagen, matrix metalloprotease 13 (MMP13), and alkaline phosphatase (ALP; [1, 2]). During development, chondrocytes follow two distinct pathways toward two distinct fates and functions. Chondrocytes following one of these distinct pathways maintains the cartilage phenotype permanently to retain normal joint function throughout life, and those following the other pathway serve as a continuous source of cartilage for conversion to bone [3]. The former is located at the epiphyseal tip of long bones and becomes the articular cartilage, and the latter is located between the epiphysis and diaphysis and becomes organized into the growth plate. Bone formation arising from a cartilaginous template at the growth plate is referred to as endochondral ossification. The growth plate consists histologically of four zones [4, 5]: (1) In the proliferative zone, immature chondrocytes proliferate actively; and the daughter cells are arranged into a column formation [1]. This column formation represents the division of chondrocytes. Elongation of the long axis of the bone occurs mainly at the proliferative zone. (2) In the zone of maturation, chondrocytes cease proliferation, lose their characteristic flat shape seen in the proliferative zone, and mature [1]. These cells enlarge to produce a large amount of matrix consisting largely of type II collagen and proteoglycans. (3) In the zone of hypertrophy, chondrocytes continue to enlarge to accumulate many glycogen stores and become hypertrophic chondrocytes. Hypertrophic chondrocytes secrete a matrix rich in type X collagen and produce ALP to start forming a calcified matrix [1, 2]. (4) In the calcified zone, cartilaginous matrices become selectively calcified, and hypertrophic chondrocytes produce vascular endothelial growth factor (VEGF) to induce the entry of new blood vessels into the hypertrophic zone [1, 6]. They also secrete MMP13 to decompose uncalcified matrix tissue. Finally, the hypertrophic chondrocytes undergo programmed cell death, and calcified cartilaginous matrix is resorbed by osteoclasts that accompany vascular vessels; and cartilage is replaced with bone tissues, thereby completing the process of endochondral ossification [6].

To regulate the dynamic organization during endochondral ossification, many kinds of hormones, such as parathyroid hormone (PTH) and active forms of vitamin D_3, as well as growth factors such as insulin-like growth factors (IGFs) and transforming growth factor (TGF)-β, have been reported to be involved in this process [7–10]. However, most of these factors stimulate the proliferation and differentiation of chondrocytes at a specific stage and inhibit them at the other stages of endochondral ossification. As such, no molecule that promotes all stage of endochondral ossification has ever been reported. We indicated earlier that the gene expression level of CCN family protein 2/connective tissue growth factor (CCN2/CTGF) is enhanced in pre-hypertrophic and hypertrophic chondrocytes [11]. By using recombinant CCN2 proteins

that we firstly succeeded to produce, we have shown that CCN2 promotes all stages of proliferation, maturation, hypertrophic differentiation, and calcification [12, 13]. In addition, we have also shown that although CCN2 stimulates proliferation and maturation (determined by protepglycan synthesis) of chondrocytes, it does not stimulate hypertrophic differentiation and calcification of articular chondrocytes [13] nor auricular chondrocytes [14], both of which do not undergo hypertrophic differentiation or calcification under physiological conditions. These findings suggest that CCN2 is a multifunctional growth factor for various types of chondrocytes, having tissue-specific harmonizing functions.

To further investigate the roles of CCN2, its derivatives and other CCN proteins in chondrocyte differentiation, in this chapter we describe some of the assays used in our laboratory to measure CCN2 functions in vitro.

2 Materials

2.1 General

1. Recombinant CCN2 (rCCN2) stock solution: purify rCCN2 produced in mammalian cells transfected with CCN2-expression vector and store 100 μg/mL of rCCN2 at −80 °C (*see* Chapter 10).

2.2 Culture of Chondrocytes

1. Dulbecco's modified Eagle's medium (DMEM), store at 4 °C (*see* **Note 1**).
2. Fetal bovine serum (FBS), store at −20 °C.
3. Phosphate-buffed saline (PBS): 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4. Sterilize with autoclaving.
4. Trypsin–EDTA; 0.25% trypsin, 0.5 mM EDTA. Sterilize the solution by filtration, and store at 4 °C.
5. HCS-2/8 cells: A human chondrosarcoma-derived chondrocytic cell line (*see* **Note 2**).
6. RCS cells: A rat chondrosarcoma-derived chondrocytic cell line (*see* **Note 3**).

2.2.1 Chondrosarcoma-Derived Cell Lines

2.2.2 Isolation of Primary Chondrocytes

1. Alpha-modified Eagle's medium (αMEM) and 10% FBS, store at 4 °C (*see* **Note 1**).
2. Collagenase solution: 0.15% collagenase in serum-free αMEM. Sterilize the solution by filtration, and store at 4 °C (*see* **Note 4**).
3. Nylon mesh (*see* **Note 5**).
4. Primary chondrocytes isolated from several animal species, such as rabbits, rats, or mice (*see* Subheading 3.1.1).

2.3 Evaluation of Chondrocyte Proliferation

2.3.1 [^{3}H]-thymidine Incorporation Assay

1. [*methyl*-^{3}H]-thymidine, 37 MBq/mL (*see* **Note 6**).
2. 5% trichloroacetic acid (TCA): Make a 5% (weight/volume) solution in distilled water.
3. Ethanol–diethyl ether (3:1, volume/volume).
4. Liquid scintillation counter (e.g., 1414 WinSpectral, Wallac) or an equivalent.

2.3.2 BrdU Incorporation Assay (e.g., Cell Proliferation ELISA, BrdU Assay Kit, Roche) or an Equivalent

1. BrdU labeling solution: Dilute 10 mM 5-bromo-2′-deoxyuridine (BrdU) reagent 1:1000 in PBS (*see* **Note 7**).
2. Fix Denat: To fix the cells and denature the DNA, use this solution provided in the kit.
3. Anti-BrdU-POD antibody: Dissolve anti-BrdU-conjugated with peroxidase (POD) in 1.1 mL double distilled water (stock solution) and dilute anti-BrdU-POD stock solution 1:100 with antibody dilution solution provided in the kit.
4. Washing buffer: Dilute washing buffer concentration provided in the kit 1:10 with double distilled water.
5. Substrate solution: 5 mg/mL tetramethyl-benzidine (TMB) provide in the kit.
6. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.3.3 Cell Proliferation Assay by Using NADH Activity

1. Water soluble disulfonated tetrazolium (WST) solution or an equivalent (*see* **Note 8**).
2. 0.5% MTT solution: Dissolve MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium) in PBS at a concentration of 5 mg/mL and store it in a brown tube at 4 °C (*see* **Note 9**).
3. Acidic isopropanol: Add 0.33 mL of concentrated HCl (11.6 M) to 100 mL of isopropanol (final concentration; 0.04 M HCl). Store it at room temperature.
4. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.4 Evaluation of Chondrocyte Maturation

2.4.1 Measurement of Collagen Synthesis

1. l-[2,3-^{3}H] proline, 37 MBq/mL.
2. Buffer A: 50 mM Tris–HCl (pH 7.2), 0.2% Triton X-100, and 100 mM phenylmethyl-sulfonyl fluoride (PMSF; stock solution: 1 M). Store it at room temperature (*see* **Note 10**).
3. Collagenase solution: 3.7 mg/mL collagenase in 25 mM Tris–HCl (pH 7.4) containing 10 mM $CaCl_2$ (*see* **Note 4**).
4. 10% TCA-0.5% tannic acid: 10% TCA containing 0.5% tannic acid.
5. Gelatin solution: Weigh 10 mg of gelatin powder and add PBS to a volume of 1 mL.
6. Liquid scintillation counter (e.g., 1414 WinSpectral, Wallac) or an equivalent.

2.4.2 Measurement of Proteoglycan Synthesis

1. [^{35}S]-sulfate, 37 MBq/mL.
2. Actinase E solution: Weigh 1 mg of actinase E and add 0.2 M Tris–HCl (pH 7.8) containing 5 mM $CaCl_2$ to a volume of 1 mL.
3. CPC solution: 1% (weigh/volume; w/v) cetylpyridinium chloride (CPC) containing 20 mM NaCl.
4. Buffer B: Weigh 0.1 mg of chondrotin-4-sulfate and add 0.2 M Tris–HCl (pH 7.8) containing 2 mM $MgSO_4$ to a volume of 1 mL.
5. Glass-filter: glass-fiber filter paper.
6. Liquid scintillation counter (e.g., 1414 WinSpectral, Wallac) or an equivalent.

2.4.3 Sulfated Glycosaminoglycan (GAG) Microassay

1. Lysis buffer: PBS containing 0.2% Triton x-100.
2. DMB stock solution: Weigh 1.6 mg of 1,9-dimethyl methylene blue (DMB) and add ethanol to a volume of 5 mL. Next, add 2.0 g of sodium formate and 2 mL formic acid, and fill up to 1 L with distilled water. Store it in a brown bottle at 4 °C.
3. Chondroitin-4-sulfate solution: Weigh 1 mg of chondroitin-4-sulfate and add distilled water to a volume of 1 mL (*see* **Note 11**).
4. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.4.4 Alcian Blue or Toluidine Blue Staining

1. PFA solution: Dilute 37% paraformaldehyde (PFA) solution with PBS to a final concentration of 3.7%.
2. 0.05% toluidine blue solution: Weigh 0.05 g of toluidine blue powder and add distilled water to a volume of 100 mL. Store it at room temperature.
3. 1% alcian blue solution (pH 2.5): Weigh 1 g of alcian blue powder and add 3% acetic acid to a volume of 100 mL (*see* **Note 12**). Store it at room temperature.
4. Lysis buffer: 0.15 M NaCl, 50 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100.
5. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.4.5 Quantitative Reverse Transcriptase (RT)-PCR (See also Chapter 4)

1. RNA isolation reagent (e.g., ISOGEN reagent, Nippon Gene) or an equivalent.
2. TE solution: 10 mM Tris–HCl (pH 8.0), 0.5 mM EDTA.
3. 10× RT buffer: 100 mM Tris–HCl (pH 8.3), 500 mM KCl (*see* **Note 13**).
4. dNTP mixture: Each 10 mM.
5. Avian myeloblastosis virus (AMV) reverse transcriptase XL, 5 U/μL.
6. RNase inhibitor: 40 U/μL.

7. Oligo dT-adaptor primer.
8. Real-time PCR master mix reagent (e.g., SYBR® green, TOYOBO) or an equivalent.
9. Specific primer sets of chondrocyte differentiation marker genes: Nucleotide sequence of the primers and predicted size of the PCR product are shown in Table 1.
10. Quantitative PCR system (e.g., StepOne plus, Applied Biosystems) or an equivalent.

2.5 Evaluation of Chondrocyte Hypertrophy

1. Specific primer sets of chondrocyte hypertrophy marker genes (*See* Subheading 2.4.5).

2.5.1 Quantitative Reverse Transcriptase-PCR

2.5.2 Measurement of ALP Activity

1. Lysis buffer: 0.9% NaCl, 0.02% Triton X-100.
2. 5 mM *p*-NPP: Weigh 18.5 mg of *p*-nitrophenyl phosphate sodium salt (*p*-NPP) and add distilled water to a volume of 10 mL (*see* **Note 14**).
3. 10 mM *p*-NP: Weigh 13.9 mg of *p*-nitrophenol (*p*-NP) and add distilled water to a volume of 10 mL (*see* **Note 15**).
4. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.6 Evaluation of Calcified Chondrocytes

1. 2 M β-glycerophosphate (β-GP): Weigh 4.32 mg of β-glycerophosphate disodium salt hydrate and add distilled water to a volume of 1 mL. Store it at −20 °C.
2. Ascorbic acid solution: Weigh 50 mg of l-Ascorbic acid powder and add distilled water to a volume of 1 mL. Store it at −20 °C (*see* **Note 16**).
3. 1% alizarin red solution: Weigh 1 g of alizarin red S powder and add distilled water to a volume of 100 mL. Adjust pH to 6.4 with 0.028% ammonium hydroxide.
4. Dissolving buffer: 10% (w/v) CPC in 10 mM sodium phosphate pH 7.0.
5. Multi-well spectrophotometer (e.g., SH-1000Lab, CORONA) or an equivalent.

2.6.1 Alizarin Red Staining

2.6.2 ^{45}Ca Incorporation Assay

1. [^{45}Ca] Cl_2: 37 MBq/mL.
2. Lysis buffer: 0.9% NaCl, 0.2% Triton X-100. Store it at room temperature.
3. Washing buffer: 0.1 M $CaCl_2$, 0.05 M Tris–HCl (pH 7.4).
4. Liquid scintillation counter (e.g., 1414 WinSpectral, Wallac) or an equivalent.

Table 1
Nucleotide sequence of the primers and predicted size of the PCR product

Gene	Accession no.	Primer sequence (bp)	Expected size
Human forward (F) and reverse (R) primers used for real-time PCR			
CCN2	NM_001901.2	(F) 5′-TGCGAGGAGTGGGTGTGTGAC-3′	124
		(R) 5′-TGGACCAGGCAGTTGCCTCTAATC-3′	
COL2a1	XM_017018831.1	(F) 5′-CAACAACCAGATTGAGAGCA-3′	166
		(R) 5′-CCATGTTGCAGAAAACCTTC-3′	
ACAN	NM_013227.3	(F) 5′-GGAGCAGGAGTTTGTCAACA-3′	186
		(R) 5′-CTTCTCGTGCCAGATCATCA-3′	
COL10a1	NM_000493.3	(F) 5′-GAATGCCTGTGTCTGCTT-3′	105
		(R) 5′-TCATAATGCTGTTGCCTGTT-3′	
MMP13	NM_002427.3	(F) 5′-TGGTGGTGATGAAGATGATTTGTCT-3	375
		(R) 5′-AGTTACATCGGACCAAACTTTGAAG-3′	
RUNX2	NM_001015051.3	(F) 5′-CCCAGGCAGTTCCCAAGCATTTC-3′	138
		(R) 5′-GGTAGTGAGTGGTGGCGGACATAC-3′	
SOX9	NM_000346.3	(F) 5′-CGTCAACGGCTCCAGCA-3′	68
		(R) 5′-TGCGCCCACACCATGA-3′	
ALP	NM_001127501.3	(F) 5′-GCACCGCCACCGCCTACC-3′	149
		(R) 5′-CCACAGATTTCCCAGCGTCCTTG-3′	
VEGF	NM_001204385.1	(F) 5′-CTTGCCTTGCTGCTCTAC-3′	88
		(R) 5′-ACCACTTCGTGATGATTCTG-3′	
GAPDH	NM_001289746.1	(F) 5′-GCCAAAAGGGTCATCATCTC-3′	214
		(R) 5′-GTCTTCTGGGTGGCAGTGAT-3′	
β-ACTIN	NM_001101.3	(F) 5′-GATCATTGCTCCTCCTGAGC -3′	100
		(R) 5′-ACTCCTGCTTGCTGATCCAC-3′	
Mouse forward (F) and reverse (R) primers used for real-time PCR			
Ccn2	NM_010217.2	(F) 5′-CCACCCGAGTTACCAATGAC-3′	168
		(R) 5′-GTGCAGCCAGAAAGCTCA-3′	
Col2a1	NM_031163.3	(F) 5′-TGGTCCTGGCATCGACATG-3′	172
		(R) 5′-GGCTGCGGATGCTCTCAAT-3′	
Acan	NM_007424.2	(F) 5′-CTTGGGCAGAAGAAAGATCG-3′	155
		(R) 5′-GTGCTTGTAGGTGTTGGGGT-3′	

(continued)

Table 1
(continued)

Gene	Accession no.	Primer sequence (bp)	Expected size
Col10a1	NM_009925	(F) 5′-CCCAGGGTTACCAGGACAAA-3′	128
		(R) 5′-GTTCACCTCTTGGACCTGCC-3′	
Mmp13	NM_008607.2	(F) 5′-TGATGAAACCTGGACAAGCA-3′	285
		(R) 5′-TCCTCGGAGACTGGTAATGG-3′	
Runx2	XM_006523548.2	(F) 5′-TCGTCAGCATCCTATCAGT-3′	141
		(R) 5′-CAGCGTCAACACCATCAT-3′	
Sox9	NM_011448.4	(F) 5′-AGGCCACGGAACAGACTCA-3′	169
		(R) 5′-AGCTTGCACGTCGGTTTTG-3′	
Alp	NM_007431.2	(F) 5′-GCTCTCCCTACGCACCCTGTTC-3′	129
		(R) 5′-TGCTGGAAGTTGCCTGGACCTC-3′	
Vegf	NM_001025250.3	(F) 5′-CCCATGAAGTGATCAAGTTC-3′	216
		(R) 5′-ATCCGCATGATCTGCATGG-3′	
Gapdh	NM_001289726.1	(F) 5′-GCCAAAAGGGTCATCATCTC-3′	214
		(R) 5′-GTCTTCTGGGTGGCAGTGAT-3′	
β-actin	NM_007393.5	(F) 5′-AAGTCCCTCACCCTCCCAAAAG -3′	96
		(R) 5′-AAGCAATGCTGTCACCTTCCC-3′	
Rat forward (F) and reverse (R) primers used for real-time PCR			
Ccn2	NM_022266.2	(F) 5′-ATCCCTGCGACCCACACAAG-3′	144
		(R) 5′-CAACTGCTTTGGAAGGACTCGC-3′	
Col2a1	NM_012929.1	(F) 5′-CCCAGAACATCACCTACCAC-3′	200
		(R) 5′-GGTACTCGATGATGGTCTTG-3′	
Acan	NM_022190.1	(F) 5′-CTTGGGCAGAAGAAAGATCG-3′	158
		(R) 5′-GTGCTTGTAGGTGTTGGGGT-3	
Col10a1	XM_008773018.1	(F) 5′-CCCAGGGTTACCAGGACCAA-3′	127
		(R) 5′-GTTCACCTCTTGGACCTGCC-3′	
Gapdh	NM_017008.1	(F) 5′-GCCAAAAGGGTCATCATCTC-3′	214
		(R) 5′-GTCTTCTGAGTGGCAGTGAT-3′	
Rabbit forward (F) and reverse (R) primers used for real-time PCR			
Ccn2	XM_008263527.1	(F) 5′-GACGGCTGCGGCTGCTGC-3′	343
		(R) 5′-CACACCCACTCCTCGCAGCA-3′	

(continued)

Table 1 (continued)

Gene	Accession no.	Primer sequence (bp)	Expected size
Acan	XM_008251726.1	(F) 5′-AACATCACTGAAGGCGAAGC-3′	148
		(R) 5′-TCTTCAGTCCCGTTCTCCAC-3′	
Col10a1	XM_002714724.2	(F) 5′-CCTGTATAAGAATGGCACGC-3′	114
		(R) 5′-CCACACCTGGTCATTTTCTG-3′	
Gapdh	NM_001082253.1	(F) 5′-TCACCATCTTCCAGGAGCGA-3′	292
		(R) 5′-CACAATGCCGAAGTGGTCGT-3′	

3 Methods

3.1 Culture of Chondrocytes

Chondrocytes are specialized cells to produce cartilage-specific proteoglycans and type II collagen [1]. However, they easily lose their differentiated phenotypes and their ability to proliferate after several passages in culture [7]. Therefore, to clarify the mechanism involved in the regulation of proliferation and differentiation of normal chondrocytes, primary or secondary cultures of chondrocytes isolated from several animal species are often used. On the other hand, to perform these studies easily, efficiently, and practically, immortalized chondrocyte cell lines are also used, although they cannot represent all phenotypes of normal chondrocytes.

3.1.1 Chondrosarcoma-Derived Cell Lines

1. Inoculate HCS-2/8 cells (*see* **Note 2**) at a density of 4.0×10^4 cells/cm^2 into several culture dishes or plates, culturing them in DMEM containing 10% FBS at 37 °C under 5% CO_2 in air.
2. Inoculate RCS cells (*see* **Note 3**) at a density of 2.5×10^4 cells/cm^2 into several culture dishes or plates and culture them in the same manner as HCS-2/8 cells.

3.1.2 Isolation Chondrocytes in Primary Culture (See Note 17)

1. Euthanasize young rabbits, weighting 300–500 g and depilate them.
2. Dissect the costal cartilage and articular cartilage from the knees and rinse them in PBS.
3. Mince these cartilage specimens and treat them with 0.1% trypsin–EDTA in PBS containing 0.2% glucose.
4. Digest the minced tissues with 0.15% collagenase solution and filter the cells through a nylon mesh to remove residual undigested cartilage.
5. Inoculate isolated chondrocytes at a density of 2.0×10^4 cells/cm^2 into several culture dishes or plates and culture them in αMEM containing 10% FBS at 37 °C under 5% CO_2 in air.

3.2 Evaluation of Chondrocyte Proliferation

These assays are basically classified into three types of measurement. The first method is the measurement of [^{3}H]-thymidine [12–16] or 5-bromo-2′-deoxyuridine (BrdU) incorporation [17] during DNA synthesis in proliferating cells. The second is the measurement of formazan dyes, which are formed by tetrazolium salt, such as MTT and WST solution, in a manner dependent on NADH-dependent dehydrogenase activity [14, 18–20]. The third method is the measurement of cell numbers by using a hemocytometer.

3.2.1 Cell Proliferation Assay by Using [^{3}H]-Thymidine Incorporation

1. Grow cultured chondrocytes in a 96-well multiwell plate; and once they have reached sub-confluence, incubate them in serum-starved medium in the presence of various concentration of rCCN2 for 22 h.
2. Add 10 μl/well of [^{3}H]-thymidine solution when the cells are cultured in 100 μL of medium/well (final concentration: 370 kBq/ml) and incubate the cells for 4 h at 37 °C.
3. Remove the medium, wash the cells once with PBS (*see* **Note 18**), and fix the cells with 5% TCA and ethanol–diethyl ether at room temperature (*see* **Note 19**).
4. Lyse the cells with 0.5 M NaOH at 37 °C for 1 h (*see* **Note 20**) and then neutralize with 6 M HCl.
5. Collect the cell lysate and measure the radioactivity by liquid scintillation counting.

3.2.2 Cell Proliferation Assay by Using BrdU Incorporation

1. Grow cultured chondrocytes in a 96-well multiwell plate and stimulate them with various concentrations of rCCN2.
2. Add BrdU labeling solution at a final concentration of 10 μM to the culture medium and incubate the cells for 2 h at 37 °C.
3. Remove the medium and fix the cells with Fix Denat for 30 min at room temperature.
4. Remove the Fix Denat from the cells, add 100 μL/well of anti-BrdU-POD antibody, and incubate the cells for 90 min at room temperature.
5. Remove antibody solution and wash the wells three times with Washing solution.
6. Remove the Washing solution by tapping, add 100 μL/well of the Substrate solution and incubate for 5 min at room temperature.
7. Measure the absorbance 370 nm by using a multi-well spectrophotometer (*see* **Note 21**).

3.2.3 Cell Proliferation Assay Monitoring NADH-Dependent Dehydrogenase Activity (See Note 22)

1. Grow cultured chondrocytes in a 96-well multiwell plate and stimulate them with various concentrations of rCCN2, as described previously.
2. Add 10 μL/well of WST or MTT solution if the cells are cultured in 100 μL of medium/well and incubate the cells for 2–4 h at 37 °C.

3. In the case of WST, measure the wells at absorbance 450 nm by using a multi-well spectrophotometer. In case of MTT, remove the medium and dissolve the cells in acidic isopropanol (*see* **Note 23**). Then measure the wells at absorbance 570 nm with the multi-well spectrophotometer.

3.2.4 Cell Proliferation Assay Using a Hemocytometer to Count the Cell Numbers

1. HCS-2/8 cells or RCS cells are inoculated into 35-mm dishes, and they are cultured in DMEM containing 10% FBS and various concentrations of rCCN2.
2. Remove the medium and wash the cells with PBS. Use trypsin–EDTA to release adherent cells, and centrifuge them at 500 × *g* for 5 min at room temperature.
3. Resuspend the cell pellet in DMEM containing 10% FBS and count the cell numbers by using a hemocytometer (*see* **Note 24**).

3.3 Evaluation of Chondrocyte Maturation

When chondrocytes mature, they cease cell proliferation and produce abundant ECM such as type II collagen and aggrecan, which is chondroitin/keratan sulfate proteoglycan. Therefore, the chondrocyte maturation assay is mainly used to measure the gene expression [14, 21–25], production [12–14, 16, 18, 20, 23, 24, 26], and accumulation [15, 17, 21] of type II collagen and aggrecan.

3.3.1 Measurement of Collagen Synthesis by Using l-[2,3-^{3}H] Proline Incorporation and Collagenase-Digestion

1. When chondrocytes reach confluence, change the DMEM or αMEM containing 10% FBS to serum-starved DMEM or αMEM and pre-incubate the cells for 24 h. Add l-[2,3-^{3}H] proline solution (final concentration; 370 kBq/ml), and continue the incubation at 37 °C for 4 h.
2. Remove the medium and wash the cells twice with PBS to remove the free radioactivity.
3. Lyse the cells with 1.0 mL of Buffer A, and sonicate.
4. Aliquot 0.5 mL of the cell lysate to each tube and digest the cell lysate with or without collagenase solution for 4 h at 37 °C.
5. Add an excess of gelatin solution as a carrier and precipitate the proteins not digested by the collagenase with 10% TCA-0.5% tannic acid.
6. Centrifuge and collect the precipitates.
7. Wash the pellet three times with ethanol–diethyl ether and solubilize it with Buffer A.
8. Collect both the samples digested with and those not digested with collagenase solution, and measure the radioactivity of both by liquid scintillation counting. To determine the radioactivity of l-[2,3-^{3}H] proline incorporated into collagen, subtract radioactivity of the collagenase-digestion samples from that of the non-digested ones.

3.3.2 Measurement of Proteoglycan Synthesis by Using [^{35}S] Sulfate Incorporation Assay (See Note 25)

1. Grow cultured chondrocytes to confluence in 48-well multiplates with wells containing DMEM or αMEM with 10% FBS.
2. Change the medium to serum-starved medium and preincubate the cells for 24 h. Stimulate these cells or not with various concentrations of rCCN2 for 5 h.
3. Add [^{35}S]-sulfate (37 MBq/mL) in PBS to the wells at a final concentration of 370 kBq/mL, and continue incubation for another 17 h.
4. Collect the culture medium and digest the cells with 1 mg/mL of actinase E solution for 21 h at 55 °C.
5. Precipitate the medium and cell lysate with CPC solution and Buffer B, respectively, for 3 h at 37 °C.
6. Collect the precipitated materials onto a glass filter and measure the radioactivity by liquid scintillation counting.

3.3.3 Measurement of Proteoglycan Accumulation by Using Sulfated Glycosaminoglycan (GAG) Microassay (See Note 26)

1. Grow cultured chondrocytes to confluence in 24-well multiplates with wells containing DMEM or αMEM with 10% FBS.
2. Treat these cells with various concentrations of CCN2 for several days.
3. Remove the culture medium and wash the cells with PBS. Collect the cells with 500 μL of lysis buffer, and transfer 100 μL aliquots of the cell lysate into 1.5-mL tubes. Determine the protein concentration of the remaining cell lysate (*see* **Note 27**).
4. Add 100 μL of actinase E solution (*see* **item 2** in Subheading 2.4.2) to the cell lysate and incubate for 90 min at 65 °C.
5. Transfer 40 μL aliquots of cell lysate digested with actinase E solution into wells of a 96-well multiplates, and add 100 μL of DMB solution to each well (*see* **Note 28**).
6. Measure the optical absorbance at a wavelength of 570 nm and quantify contents of sulfated GAG in the samples with reference to standards prepared by using chondroitin-4-sulfate solution diluted to 3, 10, 20, and 30 μg/mL (*see* **Note 29**).

3.3.4 Measurement of Proteoglycan Production by Using Alcian Blue or Toluidine Blue Staining

1. Grow cultured chondrocytes to confluence in 48-well multiplates with wells containing DMEM or αMEM with 10% FBS.
2. Treat these cells with various concentrations of CCN2 for several days.
3. Remove the culture medium and wash the cells with PBS. Fix these cells with PFA solution for 30 min at room temperature.
4. Stain the fixed cells with 1% alcian blue solution or 0.05% toluidine blue solution for 30 min at room temperature.
5. Wash the cells three times with PBS.
6. Capture photomicrographic images of alcian blue- or toluidine blue-stained matrix with a suitable camera.

7. To quantify the staining intensity of the alcian blue-stained matrix, extract the stained proteoglycan with 200 μL/well of lysis buffer overnight at room temperature. Measure the optical density of the extracted dye at a wavelength of 600 nm (*see* **Note 30**).

3.3.5 Measurement of Gene Expression of Chondrocyte Maturation Markers by Using Quantitative RT-PCR Analysis

1. Grow cultured chondrocytes to confluence in 6-well multiplates with DMEM or αMEM containing 10% FBS.
2. Add 1 ml of RNA isolation reagent (*see* **Note 31**) to each culture plate, and incubate the plates for 5 min at room temperature.
3. Add 0.2 ml of chloroform and shake vigorously for 15 s. Then, centrifuge at 9,000×*g* for 10 min and collect the aqueous phase into another tube.
4. Add an equal volume of isopropanol and vortex for 10 s.
5. Centrifuge at 9,000×*g* for 10 min at 4 °C and collect the precipitate.
6. Rinse with 70% ethanol and centrifuge at 3,500×*g* for 5 min.
7. Dry the precipitate briefly with a SpeedVac and dissolve the former with TE solution.
8. Synthesize first-strand cDNA from 1 μg total RNA by using Oligo dT-adaptor primer as a primer by AMV-derived RT at 42 °C for 30 min (*see* **Note 32**).
9. For quantification of the gene expression levels of chondrocyte differentiation markers, amplify cDNA as a template in the subsequent real-time PCR analysis with specific primers (*see* Table 1) or analyze PCR products by agarose gel electrophoresis.

3.4 Evaluation of Chondrocyte Hypertrophy

Chondrocytes undergo terminal differentiation when they become hypertrophic, at which time they produce type X collagen and ALP. Therefore, evaluation of the gene expression level of type X collagen and measurement of ALP activity are performed to determine whether chondrocytes have become hypertrophic, or not. After chondrocytes from the growth plates of rib cage are grown to confluence, they are cultured for another month, thus becoming over-confluent. Although these cells do not display the typical hypertrophic shape, they show upregulated gene expression of type X collagen [12, 13, 22–25, 27] and increased ALP activity ([12, 13, 19–25]; *see* **Note 33**).

3.4.1 Measurement of Gene Expression of Hypertrophic Chondrocyte Markers Such as Type X Collagen and ALP by Using Quantitative RT-PCR Analysis

For quantification of the gene expression levels of chondrocyte hypertrophy markers, amplify cDNA as a template in the subsequent real-time PCR analysis with specific primers (see Table 1) or analyze PCR products by agarose gel electrophoresis (*See* Subheading 3.3.5).

3.4.2 Measurement of ALP Activity in Chondrocyte Cultures

1. Grow cultured chondrocytes to over-confluence in 24-well multiplates with wells containing DMEM or αMEM with 10% FBS.
2. Treat these cells with various concentrations of CCN2 for several hours.
3. Remove the culture medium and wash the cells with PBS. Collect the cells in each well with 500 μL of Lysis buffer and transfer the cell lysate into a 1.5-mL tube.
4. Homogenize with sonication (*see* **Note 34**).
5. Transfer 200 μL of the sonicated cell lysate into a new tube and add 100 μL of 5 mM $MgCl_2$, 500 μL of 1 M Tris–HCl (pH 9.0), and 100 μL of 5 mM *p*-NPP to initiate the enzyme reaction (*see* **Note 35**).
6. Incubate each tube at 37 °C for 30 min (*see* **Note 36**).
7. To terminate the enzyme reaction, add 250 μL of 1 M NaOH.
8. Measure absorbance at 405 nm and quantify the concentration of *p*-NP generated in the samples by referring to the standard prepared with *p*-NP diluted to 50, 100, 200, 300, 400 and 500 μM.

3.5 Evaluation of Calcified Chondrocytes

Calcified chondrocytes are only seen around the last rows of hypertrophic chondrocytes and promote vascular invasion by producing several growth factors. Therefore, calcified chondrocytes accumulate calcium deposits in themselves and in their ECM. In addition, they promote the production of VEGF and MMP13 to replace cartilage with bone. Evaluation of calcified chondrocytes is performed by measuring the calcium deposits [13, 19, 28] and gene expression and production of VEGF and MMP13 (*see* **Note 33**). Chondrocytes from growth plates of rabbit rib cage are grown to confluence in αMEM containing 10% FBS as described in Subheading 3.1. These cells are then continuously grown in culture medium containing 2 mM β-GP and 50 μg/ml ascorbic acid for 50 days (*see* **Note 37**).

3.5.1 Measurement of Mineralization by Using Alizarin Red Staining

1. Grow cultured chondrocytes to over-confluence in 24-well multiplates with each well containing αMEM supplemented with 10% FBS, 2 mM β-GP, and 50 μg/ml ascorbic acid.
2. Remove the medium, wash the cells with PBS, and fix them with 95% ethanol for 15 min.
3. Stain the fixed cells with 1% alizarin red solution for 5 min at room temperature.
4. Wash three times with PBS.
5. Capture photomicrographic images of alizarin red-stained matrix with a suitable camera.
6. To quantify the staining intensity of the alizarin red-stained matrix, extract the dye with dissolving buffer for 15 min at room temperature. Measure the optical density of the extracted dye at a wavelength of 562 nm (*see* **Note 38**).

3.5.2 Determination of ^{45}Ca Uptake

1. Grow cultured chondrocytes to over-confluence (for 50 days) in 48-well multiplates with each well containing αMEM supplemented with 10% FBS, 2 mM β-GP, and 50 μg/ml ascorbic acid, to which is added various concentrations of rCCN2.
2. Add [^{45}Ca]Cl_2 in PBS to the wells at a final concentration of 185 kBq/mL, and continue incubation for another 3 h.
3. Remove the culture medium and collect the cell matrix layers with Lysis buffer. Then, centrifuge for 15 min at 9,000×*g* to separate the precipitate.
4. Wash the precipitate with Washing buffer and dissolve the former with 0.5 M HCl.
5. Measure the ^{45}Ca radioactivity by liquid scintillation counting.

4 Notes

1. Keep the powders of DMEM and αMEM at room temperature before using them. Otherwise, the powders absorb humidity, and both DMEM and αMEM are degraded.
2. HCS-2/8 cells are an immortalized chondrocyte cell lines that retains the normal human chondrocyte phenotypes during many serial passages. In addition, these cells do not calcify in their normal medium [29, 30].
3. RCS cells were isolated from a rat swarm chondrosarcoma tumor, and they produce the collagen types II, IX, and XI and alcian blue-stainable cartilage-specific proteoglycans [31].
4. Collagenase solution should be used immediately. Do not store it.
5. We have used a 100-μm cell strainer (Becton Dickinson), although other commercial devices are available. Alternatively, a nylon mesh filter can be prepared by yourself, sterilized, and ready to use.
6. Do not use the [*methyl*-^{3}H]-thymidine after long-term storage, even if the radioactivity of its solution is still high. A half life of [*methyl*-^{3}H]-thymidine is different from that of radioactivity of ^{3}H itself (12.32 years). Avoid bacterial contamination which causes degradation of thymidine. Exchange of ^{3}H between *methyl*-^{3}H and H_2O may occur.
7. Dilute BrdU labeling reagent 1:100 with PBS, and add 10 μL/well of this labeling solution to wells containing 100 μL of medium. The final BrdU concentration is 10 μM.
8. We have used TetraColor One solution Cell counting kit-8 (Seikagaku Co., Code No. 800560) with good success [20], although other commercial products also exist.
9. Aliquot this solution and store it at −20 °C.

10. PMSF stock solution is stored at −20 °C, and is added to Buffer A immediately before use.
11. Prepare chondroitin-4-sulfate solution each time. Do not store it.
12. Alcian blue (pH 2.5) stains sulfated and carboxylated acidic polysaccharides such as GAGs in cartilages; and alcian blue (pH 1.0), strongly sulfated polysaccharides. Generally, a solution at pH 2.5 is used for the cytohistological visualization of cartilaginous proteoglycans.
13. These reagents from items 3 to 7 in Subheading 2.4.5 are contained in TaKaRa RNA PCR kit (AMV) ver.3.0 (Takara–Bio). We have used this kit with good success [12, 17, 19–24, 27], although other commercial kits are also available.
14. Prepare *p*-NPP solution each time. Do not store it.
15. Storage of *p*-NP solution is possible at room temperature.
16. Aliquot this solution and store it at −20 °C. Repeated freezing and thawing may result in degradation of ascorbic acid.
17. We describe here how to isolate chondrocytes in primary cultures from young rabbits [12–14, 16]. However, primary chondrocytes from other animal species, such as mice [15] and rats [17], are isolated by use of similar methods.
18. Take care not to detach the cells when they are washed with PBS.
19. Fix the cells with 5% TCA until they become white. Then, dry up the plates until the smell of the ethanol–diethyl ether has disappeared completely.
20. If the cells are incompletely dissolved in 0.5 M NaOH after incubation, try to dissolve the cell lysate completely by pipetting very well.
21. Unless the substrate reaction is stopped, repeated measurement at various time points is possible. Appropriate time points must be determined experimentally.
22. Although these assays are used as a cell proliferation assay, be careful in interpreting the results of these assays because of the measurement of cell viability.
23. Pipetting the solution very well because of the difficulty in dissolving the cells with acidic isopropanol.
24. If the cell numbers are too large, dilute the cell suspension with fresh medium to achieve the optimal range for counting.
25. This assay is used to measure incorporation of [^{35}S] sulfate over 17 h into both culture medium and cell lysate of chondrocytes. Therefore, this result represents total proteoglycan synthesis.
26. This assay is conducted for the measurement of the amount of sulfated GAG bound to DMB solution [32]. Therefore, this

result indicates proteoglycan accumulation from the start of the culture to the collection of cell lysate.

27. The cell lysate may be diluted with PBS in order to fall within the optimal range of the assay.
28. If not analyzed immediately, the sample can be stored at –20 °C.
29. Measure within 10 min, otherwise insoluble debris appears in the sample.
30. It is easier to use alcian blue-staining rather than metachromatic staining by toluidine blue for quantification of proteoglycan accumulation. The former staining stains only cartilage proteoglycan with one (blue) color, whereas the latter staining produces complex of metachromatic purple colored materials (cartilage proteoglycan) and blue colored materials (other types of glycosaminoglycans such as hyaluronic acid, cell membrane, etc.)
31. To check the grade quality of total RNA, perform not only the measurement of optical absorbance at the wavelength of 260/280 nm but also electrophoresis to examine degradation of RNA.
32. Do not store cDNA for long time even if it is stored at –20 °C, because cDNA easily becomes degraded.
33. For articular chondrocytes, these methods can be used to evaluate pathological changes in chondrocytes toward osteoarthritis.
34. Because ALP is a group of enzymes localized to the cellular membrane, homogenize the samples very well.
35. Determine total protein concentration of the samples by using the remaining cell lysate.
36. Measurement time of the enzyme reaction may need to be optimized.
37. Take care not to detach the cells, because they have been cultured for a long time.
38. Dissolve the cell lysate completely by pipetting very well. If the optical density of alizarin red-stained extracts does not fall within the optimal range of the assay, dilute with dissolving buffer.

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Chapter 22

Cell Biological Assays for Measuring Angiogenic Activities of CCN Proteins

Tsuyoshi Shimo and Masaharu Takigawa

Abstract

Angiogenesis, the process of generating new blood vessels from an existing vasculature, is essential in normal developmental processes such as endochondral ossification and in numerous kinds of pathogenesis including tumor growth. A part from the action of angiogenic factor or antiangiogenic factor, it is still unknown at which stage of the angiogenic cascade these agents affect angiogenesis. Here, we describe methods for the use of connective tissue growth factor (CTGF/CCN2) and CCN2 neutralizing antibody in the currently used principal angiogenesis assays, including those in vitro ones for the proliferation, migration, adhesion, and tube formation of endothelial cells and in vivo assays such as those utilizing type I collagen implantation and the chick chorioallantoic membrane (CAM).

Key words Cell proliferation, Adhesion, Migration, Tube formation

1 Introduction

Endothelial cell proliferation, adhesion, migration, and tube formation are fundamental processes in angiogenesis [1, 2]. In vitro models of angiogenesis have been performed predominantly to examine the effects of exogenous CCN2 protein (e.g., treatment with recombinant CCN2) or CCN2 neutralizing antibody on proliferation, migration, adhesion, and tube formation by vascular endothelial cells.

Cell proliferation assay can be used to assess endothelial cell number by use of a hemocytometer.

Another method for determining cell numbers is the use of Cell Counting Kit–SK (CCK-SK) assays utilizing a highly water-soluble tetrazolium salt. One such salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4- disulfophenyl)-*2H*-tetrazolium, monosodium salt], produces a water-soluble formazan dye upon reduction in the presence of an electron mediator [3]. CCK-SK, being a nonradioactive assay, allows sensitive colorimetric determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange-colored product

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(formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells.

Alternatively, [^{3}H] thymidine which is incorporated into new strands of chromosomal DNA during mitotic cell division, can be used to measure DNA synthesis. The advantage of this incorporation assay is that it measures proliferation directly. Endothelial cell adhesion and migration are also critical in the formation of vessels.

Migration assays, performed in a modified Boyden chamber [4], have been used to assess endothelial cell migration in a gradient of CCN2 protein.

There is also the Scratch assay, one of the earliest developed methods to study directional cell migration; and it is particularly suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration [5].

Adhesion assay is appropriate analysis of endothelial cells adhesion to extracellular matrix protein [6]. Cell adhesion is the fundamental process that is critically involved in the initial step of angiogenesis. We describe the methods for measurement of adhesion ability of endothelial cells to CCN2 protein.

The tube formation assay, which is the most specific test for studying the reorganization stage of angiogenesis, measures the ability of endothelial cells to spontaneously form a three-dimensional structure (tubules) on appropriate extracellular matrix components [7].

Angiogenesis does not involve endothelial cells alone; and as in vivo models, the chick chorioallantoic membrane (CAM) assay and subcutaneous implantation assay can be performed. The CAM assay is the most widely used assay for screening for both angiogenesis and antiangiogenesis activity [8]. For the latter assay, type I collagen gel containing CCN2 protein is injected subcutaneously; and the injection site is then examined histologically to determine the extent to which blood vessels have entered the gel [9].

In this chapter, we describe in vitro and in vivo angiogenesis assays methods that can be used for elucidation of the function of CCN2 protein and for assessing the effects of anti-CCN2 neutralizing antibody. These assays could be applied to similar studies on other CCN proteins as well.

2 Materials

In vitro angiogenesis assay, inoculate the bovine aorta endothelial (BAE) cells at a density of $2–7 \times 10^4/cm^2$ into 10 cm-diameter dishes, 6-well plates or 96-well plates and then culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM) and 10% heat incubated fetal bovine serum (FBS) at 37 °C under 5% CO^2 in air. It is also available for human umbilical endothelial cells (HUVEC) using EGM-2 culture medium with growth factors (LONZA).

2.1 Cell Counting Kit SK Assay

1. Tissue culture plates (96-well).
2. Dulbecco's modified Eagle's medium (DMEM) with 0.5 and 10% FBS.
3. Cell counting kit (CCK-SK) solution (Dojindo Molecular Technologies Inc).
4. Microplate reader (450 nm).

2.2 [^{3}H] Thymidine Incorporation Assay

1. Tissue culture plates (96-well).
2. Dulbecco's modified Eagle's medium (DMEM) with 0.5 and 10% FBS.
3. [^{3}H]-Thymidine (e.g., TRK120, Amersham Biosciences).
4. Phosphate buffered saline (PBS).
5. 0.25% Trypsin + 0.02% EDTA (TE).
6. Glass fiber filtermat (96-position. 8 × 12 format, Perkin Elmer).
7. Cell harvester (Perkin Elmer).
8. MeltLex solid scintillator (Perkin Elmer).
9. Hot plate.
10. MicroBeta cassette (Perkin Elmer Wallac).
11. 1450 MicroBeta PLUS (Perkin Elmer Wallac).

2.3 Migration Assay

1. Tissue culture plate (12 wells).
2. Dulbecco's modified Eagle's medium (DMEM) with BSA (0.2 mg/ml).
3. Chemotaxicell (8-μm pore size).
4. Cotton swab.
5. Methanol.
6. Giemsa stain solution.
7. Glass slide (76 × 26 mm) and cover glass (50 × 24 mm).
8. Mounting medium.

2.4 Scratch Assay

1. Tissue culture dishes (35 mm).
2. Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.
3. Phosphate buffer saline (PBS).
4. Cell scraper.
5. Hemocytometer.
6. Phase-contrast microscope.
7. Camera.
8. Image analysis software.

2.5 Cell Adhesion Assay

1. Tissue culture plate (96-well).
2. Dulbecco's modified Eagle's medium (DMEM) with or without 10% FBS.

3. Phosphate buffered saline (PBS).
4. 6% bovine serum albumin (BSA) solution.
5. Cell counting kit (CCK-SK) solution.
6. Microplate reader (450 nm filter).

2.6 Tube Formation Assay

1. Tissue culture plate (35 mm diameter dishes).
2. Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.
3. Phase-contrast microscope.

2.7 Chorioallantoic Membrane (CAM) Assay

1. Fertilized E8 chicken embryos.
2. 4% paraformaldehyde (PFA).
3. Sterile Whatman GB/B glass fiber disks (6 mm in diameter).
4. Paraffin film.
5. Incubator.
6. Bench.
7. File.
8. Forceps.
9. Camera.

2.8 Subcutaneous Implantation Assay

1. Type I collagen derived from bovine skin.
2. Wister male 5 weeks' rats.

3 Methods

3.1 Cell Counting kit SK Assay

1. Prepare a suspension of BAE cells in DMEM containing 10% FBS and then inoculate the cells at a density of 5×10^3/well into 96-well plates and then culture them for 24 h at 37 °C under 5% CO_2 in air (*see* **Note 1**).
2. Change the medium to DMEM containing 0.5% FBS and 30 ng/ml rCCN2 or anti-CCN2 antibodies (IgG, 25 μg/ml) (day 0), and then culture the cells for 48 h.
3. Change the medium to fresh DMEM containing 0.5% FBS with the rCCN2 or anti-CCN2 antibodies.
4. On day 1, 2, 3, 4, or 5 add 10 μl of the CCK-CK solution to each well containing 100 μl of culture medium (*see* **Note 2**).
5. Incubate the wells for 1 h at 37 °C under 5% CO_2 in air.
6. Measure the absorbance at 450 nm by using a microplate reader (Fig. 1a).

3.2 [^{3}H] Thymidine Incorporation Assay

1. Inoculate BAE cells (prepared in DMEM containing 10% FBS) at a density of 5×10^3/well into 96-well plates and then culture the cells for 24 h at 37 °C under 5% CO_2 in air.

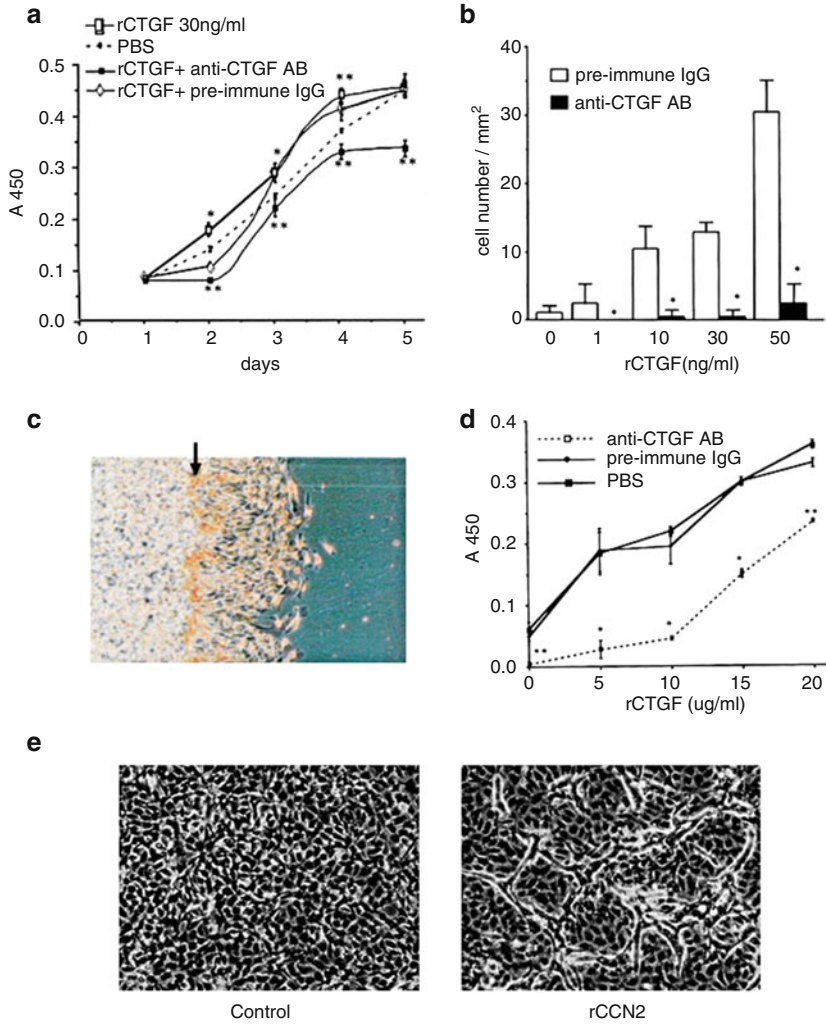

Fig. 1 In vitro angiogenesis assay. (**a**) Effect of CCN2 protein and CCN2 neutralizing antibodies on the proliferation of BAE cells (Cell counting kit SK assay). (**b**) The migration of cells was measured by use of a modified Boyden Chamber. (**c**) Migration assay. Immunohistochemical staining of CCN2 in BAE cells that had migrated. Intense staining is observed in the cytoplasm of the migrating cells. *Arrow*, scratch line. (**d**) Adhesion assay of BAE cells on CCN2 protein. (**e**) Induction of capillary-like tube formation upon the addition of rCCN2. The data are from Shimo et al. [10] (**a**, **b**, **d** and **e**) and [11] (**c**) by permission of Oxford University Press

2. Change the medium to DMEM containing 0.5% FBS and 30 ng/ml rCCN2 or anti-CCN2 antibodies (IgG, 25 μg/ml), and then culture the cells for 48 h.
3. Change the medium to fresh DMEM containing 0.5% FBS and either factor.
4. Add [^{3}H] thymidine (10 μCi/ml) to each well and incubate the cells for 4 h.

5. Wash the cells twice with PBS and then treat with 100 μl TE for 5 min at 37 °C.
6. Harvest the detached endothelial cells onto glass fiber filters by using a cell harvester.
7. Dry the filter mat and fix the cells with MeltiLex solid scintillator (*see* **Note 3**).
8. Count the labeled cells (cpm) in the MicroBeta (*see* **Note 4**).

3.3 Migration Assay

1. Place a BAE cell suspension 4×10^4 cells/400 μl containing BSA (0.2 mg/ml) inside each modified Boyden chamber lined with polyvinylpyrrolidone filters (pore size: 8 μm diameter, *see* **Note 5**).
2. Place the chambers in a 24-well plate containing 800 μl of DMEM containing BSA (0.2 mg/ml) and rCCN2 (1, 10, 30, or 50 ng/ml) with or without neutralizing anti-CCN2 antibodies (50 μg/ml) or pre-immune IgG (50 μg/ml) and incubate the chambers for 4 h at 37 °C (*see* **Note 6**).
3. Remove the filter from each chamber, fix the cells that had migrated in 100% methanol, wash them once with PBS, and then stain them with Giemsa solution (*see* **Note 7**).
4. Mount the filter on glass slides, and examine the cells that had migrated through the filters under a microscope (*see* **Note 8**).
5. Chose five high-power fields (×200) at random and evaluate the number of cells that had migrated (Fig. 1b).

3.4 Scratch Assay

1. Incubate BAE cells at a density of 1×10^5 cells in 35-mm dishes and then culture the cells in DMEM containing 10% FBS to create a confluent monolayer (*see* **Note 9**).
2. Peel one-half of the monolayer off with a cell scraper (*see* **Note 10**).
3. Wash the cells with PBS and culture the remaining half of the cell layer in the medium for 12 or 24 h (*see* **Note 11**).
4. Place the dish under a phase-contrast microscope, photograph the desired region, and analyze it quantitatively by using computer software (*see* **Note 12**, Fig. 1c).

3.5 Cell Adhesion Assay

1. Coat immunological 96-well plates with 5–20 μg/ml rCCN2 containing 0.1% BSA in PBS at 4 °C for 24 h (*see* **Note 13**).
2. Block the plates with 6% BSA in PBS for 1 h at RT, and then wash them with PBS three times.
3. Incubate the plates with anti-CCN2 antibody or the IgG fraction of pre-immune serum for 15 min at RT.
4. Plate BAE cells at 3×10^4/well in serum-free DMEM and incubate the cells for 1 h (*see* **Note 14**).

5. Add DMEM containing 10% FBS to fill the wells (*see* **Note 15**).
6. Cover the plates with paraffin film, place them bottom up, and keep them in that position for 15 min at RT (*see* **Note 16**).
7. Discard the floating cells, and culture the attached cells for 1 h at 37 °C under 5% CO_2 in air in 100 μl of DMEM containing 10% DMEM and 10% CCK-SK solution. Determine the attachment efficiency by measuring the OD of the extract at A450 (Fig. 1d).

3.6 Tube Formation Assay

1. Inoculate BAE cells at a density of 3×10^5/well into 35-mm-diameter dishes containing 3 ml of DMEM supplemented with 10% FBS (*see* **Note 17**).
2. Change the medium on day 3, and culture the cells for another 5 days until monolayers have formed.
3. Change the medium to serum-free DMEM and incubate the cells for 24 h.
4. Add rCCN2 to the cells and culture the cells for 12 h (*see* **Note 18**).
5. Examine the tube formation under a microscope (Fig. 1e).

3.7 Chorioallantoic Membrane (CAM) Assay

1. Clean fertilized E8 chicken embryos with 0.1% benzalkonium chloride by spraying and preincubate the egg at 37.5 °C in 85% humidity for 2 days (*see* **Note 19**).
2. Disinfect the shell with 0.1% benzalkonium chloride, and make a hole with forceps on the top of the air sac (*see* **Note 20**).
3. Make a 1 cm × 1 cm window by using a file and remove the shell with forceps.
4. Shake the egg transversely (*see* **Note 21**).
5. Separate the inner cell membrane from the CAM by using forceps.
6. Place a sterile glass fiber disk prepared with 20 μl of the desired factor upside down on then CAM (*see* **Note 22**).
7. Seal upon the openings with sterile Parafilm, and incubate the eggs further for 5 days.
8. Add 4% PFA in PBS directly to immerse and fix the blood vessels in the experimental area.
9. Separate the CAM from the embryo, spread it on a 10-cm dish, and then examine the blood vessels under a microscope (Fig. 2).
10. Evaluate the angiogenesis response of the CAM by using image analysis software and analyze the length of the microvessels statistically.

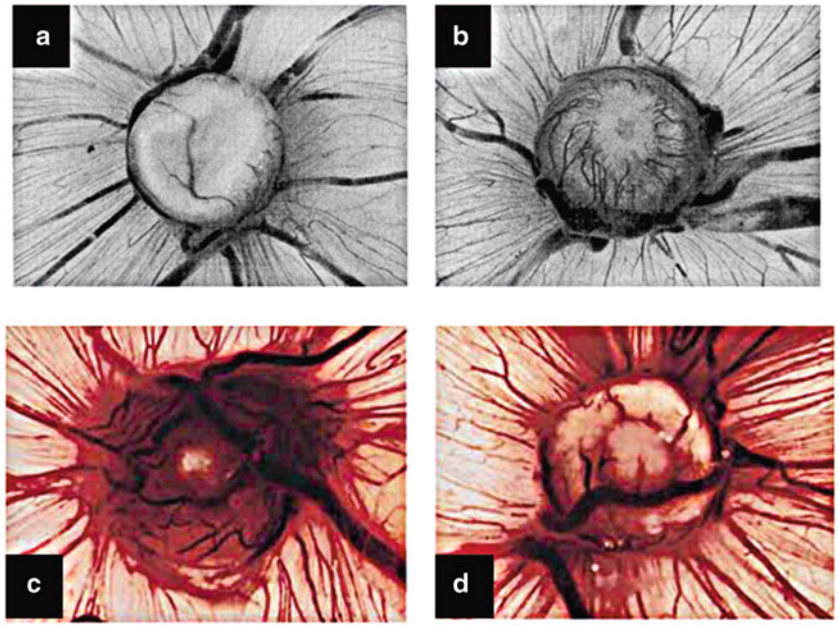

Fig. 2 Chorioallantoic membrane (CAM) assays. (**a** and **b**) PBS as a control (**a**), 2 μg of rCCN2 (**b**). (**c** and **d**) Embryonic CAMs were treated with glass fiber filter disks containing nu/nu xenografts and 200 μg of anti-CTGF antibody. Figures indicate the results on day 5 after the addition of glass fiber filter disks containing MDA231 cells treated with rabbit IgG (**a**) or anti-CTGF IgG antibody (**b**). The data are from Shimo et al. [10, 12] by permission of Oxford University Press and S. Karger AG

3.8 Subcutaneous Implantation Assay

1. Mix 10 μg rCCN2 with 10 mg of type I collagen in a centrifuge tube and then lyophilize them.
2. Press the rCCN2 and carrier into globular pellets (*see* **Note 23**).
3. Implant the rCCN2/carrier into the dorsal subcutaneous tissues of rats (*see* **Note 24**).
4. Sacrifice the rats on day 7 and fix the explants of rCCN2/carrier in 10% paraformaldehyde in PBS for 24 h (*see* **Note 25**, Fig. 3a, b).
5. Dehydrate the explants in alcohol and embed them in paraffin.
6. Deparaffinize sections of 5-μm thickness with xylene and rehydrate them by ethanol treatment. For routine histology, stain the sections with hematoxylin and eosin.
7. Stain the sections with anti-factor VIII antibodies for immunohistochemistry (Fig. 3c, d).

4 Notes

1. At least 1000 cells are necessary per well (100 ml medium).
2. Be careful not to introduce bubbles into the wells, since they interfere with the O.D. reading.

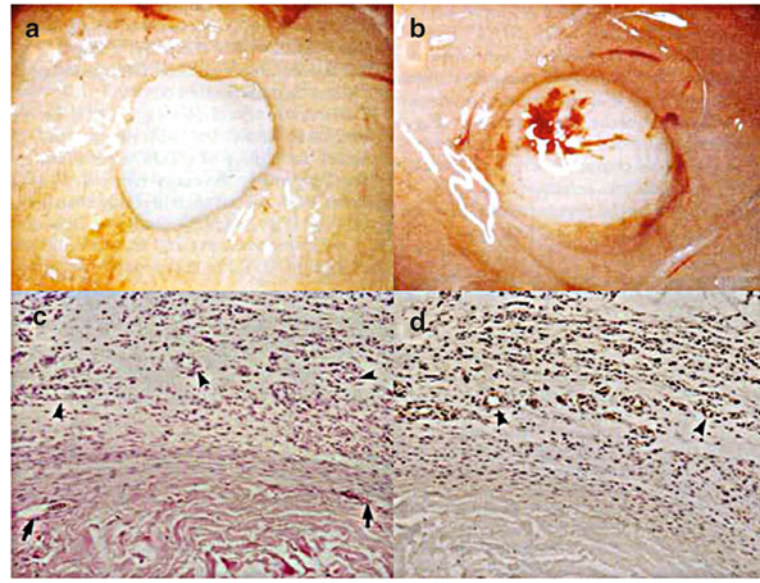

Fig. 3 Macroscopic appearance and immunohistochemical analysis of type I collagen/CCN2 implants. (**a** and **b**) Macroscopic appearance of control (**a**) and rCCN2 (**b**). (**c** and **d**) Hematoxylin–eosin staining (**c**) and immunohistochemical staining for factor VIII (**d**). *Arrowheads*, endothelial cells. *Arrows*, blood vessels. The data are from Shimo et al. [10] by permission of Oxford University Press

3. If the filters are not completely dry prior to the addition of solid scintillator, the residual water present in the filter can interact with the scintillator to reduce the counting efficiency.
4. The MicroBeta cassette is designed to hold a glass-fiber filter and to allow it to be moved from the rack to the conveyor and back again.
5. Pre-warm the BSA medium at 37 °C in a water bath.
6. Make sure the medium touches the bottom surface of the filter and remove the bubbles under the filter.
7. Gently clean the filter side of the upper chamber with a cotton swab, turn the chamber upside-down and gently submerge it in PBS to remove unattached cells.
8. Gently cut the filter from the chamber by using a cutter.
9. It is important to wait until the cells reach confluence, otherwise one can not draw clear scratched line.
10. The diameter of the 35-mm culture dish is traced onto a piece of paper, on which a straight line is then drawn through its center. The paper is then superimposed on the monolayer in the dish and a straight cut is made through the monolayer by cutting through the line on the paper with a cell scraper.
11. Wash the cell layer at least a second time with PBS to remove the floating cells.

12. For further analysis of the expression pattern of CCN2 in the migrating cells, fix the cell layers with methanol and stain them with anti-CCN2 antibody (Fig. 1c).
13. ELISA 96-well plates have the advantages of minimizing the amount of protein. The time of CCN2 treatment can be extended up to 48 h in the case of weak binding of the cells.
14. Incubation time during which the cells attach to the CCN2 protein should be experimentally determined for each cell type.
15. The wells should be filled with DMEM without bubbles being formed.
16. The bubbles should be removed by manually rubbing the Parafilm lightly.
17. The tube-forming capability of endothelial cells greatly participates in this system.
18. Once tube formation has occurred, the networks are usually maintained for approximately 24 h; and so timely observation of the cells is necessary.
19. On day 9, eggs were confirmed to have an embryo or not by holding the eggs one by one up to an incandescent lamp.
20. Egg morphology is an ellipse with a relatively larger end and a smaller one, and the air sac is located at the larger end directly under the shell.
21. The air sac located on the larger end of the ellipse-shaped egg is moved by this shaking to the opened window side.
22. For evaluation of the effect of anti-CCN2 antibody on tumor xenograft-induced angiogenesis, MDA231 or HT1080 tumor nu/nu mice xenografts ($3 \times 3 \times 3$ mm) are put on the filter which have been absorbed by the 200 ng of anti-CCN2 neutralizing antibody.
23. rCCN2/carrier pellets need to be prepared immediately before implantation.
24. The pellet is injected by using a cancer cell transplantation needle with dimensions of $\varphi 3.5 \times 9.5$ mm (Chubu Kagaku Shizai, Nagoya, Japan).
25. Macroscopic appearance of neovascularization around the pellet is observed in the CCN2-treated group.

Acknowledgements

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Chapter 23

Cell Biological Assays for Measuring Odontogenic Activities of CCN Proteins

Koichiro Muromachi, Hiroshi Sugiya, and Nobuyuki Tani-Ishii

Abstract

In postnatal dentin formation, odontoblast differentiation occurs in the pulp tissue regenerative process under pathological condition. Odontoblasts and newly differentiated odontoblast-like cells beneath the caries lesion form tertiary dentin and are highly odontogenic. To observe the activity of dentinogenesis occur within the hard tissue, a combination of immunohistological analysis and immunodetection of dentinogenesis specific molecules, such as dentin sialophosphoprotein (DSPP) and/or its cleaved products dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), is a reliable approach. Besides, recent studies have revealed that the expression of CCN family member 2 (CCN2), a member of the CCN family protein, is confirmed in accordance with tooth development and reparative dentin formation. Therefore, CCN2 could serve as a marker for dentinogenesis. Here, we describe a method for visualizing the CCN2 signal as an odontogenic activity in formalin-fixed paraffin-embedded (FFPE) sections of demineralized human teeth and human dental pulp cells.

Key words Immunohistochemistry, Western blot, Human teeth, Dentin–pulp complex, Odontoblasts, Dentinogenesis

1 Introduction

Odontoblasts are one of the most unique cells in orofacial tissues due to its morphological and functional features. These cells are aligned along the predentin and all of them extend its cellular processes in an outward direction [1]. Previous immunohistochemical studies have revealed that odontoblast is divided into several stages by their metabolic status [2, 3]. Once primary dentin formation is settled, dentin-secreting activity of odontoblast is reduced in accordance with aging [3]. In this resting phase, odontoblasts deposit secondary dentin faintly onto originally formed dentin. The rate of secondary dentin secretion is limited from one-tenth to one-fiftieth than that of primary dentinogenesis [4]. However, even after the tooth eruption, tertiary dentinogenesis recurs upon harmful external stimuli such as dental caries, trauma, and cavity preparation during restorative procedures in dentistry as a protective response of pulp tissue [4].

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Concerning the status of odontoblast activity and differentiation, it is thought that dentin sialophosphoprotein (DSPP) and/or its processed products dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are valuable markers for characterizing the phase of odontoblast maturation, especially dentin secretion [2]. DSPP, DSP, and DPP are the most prominent component of noncollagenous protein in dentin [5]. The two DSPP derived products play different roles; DSP involves in the initiation of dentin mineralization, and DPP facilitates the dentin maturation [6].

In developing tooth germs, the epithelial–mesenchymal interaction is necessary for tooth growth [7]. This interplay between each type of cells leads the CCN family member 2 (CCN2) expression [8]. In addition, experimentally neutralized CCN2 function provides the impairment of tooth growth, as a result of suppression of ameloblasts and odontoblasts differentiation [8]. Meanwhile, the expression of CCN2 is increased in odontoblast-like cells beneath the reparative dentin in carious teeth [9]. Thus, we hypothesized that the expression patterns of CCN2 and DSPP in pathological conditioned dental pulp will be instrumental in understanding the odontogenic activity in mature teeth [10]. To this end, we applied a methodology of immunohistochemistry and western blot. This document provides the full methods we have employed as a demonstration of CCN2 expression in dentin–pulp complex to measure the odontogenic activity.

2 Materials

2.1 Immunohistochemistry

2.1.1 Tissue Preparation Reagents

1. 4% paraformaldehyde phosphate buffer solution.
2. Decalcifying solution B (EDTA method, Wako) (*see* **Note 1**).
3. Sample Pack.
4. Series of graded ethanols (70, 80, 90, 95, and 100% in DW).
5. Xylene.
6. Tissue-Tek® MEGA-CASSETTE® (Sakura Finetek) or equivalent.
7. Automatic embedding equipment Sakura Rotary (Sakura Finetek, RH12-DM) or equivalent.
8. Tissue-Tek TEC Embedding Center (Sakura Finetek) or equivalent.

2.1.2 Tissue Sectioning Equipments, Deparaffinization and Rehydration Reagents

1. Sliding microtome (Leica, SM2000R) or equivalent.
2. Disposable microtome blade (Feather, A35).
3. Water bath.
4. Superfrost Micro Slide Glass (MAS-coated).
5. Tissue-Tek Slide Warmer (Sakura Finetek) or equivalent.

6. Small paintbrush.
7. Xylene.
8. Series of graded ethanols (100, 95, 90, 80, and 70% in DW).

2.1.3 Blocking Reagents for Endogenous Peroxidase and Nonspecific Antibody Interaction

1. Endogenous Peroxidase Blocking Reagent: Add 30% of Hydrogen peroxide to Methanol just before use.
2. Blocking buffer: Dissolve 4 g of Block Ace® (DS Pharma Biomedical) powder in a 100 ml of Millipore water. Add 0.5 ml of Tween 20 and make up to a volume of 1 l with Millipore water (*see* **Note 2**).

2.1.4 Primary and Secondary Antibodies

1. Anti-human CCN2/CTGF rabbit polyclonal antibody (Peprotech) (*see* **Note 3**).
2. Anti-human DSPP mouse monoclonal antibody (LFMb-21) (Santa Cruz Biotechnology) or equivalent (*see* **Note 4**).
3. Dako pen (Dako).
4. Dako REAL™ EnVision™ Detection Reagent Peroxidase Rabbit/Mouse (Dako).

2.1.5 Detection and Counterstaining Reagents

1. Dako EnVision™ kit (Dako) containing the substrate buffer and the DAB + Chromogen Solution.
2. Mayer's hematoxylin solution.
3. Series of graded ethanols (70, 80, 90, 95, and 100% in DW).
4. Xylene.
5. Entellan® (Merck) or equivalent.
6. Coverslip (24 × 50).

2.2 Western Blot

2.2.1 Cell Culture

1. α-MEM supplemented with 10% FBS and antibiotics (20 U/ml penicillin G, 100 μg/ml kanamycin, 250 ng/ml fungizone).
2. Trypsin.

2.2.2 Cell Preparation

1. Recombinant human BMP-1 (R&D systems) (*see* **Note 5**).
2. Recombinant human MMP-3 (Acris Antibodies) or equivalent (*see* **Note 5**).
3. Protease inhibitor cocktail solution: Dissolve one tab of Complete Mini EDTA-free (Roche) in a 500 μl of CelLytic™ M Cell Lysis Reagent (Sigma-Aldrich).
4. Cell lysis buffer: Mix the Protease inhibitor cocktail solution and the CelLytic™ M Cell Lysis Reagent in a 1 : 20 ratio. Add 100 μM PMSF (Cell Signaling Technology), 0.2 mM EGTA, and 2 mM EDTA.
5. Cell scraper.

6. Sample buffer: We routinely use the Red Loading Buffer pack (New England BioLabs) containing 3× SDS sample buffer and 30× DTT. Mix the 3× SDS sample buffer and the 30× DTT in a 9:1 ratio before using.

2.2.3 SDS-PAGE and Immunoblot

1. 7.5% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad).
2. Precision Plus Protein Dual Color Standard (Bio-Rad).
3. Running buffer: Dissolve one pack of Tris-Glycine-SDS powder (Takara) in a 500 ml of Millipore water.
4. Transfer buffer: Mix 100 ml of 10× Tris Glycine buffer (Bio-Rad) to 700 ml of Millipore water and 200 ml of Methanol.
5. Supported Nitrocellulose Membranes 0.2 μm (Bio-Rad) or equivalent.
6. TBS buffer: Mix 5 ml of 10× TBS Buffer pH 7.5 (Rockland Immunochemicals) and 45 ml of Millipore water.
7. Blocking buffer: Dissolve 4 g of Block Ace® powder in a 100 ml of Millipore water (*see* **Note 2**).
8. Anti-human CCN2/CTGF and anti-human DSPP antibodies: the same as in Subheading 2.1.4.
9. Anti-β-actin antibody (e.g., anti-β-actin rabbit monoclonal antibody, Cell Signaling Technology).
10. PE/PET pouch (Yamamoto Manufacturing).
11. Wash buffer: Add 0.5 ml of Tween 20 to 100 ml of blocking buffer. Make up to a volume of 1 l with Millipore water.
12. Anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) or equivalent.
13. Anti-mouse IgG HRP-linked antibody (Cell Signaling Technology) or equivalent.
14. ECL Prime Western Blotting Detection Reagent (GE).
15. Hyperfilm ECL (GE).
16. LAS 3000 (Fuji Film) or equivalent.

3 Methods

All steps are performed at room temperature unless noted otherwise. The procedure to obtain the tooth samples must be approved by the ethics committee.

3.1 Immunohistochemical Staining of CCN2 in Odontoblasts Under Pathological Condition

3.1.1 Preparation of Teeth

1. After extraction, cut the intact and carious teeth horizontally at the cervical region in order to obtain a secure fixation of dental pulp.
2. Immerse the samples immediately with 4% paraformaldehyde for 1 day at 4°C (*see* **Note 6**).
3. After fixation, rinse the samples with distilled water.
4. Put the samples into the Sample Packs.
5. Hang the Sample Packs and demineralize the samples with decalcifying solution B for 4 weeks at 4 °C with stirring (*see* **Note 7**).
6. Process the demineralized tissues through a series of graded ethanols (70, 80, 90, 95, and 100%) for dehydration, immerse in xylene, and infiltrate in paraffin using the automatic embedding equipment Sakura Rotary.
7. Embed the samples in paraffin using Tissue-Tek TEC Embedding Center.

3.1.2 Sectioning, Deparaffinization, and Rehydration of Samples

1. Cut sections of 4-μm thickness from paraffin blocks on a microtome with a disposable blade suited for hard tissue block.
2. Pick up the cut sections from the microtome using wetted paintbrush.
3. Float the thin sections on water bath at 40 °C to stretch them.
4. Mount the stretched sections on slide glasses using paintbrush.
5. Wipe off the excess water on the slide glasses.
6. Dry the tissue mounted slides using a paraffin stretching plate (Slide Warmer) at 37 °C for 1 day.
7. To melt the paraffin, take the slides on paraffin stretching plate at 37 °C for 30 min or in paraffin oven at 60 °C for 10 min.
8. Deparaffinize the slides two times with xylene for 3 min.
9. Rehydrate the slides by 3 min processes through a series of graded ethanols (100, 95, 90, 80, and 70%).

3.1.3 Inactivation of Endogenous Peroxidases and Blocking Nonspecific Interactions

1. Incubate the sections with Endogenous Peroxidase Blocking Reagent for 30 min.
2. To block nonspecific antibody interactions, rinse the sections with blocking buffer for 3 min, at least three times.

3.1.4 Primary and Secondary Antibodies

1. Wipe off the excess buffer on the slide glasses.
2. Encircle the sections with a Dako pen.
3. Add 40 μl/slide of primary antibody diluted in blocking buffer (*see* **Note 8**).
4. Put the slides into the moist chamber and incubate overnight at 4 °C.
5. After incubation, remove the primary antibody solution carefully using blotting paper (*see* **Note 9**).

6. Wash the slides with blocking buffer for 3 min, at least three times.
7. Wipe off the excess buffer on the slide glasses.
8. Add one to two drops of the Dako REAL™ EnVision™ Detection Reagent Peroxidase Rabbit/Mouse.
9. Put the slides into the moist chamber and incubate for 1 h.

3.1.5 Detection and Counterstaining

1. After incubation, remove the secondary antibody solution carefully using blotting paper.
2. Wash the slides with blocking buffer for 3 min, at least three times.
3. Wipe off the excess buffer on the slide glasses.
4. Mix the substrate buffer and the DAB + Chromogen Solution just before use.
5. Add 40 μl/slide of the DAB mixture solution.
6. Incubate for 1–5 min and visualize the antigen–antibody reaction under a microscope.
7. Wash the slides with distilled water for 5 min to stop the reaction.
8. Immerse the slides into Mayer's Hematoxylin for 2 min.
9. Wash the slides with distilled water for 5 min.
10. Dehydrate the slides by 10 s processes through a series of graded ethanols (70, 80, 90, 95, and 100%) (*see* **Note 10**).
11. To permeate, process the sections two times with xylene for 10 s.
12. Add 1–2 drops of the Entellan® onto the sections and put a coverslip on the mounting solution.
13. Dry the slides for 1 h on slide tray.
14. Observe the slides using the microscope.

3.2 Measuring CCN2 Expression in Human Dental Pulp Cell Lysate by Western Blot

3.2.1 Cell Culture

1. After extraction, cut the intact teeth horizontally at the cervical region.
2. Strip off the pulp tissue from pulp chamber using tweezer under aseptic conditions.
3. Mince the dental pulp using scalpel with one drop of the α-MEM supplemented with 10% FBS.
4. Place the minced tissue on a tissue culture dish and cover with a sterilized glass coverslip.
5. Culture the explants in α-MEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 in air.
6. Once cell growth from the explants had reached confluence, detach the cells with 0.025% trypsin in PBS.

7. Add same volume of α-MEM and harvest the floating cells.
8. After centrifugation, subculture the pelleted cells in culture dishes.

3.2.2 Cell Preparation

1. Seed human dental pulp cells (passages 3–5) in 100-mm tissue culture dishes at 2×10^5 cells/ml in α-MEM containing 10% FBS.
2. For the experiments, starve the cells in medium containing 1% FBS for 24 h before stimulation.
3. After serum starvation, stimulate the cells with rhBMP-1 or rhMMP-3 at appropriate concentrations.
4. After stimulation, aspirate the medium and wash the cells twice with PBS.
5. Add 250 μl/dish of the cell lysis buffer and collect the cell lysates using cell scraper (*see* **Note 11**).
6. Centrifuge for 1 min at 10,000 × *g* and transfer the supernatant to new 1.5 ml sample tubes.
7. Mix the supernatant and the sample buffer in a ratio of 2:1.
8. Then, boil the samples at 95 °C for 5 min.
9. Storage the heated samples at −80 °C prior to SDS-PAGE.
10. Adjust the protein concentration of samples using the method of Bradford [11].

3.2.3 SDS-PAGE and Immunoblot

1. Set the 7.5% Mini-PROTEAN® TGX™ Precast Gels into the electrode assembly.
2. Fill the buffer chamber with the running buffer.
3. Load an equal concentration of samples to gels.
4. Run the gels with the running condition at 200 V.
5. After electrophoresis, cut the excess part of gels using wide and flat-ended spatula.
6. Remove the gels gently from the gel cassettes and overlay the 0.2 μm nitrocellulose membrane onto the gel (*see* **Note 12**).
7. Set the gel and the nitrocellulose membrane to transfer assembly.
8. Fill the transfer module with the transfer buffer.
9. Transfer the proteins with a condition at 12.5 V for overnight.
10. After transfer, trim the nitrocellulose membranes to avoid the nonspecific reaction of primary antibody.
11. Incubate the membranes with the TBS buffer at 60 rpm shaking for 10 min.
12. Incubate the membranes with blocking buffer at 60 rpm shaking for 50 min.

13. Dilute the primary antibodies in wash buffer (excluding Tween 20) at appropriate ratio (*see* **Note 13**).
14. Place the membrane into heat-sealed PE/PET pouch and add primary antibody solution (*see* **Note 14**).
15. Incubate at 200 rpm shaking for 2 h.
16. Dilute the secondary antibodies in wash buffer (excluding Tween 20) at appropriate ratio (*see* **Note 15**).
17. Pick out the membranes from the pouch and wash with the wash buffer for 3 min at least three times.
18. Incubate the membranes with secondary antibodies at 60 rpm shaking for 90 min.
19. Wash the membranes with the wash buffer for 3 min at least three times.
20. After the final wash, detect the immunoreactivity using an ECL Prime Western Blotting Detection Reagent.

4 Notes

1. We recommend the Decalcifying solution B (EDTA method, Wako) for demineralizing the tooth samples with good success [9, 10], although we have also tried other acidic decalcifying reagents (e.g., formic acid).
2. We routinely use the Block Ace® (DS Pharma Biomedical) for blocking buffer to prevent the nonspecific antibody interaction. This method is easy and displays good results [9, 10] than skim milk. Other commercially available blocking reagents can also be used.
3. We recommend the anti-human CCN2/CTGF rabbit polyclonal antibody (Peprotech) with excellent results [9, 10], although we have also tried other anti-human CCN2/CTGF mouse monoclonal antibody (Data not shown). We think that it is worth trying other commercially available antibodies.
4. We have also tried to observe the immunoreaction for dentin matrix protein-1 (DMP-1) in human teeth, but the specific signals were shown in overall the dentin and pulp tissue (Data not shown). Hence, we thought that DMP-1 is not suitable as an odontoblast specific marker.
5. To induce the expression of CCN2, we routinely stimulate the dental pulp cells with rhMMP-3 or rhBMP-1 [10]. The expression of both metalloproteases under pathological conditioned dental pulp is well investigated [9].
6. We recommend that the volume of paraformaldehyde should be at least ten times greater than the size of samples.

7. We recommend that the volume of decalcifying solution B should be at least 50 times greater than the size of samples. When the surface of samples is softened, cut off the redundant area of samples using a scalpel for secure permeation of the decalcifying solution.
8. We routinely dilute the primary antibodies (anti-CCN2/CTGF and anti-DSPP) at 1:100 ratio.
9. Do not touch the tissue section. Carefully blot the antibody solution only.
10. Shrinkage of specimens and separation of dentin and odontoblast layer may occur owing to long time exposure to dehydration.
11. Place the sample tubes on ice to avoid the sample degradation.
12. To prevent the gel drying, we perform this procedure in the stainless steel tray filled with transfer buffer. When overlaying the nitrocellulose membrane on the gel, carefully remove the bubbles between the membrane and the gel.
13. We routinely dilute the primary antibodies at following ratio; anti-CCN2/CTGF and anti-DSPP (diluted 1:1000), anti-β-actin (diluted 1:2000).
14. Remove bubbles carefully before seal the pouch.
15. We routinely dilute the secondary antibodies at 1:2000 ratio.

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Chapter 24

Separation and Enrichment of Hematopoietic Stem Cells for CCN Studies

Lisa J. Crawford and Alexandra E. Irvine

Abstract

The regulation of blood cell production (hematopoiesis) by CCN proteins is an area of increasing interest to hematologists. There is some discordance in the literature in this area due to the use of mixed or ill-defined cell populations for experiments. Expression of, and response to, CCN proteins is specific to both cell type and differentiation status. Here, we describe methods to prepare defined hematopoietic cell populations and associated functional assays.

Key words Hematopoietic stem cells, CFU-GM, LTC-IC

1 Introduction

Hematopoietic stem cells (HSCs) give rise to myeloid and lymphoid progenitor cells in the bone marrow which then differentiate into the mature circulating blood cell. Homeostasis is maintained by a carefully regulated balance between the processes of HSC self-renewal and differentiation. The stem cells reside in the bone marrow microenvironment where their development and function are controlled by numerous interactions including with the extracellular matrix [1]. The matricellular CCN proteins have been reported to be key regulators in hematopoiesis [2–5].

HSCs are very rare (0.01% of bone marrow nucleated cells) but in theory one HSC could repopulate the entire bone marrow. Whilst purification of HSCs is a major research goal, it is generally agreed that HSCs and progenitor cells reside within a population of cells characterized by CD 34+ and CD38−. We have previously shown that CCN3 expression and responsiveness changes with HSC differentiation. Experiments investigating the role of CCN proteins in hematopoiesis must be carried out using well-defined cell populations under carefully controlled assay conditions to produce clear, unambiguous results.

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This chapter describes the separation and enrichment of human HSCs, the production of CCN3 overexpressing cell lines and the application of specialized functional assays to evaluate response to CCN treatment.

2 Materials

2.1 HSC Enrichment Components

1. Ficoll-Paque PLUS.
2. Hank's balanced salt solution (HBSS).
3. MACS CD34 Microbead Kit (Miltenyi Biotech Ltd, Surrey, UK).
4. MACS separation buffer: Phosphate Buffered Saline (PBS) with 0.5% BSA and 2 mM EDTA.
5. 60 μm nylon net filter roll (Merck Millipore, Watford, UK).
6. Sterile PBS with 2% fetal calf serum (PBS-FCS).
7. Flow cytometry antibodies: CD34 APC, CD38 FITC.
8. Serum free media (SFM): IMDM supplemented with serum substitute (bovine serum albumin, insulin, and transferrin: 'BIT', StemCell™ Technologies, Cambridge, UK), glutamine, and penicillin/streptomycin.

2.2 Transfection Components

1. K562 cells.
    1. RPMI1640 medium supplemented with 10% fetal calf serum.
    2. Amaxa® Cell Line Nucleofector® Kit V—contains Nucleofector® solution, pmaxGFP® vector, and certified cuvettes (Lonza Cologne AG, Cologne, Germany).
    3. G418.

2.3 CFU-GM Materials

1. Methocult™ H4536 medium (StemCell Technologies, Cambridge, UK).
2. 16-G blunt end needles.
3. Luer lock syringes.
4. IMDM with 2% FCS.
5. 35 mm culture dishes.

2.4 LTC-IC Assay Materials

1. Hydrocortisone . Dissolve hydrocortisone powder in Alpha MEM to a working concentration of 100 μM, filter-sterilize using a 0.2 μm low protein binding filter and store at 4 °C for up to a week.
2. Human long term culture medium (HLTM): Myelocult™ H5100 (StemCell™ Technologies, Cambridge, UK). Thaw HLTM in a 37 °C water bath, at room temperature, or overnight at 4 °C. Once thawed mix well and store at 4 °C for up to 1 month.

3. HLTM with hydrocortisone: This medium is prepared by diluting the 100 μM working stock of hydrocortisone one in 100 in HLTM (e.g., 1 ml of 100 μM hydrocortisone in 99 ml HLTM) to yield a final hydrocortisone concentration of 1 μM. This medium can be stored at 4 °C for up to 1 week.
4. 16-G blunt-end needles.
5. Luer lock syringes.
6. 35 mm culture dishes.
7. Methocult™ H4435 (StemCell™ Technologies, Cambridge, UK).
8. IMDM with 2% FCS.
9. Hank's Balanced Salt Solution (HBSS), trypsin with 0.25% citrate solution or 0.25% EDTA.
10. Collagen solution.
11. M2-10B4 murine fibroblast cell line. M2-10B4 cells are routinely maintained in RPMI 1640 medium containing 10% FCS.
12. Serum free medium with growth factors (SFM + GF): IMDM supplemented with serum substitute (bovine serum albumin, insulin and transferrin: 'BIT', StemCell™ Technologies, Cambridge, UK), glutamine, penicillin/streptomycin and growth factor cocktail [IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml), Flt3 (100 ng/ml), SCF (100 ng/ml)].

3 Methods

3.1 Enrichment of Human HSC and Progenitor Cells From Primary Clinical Samples

Due to the low frequency of CD34+ CD38− HSCs in peripheral blood and bone marrow samples, we enrich for this cell type using a three step process. The first step is to isolate mononuclear cells from the sample by density gradient, the next step is to enrich for CD34+ cell by magnetic cell sorting and finally to enrich for CD34+ and CD38− cells by fluorescence activated cell sorting.

3.1.1 Mononuclear Cell Isolation

1. Dilute heparinized peripheral blood sample at a ratio of 1:1 with HBSS and mix well (*see* **Note 1**).
2. Aliquot 3 mls Ficoll-Paque PLUS solution into a 15 ml centrifuge tube and carefully layer 4 ml of dilute blood sample over the Ficoll-Paque PLUS ensuring there is no intermixing (*see* **Note 2**) and centrifuge at 400 × *g* for 30 min at room temperature with the centrifuge brake off.
3. During centrifugation erythrocytes sediment through the Ficoll-Paque PLUS and mononuclear cells are found at the interface between the Ficoll-Paque PLUS and plasma. Collect mononuclear cells using a Pasteur pipette, transfer to a 15 ml centrifuge tube and wash twice by centrifugation in 5 ml HBSS

to remove residual plasma, Ficoll-Paque PLUS or platelets. At this stage mononuclear cells can be frozen down for later use (*see* **Note 3**), or proceed to CD34+ cell enrichment.

3.1.2 CD34+ Cell Enrichment

1. Prepare sufficient buffer before beginning, filter-sterilize using a 0.2 μm filter and store at 4 °C until required (*see* **Note 4**).
2. Ensure cells are in a single cell suspension by passing through sterile 60 μm nylon mesh, harvest cells by centrifugation at $300 \times g$ for 10 min, aspirate supernatant and resuspend cell pellet in 300 μl buffer for up to 1×10^8 cells.
3. Add 100 μl FcR blocking reagent and 100 μl CD34+ microbeads for up to 1×10^8 cells, mix well and incubate for 30 min at 4 °C.
4. Wash cells by adding 5 ml of buffer per 1×10^8 cells and harvest by centrifugation at $300 \times g$ for 10 min, aspirate supernatant and resuspend in 500 μl buffer per 1×10^8 cells.
5. Proceed to magnetic separation using MS columns (for up to 2×10^8 total cells), LS columns (for up to 2×10^9 total cells) or AutoMACS pro separator as described below. If required, after magnetic separation cells can be maintained overnight in serum free media at 37 °C in a humidified incubator with 5 % CO_2.

3.1.3 Magnetic Separation with MS or LS Columns

1. Place column on suitable MACS separator (*see* **Note 5**) and prepare column by rinsing with buffer (500 μl for MS column/3 ml for LS column).
2. When buffer has completely run through the column, apply cell suspension to the column and collect the flow-through containing unlabelled CD34– cells.
3. Wash cells with buffer (500 μl for MS column/3 ml for LS column), collect flow-through containing unlabelled cells and combine with flow-through from previous step. Repeat for a total of three washes.
4. Remove column from separator, place in a 15 ml centrifuge tube, pipette buffer onto the column (1 ml for MS column/5 ml for LS column) and flush out magnetically labelled CD34+ cells by firmly pushing the plunger into the column.

3.1.4 Magnetic Separation with AutoMACS Pro Separator

1. Prepare the separator by ensuring there is sufficient running buffer for the separation (*see* **Note 4**) and prime instrument by selecting the "quickwash" program.
6. Place cells in a 15 ml tube in row A of pre-cooled tube rack and place empty 15 ml tubes in rows B and C to collect cells.
7. Select "Posseld" program, enter volume of cell suspension for each sample and begin the separation. Unlabelled CD34-cells will be eluted into the collection tube in row B and the CD34+ fraction will be eluted into the collection tube in row C.

3.1.5 CD34+CD38– Cell Sorting

1. Perform a cell count, collect cells by centrifugation (300 × *g*, 5 min) and resuspend in 100 µl PBS + 2% FCS per 1×10^6 cells (*see* **Note 6**).
2. Take an aliquot of cells for controls and make up to 100 µl with PBS-FCS. The following controls are suggested:

   Unstained control

   CD34 APC positive

   CD38 FITC positive

   APC isotype control

   FITC isotype control
3. Stain controls with appropriate antibody or isotype control and stain the remaining cells with CD34 APC (5 µl per 1×10^6 cells) and CD38 FITC (10 µl per 1×10^6 cells). Incubate in the dark at room temperature for 20 min.
4. Wash twice with 1 ml PBS-FCS (300 × *g*, 5 min) and aspirate supernatant. Resuspend controls in 100 µl PBS-FCS and resuspend stained cells in PBS-FCS at a concentration of approximately 1×10^7 cells per ml. Sterile filter cell suspensions through 60 µm nylon mesh immediately prior to cell sorting.
5. Complete in-house quality control procedures for the flow cytometer and run isotype controls and antibody labelled controls to establish sort gates for CD34+ and CD38– cells.
6. Sort stained cells into CD34+ CD38– and CD34+ CD38+ fractions and collect cells in 15 ml centrifuge tubes containing 3 ml serum free media.

3.2 Transfection

The effects of overexpression of a CCN family member in hematopoietic cell lines can be studied using transient or stable overexpression systems. We describe using the full length sequence of CCN3 cloned into the pCB6 vector to transfect a chronic myeloid leukemia cell line called K562. Hematopoietic cells are notoriously difficult to transfect and we used Nucleofector® technology. Cells are transfected following the manufacturer's (Amaxa) instructions.

3.2.1 Generation of Cell Line Transiently Expressing CCN3

1. Prepare 12-well plate by filling appropriate number of wells with 1.5 ml culture medium and incubate in 5% CO^2 at 37 °C until required (*see* **Note 7**).
2. Cells to be transfected should be in exponential growth phase. Take 1×10^6 cells, centrifuge at 200 × *g* for 5 min, discard supernatant and resuspend in 100 µl Nucleofector™ Solution V at room temperature (*see* **Note 8**).
3. Add 5 µg control vector or vector containing CCN sequence and transfer to an Amaxa certified cuvette (*see* **Note 9**).
4. Transfection is carried out using program T-16

5. Remove the cuvette from the holder once the program is finished and add 500 µl pre-warmed culture medium.
6. Transfer the sample into the prepared 12-well plate (*see* **step 1** above)
7. Incubate the cells in 5 % CO^2 at 37 °C for 24 h before examining effects on cell function (*see* **Note 10**).

3.2.2 Generation of Cell Line Stably Expressing CCN3

1. Following transfection, cells are maintained in non-selective standard medium for 24 h before selection.
2. The pCB6 vector contains a G418 resistance gene to allow selection of positively transfected cells. Cells are transferred into fresh medium containing 800 µg/ml G418 for selection (*see* **Note 11**).
3. Medium is changed every 2 days for up to 2 weeks to eliminate dead cells and debris.
4. Viable transfected cells are counted and diluted to a density of 50 cells per 10 ml in selective medium (*see* **Note 12**).
5. Diluted samples (100 µl) are pipetted into each well of a 96-well round bottom plate. Ten plates are performed for each transfection and maintained in a humidified atmosphere with 5 % CO^2 at 37 °C (*see* **Note 13**).
6. After approximately 10 days 100 µl of fresh selection medium is added to maintain the G418 selection.
7. Following 3 weeks incubation the medium in certain wells appears yellow and large numbers of cells are visible under the microscope. These wells are marked, transferred to a 24-well plate and allowed to grow on in selective medium.
8. Potential stable cell lines derived from a single cell are screened and their identity confirmed using RQ-PCR and Western blotting.
9. Aliquots of cells which are confirmed as stably transfected are frozen for future studies.

3.3 Functional Assays to Determine Response to CCN3

In this section we describe in vitro assays to measure the frequency of hematopoietic progenitor cells (granulocyte and macrophage colony forming units—CFU-GM) and stem cells (long term culture-initiating cell—LTC-IC). CFU-GM is an in vitro assay to assess the ability of hematopoietic progenitor cells to proliferate and differentiate into colonies when grown in a semisolid culture system that mimics the in vivo bone marrow environment. LTC-IC assays are used to quantify primitive hematopoietic progenitors or stem cells. Quantification of stem cells or analysis of stem cell function in the LTC-IC assay requires culturing hematopoietic cells on a supportive feeder layer of stromal cells for ≥5 weeks during which time committed hematopoietic progenitors will mature. More primitive

hematopoietic progenitors can then be quantified using a CFU assay. Both assays provide a robust method to screen for the inhibitory or stimulatory effects of CCN3 proteins on hematopoietic cells.

3.3.1 CFU-GM Assay

1. Thaw Methocult™ medium at room temperature or overnight at 4 °C, shake bottle and let stand for 5 min to allow air bubbles to rise to the top. Once thawed the medium can be used immediately or stored at 4 °C for up to 1 month.
2. For each triplicate culture condition dispense 4 ml Methocult™ into a tube using a 16-G blunt end needle with a Luer lock syringe. To avoid bubbles take up excess medium into the syringe so you do not need to expel all medium in the syringe when aliquoting (*see* **Note 14**).
3. Separate mononuclear cells by density gradient as described in Subheading 3.1, perform a cell count and dilute cells in 0.4 ml IMDM with 2% FCS to ten times the final concentration required dependent on cell type as outlined in Table 1. Add required concentration of recombinant CCN protein to the cell mixture.
4. Add 0.4 ml of diluted cells to tube containing 4 ml Methocult™ medium, vortex tube to mix contents thoroughly and let stand for 5 min to allow air bubbles to rise.
5. Dispense 1.1 ml of the Methocult™–cell mixture into triplicate 35 mm culture dishes using a 16-G needle attached to a Luer lock syringe. As before draw excess medium into the syringe and do not expel all medium when dispensing. Ensure even coverage of the medium across the surface of the culture dish by gently swirling and rotating the dish.
6. Place the 35 mm dishes in a 100 mm petri dish or a tray with a lid. Also include an uncovered 35 mm dish containing 3 ml of sterile water to provide humidity. Incubate at 37 °C with 5% CO_2 in a humidified atmosphere for 7–14 days.
7. At the end of the culture period count all colonies in each dish.CFU-GM produce colonies containing at least 20 cells. CFU-G, CFU-M or CFU-GM colony descriptions can be found in the Methocult™ product manual at www.stemcell.com.

Table 1
Cell numbers required for clonogenic CFU-GM assays

Cell source	Cells per culture (35 mm dish)
Bone marrow mononuclear cells	$1–5 \times 10^4$
Peripheral blood mononuclear cells	$1–2 \times 10^5$
CD34+ enriched mononuclear cells	500

3.3.2 Preparation of Irradiated M2-10B4 Feeder Layers for LTC-IC Assay

1. Collagen coat 35 mm culture dishes by evenly spreading 1 ml collagen solution over the surface of the dish for 1–2 min. Remove excess collagen (*see* Subheading 3.2) and air-dry dish at room temperature for a minimum of 1 h and rinse with sterile PBS before adding M2-10B4 cells (*see* **Note 15**).
2. Trypsinize M2-10B4 cells and wash twice with RPMI 1640 culture medium containing 2 % FCS.
3. Resuspend cells in 1–2 ml HLTM with hydrocortisone and count. Ensure there are sufficient cells for required experiment and, using an X-ray or γ-irradiation source, irradiate cells with 8000 cGy.
4. Plate M2-10B cells at 3×10^5 cells in 2.5 ml per collagen-coated 35 mm culture dish and incubate at 37 °C in 5 % CO2 in a humidified incubator. Incubate irradiated feeder cultures for a minimum of 24 h prior to the addition of test cells (*see* **Note 16**).

3.3.3 LTC-IC Assay

1. Isolate mononuclear cells from bone marrow or peripheral blood by density gradient as described in Subheading 3.1. LTC-IC assays can be performed using mononuclear cells from bone marrow or peripheral blood, or using CD34+ enriched mononuclear cells.
2. Culture mononuclear cells for 24 h in SFM + GF under the required test conditions (e.g., control verses coculture with recombinant CCN protein of interest). Harvest cells and resuspend in 2.5 ml HLTM with hydrocortisone at concentration required for cell source, as outlined in Table 2.
3. Carefully remove medium from the 35 mm culture dishes containing irradiated M2-10B4 cells and replace with HLTM medium containing test cells, taking care not to disturb the adherent layer. Place the 35 mm culture dishes within a 100 mm petri dish or covered tray containing an additional uncovered 35 mm dish containing 3 ml sterile water to maintain humidity and incubate at 37 °C in 5 % CO_2 for 5 weeks.
4. Perform weekly half medium changes by gently swirling the culture dishes. Carefully remove and discard 1 ml of culture

Table 2
Cell numbers required for LTC-IC assays

Cell source	Cells per culture (35 mm dish)
Bone marrow mononuclear cells	1×10^6
Peripheral blood mononuclear cells	8×10^6
CD34 + enriched mononuclear cells	3000–5000

medium and replace with 1 ml of freshly prepared HLTM, taking care not to disturb the adherent layer. After 5 weeks incubation harvest all cells from 35 mm culture dishes.

3.3.4 Harvest of LTC-IC Cultures

1. Pipette non-adherent cells and medium from 35 mm culture dish into a sterile 15 ml centrifuge tube. Rinse the adherent layer of cells twice with 1 ml sterile HBSS and add to 15 ml centrifuge tube.
2. Add 1 ml of trypsin and incubate at 37 °C for a maximum of 10 min, checking cultures at regular intervals using an inverted microscope for detachment of adherent cells. Once adherent layer has begun to detach add 0.2 ml FCS to neutralize the trypsin.
3. Repeatedly pipette the trypsin/FCS solution over the surface of the 35 mm culture dish to ensure all cells are detached and in a single-cell suspension and transfer to the 15 ml tube containing the non-adherent cells.
4. Rinse the 35 mm culture dish twice with IMDM with 2% FCS and add cells and medium to the 15 ml tube, centrifuge cells at $300 \times g$ for 10 min, resuspend in 500 µl IMDM with 2% FCS and perform a cell count. Dilute cells to $2–5 \times 10^5$ cells in 0.4 ml IMDM with 2% FCS, add to tube containing 4 ml Methocult™ H4435 medium and plate in triplicate as described previously for the CFU-GM assays. Score colonies after 14 days in culture.

4 Notes

1. Dilution of the blood samples gives a better yield, however if you wish to use the plasma for downstream analysis the sample can be processed undiluted. We normally collect bone marrow aspirate in heparinized tissue culture medium; equivalent volumes of bone marrow would then be used in the separation procedure.
2. Good separation is dependent on the height of the Ficoll-Paque PLUS (2.4 cm) and layered blood sample (3 cm). Larger volumes of blood can be separated in a 50 ml centrifuge tube with 12 ml Ficoll-Paque PLUS and 18 ml blood.
3. Freezing and thawing of mononuclear cells. Freeze mononuclear cells at a concentration of $1–10 \times 10^7$ cells per ml in a cryopreservation medium containing 10% DMSO, 40% FCS and 50% RPMI 1640 or IMDM. Thaw cells quickly, transfer to a 50 ml centrifuge tube and add 15–20 ml medium dropwise, while swirling the tube. Centrifuge at $300 \times g$ for 10 min, remove supernatant, resuspend cells in 1 ml medium containing 100 µg DNase I to stop clumping and incubate at room temperature for 15 min. Wash in 15 ml medium, perform viable cell count, and continue with CD34+ cell enrichment.

4. Separation buffer (PBS + EDTA + BSA) must be prepared fresh on the day of use. The following volumes are required depending on type of columns used:

   MS columns—10 mls per sample.

   LS columns—25 ml per sample.

   AutoMACS Pro Separator—500 ml for up to four samples.

5. There are a range of different separators available for manual cell separation, details of which can be found on the manufacturer's website (http://www.miltenyibiotec.com). The following are suitable for use with MS/LS columns:

   MS columns—MiniMACS, OCtoMACS, VarioMACS, SuperMACS II.

   LS columns—MidiMACS, QuadroMACS, VarioMACS, SuperMACS II.

6. Ensure you have enough cells to sort to have sufficient cells for downstream application. CD34+ CD38– enriched populations will constitute approximately 5 % of the total CD34+ cell population and therefore if you are aiming to have 1×10^6 CD34+ CD38– enriched cells you will need to sort at least 2×10^7 stained cells.

7. Use whichever tissue culture medium you normally grow your cells in. We use RPMI-10 % FCS.

8. We found $1x10^6$ cells to be optimal. Using lower or higher cell numbers may lead to lower transfection efficiency. Do not leave the cells in the Nucleofector solution for more than 15 min as it can affect cell viability and transfection efficiency.

9. Changes may be seen earlier with different CCN constructs and cell lines but we found 24 h to be optimal for this system.

10. A sample including 2 μg pmaxGFP® vector should also be included to check transfection efficiency; the green fluorescent protein acts as a positive control. Make sure the sample covers the bottom of the cuvette without any air bubbles.

11. Each cell line has varied sensitivity to G418. The optimal concentration for selection was determined by plotting survival curves over 15 days. The lowest concentration that begins to kill significant numbers of cells in 3 days and kills all untransfected cells within 2 weeks was chosen.

12. The cells of the un-transfected controls were examined by trypan blue exclusion to ensure they were all dead.

13. This should theoretically position a single cell in each well. This enhances the potential for the resulting cell colonies to be the progeny of a single clone.

14. Remove excess collagen from 35 mm culture dishes using a sterile pipette, this excess collagen can then be reused to coat additional dishes.

15. Collagen-coated dishes can be used immediately or wrapped in Parafilm and stored at 4 °C for up to 2 weeks.
16. Irradiated M2-10B4 feeder cultures can be incubated at 37 °C in 5 % CO_2 in a humidified atmosphere for up to 10 days before adding test cells. If the time before use exceeds 7 days, the HTLM with hydrocortisone should be changed.

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Chapter 25

In Vivo Evaluation of Cartilage Regenerative Effects of CCN2 Protein

Takashi Nishida, Satoshi Kubota, and Masaharu Takigawa

Abstract

CCN family protein 2/connective tissue growth factor (CCN2/CTGF) is a unique growth factor that promotes the proliferation and differentiation, but not the hypertrophy of articular chondrocytes in vitro. Based on these findings, we previously evaluated the cartilage-regenerative effects of recombinant CCN2 protein (rCCN2) by using both mono-iodoacetate (MIA) injection into the rat joint cavity and formation of full-thickness defects of rat articular cartilage in vivo, and our results suggested the utility of CCN2 in the regeneration of articular cartilage. This chapter entails helpful tips to apply these two animal models for the evaluation of cartilage-regenerative effects of CCN2 or its derivatives.

Key words Recombinant CCN2 protein (rCCN2), Articular cartilage, Regeneration and repair, Gelatin hydrogels, Collagen sponge, Mono-iodoacetic acid (MIA), Full-thickness defects, Micro drill

1 Introduction

Articular cartilage is a hyaline cartilage that covers the heads of bones, and its most important function is to provide a frictionless and pain-free movement of the joint throughout life [1]. Articular cartilage is composed of a large amount of extracellular matrix (ECM) without blood vessels and nerves and a sparse distribution of highly differentiated cells [1]. Because blood vessels required in tissue regeneration are not present in articular cartilage and the cells therein do not have adequate ability to proliferate and migrate due to the large amount of ECM, when articular cartilage is injured, the capacity for intrinsic healing and repair is limited [2, 3]. Therefore, present treatments for damaged articular cartilage are not sufficient to repair it. To overcome these issues, novel regenerative strategies are desirable for the restoration of damaged articular cartilage. Recently, it has been reported that autologous chondrocyte implantation, stem cell transplantation, and osteochondral autograft or allograft transplantation are clinically useful because these cells can proliferate in culture without losing their

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ability to form bone or cartilage [4, 5]. However, although recent biochemical studies have shown that bone marrow stromal cells or cultured chondrocytes may be useful for cartilage healing, more information is needed regarding the variety of growth factors produced by differentiated chondrocytes and how these factors regulate chondrogenic differentiation, in order to move such research toward clinical application [6].

Previously we found that recombinant CCN family protein 2/connective tissue growth factor (CCN2/CTGF) promotes the proliferation and differentiation of articular cartilage cells but has no effect on the terminal hypertrophy or calcification of these cells [7]. These findings indicate that this growth factor plays an important role in the expression and maintenance of the specific phenotypes of these cells. Therefore, we focused on CCN2 functions in articular cartilage repair and examined the possibility of therapeutic application of rCCN2 for the treatment of damaged articular cartilage.

In this chapter, we describe two strategies used in our laboratory to investigate the regenerative effect of rCCN2 on articular cartilage in vivo [8]. As the first strategy, we injected monoiodoacetate (MIA) into the joint cavities of rats to produce experimental osteoarthritis (OA)-like lesions like those characteristic of human OA [8, 9]. MIA is an inhibitor of glycolysis, and intra-articular injection of MIA induces the loss of proteoglycans and results in chondrocyte death. Using this model, we investigated whether the injection of rCCN2 into the joint cavities of such rats would stimulate articular cartilage repair, or not. Our findings clarified that the histological appearance of OA-like lesions was lessened or reversed toward normal by the injection of rCCN2 incorporated into the gelatin hydrogel [8]. Next, in order to clarify the direct effect of rCCN2 on the repair of articular cartilage, as a second strategy we investigated whether or not full-thickness defects made in rat articular cartilage could be refilled with hyaline cartilage-like tissue after the implantation of a collagen sponge bearing rCCN2 incorporated into a gelatin hydrogel [8, 10]. As a result, we clarified that such full-thickness defects are filled with hyaline-like cartilage tissues in response to the implantation of a collagen sponge containing rCCN2 incorporated into the gelatin hydrogel [8]. Furthermore, using these two methods, we recently also evaluated the cartilage regenerative-effect of each of the independent modules of CCN2 protein [11]. Based on these experiences, in this chapter we describe how to evaluate the cartilage-regenerative effects of CCN2 protein [8] and its fragments [11]. These methods might be applied for evaluation of other CCN proteins, as well.

2 Materials

2.1 General

1. Recombinant *CCN2* (rCCN2) stock solution: purify rCCN2 produced in mammalian cells transfected with *CCN2*-expression vector and store 100 μg/mL of rCCN2 at −80 °C (*see* **Chapter 10**).
2. Gelatin hydrogel microspheres (gelatin hydrogels): 95% of water content is used for these experiments (*see* **Note 1**).

2.2 Tissue Preparation

1. 0.2 M phosphate buffer (pH 7.4): Weigh 57.4 g of disodium hydrogen phosphate 12-water ($Na_2HPO_4 \cdot 12H_2O$) and weigh 5.96 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$). Mix to dissolve with distilled water to a volume of 1-L. Store it at room temperature.
2. 10% neutral buffered formalin: Weigh 20 g of paraformaldehyde and add distilled water to a volume of 200 mL. Mix the solution and heat it at 70–80 °C. Then, add several drops of 1 M NaOH and mix the solution until it becomes clear. After cooling down on ice, add 0.2 M phosphate buffer at a final concentration of 0.1 M and distilled water to a volume of 500 mL.
3. 10% EDTA: Weigh 100 g of Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and 12.1 g of trisaminomethane, and add distilled water to a volume of 800 mL. Mix it and adjust pH to 7.4 with 1 M KOH.
4. Xylene.
5. Ethanol for dehydration: Dilute Ethanol with distilled water at 70, 80, and 90%.
6. Microtome.

2.3 MIA Injection into Femorotibial Joint

1. Mono-iodoacetate (MIA) solution: Weigh 60 mg of MIA powder, and add phosphate buffered saline (PBS) to a volume of 1 mL. Sterilize the solution by filtration.
2. Diethyl ether: For anesthesia, diethyl ether is used (*see* **Note 2**).
3. Wistar rats (8 weeks-old).

2.4 Making of Full-Thickness Defects in Rat Articular Cartilage

1. Nembutal sodium solution: This solution is one of the most common injectable anesthetic agents used in rodents, and contains 50 mg/mL pentobarbital sodium. For anesthesia via intraperitoneal injection. Nembutal solution is diluted by ten times with normal saline solution (*see* **Note 3**).
2. Collagen sponge: Collagen solution for tissue culturing (e.g., Atelocollagen, Koken Co.) or an equivalent. Collagen sponge is prepared by lyophilization of collagen solution.
3. Retired Wistar rats (32 weeks old) (*see* **Note 4**).
4. Micro drill: Micromotor dental drill.

3 Methods

3.1 Preparation of rCCN2-Incorporated Gelatin Hydrogel

1. Weigh 1 mg of freeze-dried gelatin hydrogel and drop 10 μL of PBS containing 0.5, 1.0, 1.5 mg of rCCN2 onto it (*see* **Note 5**). Control gelatin hydrogels are prepared by using 10 μL of PBS instead of the rCCN2.
2. Leave the rCCN2 solution on the gelatin hydrogel for 1 h at room temperature.
3. After incubation, add 100 μL of PBS or collagen solution.
4. After incubation for 1 h, soak the rCCN2 and gelatin hydrogel mixture in PBS for 24 h. Perform Western blot analysis using the supernatant and the pellet of the mixture to confirm the interaction of rCCN2 with the gelatin hydrogel.

3.2 Effect of rCCN2-Gelatin Hydrogels on MIA-Induced OA-Like Lesions

MIA is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, and therefore MIA inhibits glycolysis. Previously we showed that inhibition of glycolysis by sodium fluoride (NaF) induces chondrocyte death [12]. These findings suggest that intra-articular injection of MIA would cause chondrocyte degeneration. In fact, histological analysis after injection of MIA revealed chondrocyte necrosis, cluster formation, fibrillation, and loss of stainable proteoglycan matrix in articular cartilage lesions. These histological appearances mimic those of human OA, and this model offers a rapid and minimally invasive method to induce OA-like lesions in a rodent species.

3.2.1 Induction of OA by MIA and Tissue Preparation

1. Anesthetize Wistar rats (8 weeks-old) with diethyl ether (*see* **Note 6**).
2. Inject the rats with MIA at a dose of 6 mg (100 μL of MIA solution) once into the right knee joint (*see* **Note 7**; Fig. 1a).
3. The left knee joint is injected with PBS as a control (*see* **Note 8**; Fig. 1a).
4. At 14 days after the injection of MIA, euthanize the rats and dissect the whole knee joints and further fix them with 10% neutral buffered formalin overnight at 4 °C.
5. Wash the joints with PBS twice, and decalcify them with 10% EDTA at room temperature for 2 weeks.
6. Dehydrate the joint tissues with ethanol followed by xylene. Then, embed the tissues in paraffin.
7. Cut the paraffin-embedded tissue blocks on a microtome at a thickness of 5 μm and mount the sections on silane-coated slides.
8. For histological evaluation of these sections, stain them with toluidine blue (*see* Fig. 1b).

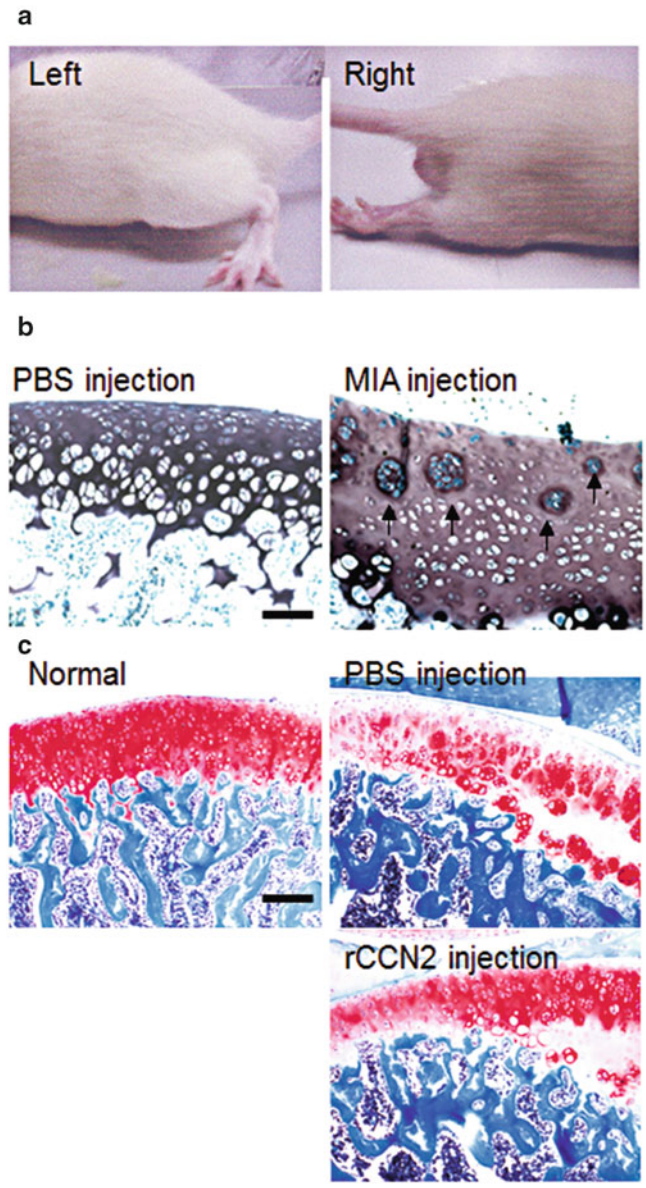

Fig. 1 Effect of gelatin hydrogels incorporating rCCN2 on the repair of experimental OA induced by MIA. (**a**) Photographs of rat left and right hind legs. MIA is injected into the right knee joint; and PBS, into the left one. The rat can still use the left hind leg but not the right leg. (**b**) Histological analysis of toluidine blue-stained articular cartilage from rats injected with PBS or MIA. After injection with MIA, reduced toluidine blue staining is observed, and clusters of chondrocytes are evident in the articular cartilage (*arrows*). The bar represents 250 μm. (**c**) Histological findings of MIA-induced OA-like lesions after injection of rCCN2-containing gelatin hydrogels. These sections were stained with safranin O–fast green. Although histological findings of OA-like lesions are observed after injection of PBS-containing gelatin hydrogels, histological findings after injection of rCCN2 containing ones are similar to those for normal articular cartilage. The bar represents 250 μm

3.2.2 Evaluation of the Effect of rCCN2-Gelatin Hydrogel on MIA-Induced Articular Cartilage Degradation

1. Induce experimental OA in the right knee joints of Wistar rats by injecting the joints with MIA, as described in Subheading 3.2.1.
2. At 14 days after the injection, anesthetize the rats with diethyl ether and inject their right knee joints with a 100 μL volume of rCCN2-gelatin hydrogel soaked in PBS. As a control, inject the left knee joint with PBS-incorporated gelatin hydrogel. At 7 days after the injection, euthanize the rats and dissect the whole knee joints and further fix them with 10% neutral buffered formalin overnight at 4 °C.
3. Prepare tissue sections as described in Subheading 3.2.1 (*see* Fig. 1c).
4. The sections from each animal are examined and scored by reference to a histological scale. The cell morphology is graded from 0 (for tissues equivalent to the normal cartilage) to 3 points (for the presence of dead chondrocytes). Matrix staining is graded from 0 (for tissues with metachromatic staining comparable to that of normal cartilage) to 3 points (for those without metachromatic staining). The two histological scores are added together, resulting in a score ranging from 0 to 6 points (*see* **Note 9**).

3.3 Effect of rCCN2-Gelatin Hydrogels on the Repair of Full-Thickness Defects

It is believed that full-thickness defects of articular cartilage undergo regeneration more easily than partial-thickness ones. This reason is thought that bone marrow-derived stromal cells supplied from the bone marrow differentiate into chondrocytes when stimulated by several growth factors. However, in the rat knee joint, it was reported that an articular cartilage defect of more than 1.5 mm in diameter can not be repaired by hyaline cartilage spontaneously [13]. Therefore, for evaluation of the effect of rCCN2-gelatin hydrogel on the repair of full-thickness defects on the femoral patellar groove, the defects (2-mm diameter and 2-mm depth) were created by using a dental round bar (2 mm diameter) and micro-motor dental drill.

1. Anesthetize retired Wistar rats (32 weeks-old) with an intraperitoneal injection of Nembutal sodium solution given at dose of 2.7 mg/kg body weight (*see* **Note 10**).
2. Dissect the skin with scalpel to expose the capsula.
3. Dissect the capsula and dislocate the patella laterally to see the femoral patellar groove.
4. Prepare a defect of 2-mm diameter on the patellar groove in the femur with the micro drill (*see* **Note 11**; Fig. 2a).
5. Implant a collagen sponge containing 1 μg of rCCN2-gelatin hydrogel into the defect (*see* **Note 12**).
6. Relocate the patella and close the capsula and skin with a nylon suture.

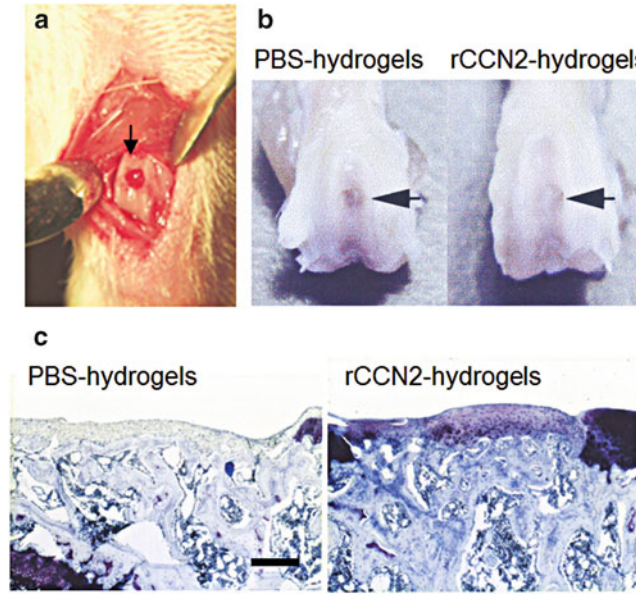

Fig. 2 Effect of collagen sponge containing rCCN2-bearing gelatin hydrogel on the repair of full-thickness defects formed in retired rats. (**a**) Photographs of full-thickness defect on femoral patellar groove (*arrow*). The defect of 2-mm in diameter is formed by using a micro drill. Evidence of bleeding is observed. (**b**) At 4 weeks after the operation, the femurs are collected and fixed with 10 % neutral buffered formalin. These photographs indicate the femurs treated with PBS- or rCCN2-containing gelatin hydrogel-collagen sponge. *Arrows* indicate the area of the treated full-thickness defect. Although the defect remains after PBS-gelatin hydrogel treatment, no defect is recognized after the treatment with the rCCN2-gelatin hydrogel. (**c**) Histological findings of the full-thickness defect after treatment with PBS- or rCCN2-hydrogels at 4 weeks after the operation. These sections were stained with toluidine blue. The full-thickness defect treated with the rCCN2-gelatin hydrogel is filled by hyaline cartilage-like tissue, but the PBS-treated defect remains filled with soft tissue. The bar represents 250 μm

7. At 4 weeks postoperatively, euthanize the rats and dissect the whole knee joints and then fix the femurs in 10 % neutral buffered formalin overnight at 4 °C (*see* Fig. 2b).
8. Prepare tissue sections as described in Subheading 3.2.1.
9. To evaluate the effect of rCCN2-gelatin hydrogel in the collagen sponge on the repair of the defect, examine the site of the defect on the sections by light microscope (*see* Fig. 2c).

4 Notes

1. Gelatin hydrogels were kindly provided by Dr. Tabata, Y. (Institute for Frontier Medical Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan). Gelatin hydrogel

interacts with several growth factors electrostatically, and the gel is degraded in vivo. The degradation period of the hydrogel depends on its water content, and gelatin hydrogels with a higher water content are degraded more quickly than those with a lower water content. Therefore, the release period of growth factors that have interacted with the gelatin hydrogel can be controlled by changing the water content of the hydrogel. Because it was reported that gelatin hydrogels with a water content of 94% are degraded completely by 20 days after implantation into the subcutaneous tissues of mice, it is thought that gelatin hydrogels with a water content of 95% would be degraded by less than 20 days after injection into an articular joint [14, 15]. Recently, this material has become available from MedGEL, Co. (Kyoto, Japan). We use gelatin hydrogels with an isoelectric point of 5 for CCN2.

2. Although diethyl ether is cheap and is widely used as an inhalation anesthetic for rodents, it is difficult to control the level of anesthesia. Take care that the level of anesthesia is not too deep so that the experimental animals do not die.
3. At the present, Nembutal sodium solution is not available. Instead of Nembutal solution, we use Somnopentyl solution (Kyoritsu Seiyaku Co., Tokyo, Japan). This injectable anesthetic solution contains 64.8 mg/mL pentobarbital sodium.
4. We used retired rats that are bigger than 8-week-old rats as it is easier to form the defects on their patellar groove. In addition, we also investigated the effect of rCCN2 on the repair of full-thickness defects made in aged rats.
5. For incorporation of rCCN2 into gelatin hydrogels, the relationship between the volume of rCCN2 solution and the weight of the gelatin hydrogel microspheres is important. Weigh 1 mg of gelatin hydrogels and soak them in 10 μL of rCCN2 solution. If 2 mg of gelatin hydrogel is desired, soak them in 20 μL of the rCCN2 solution. The concentration of the rCCN2 solution does not affect the incorporation of rCCN2 into the gelatin hydrogel. Moreover, to investigate the effect of CCN2 fragments or other CCN proteins, it is important to evaluate retention time of the proteins in the gelatin hydrogels [11, 14]. Depending on the isoelectric point of the proteins being investigated, gelatin hydrogels with a different isoeclectric point must be selected.
6. Clear glass or plastic jars or chambers are often used to anesthetize rodents. Animals should not come into direct contact with the source of the anesthetic agent. They should be exposed only to the vapors.
7. After anesthesia, the knee joint is bent to confirm the edges of both patella and tibiae by palpation. The space between the patella and tibiae contains the intra-patellar ligament of the

knee. After disinfection with 70% ethanol, inject MIA solution via a 27-G needle through the intra-patellar ligament into the joint space of the knee. Take care not to allow the MIA solution to leak from the joint space while inserting or withdrawing the needle. In addition, take care not to injure the articular cartilage by the needle insertion.

8. When we injected MIA solution into both right and left knee joints, the rat immediately died. Therefore, do not inject MIA solution in both knee joints.
9. These histological scales are composed of cell morphology and matrix staining with toluidine blue, which is modification of that described by Wakitani et al. [4, 5, 8].
10. After brief inhalation anesthesia (diethyl ether), inject retired rats with Nembutal solution into abdominal cavity. The injection site of the anesthetic agent is at the lower-right quadrant of the abdomen.
11. It was previously reported that cartilaginous repair responses fail to occur in the full-thickness defects of the larger 1.5 mm in diameter [13]. Therefore, we think that our experimental method is not suited to evaluate spontaneous cartilage repair. However, this method is suitable for evaluation of regeneration of articular cartilage stimulated by a growth factor and biomaterials. The depth of full-thickness defects on the femoral patellar groove is decided based on the diameter size of the dental bar, and bleeding from the bone marrow is also recognized. Furthermore, the articular cartilage must continue to be cooled with normal saline while the defects are prepared with the micro drill. Otherwise, articular cartilage cells become necrotic due to the heating.
12. Because a collagen solution changes to sponge-shaped collagen by lyophilization, this sponge-shaped collagen containing rCCN2-incorporated gelatin hydrogel can be inserted into the defect made in the articular cartilage. In addition, relocation of the patella over the femoral groove prevents the collagen sponge containing the rCCN2-gelatin hydrogel from becoming dislodged from the defect.

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Chapter 26

Gene Expression Analysis of CCN Protein in Bone Under Mechanical Stress

Teruko Takano-Yamamoto, Tomohiro Fukunaga, and Nobuo Takeshita

Abstract

To investigate mechanical-dependent bone remodeling, we had previously applied various types of mechanical loading onto the teeth of rats and mice. In vitro cultured bone cells were then used to elucidate the mechanisms underlying the specific phenomenon revealed by in vivo experiments. This review describes the techniques used to upregulate CCN2 expression in bone cells produced by different types of mechanical stress, such as fluid shear stress and substrate strain in vitro, and compression or tension force in vivo.

1 Introduction

Cells in all organisms have conserved mechanosensory mechanisms. Essentially, all forms of life have the capacity to adapt to physical signals [1–4]. Physical forces regulate physiological processes to maintain homeostasis and repair subcellular, cellular, tissue, and organ systems. Cells such as vascular endothelial cells [5], myocytes [6], cardiomyocytes [7], periodontal ligament cells [8, 9], fibroblasts [10], chondrocytes [11, 12], and bone cells [13, 14], are thought to have mechanosensitive properties and respond to various mechanical forces to induce proliferation [15, 16] and differentiation [16, 17].

Bone is a dynamic organ consistently subjected to mechanical loading during physical activities such as walking or exercise, and has the capacity to adapt structurally in response to these mechanical loads [18–20]. The structural integrity of skeletal tissue is maintained by formation/resorption and the remodeling cycle in response to the mechanical environment [21, 22]. Remodeling of bone is regulated by bone-forming osteoblasts, bone-resorbing osteoclasts, and their progenitors [23]. We have previously reported that osteocytes, which are terminally differentiated osteoblasts, act as mechanosensory cells during the early stages of bone remodeling [24]. Furthermore, osteopontin (OPN) is transiently produced by osteocytes in response

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to mechanical compression, and is an important factor that triggers osteoclastic bone resorption during experimental tooth movement [24]. These findings support that OPN and other extracellular matrix (ECM) molecules play essential roles in skeletal tissue structure and mediate crucial autocrine and paracrine functions in the regulation of tissue formation and remodeling [25].

A subset of ECM proteins, known as matricellular proteins, include the CCN family of proteins which are dynamically expressed and serve primarily regulatory rather than structural roles [26, 27]. Similar to OPN, CCN family proteins are involved in bone remodeling, wound healing, inflammation, and injury repair [26]. For example, CCN2/CTGF is a 38 kDa, cysteine-rich ECM protein, involved in different cellular events including angiogenesis, skeletogenesis, wound healing [27], adhesion, migration, proliferation, and differentiation [28–32]. The CCN2 gene is upregulated or downregulated in response to mechanical stress, such as hydrostatic pressure [33], stretching [34], compression [35], and shear stress [36], depending on the cell type.

To investigate mechanical-dependent bone remodeling, we applied various types of mechanical loading onto the teeth of rats and mice using elastic [24, 37, 38], NiTi wire spring [39], and coil spring [40–42]. Furthermore, cultured bone cells, including osteocytes, were used to elucidate the mechanisms underlying the specific phenomenon revealed by the in vivo experiments. We found that CCN2/CTGF mRNA expression was markedly increased in osteocytes, followed by an increase in osteoclastic bone resorption. This was especially observed on the pressured side of the alveolar bone [37, 39] and on the compressed calvarial suture in vivo [35]. Furthermore, CCN2/CTGF protein produced by the compression force was found to induce apoptosis, mediated through mitogen-activate protein kinases (MAPKs) in osteocytes in vitro [35]. Thus, changes in the expression levels of certain osteocyte genes during bone modeling and/or remodeling suggest that complex and interdependent signaling networks are likely involved in their response to mechanical loading.

This review describes the techniques used to upregulate CCN2 expression in bone cells, following stimulation by different types of mechanical stress, such as fluid shear stress or substrate strain in vitro, and compression [35] or tension force [37, 39, 43] in vivo.

2 Materials

2.1 Application of Mechanical Stress to Periodontal Ligament and Alveolar Bone

1. Male C57BL/6J mice.
2. Male Wistar rats.
3. Dental round steel bur in a diameter of 0.8 mm.

4. Dental laboratory micromotor handpiece.
5. Nickel-titanium closed coil spring (e.g., Tomy Inc., Fukushima, Japan).
6. 0.1 mm diameter stainless steel wire.
7. Howe utility pliers.
8. Silicone impression material.
9. Self-curing acrylic resin.
10. Nickel-titanium wire.
11. Composite resin for dental filling: Light-cured self-etch adhesive and light-cured nano hybrid flowable composite.
12. High strength dental stone.
13. Orthodontic elastic module.
14. Elastic separating pliers.
15. 0.2 M PB: Add about 200 mL of water to glass beaker. Weigh 1.4825 g of $NaH_2PO_4 2H_2O$ and 14.95 g of $Na_2HPO_4 12H_2O$, and transfer to the beaker. Mix and make up to 250 mL with water.
16. 4% PFA: 4% paraformaldehyde, 0.1 M PB, pH 7.4. Add about 100 mL of water to a glass beaker and heat it to about 60 °C. Weigh 20 g of paraformaldehyde and transfer to the beaker (*see* **Note 1**). Mix and add NaOH until the solution is clear (*see* **Note 2**). When it is dissolved, cool it. Add 250 mL of 0.2 M PB and make up to 500 mL with water (*see* **Note 3**).

2.2 Application of Mechanical Stress to Murine Sutures and Cranial Bones

1. Six-week-old male ICR mice.
2. Orthodontic wires (0.012-in. diameter nickel-titanium wire, 0.016-in. diameter beta-titanium wire).
3. Rabbit polyclonal antibody against CCN2/CTGF (PeproTech, 500-P252, Rocky Hill, NJ, USA) (*see* **Note 4**).
4. Rabbit IgG.
5. Dental drill with round bur (0.5 mm of diameter).
6. Surgical instruments such as scissors, forceps, and sutures with needle.
7. Dial tension gauge (e.g., Teclock, Nagano, Japan).

2.3 Mandibular Ramus Fracture Model

1. Male Wistar rats.
2. Straight iris scissors with tungsten carbide insert.
3. Surgical blade No. 11.
4. 0.2 M PB: *see* Subheading 2.1, **item 17**.
5. 4% PFA: *see* Subheading 2.1, **item 18**.

2.4 In Vitro Application of Tension or Compression Force

1. Murine long bone osteocyte Y4 (MLO-Y4) cells.
2. Flexible silicone membrane culture chamber (e.g., Stretch chamber, Strex Inc., Osaka, Japan).
3. Uniaxial cell stretching instrument (e.g., STREX Cell stretching system, Strex Inc.).
4. Fibronectin: Prepare 0.05 mg/mL of fibronectin with DPBS.
5. Dulbecco's Phosphate Buffered Saline (DPBS, 10×): 26.7 mM KCl, 14.7 mM KH_2PO_4, 1370 mM NaCl, 81 mM Na_2HPO_4.
6. α-minimal essential medium (α-MEM).
7. Fetal bovine serum (FBS).
8. Bovine serum (BS).
9. 10000 unit/mL penicillin and 10000 μg/mL streptomycin.

2.5 In Vitro Application of Compression Force

1. Loading culture device: We used our original culture device (Fig. 1) described in the previous report (*see* **Note 5**) [44].
2. Fifteen-day-old fetal chicken.
3. OB7.3 monoclonal antibody: This is kindly provided by Dr. J Klein-Nulend (ACTA University, Amsterdam, The Netherlands).
4. α-MEM.
5. FBS.

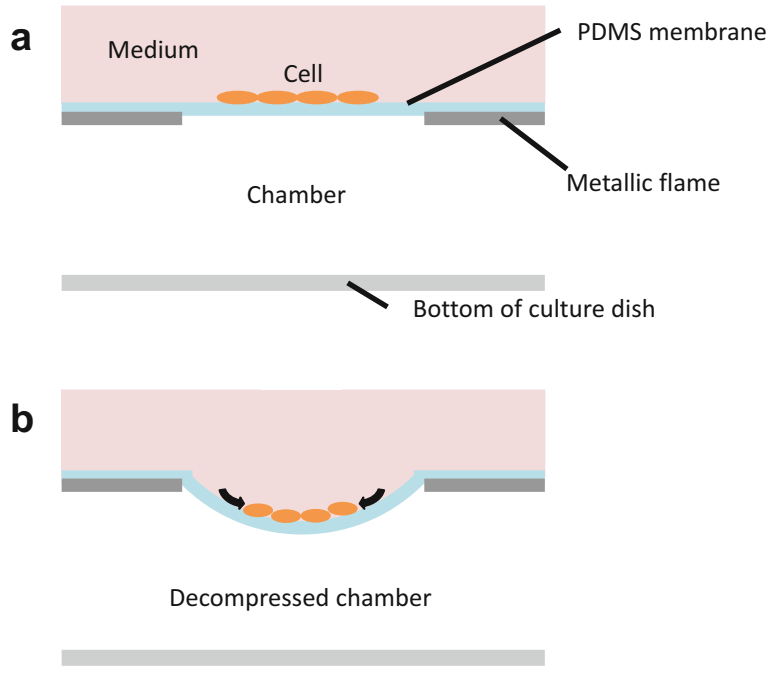

Fig. 1 Schematic representation of loading culture device before (**a**) and after (**b**) application of compression force. By decompressing a chamber under the culture wells at −18.7 kPa using an air pump, the PDMS membrane can be dented and compression force is loaded to cultured cells

2.6 In Vitro Application of Cyclic Tensile Force

1. Flexible silicone bottomed 6-well plate (e.g., BioFlex® plates, Flexcell® International, Burlington, NC, USA).
2. Computer-controlled vacuum-operated instrument (e.g., Flexcell® tension system, Flexcell® International).
3. Fibronectin: Prepare 0.05 mg/mL of fibronectin with DPBS.
4. DPBS (10×): *see* Subheading 2.4, **item 5**.
5. α-MEM.
6. FBS.
7. BS.
8. 10000 unit/mL penicillin and 10000 μg/mL streptomycin.

2.7 In Vitro Application of Fluid Shear Stress

1. MC3T3-E1: This is purchased from Riken Cell Bank (Tsukuba, Japan).
2. Flow meter (e.g., Alicat Scientific Inc., Tucson, AZ).
3. Flow chamber (e.g., Catch Burger; Nepagene, Chiba, Japan)
4. Flow apparatus (e.g., Micro Tube Pump; Icomes Lab, Iwate, Japan).
5. Poly-D-lysine.
6. Fibronectin.
7. α-MEM.
8. FBS.
9. Slide glass.

2.8 Immunohistochemistry Components

1. Xylene.
2. Ethanol: ethyl alcohol.
3. PBS (10×): 1370 mM NaCl, 81 mM Na_2HPO_4, 26.8 mM KCl, 14.7 mM KH_2PO_4, pH 7.4.
4. Hydrogen peroxide solution: 30% solution in water.
5. Methanol: methyl alcohol.
6. Blocking solution: 3% goat serum in PBS. Store at 4 °C.
7. Immunoreaction enhancer solution (e.g., Can Get Signal® immunostain Immunoreaction Enhancer Solution, Toyobo Co., Osaka, Japan).
8. Peroxidase-conjugated secondary antibody (e.g., Histofine® Simple Stain™ Rat MAX PO (R) or Mouse MAX PO (R), Nichirei Biosciences Inc., Tokyo, Japan).
9. 3,3-diaminobenzidine tetrahydrochloride (DAB) containing 0.02% hydrogen peroxide (e.g., Histofine® DAB Substrate kit, Nichirei Biosciences Inc.).
10. Mounting medium for microscopy (e.g., Entellan® new, Merck, Darmstadt, Germany).

2.9 In Situ Hybridization Components

1. Xylene.
2. Ethanol: ethyl alcohol.
3. PBS (10×): *see* Subheading 2.8, **item 3**.
4. Proteinase K in PBS: Dilute proteinase K (e.g., Roche, Mannheim, Germany) to a concentration of 2 μg/mL with PBS.
5. 4% PFA in PBS: Add about 35 mL of water to 50 mL centrifuge tube. Add 12.5 μL of 8 N NaOH. Weigh 2 g paraformaldehyde and transfer to the centrifuge tube. Heat to about 60 °C and vortex. When dissolved, add 5 mL of PBS (10×). Make up to 50 mL with water.
6. 5 N HCl
7. 20× SSC: 3 M NaCl, 0.3 M sodium Citrate.
8. Formamide.
9. Hybridization solution: Mix 25 mL of formamide, 0.5 mL of 1 M Tris–HCl (pH 7.5), 1 mL of tRNA (10 mg/mL), 1 mL of 50× Denhardt's solution, 5 mL of 50% dextran sulfate sodium, 6 mL of 5 M NaCl, 1.25 mL of 10% SDS, 0.1 mL of 0.5 M EDTA (pH 8.0), and 10.95 mL of water. Make aliquots and store at −20 °C.
10. Moisture chamber.
11. Plastic paraffin film (e.g., Parafilm M® film, Bemis flexible packaging, Neenah, WI, USA).
12. TNE: Mix 5 mL of 1 M Tris–HCl (pH 7.5), 50 mL of 5 M NaCl, 1 mL of 0.5 M EDTA (pH 8.0), and 444 mL of water.
13. RNase A.
14. DIG buffer 1: Mix 50 mL of 1 M Tris–HCl (pH 7.5), 15 mL of 5 M NaCl, and 435 mL of water.
15. Tween 20.
16. DIG buffer 3: Mix 15 mL of 1 M Tris–HCl (pH 9.5), 3 mL of 5 M NaCl, 7.5 mL of 1 M $MgCl_2$, and 124.5 mL of water.
17. Blocking solution: 1.5% blocking reagent (e.g., Blocking reagent, Roche) in DIG buffer 1.
18. Anti-digoxigenin antibody conjugated with alkaline phosphatase (e.g., Anti-Digoxigenin-AP, Fab fragments, Roche).
19. NBT/BCIP solution: Mix 20 μL of NBT/BCIP Stock Solution (e.g., Roche) and 1 mL of DIG buffer 3 just before adding to the sections.
20. Aqueous permanent mounting medium.

2.10 Real-Time Polymerase Chain Reaction (PCR) Components

1. Reagent for total RNA isolation (e.g., TRIzol Reagent, Invitrogen, Carlsbad, CA, USA).
2. PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) (*see* **Note 6**).

3. Premix reagent for real-time PCR (e.g., SYBR Premix Ex Taq II, Takara).
4. Primers.
5. 96-well plate for real-time PCR.
6. Thermal cycler

2.11 Western Blotting Components

1. Lysis buffer (e.g., Cell Lytic M Lysis Reagent, Sigma-Aldrich, St. Louis, MO, USA).
2. Proteinase inhibitor (e.g., Proteinase Inhibitor Cocktail, Sigma-Aldrich).
3. Phosphatase inhibitor (e.g., Phosphatase Inhibitor Cocktail, Sigma-Aldrich).
4. Protein assay kit (e.g., BCA Protein Assay Kit, Pierce, Rockford, IL, USA).
5. 12% polyacrylamide gel.
6. Polyvinylidene difluoride (PVDF) membrane.
7. Tris buffered saline (TBS) containing 3% bovine serum albumin (BSA): 10 mM Tris–HCl, 150 mM NaCl, 3% BSA, pH 7.4.
8. TBS containing 0.1% Tween 20.
9. Antibody against CCN2/CTGF (*see* **Note** 7).
10. Secondary antibodies conjugated with horseradish peroxidase.
11. Chemiluminescent substrate (e.g., SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA).
12. Semidry blotting apparatus.
13. Image analyzer.

2.12 Tartrate-Resistant Acid Phosphatase (TRAP) Staining Components

1. Xylene.
2. Ethanol: ethyl alcohol.
3. TRAP staining kit (e.g., Leukocyte acid phosphatase (TRAP) kit, Sigma-Aldrich, St. Louis, MO, USA). Naphthol AS-BI phosphoric acid solution: 12.5 mg/mL Naphthol AS-BI phosphoric acid. Fast garnet GBC base solution: 7.0 mg/mL Fast garnet GBC base, 0.4 M hydrochloric acid. Acetate solution: 2.5 M acetate buffer, pH 5.2. Tartrate solution: 0.335 M L(+)-Tartrate buffer, pH 4.9. Sodium nitrite solution: 0.1 M sodium nitrite.
4. Moisture chamber.
5. Aqueous permanent mounting medium.

2.13 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

1. Kit for detection of apoptosis (e.g., In Situ Cell Death Detection Kit Fluorescein, Roche, Mannheim, Germany).
2. 4,6-diamidine-2-phenylindole, dihydrochloride (DAPI).

3. Fluorescence microscope.
4. Charge-coupled device camera.
5. Software for image analysis (e.g., Image-Pro Plus, MediaCybernetics, Bethesda, MD, USA).

3 Methods

3.1 Application of Mechanical Stress to Periodontal Ligament and Alveolar Bone

3.1.1 Experimental Tooth Movement by Coil Spring

Move maxillary first molar of mice or rats mesially with continuous light orthodontic force [40–42] (*see* **Note 8**).

1. Anesthetize the mice or rats with an intraperitoneal injection.
2. Drill a hole with a diameter 0.8 mm in the alveolar bone in front of both maxillary incisors using a dental micromotor handpiece.
3. Tie a one end of nickel-titanium (Ni-Ti) closed coil spring to the maxillary first molar with a 0.1-mm diameter stainless steel wire using Howe utility pliers.
4. Tie another end of Ni-Ti closed coil spring to the hole drilled in the alveolar bone with a 0.1-mm diameter wire (Fig. 2) (*see* **Note 9**).
5. After 12 days of tooth movement, perfuse the mice or rats with 4% PFA under deep anesthesia, and then dissect the maxilla containing the experimental teeth.
6. Take an impression of the teeth and maxilla with a silicone impression material using individual resin tray for preparing a plaster dental cast. Make a plaster model of the maxillary dentition by filling the impression with the dental stone.

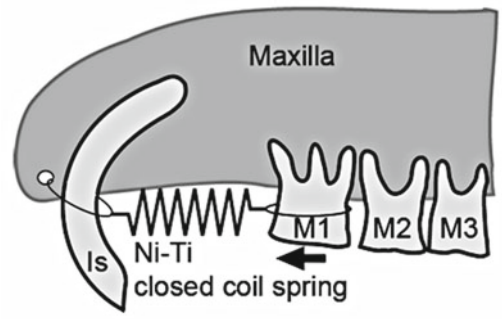

Fig. 2 Schematic drawings of experimental tooth movement by coil spring. Maxillary first molar (M1) was moved mesially by a nickel-titanium (Ni-Ti) closed coil spring ligated between M1 and hole in the alveolar bone with maxillary incisors (Is). *Arrow* indicates the direction of the tooth movement. *M2* maxillary second molar, *M3* maxillary third molar

7. Measure the amount of tooth movement by the distance between first and second molar on the plaster model under a microscope at a magnification of 40× using a digital caliper (*see* **Note 10**).
8. Fix the teeth and maxilla with 4% PFA overnight at 4 °C for examination.

3.1.2 Experimental Tooth Movement by Wire Spring

Move maxillary first molar of mice or rats toward the palatal or buccal side with continuous light force by simply designed orthodontic wire [39, 45, 46] (*see* **Notes 8** and **11**).

1. Anesthetize the mice or rats with an intraperitoneal injection.
2. Cut thin circumferential groove on the labial surface of the maxillary incisor at the level of near the gingiva with dental bur in a high speed handpiece (*see* **Note 12**).
3. Insert the orthodontic nickel-titanium (Ni-Ti) wire between the maxillary incisors, and place the Ni-Ti wire along the groove cut on the labial surface of the maxillary incisor (*see* **Note 13**).
4. Fix the orthodontic Ni-Ti wire to the maxillary incisor using composite resin for dental filling.
5. Place the end of the orthodontic wire on the buccal or palatal surface of the maxillary first molar to move it toward the palatal or buccal side, respectively (Fig. 3) (*see* **Notes 14** and **15**).
6. Take an impression of the teeth and maxilla with a silicone impression material using individual resin tray under isoflurane inhalation anesthesia at every few days (*see* **Note 16**). Make a plaster model of the maxillary dentition by filling the impression with dental stone.
7. Read the occlusal surface view of the plaster model of the maxillary dentition with the scanner after trimming of the plaster model.
8. Print out the scanned picture in the size of ten times, and trace out teeth, cusps of teeth, and palate folds.
9. Superimpose the traces before and after the tooth movement on the basis of molars of the contralateral side.
10. Measure the distance between the bilateral palatal cusps of the maxillary first molars with digital calipers. One tenth the measured distance is amount of tooth movement.
11. At desired number of days of tooth movement, perfuse the mice or rats with 4% PFA under deep anesthesia, and then dissect the maxilla containing the teeth. Further fix the maxilla with 4% PFA overnight at 4 °C for examination (*see* **Note 17**).

3.1.3 Experimental Tooth Movement by Elastic Module

Move maxillary first molar of mice or rats mesially with technically easy way and little stress to the animals by insertion of elastic module between maxillary first and second molars [37, 38, 47–49] (*see* **Note 8**).

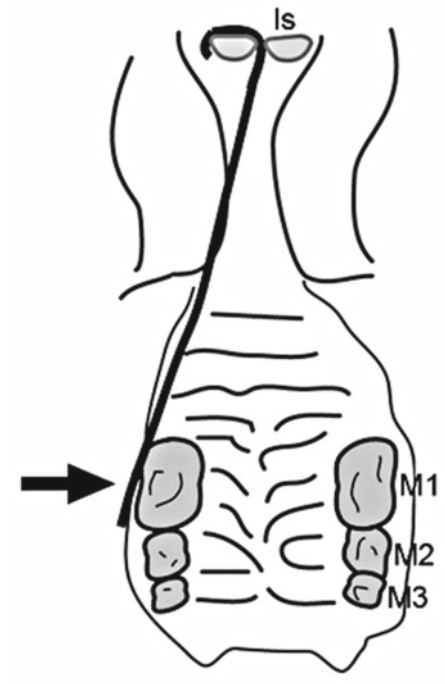

Fig. 3 Schematic drawings of experimental tooth movement by wire spring. Maxillary first molar (M1) was moved palatally by nickel-titanium wire, 0.012 or 0.014 in. in diameter, which was fixed to maxillary incisors (Is). Arrow indicates the direction of the tooth movement. *M1* maxillary first molar, *M2* maxillary second molar, *M3* maxillary third molar

1. Anesthetize the mice or rats with an intraperitoneal injection.
2. Stretch an orthodontic elastic module by elastic separating pliers and insert it interproximally between maxillary first and second molars until just cervical to the contact area.
3. Cut the elastic module at the both palatal and buccal corner of interproximal space by a small scissors (Fig. 4).
4. At desired number of days of tooth movement, perfuse the mice or rats with 4% PFA under deep anesthesia, and then dissect the maxilla containing the teeth (*see* **Note 18**). Further fix the maxilla with 4% PFA overnight at 4 °C for examination.

3.2 Application of Mechanical Stress to Murine Sutures and Cranial Bones

1. Anesthetize 6-week-old male ICR mice by intraperitoneal injection.
2. Shave hair in sagittal suture area and make an 8–9 mm of skin incision to expose the parietal bones.
3. Make two holes equidistant from a sagittal suture at the anteroposterior middle area of each parietal bone using a round bur attached to a dental drill (*see* **Note 19**). The distance between two holes was 3 mm.

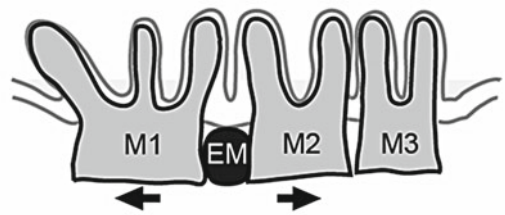

Fig. 4 Schematic drawings of experimental tooth movement by elastic module. An elastic module (EM) was inserted between maxillary first (M1) and second (M2) molars. *Arrows* indicate the directions of the tooth movement. *M3* maxillary third molar

4. Make tensile or compression force-loading springs by bending 0.012-in. diameter nickel-titanium or 0.016-in. diameter beta-titanium orthodontic wires, respectively (*see* **Note 20**) (Fig. 5).
5. Set the mechanical stress-loading spring within the holes in the parietal bones (*see* **Notes 21** and **22**) (Fig. 6).
6. Cover the spring by the skin, and close the incision by suturing.
7. To investigate CCN2/CTGF protein expression by immunohistochemical analysis (*see* Subheading 3.8), perfuse the mice with 4% PFA under deep anesthesia after application of mechanical stress, and then dissect calvarial bones including sagittal suture (*see* **Note 23**).

3.3 Mandibular Ramus Fracture Model

The edges of the non-immobilized fractured mandibular bone are subjected to mechanical stress by physiological jaw movement during the process of fracture healing [50, 51]. Chondrogenesis and endochondral ossification under mechanical stress could be investigated in this process.

1. Anesthetize the rats with an intraperitoneal injection of pentobarbital sodium and inhalation of isoflurane.
2. Make an incision on the right side of the mandible to the periosteum with a sterile condition.
3. Expose the mandibular ramus and cut the mandible perpendicular to the axis of the mandibular ramus at the posterior notch using scissors (Fig. 7) (*see* **Note 24**).
4. Place the condylar segment in the correct anatomic position after osteotomy, and then sew up and close the wound without the fixation of cut mandibular ramus (*see* **Notes 25** and **26**).
5. At 0, 3, 7, 14, and 28 days after the operation, perfuse the rats with 4% PFA under deep anesthesia, and then dissect the mandible with surrounding tissue (*see* **Note 27**). Further fix the mandible with 4% PFA overnight at 4 °C for examination (*see* **Note 28**).

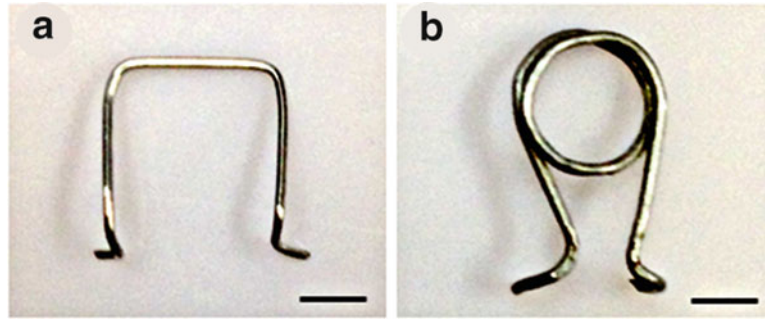

Fig. 5 Springs to load tensile (**a**) and compression (**b**) force. Scale bar: 2 mm

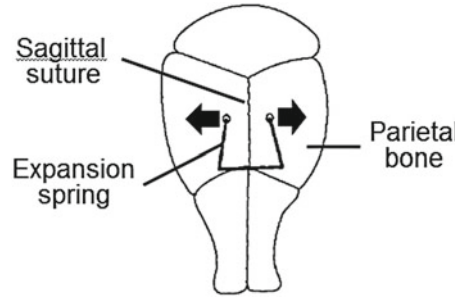

Fig. 6 Schematic representation of tensile force loading to murine sagittal suture. *Arrows* indicate the direction of tensile force

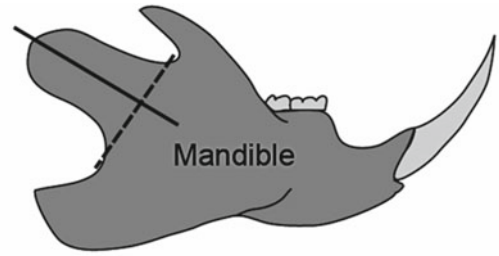

Fig. 7 Schematic drawings of rat mandibular ramus fracture model. The *solid line* represents the axis of mandibular ramus. The *dotted line* represents the fracture line

3.4 In Vitro Mechanical Tension or Compression Force Application

1. Coat flexible silicone membrane culture chambers with 0.05 mg/mL fibronectin for 12 h at room temperature (*see* **Notes 29** and **30**).
2. Set the flexible silicone membrane culture chambers to uniaxial cell stretching instrument (*see* **Note 31**).
3. Seed the MLO-Y4 cells into the wells of the flexible silicone membrane culture chambers in α-MEM containing 5% FBS,

5 % BS, 100 unit/mL penicillin and 100 μg/mL streptomycin, and incubate for 48 h at 37 °C in 5 % CO_2.

4. Apply the uniaxial mechanical tension or compression force to the cells by increasing or decreasing the width of culture chambers at desired percent of initial width (*see* **Note 32**).
5. Lyse cells for isolation of RNA or protein at desired time after application of mechanical stress.

3.5 In Vitro Application of Compression Force

1. Isolate primary osteocytes from 15-day-old fetal chicken calvariae according to the previous report (*see* **Note 33**) [52].
2. Seed osteocytes at a density of 1.3×10^4 cells/cm^2 onto the central area of the well of loading culture device, 4 mm in diameter.
3. Incubate cells in α-MEM containing 2 % FBS at 37 °C in a humidified 5 % CO_2 atm for 24 h.
4. Decompress under the culture wells at −18.7 kPa using an air pump. The PDMS membrane can be dented and compression force is applied to the osteocytes (*see* **Note 34**).
5. After application of compression force, lyse osteocytes to isolate total RNA or protein to examine CCN2/CTGF expression (*see* **Note 35**).

3.6 In Vitro Application of Cyclic Tensile Force

Apply cyclic mechanical force, which represents the physiological daily function such as walking and mastication, to the cultured cells [9, 53, 54].

1. Coat a flexible silicone bottomed 6-well plate with 0.05 mg/mL fibronectin for 12 h at room temperature (*see* **Note 30**).
2. Seed cells into a flexible silicone bottomed 6-well plate in α-MEM containing 5 % FBS, 5 % BS, 100 unit/mL penicillin, and 100 μg/mL streptomycin, and incubate for 2 or 3 days at 37 °C in 5 % CO_2.
3. Apply cyclic stretch and relaxation to the cells using a computer-controlled vacuum-operated instrument at various strain and frequency (*see* **Note 32**).
4. Lyse cells for isolation of RNA or protein at desired time after application of mechanical stress.

3.7 In Vitro Application of Fluid Shear Stress

1. Coat a slide glass with 20 μg/mL of poly-D-lysine and fibronectin at 37 °C in a cell culture incubator for 30 min.
2. Seed 8×10^5 osteogenic cell line MC3T3-E1 cells onto the slide glass and culture in α-MEM containing 10 % FBS for 3 days before the fluid shear stress loading.
3. Substitute the culture medium for that containing 0.5 % FBS 24 h before the stimulation of the fluid shear stress (*see* **Note 36**).

4. Load fluid flow in a flow chamber connected to a flow apparatus (Fig. 8). The cells are exposed to a defined laminar shear stress of 1.25 Pa for 2 h, as monitored by a flow meter (*see* **Notes 37** and **38**).
5. Harvest cells for analysis of CCN2/CTGF expression (*see* **Note 39**).

3.8 Immunohistochemistry

1. Deparaffinize the sections in three changes of xylene for 5 min each.
2. Hydrate the sections in three changes of 100% ethanol, 90, 80, and 70% ethanol for 3 min each. Leave the sections in distilled water for 5 min.
3. Wash with three changes of PBS for 3 min each.
4. Incubate the sections with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity for 15 min at room temperature.
5. Wash with PBS for 2 min twice.
6. Block the sections with 3% goat serum for 1 h at room temperature.
7. Wash with three changes of PBS for 5 min each.
8. Incubate the sections with appropriate rabbit antibody in immunoreaction enhancer solution for 16 h at 4 °C (*see* **Note 40**).
9. Wash with PBS for 2 min twice.
10. Incubate the sections with peroxidase-conjugated secondary antibody for 30 min at room temperature.
11. Wash with PBS for 2 min twice.

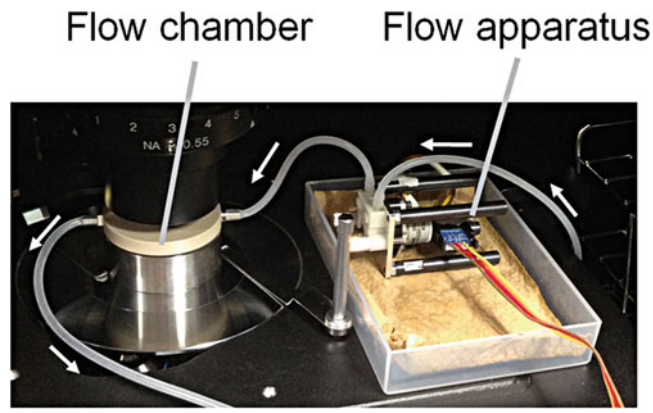

Fig. 8 Fluid chamber and apparatus to apply fluid shear stress to cells. *Arrows* indicate the direction of fluid flow

12. Visualize the signals by immersion of the sections in 3,3-diaminobenzidine tetrahydrochloride (DAB) containing 0.02% hydrogen peroxide.
13. Wash the sections with distilled water, and dehydrated through graded ethanols.
14. Rinse the sections in three changes of xylene and mount by mounting medium for microscopy (*see* **Note 41**).

3.9 In Situ Hybridization

Carry out all the procedures before hybridization in RNase free [24, 37, 39, 50, 55].

1. Deparaffinize the sections in three changes of xylene for 10 min each.
2. Hydrate the sections in three changes of 100% ethanol, 90, 80, and 70 ethanol for 1 min each.
3. Rinse with PBS for 1 min twice.
4. Incubate the sections with 2 μg/mL proteinase K in PBS for 15 min at 37 °C.
5. Fix the sections with 4% PFA in PBS for 10 min at room temperature.
6. Wash with PBS for 1 min.
7. Treat the sections with 0.2 N HCl for 20 min.
8. Wash with PBS for 1 min.
9. Rinse the sections in two changes of 4× SSC for 10 min at room temperature.
10. Incubate the sections with 50% formamide in 2× SSC for 3 h at 42 °C.
11. Heat the hybridization solution containing DIG-UTP labeled sense or antisense probes for 3 min at 85 °C.
12. Hybridize the probes to the sections by placing 50 μL of hybridization solution on the sections and cover it with plastic paraffin film.
13. Incubate the sections in a moisture chamber for 16 h at 42 °C (*see* **Note 42**).
14. Remove the plastic paraffin film in 5× SSC at 45 °C (*see* **Note 43**).
15. Wash the slides with three changes of 50% formamide in 2× SSC for 20 min at 45 °C.
16. Incubate the sections with TNE for 10 min at 37 °C.
17. Incubate the sections with 20 μg/mL RNase A in TNE for 30 min at 37 °C.
18. Incubate the sections with TNE for 10 min at 37 °C.
19. Wash with 2× SSC and 0.2× SSC for 20 min at 45 °C.

20. Rinse the sections in DIG buffer 1 for 5 min.
21. Block the sections with 1.5 % blocking reagent in DIG buffer 1 for 60 min at room temperature.
22. Wash with DIG buffer 1 for 3 min.
23. Incubate the sections with sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (1:2000) in DIG buffer 1 for 40 min at room temperature.
24. Wash with DIG buffer 1 containing 0.2 % Tween 20 for 15 min twice.
25. Wash with DIG buffer 1 for 15 min.
26. Equilibrate the sections with DIG buffer 3 for 3 min at room temperature.
27. Visualize the signals by incubation of the sections in the dark with NBT/BCIP in DIG buffer 3 at room temperature.
28. Stop the color reaction by washing in distilled water and mount by aqueous permanent mounting medium (*see* **Note 44**).

3.10 Real-Time PCR

1. Harvest cells or tissues, and isolate total RNA using a reagent, such as TRIzol Reagent, according to the manufacture's protocol.
2. Synthesize cDNA from 0.3 μg of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser according to the manufacture's protocol.
3. Prepare reaction mixtures and apply them into a 96-well plate for real-time PCR (*see* **Note 45**).
4. Perform real-time PCR using a thermal cycler. The reactions consisted of 40 cycles of 5 s at 95 °C and 30 s at 60 °C.
5. Relative mRNA levels were normalized to those of housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (*see* **Note 46**).

3.11 Western Blotting

1. Prepare total cellular proteins by using a lysis buffer containing protease and phosphatase inhibitors (*see* **Notes 47** and **48**).
2. Determine protein concentration by a protein assay kit according to the manufacture's protocol.
3. Separate 20 μg of extracted cellular proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12 % polyacrylamide gel.
4. Transfer the separated proteins to PVDF membrane using a semidry blotting apparatus.
5. Incubate the membrane in TBS containing 3 % BSA 30 min at room temperature.

6. Incubate the membrane overnight at 4 °C in a primary antibody specific for CCN2/CTGF diluted 1:200 with PBS containing 1% BSA.
7. Wash the membrane with TBS containing 0.1% Tween 20 for 5 min at room temperature three times.
8. Incubate the membranes with secondary antibodies conjugated with horseradish peroxidase diluted 1:1000 with TBS containing 3% BSA for 1 h at room temperature.
9. Wash the membrane with TBS containing 0.1% Tween 20 for 5 min at room temperature 3 times.
10. Developed the membrane with a chemiluminescent substrate.
11. Capture chemiluminescent signals by an image analyzer.

3.12 TRAP Staining

1. Warm the TRAP staining kit to room temperature.
2. Deparaffinize the sections in three changes of xylene for 5 min each.
3. Hydrate the sections in three changes of 100% ethanol, 90, 80, and 70% ethanol for 1 min each. Leave the sections in distilled water for 5 min.
4. Mix 10 μL of sodium nitrite solution and 10 μL of fast garnet GBC base solution in 1.5 mL tube, and pipette up and down for 1 min thoroughly.
5. Let sit at room temperature for 2 min.
6. Add 930 μL of deionized water, 10 μL of naphthol AS-BI phosphoric acid solution, 40 μL of acetate solution, and 20 μL of tartrate solution, and mix the solution by pipetting.
7. Transfer the mixture to sections on slides (*see* **Note 49**).
8. Incubate the slide at room temperature for 20 min in the moisture chamber (*see* **Note 50**).
9. Wash the sections for 5 min with deionized water, and mount by aqueous permanent mounting medium.

3.13 TUNEL Staining

1. TUNEL staining of cultured cells was performed with a kit for detection of apoptosis according to the manufacturer's instructions.
2. Treat cells with DAPI (diluted 1:1000 with PBS) for nuclear staining.
3. Observe stained cells on a fluorescence microscope and images of the cell culture area were acquired with a charge-coupled device camera DP71.
4. Count TUNEL-positive cells using a software for image analysis.

4 Notes

1. To avoid exposing paraformaldehyde, wear a mask and measure the paraformaldehyde in the fume hood. Transfer the weighed paraformaldehyde to the glass beaker inside the fume hood.
2. Using hot water helps to dissolve paraformaldehyde and working the magnetic stir bar. To avoid exposing paraformaldehyde, mix on a stirrer placed inside the fume hood.
3. We find that it is best to prepare this fresh each time.
4. We use this product as a neutralizing antibody.
5. Loading culture device has eight culture wells composed of polydimethylsiloxane (PDMS) membrane of 16 mm in diameter and 1 mm in thickness. By decompressing under the culture wells at −18.7 kPa using an air pump, the PDMS membrane can be dented. Finite element modeling analysis quantifies the strain in the PDMS membrane. As a result, the concave culture wells have gradation strains, with the maximal compressive strains (2.9%) in the central area and the maximal tensile strains (2.9%) in the peripheral areas.
6. Contamination of genomic DNA in total RNA sample results in nonspecific amplification of genes in PCR analysis. Because gDNA Eraser in PrimeScript RT Reagent Kit degrades the genomic DNA, it can prevent the nonspecific amplification of genes.
7. We use a rabbit polyclonal antibody against CCN2/CTGF, which is purchased from Abcam (Cambridge, UK).
8. By experimental tooth movement, the trabecular bone is compressed one side and tensioned on the opposite side. Therefore, gene expression associated with both compressive and tension force could be investigated simultaneously in vivo.
9. The loaded force magnitude is measured using a dial tension gauge, and should be 10 g for mice or 10–20 g for rats.
10. For evaluating the intra-measurer error, measure the distance between first and second molar of each animal three times by three well-trained measurers.
11. The direction of tooth movement might be better in palatal direction, because of thinner alveolar bone on the buccal side in mice.
12. During the experimental period, orthodontic Ni-Ti wire is sometimes detached from maxillary incisors. Thin circumferential groove helps to retain the appliance and to facilitate the placement the appliance on incisors.
13. Use 0.012 in. in diameter of Ni-Ti wire for mice, and 0.014 in. for rats.

14. The loaded force magnitude is directly measured on the plaster model using a dial tension gauge, and should be 10 g for mice or 10–20 g for rats.
15. Ni-Ti wire has the super elastic property, and an appliance made with this wire exerts a stable light force on the teeth [39, 45, 46]. Once this appliance made with Ni-Ti wire has been adjusted to produce a certain magnitude of force, no further adjustment is needed until 21 days of experiment.
16. During taking an impression, the edge of wire is positioned away from the molar to non-activated position of wire. After the impression has been taken, the wire is adjusted to be touched to the molar again.
17. We show that the periodontal ligaments were compressed in the compression side and stretched in the tension side on day 9 after the start of experimental tooth movement of rats (Fig. 9).
18. Elastic module could remain in place for 5–7 days [47, 48].
19. The biological response of sutures to mechanical stress is dependent on magnitude of the mechanical stress [56, 57]. Therefore, it is important to load a certain magnitude of mechanical stress and making holes is a critical step to achieve this.

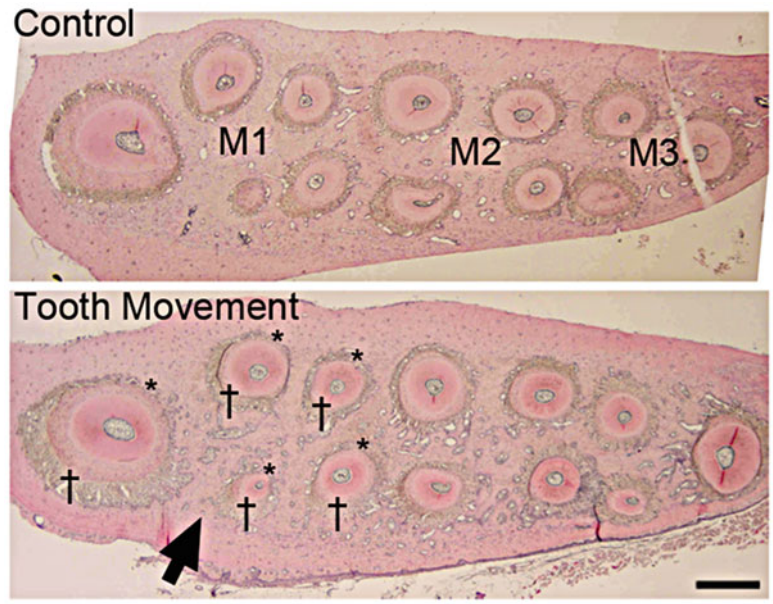

Fig. 9 Histology of experimental tooth movement of rat. Horizontal sections were obtained in the middle level of the root. On day 9 after the start of experimental tooth movement, the periodontal ligaments were compressed in the compression side (*asterisk*), and stretched in the tension side (*dagger*). *Arrow* indicates the direction of the tooth movement. *M1* maxillary first molar, *M2* maxillary second molar, *M3* maxillary third molar. Scale bar = 1 mm

20. The relationship between force and spring deflection should be confirmed using a dial tension gauge.
21. The springs should be calibrated to load the desired force using a dial tension gauge prior to each experiment.
22. To examine the effects of inhibition of CCN2/CTGF on mechanobiological response of sutures, inject a 100 μL volume of 10 μg/mL rabbit polyclonal antibody against CCN2/CTGF subcutaneously into the sagittal suture area. As a negative control, inject the same concentration of rabbit IgG. Install the expansion springs to sagittal sutures 6 h after the injection.
23. In our study, there are many CCN2/CTGF immuno-positive osteocytes in parietal bones after application of 20 g compressive force for 6 h (Fig. 10).
24. To avoid damaging surrounding soft tissue, expose the small area of mandibular ramus around the posterior notch.
25. Splint is not used for immobilizing bone fragments. Not fracture the contralateral side (left side) of the mandible.
26. After surgery, give the powder feed of rodent chow to the rats for 1 week, and feed solid form thereafter.
27. In general, by day 3 after fracturing, undifferentiated mesenchymal cells envelope the bone surface around the fracture site. By day 7 after fracturing, a certain amount of chondroid tissue appears in the central part of fibrous tissue. By day 14 after fracturing, the amount of chondroid tissue is increased and the newly formed bone trabeculae is seen. By day 28 after fracturing, almost all of the chondroid tissue has been replaced by bone.
28. We show the histology of rat mandibular fracture healing at day 3 after fracture. The fracture ends were at an angle to each other and had slid laterally, because of non-immobilizing the fractured bone (Fig. 11a, b).

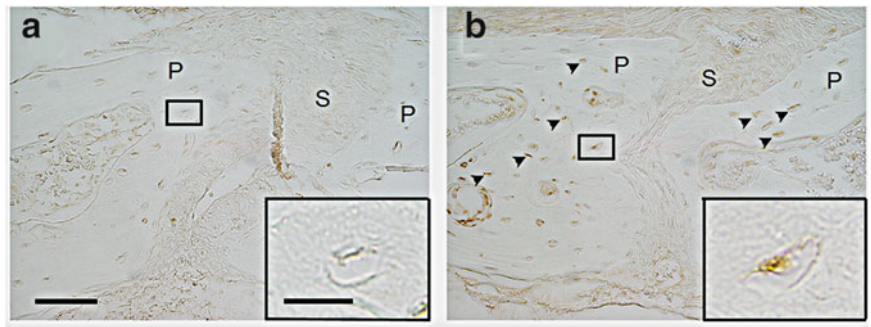

Fig. 10 Expressions of CCN2/CTGF protein in the compression force-nonloaded (**a**) and -loaded (**b**) parietal bones were examined by immunohistochemistry 6 h after loading. A magnified picture of the rectangular area is shown on the *lower right corner* of each picture. *Arrowheads* indicate immunopositive osteocytes. Scale bars = 50 μm, and 10 μm in *insets*. *P* parietal bone, *S* sagittal suture

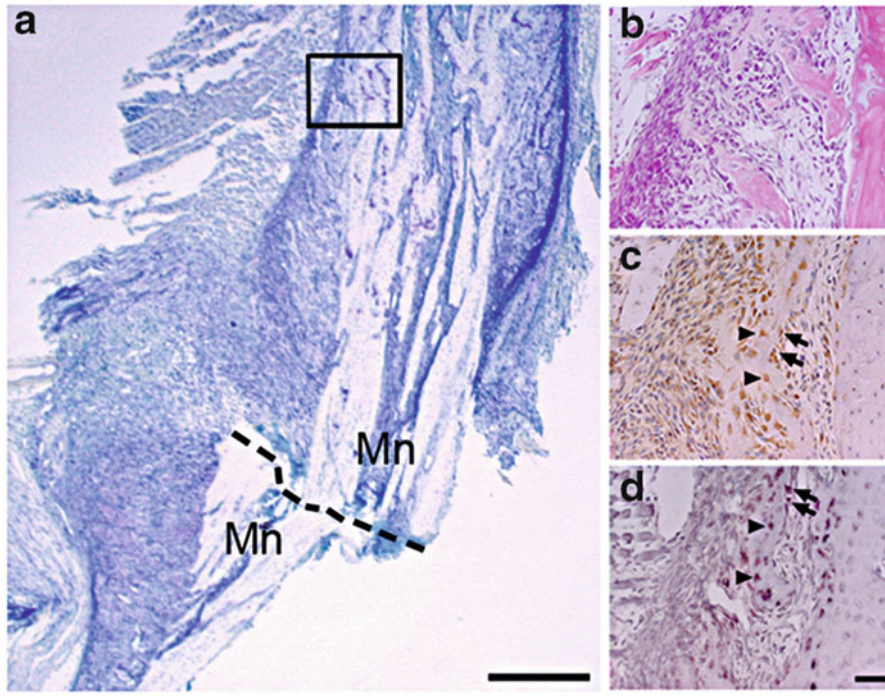

Fig. 11 Localization of CCN2/CTGF protein and mRNA on day 3 after fracturing of the mandibular ramus of rat. (**a**, **b**) Histology of mandibular ramus fracture healing of rat. (**c**) CCN2/CTGF protein was detected in osteoblasts (*arrows*) and osteocytes (*arrowheads*) in newly formed bone near the fracture site. (**d**) CCN2/CTGF mRNA was detected in osteoblasts (*arrows*) and osteocytes (*arrowheads*) in newly formed bone near the fracture site. A *black box* in (**a**) indicates the areas enlarged in **b**, **c**, and **d**. The *dotted line* indicates the fracture site. *Mn* mandibular bone. Scale bar = 500 μm (**a**), 50 μm (**d**)

29. Autoclave the stretch chambers at the first time of use and disinfect with 70% ethanol for 10 min after second time.
30. Employed extracellular matrix coating is dependent on cell type. Usually, the membrane of stretch chamber is coated with extracellular matrix such as fibronectin, collagen, or gelatin.
31. If you would like to apply compressive force, stretch the chambers before seeding cells. After cells adhere to the stretch chambers, relax the stretch chambers to compress the cells.
32. Incubate unstretched cells in the same condition as a control.
33. To identify osteocytes in isolated cells, we use the OB7.3 monoclonal antibody, which is specific for chicken osteocytes. More than 90% of the dendritic cells are regarded as osteocytes.
34. We apply compressive strain ranging from 1.2 to 2.9%.
35. We showed that the CCN2/CTGF mRNA and protein levels are significantly increased at 1 and 2 h after loading, respectively [35].

36. When you examine the effect of mechanical stress on expression and function of CCN2/CTGF, the cellular activities induced by serum would mask the effect of mechanical stress. Thus, serum starvation is performed in our protocol.
37. The control cells are maintained in the same flow chamber without fluid flow.
38. This level of shear stress is within the predicted physiological range [58].
39. We showed that the *Ccn2/Ctgf* mRNA level is significantly increased at 1–2 h after loading [36].
40. Controls are obtained by replacement of the primary antibody by nonimmune rabbit IgG.
41. We show that the CCN2/CTGF protein was detected in osteoblasts and osteocytes in newly formed bone near the fracture site (Fig. 11c).
42. Moisturize the moisture chamber with 50% formamide.
43. Wait until the plastic paraffin film become detached by itself.
44. We show that the CCN2/CTGF mRNA was detected in osteoblasts and osteocytes in newly formed bone near the fracture site (Fig. 11d).
45. Our reaction volume is 25 μL, which contained 2 μL of cDNA, 12.5 μL of SYBR Premix Ex Taq II, and 0.4 μM of sense and antisense *Ccn2/Ctgf* primers (Table 1).
46. In our study, *Ccn2/Ctgf* mRNA expression in sutures is significantly upregulated by tensile force 3 h after loading (Fig. 12).
47. To prevent degradation of proteins, lyse cells on ice using prechilled reagent and store cell lysates at −80 °C.
48. We generally add Proteinase Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 and 3, which are purchased from Sigma-Aldrich (St. Louis, MO, USA), into the lysis buffer according to the manufacture's protocol.

Table 1
***Ccn2/Ctgf* primer sequences used for real-time PCR**

Species	Primer sequence	Accession no.
Chicken [25]	5′-CACCAACGATAATGCTTTC-3′ (S) 5′-ACTTAGCTCTGTACGTCTTCA-3′ (AS)	NM_ 20427
Mouse [27]	5′-AGAAGGGCAAAAAGTGCATCCG-3′ (S) 5′-GC CATGTCTCCGTACATCTTCCTG-3′ (AS)	NM_010217

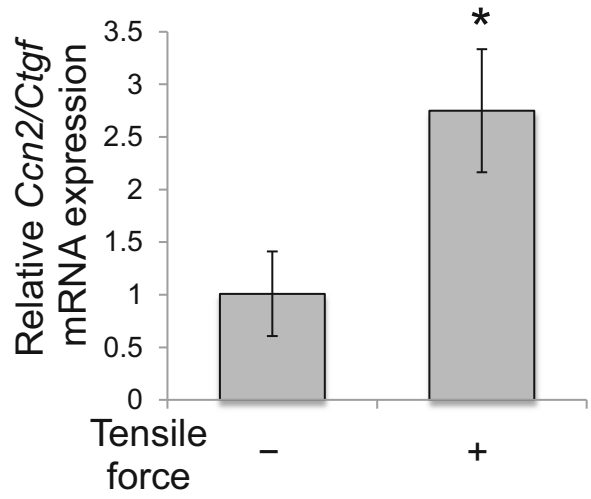

Fig. 12 Expression of *Ccn2/Ctgf* mRNA in suture 3 h after tensile force application was analyzed by real-time PCR. Significant difference from the tensile force-nonloaded group: $*p < 0.05$; $n = 6$

49. The amount of 150 μL is enough for a section of rat unilateral maxilla.
50. If the red color reaction is low, incubation time can be extended up to 1 h.

Acknowledgement

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Chapter 27

The Bone Regeneration Model and Primary Osteoblastic Cell Culture Used in the Analysis of *Ccn3* Transgenic and Knockout Mice

Kei Sakamoto, Yuki Matsushita, Tokutaro Minamizato, Yuko Katsuki, Ken-ichi Katsube, and Akira Yamaguchi

Abstract

Bone tissue is intrinsically hard and thus, it is more difficult to handle, process, and examine than soft tissues. Here, we describe an experimental model of bone regeneration and several selected protocols useful for investigating mRNA and protein expression in bone. The inhibitory function of CCN3 on membranous bone formation has been confirmed by following the protocols described herein (Fig. 1).

Key words Bone regeneration, Drill hole injury, HE staining, Immunohistochemistry, Primary osteoblastic cell culture

1 Introduction

We identified *CCN3* as a candidate gene that modulates bone regeneration [1–3]. In this chapter, we introduce several selected protocols that we followed for analysis of bone phenotypes of *Ccn3* knockout mice and *CCN3* transgenic mice. Bone tissue, by nature, is more difficult to handle, process, and examine than other tissues and so presents unique challenges requiring the development of specialized protocols. Bone formation is regulated by many biological factors acting through complex mechanisms that include synergistic, competitive, and suppressive feedbacks. Thus, the role of a specific factor may be uncovered solely by using a standardized experimental model. Our protocols are not unique for analysis of CCN3, and the procedures and tips would be useful for investigating general mRNA and protein expression in bone. Since the effects of gene overexpression and knockout are compromised during development, the inhibitory function of CCN3 on membranous bone formation has been determined only via the bone

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regeneration model described herein [2]. For convenience, some of these protocols are slightly modified versions of the ones used in the abovementioned references [1–3].

2 Materials

2.1 Bone Regeneration Model of a Drill Hole Injury in the Mouse Femur

1. Three mix anesthetic: Domitor® (medetomidine hydrochloride) 1.0 mg/mL, Dormicum® (midazolam) 5.0 mg/mL, Vetorphale® (butorphanol tartrate) 5.0 mg/mL). Mix 0.75 mL of Domitor®, 2.0 mL of Dormicum®, 2.5 mL of Vetorphale®, and 19.75 mL of distilled water. Store at 4 °C. Stable at least 8 weeks (Fig. 1).
2. Normal saline: Dissolve 9.0 g of NaCl in 1000 mL of water. Sterilize the solution by autoclaving for 15 min at 12 °C. Store at room temperature.
3. 70% ethanol: Mix 70 mL of ethanol and 30 mL of water. Store at room temperature.
4. Syringe (10 mL).
5. 23 G needle.
6. Electric hair clipper.
7. Scissors (Fig. 2).
8. Forceps (Fig. 2).
9. Scalpel (Fig. 2).

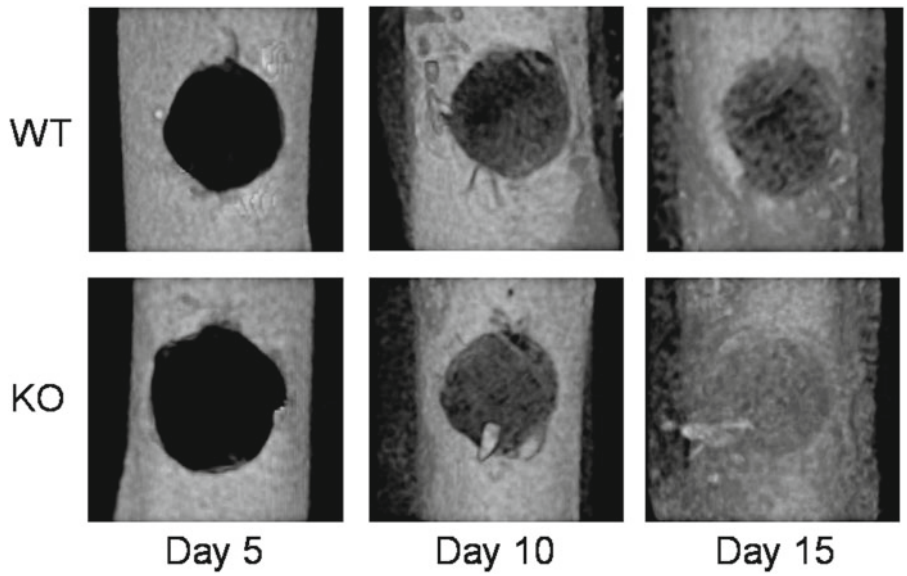

Fig. 1 Bone regeneration of drill hole injury. Micro-CT images of drill hole injury on days 5, 10, and 15 after surgery. The micro-CT protocol is not described in this chapter

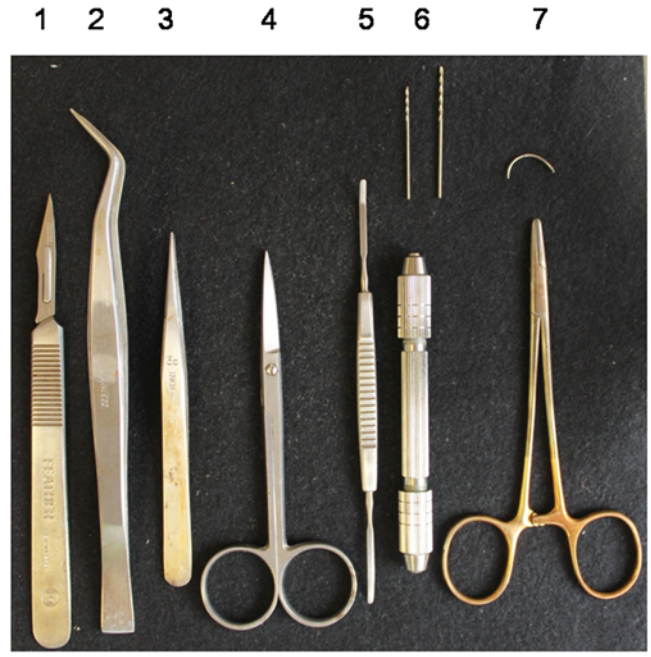

Fig. 2 Surgical instruments to make a drill hole injury. *1*: scalpel, *2*: forceps (large), *3*: forceps (small), *4*: scissors, *5*: periosteum elevator, *6*: screwholder and drill bits, *7*: suture needle and needle holder

10. Periosteum elevator (Fig. 2).
11. Screwholder (Fig. 2).
12. Drill bits (0.8 and 1.2 mm) (Fig. 2).
13. Suture needle (Fig. 2).
14. Needle holder (Fig. 2).
15. 4-0 silk surgical suture.
16. Paper towel.
17. Three gem-type paper clips (to prepare self-made muscle hooks and a ruler. Two for the muscle hooks, one for the ruler) (Fig. 3) (*see* **Note 1**).

2.2 Immunohistological Examination of the Femur

1. Phosphate buffered saline (PBS; 10×): Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, and 2.4 g of KH_2PO_4 in 800 mL of water. Adjust the pH to 7.4 with HCl and make up to 1 L. Sterilize the solution by autoclaving for 15 min at 121 °C. Store at room temperature. Prepare 1:10 dilution for use.
2. 10% neutral buffered formalin.
3. Decalcification solution (20% ethylenediaminetetraacetic acid (EDTA)): Dissolve 200 g of EDTA·2Na and 24 g of Tris in 1 L of water. Adjust the pH to 7.4 by NaOH. Sterilize the

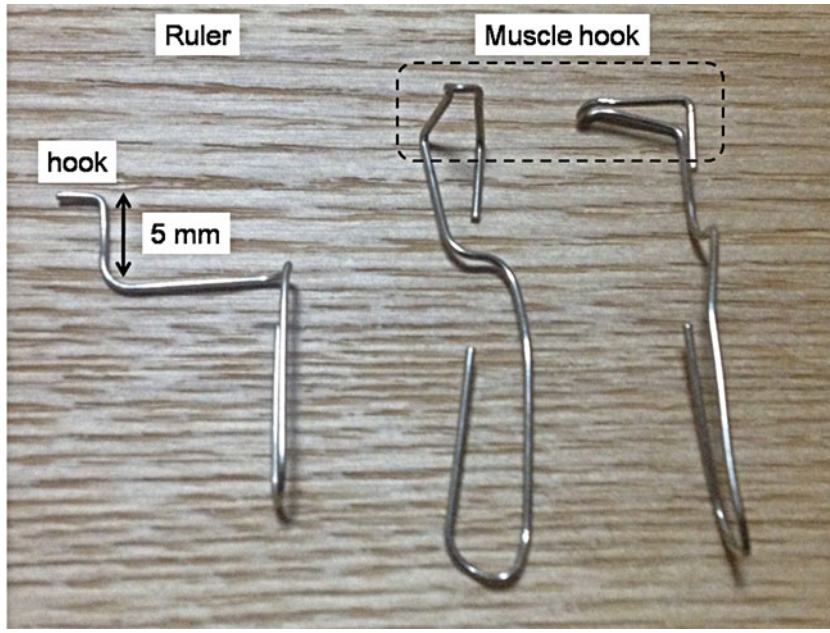

Fig. 3 Self-made ruler and muscle hooks

solution by autoclaving for 15 min at 121 °C. Store at room temperature.

4. Tris buffered saline (TBS; 10×): Dissolve 87.7 g of NaCl and 24.2 g of Tris in 900 mL water. Adjust the pH to 7.4 with HCl and make up to 1 L. Sterilize the solution by autoclaving for 15 min at 121 °C. Store at room temperature. Prepare 1:10 dilution for use.
5. TBST: TBS containing 0.1% Tween 20.
6. Antigen retrieval buffer: 10 mM Tris–HCl (pH = 9.0), 1 mM EDTA.
7. Ethanol (100, 95, 90, 80, 70%).
8. Chloroform.
9. Xylene.
10. Paraffin wax.
11. Hydrogen peroxide.
12. DAB stock solution: Dissolve 2 g of 3,3′-Diaminobenzidine-tetrahydrochloride in 20 mL of distilled water and add 80 mL of ethyleneglycol monomethylether. Store protected from light at −20 °C.
13. Hematoxylin solution, Mayer's.
14. Eosin Y solution, alcoholic.
15. Xylene-based mounting medium.

16. EnVision™ (Dako, Glostrup, Denmark) or an equivalent.
17. Scissors, forceps (*see* Subheading 2.1).
18. Razor blade.
19. Tissue cassette.
20. Embedding mold.
21. Sectioning machine.
22. Silane-coated slide glass.
23. Coverslip.
24. Microwave processor.
25. Shandon™ Sequenza™ Immunostaining Center (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 RNA Extraction from Bone Tissue

1. Liquid nitrogen.
2. Multi-beads Shocker® (Yasui Kikai Co. Ltd., Osaka, Japan) (Including 2 mL plastic tubes and homogenizing bullets. Fig. 4, *see* **Note 2**).
3. Acid guanidinium thiocyanate-phenol-chloroform solution (e.g., TRIzol® (Thermo Fisher Scientific, Waltham, MA, USA), *see* **Note 3**).
4. RNA extraction spin column (e.g., NucleoSpin® RNA (Takara, Shiga, Japan) , *see* **Note 3**).
5. Chloroform.
6. Isopropanol.

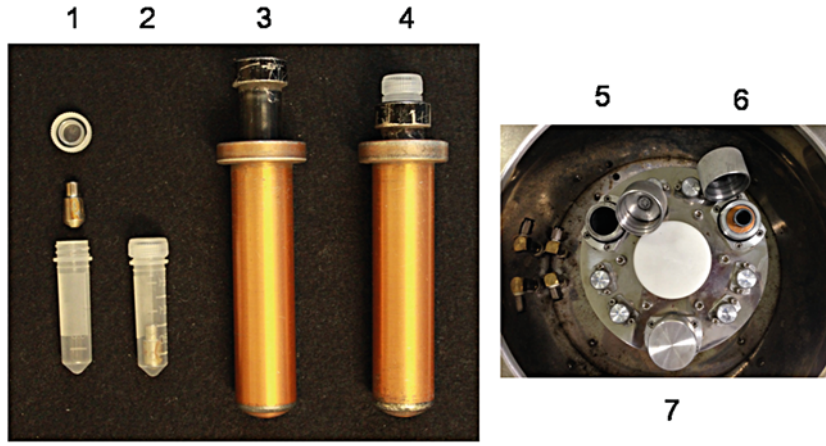

Fig. 4 Plastic tubes, homogenizing bullets, and tube holders of Multi-beads Shocker®, illustrating how to set the tube and the homogenizing bullet ready for operation. *1*: Tube and homogenizing bullet, *2*: Homogenizing bullet in the tube (with a tissue sample inside), *3*: Holder consisting of outer casing and inner casing. *4*: The tube in the inner casing inserted into the outer casing. *5*: There are stations for three holders in the machine. The lid is open. *6*: The tube and the holder is inserted into a fixed position. *7*: The lid is closed. During operation, the stage is vigorously vibrated and the tissues are homogenized

7. Ethanol.
8. Diethylpyrocarbonate (DEPC)-treated water: Add 0.1 mL of DEPC to 1 L of double distilled water. Shake vigorously and let the solution sit at room temperature overnight. Autoclave for 15 min at 121 °C (*see* **Note 4**).
9. Normal saline (*see* Subheading 2.1).
10. 2-mercaptoethanol.
11. Scissors, forceps (*see* Subheading 2.1).
12. Syringe (10 mL).
13. 18 G needle.

2.4 Protein Extraction from Bone Tissue

1. Liquid nitrogen.
2. Laemmli buffer: 50 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol).
3. cOmplete™ protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) or an equivalent.
4. Normal saline (*see* Subheading 2.1).
5. Multi-beads Shocker® (*see* Subheading 2.3).
6. Scissors, Forceps, Syringe (10 mL), 18 G needle (*see* Subheading 2.3).

2.5 Primary Culture of Osteoblastic Cells from the Calvariae of Neonatal Mice

1. Hanks' balanced salt solution.
2. Collagenase/dispase solution: Dissolve 25 mg of collagenases II and 50 mg of dispase II in 25 mL of Hank's Balanced Salt Solution. Sterilize the solution by filtration using the Syringe filter (0.45 μm pore size). Transfer to a new 50 mL conical tube and store at −20 °C until usage.
3. Antibiotics: penicillin–streptomycin (100×) for cell culture.
4. 70% ethanol.
5. Minimum essential medium Eagle with alpha-modification (αMEM).
6. Fetal bovine serum (FBS).
7. Syringe (10 mL).
8. Syringe filter, 0.45 μm pore size.
9. Falcon® 70 μm Cell Strainer (Thermo Fisher Scientific, Waltham, MA, USA) or an equivalent.
10. Scissors, Forceps (*see* Subheading 2.1).
11. Culture dish (3.5 and 6 cm in diameter).
12. Conical tubes (50, 15 mL).
13. Stereomicroscope.
14. Hemocytometer.

15. Parafilm®.
16. Crush ice.

3 Methods

All the surgical instruments should be sterilized by dry heat sterilization or autoclaving.

3.1 Bone Regeneration Model of a Drill Hole Injury in the Mouse Femur

1. Inject 0.1 mL three-mix anesthetic per 10 g body weight intraperitoneally to 8-week-old male mice using a 10 mL syringe and a 23 G needle. Wait until the mouse goes down under anesthesia.
2. Shave the leg at the site for incision by the electric hair clipper. Disinfect the shaved area by wiping with a paper towel soaked with 70% ethanol (Fig. 5a).
3. Using scissors, make a 5-mm incision in the front skin of mid-femur (Fig. 5b).
4. Exfoliate the dermis laterally by scissors, and expose the biceps femoris muscle and the quadriceps femoris muscles (Fig. 5c).
5. Incise the fascia by the scalpel between the biceps femoris muscle and the vastus lateralis muscle (Fig. 5d). Insert the

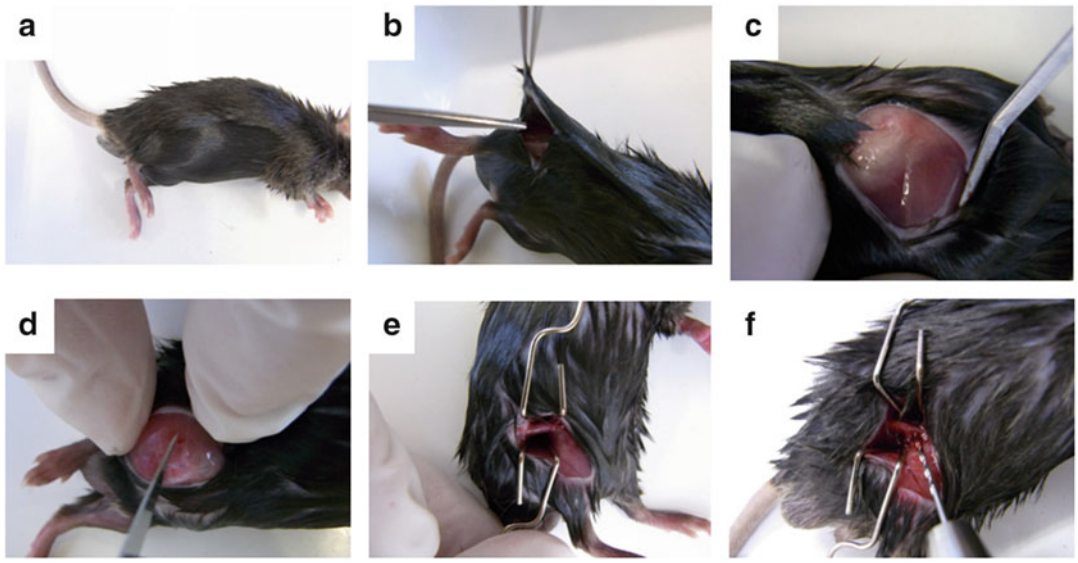

Fig. 5 Surgical procedures. (**a**) Shave and disinfect the skin of leg. (**b**) Make a 5-mm incision in the front skin of the mid-femur. (**c**) Exfoliate the dermis laterally by scissors, and expose the biceps femoris muscle and the quadriceps femoris muscles. (**d**) Incise the fascia by scalpel between the biceps femoris muscle and the vastus lateralis muscle. (**e**) Using the self-made muscle hooks, your assistant pulls apart the biceps femoris muscle and the vastus lateralis muscle to expose the femur. (**f**) Make a drill hole using a drill bit of 0.8 mm in diameter first, then a drill bit of 1.2 mm in diameter, in the anterior portion of the diaphysis of the femur

periosteum elevator between the muscles and separate the muscles until the periosteum elevator reaches the femur.

6. Using the self-made muscle hooks, pull apart the biceps femoris muscle and the vastus lateralis muscle to expose the femur (Fig. 5e). You need an assistant who keeps pulling the muscles apart while you handle the drill.
7. While your assistant is holding the muscles apart, exfoliate the periosteum by scraping the bone surface with the periosteum elevator to expose the femoral surface (*see* **Note 5**).
8. Make a drill hole using the drill bit of 0.8 mm in diameter, in the anterior portion of the diaphysis of the femur, at 5 mm above the knee joint (Fig. 5f) (*see* **Note 6**). Change the drill bit to that of 1.2 mm in diameter and enlarge the hole (*see* **Note 7**).
9. Irrigate the surgical field thoroughly with normal saline using the syringe (*see* **Note 8**).
10. Suture the skin by one or two simple interrupted stitches.

3.2 Histological andmmunohistological Examination of the Femur

3.2.1 Preparation of Tissue Section

1. Sacrifice the mice by cervical dislocation at days 5, 10, and 15 after surgery.
2. Make an incision in the skin of the leg by scissors and peel off the skin. Dissect the femur by separating it at the femur head and at the knee joint using scissors.
3. Cut the femur by scissors at the proximal end so as to expose the bone marrow, taking care not to disturb the drill hole. Remove the muscles by scissors and forceps (*see* **Note 9**). Fix the femur sample in 10% neutral buffered formalin at 4 °C with gentle shaking for 12 h.
4. Rinse the femur in PBS at 4 °C with gentle shaking for 12 h.
5. Decalcify the femur in the decalcification solution at 4 °C with gentle shaking for 10–14 days. Change the decalcification solution to fresh one next day, and then every 3 days.
6. Rinse the femur in PBS at 4 °C with gentle shaking for 12 h.
7. Trim the femur to facilitate sectioning. Place the femur on a paper towel, the drill hole side up, and cut the femur sagittally by the razor blade, at an eccentric position that exposes the bone marrow but does not cross the drill hole (*see* **Note 10**). This is to avoid demolition of the restorative tissue (*see* **Note 11**).
8. Dehydration. Put the femur in a tissue cassette and close the lid. Transfer the cassette at room temperature in series of increasing alcohol concentrations with gentle shaking each for 2 h (*see* **Note 12**); 70, 80, 90, 95% ethanol and three changes of 100% ethanol.

9. Transfer the cassette to three changes of chloroform at room temperature, then to five changes of melted paraffin at 60 °C each for 2 C.
10. Embedding. Take out the femur from the cassette by warmed forceps and place in an embedding mold filled with melted paraffin. Using warmed forceps (*see* **Note 13**), adjust the location and direction of the femur. The trimmed side of the femur must be facing to the bottom of the embedding mold and the drill hole side must be facing horizontally to the side wall of the embedding mold (*see* **Note 14**). Wait until the paraffin solidifies.
11. Cut 4–5 μm thick sections and place them on silane-coated glass slides (*see* **Note 15**).
12. Air-dry the sections. Proceed to staining or store at −20 °C (*see* **Note 16**).

3.2.2 Hematoxylin and Eosin Staining (see Note 17).

1. Deparaffinize the sections by passing them in two changes of xylene for 10 min each (*see* **Note 18**).
2. Rehydrate the sections by passing them in two changes of 100% ethanol, then in two changes of 70% ethanol, and then in running tap water (*see* **Note 19**) for 5 min each.
3. Rinse briefly in distilled water.
4. Stain in hematoxylin solution for 5 min.
5. Wash in running tap water for 10 min.
6. Rinse briefly in distilled water.
7. Rinse briefly in 95% ethanol.
8. Stain in eosin Y solution for 2 min.
9. Dehydrate through 95% ethanol and then two changes of 100% ethanol for 5 min each.
10. Pass two changes of xylene for 5 min each.
11. Mount with mounting medium and a coverslip.

3.2.3 Immunohistochemical Staining

1. Deparaffinize the sections by passing them in two changes of xylene for 10 min each.
2. Rehydrate the sections by passing them in two changes of 100% ethanol, then in two changes of 70% ethanol, and then in running tap water for 5 min each.
3. Rinse in tap water and then in distilled water.
4. Antigen retrieval (*see* **Note 20**). Place the glass slides in the antigen retrieval buffer, and run the microwave processor MI-77 at 80 °C for 30 min. Let the specimens cool at room temperature.
5. Mount the glass slides on Shandon™ Sequenza™ cassette.

6. Apply 2 mL of TBST for rinse.
7. Apply 100 μL of 3% hydrogen peroxidase (dilute 30% H_2O_2 to 1:10) and incubate for 10 min at room temperature to deactivate endogenous peroxidase.
8. Apply 2 mL of TBST for wash.
9. Dilute the primary antibody at a 1:500 dilution in TBST and apply 100 μL antibody solution per slide (*see* **Note 21**).
10. Incubate at 4 °C overnight.
11. Apply 2 mL of TBST twice for wash.
12. In case that the primary antibody is a rabbit antibody, apply 100 μL of EnVision™ and incubate for 1 h.
13. Apply 2 mL of TBST twice for wash.
14. Mix 10 mM Tris–HCl (pH = 7.4), the DAB stock solution and hydrogen peroxide at the ratio of 1000:10:1 and apply. Brown color should be visible within 5 min. Proceed to the next step when sufficient color develops or 10 min passes.
15. Remove the glass slide from the cassette. Rinse in tap water briefly.
16. Counterstain with hematoxylin solution for 5 s (*see* **Note 22**).
17. Rinse in tap water and then in distilled water.
18. Dehydrate through 95% ethanol and then two changes of 100% ethanol for 5 min each.
19. Pass two changes of xylene for 5 min each.
20. Mount with mounting medium and a coverslip.

3.3 RNA Extraction from Bone Tissue

1. Sacrifice the mice by cervical dislocation at days 5, 10, and 15 after surgery.
2. Make an incision in the skin of the leg by scissors and peel off the skin. Dissect the femur by separating it at the femur head and at the knee joint using scissors. Remove the muscle as much as possible.
3. Trim the femur so that it involves the drill hole with 1 mm of proximal and distal margins using scissors.
4. Flush out the bone marrow briefly with 5 mL of normal saline using a syringe with an 18 G needle (*see* **Note 23**).
5. Put the femur into a 2 mL plastic tube. Place the homogenizing bullet on the tissue and close the lid tightly (Fig. 4). Sink the tube in liquid nitrogen to freeze.
6. Homogenization: Set the frozen tube in Multi Beads Shocker® (Fig. 4) and operate the machine with the setting of 2000 rpm, 15 s.
7. Pipet 1 mL of TRIzol® into the tube. Wash the wall of the tube and the homogenizing bullet by pipetting and transfer the tissue suspension to a new 1.5 mL tube.

8. Vortex for 10 s and incubate for 30 min at 4 °C.
9. Centrifuge at 14,000 × *g* at 4 °C for 1 min.
10. Transfer the supernatant to a clean 1.5 mL tube.
11. Centrifuge at 14,000 × *g* at 4 °C for 10 min.
12. Transfer the supernatant to a clean 1.5 mL tube.
13. Add 200 μL of chloroform. Vortex for 15 s and leave at room temperature for 3 min.
14. Centrifuge at 14,000 × *g* at 4 °C for 15 min.
15. Transfer the supernatant to a clean 1.5 mL tube. Take care not to let the intermediate layer contaminate into the upper aqueous layer.
16. Add 500 μL of isopropanol. Vortex for 15 s and leave at 4 °C for 15 min.
17. Centrifuge at 14,000 × *g* at 4 °C for 30 min.
18. Aspirate the supernatant.
19. Pipet 1 mL of 75% ethanol. Wash the wall of the tube by pipetting.
20. Centrifuge at 14,000 × *g* at 4 °C for 5 min. Aspirate the supernatant.
21. Spin down the liquid on the wall by brief centrifugation and remove the remaining 75% ethanol by a pipet. Air-dry the pellet for 5 min.
22. Pipet 100 μL of DEPC water.
23. Incubate the pellet at 60 °C for 10 min with occasional vortexing and spinning down.
24. Pipet up and down, confirming that the pellet has been completely dissolved. This solution is a crude RNA extract.
25. To the crude RNA extract, add the mixture of 600 μL of RA1 supplied in NucleoSpin® RNA and 6 μL of 2-mercaptoethanol.
26. Vortex for 10 s and spin down by brief centrifugation.
27. Add 600 μL of 70% ethanol. Vortex for 10 s.
28. Transfer 650 μL of the sample each to two adsorption columns.
29. Centrifuge at 10,000 × *g* at 4 °C for 30 s. Discard the flow-through.
30. Pipet 350 μL of the membrane desalting buffer. Centrifuge at 10,000 × *g* at 4 °C for 1 min.
31. Mix 90 μL of reaction buffer and 10 μL of DNase solution, and pipet the DNase reaction mixture to the membrane of the column. Leave for 15 min at room temperature.
32. Centrifuge at 10,000 × *g* at 4 °C for 30 s. Discard the flow-through.

33. Pipet 200 μL of RA2. Leave for 5 min at room temperature.
34. Centrifuge at 10,000 × *g* at 4 °C for 30 s. Discard the flow-through.
35. Pipet 600 μL of RA3.
36. Centrifuge at 10,000 × *g* at 4 °C for 30 s. Discard the flow-through.
37. Pipet 250 μL of RA3.
38. Centrifuge at 10,000 × *g* at 4 °C for 2 min. Place the column to a clean 1.5 mL tube.
39. Preheat RNase-free water at 60 °C and pipet 20 μL to the membrane of the column. Leave for 5 min at 60 °C.
40. Centrifuge at 10,000 × *g* at 4 °C for 1 min. Discard the column. Store the purified RNA solution at −80 °C until ready for analysis.

3.4 Protein Extraction from Bone Tissue

1. Sacrifice the mice by cervical dislocation at days 5, 10, and 15 after surgery.
2. Make an incision in the skin of the leg by scissors and peel off the skin. Dissect the femur by separating it at the femur head and at the knee joint using scissors. Remove the muscle as much as possible.
3. Flush out the bone marrow briefly with 5 mL of normal saline using a syringe with an 18 G needle.
4. Put the femur into a 2 mL plastic tube. Place the homogenizing bullet on the tissue and close the lid tightly (Fig. 4). Sink the tube in liquid nitrogen to freeze.
5. Homogenization: Set the frozen tube in Multi Beads Shocker® (Fig. 4) and operate the machine with the setting of 2000 rpm, 15 s.
6. Pipet 200 μL of the Laemmli buffer into the tube. Wash the wall of the tube and the homogenizing bullet by pipetting and transfer the tissue suspension to a new 1.5 mL tube.
7. Incubate the mixture on ice for 1 h with occasional vortexing and spinning down.
8. Transfer the lysates to a clean 1.5 mL tube.
9. Clear the lysates by centrifugation at 14,000 × *g* at 4 °C for 10 min.
10. Transfer the supernatant to a clean 1.5 mL tube.
11. Store the bone tissue protein extract at −80 °C until ready for analysis.

3.5 Primary Culture of Osteoblastic Cells from the Calvariae of Neonatal Mice

1. Prepare Hank's-based reagents as described below.

   H1: Hanks 50 mL + Antibiotics 500 μL.

   H2: Hanks 30 mL + Antibiotics 600 μL.

Hanks/10% FBS: Hanks 45 mL + FBS 5 mL.

2. Setup for dissection: Prepare the reagents and instruments in the laminar flow cabinet as described below.

   30 mL of 70% ethanol in a 100 mL beaker.

   30 mL of H2 in a 100 mL beaker.

   3 mL of H1 in two 3.5 cm dishes (referred to H1a; for provisional storage of dissected calvariae, and H1b; for provisional storage of dissected parietal bones) on ice.

   Scissors.

   Forceps.

3. Take siblings of 1-day-old infants (5–7 pups on average in case of C57BL/6).
4. Anesthetize the pups by burying them into crush ice and wait for 5 min.
5. Sterilize the pup briefly in 70% ethanol.
6. Rinse the pup briefly in H2.
7. Make an incision at the nuchal skin by scissors and peel the skin of head using forceps to the level of eyes. Decapitate by scissors. Pierce the forceps into the nostril to hold the head and dissect the calvaria by scissors along the dashed line indicated in Fig. 6, taking care not to touch the parietal bone.
8. Place the dissected calvaria in H1a on ice.
9. Dissect the calvariae from all the infants.
10. Under a stereomicroscope, carefully dissect the parietal bones by scissors along the dashed line indicated in Fig. 6 and collect them in H1b on ice.
11. Place the dissected parietal bones onto the wall of 50 mL conical tube. Pour half of the remaining H1 (approximately 22 + 22 mL) into the tube and rinse the parietal bones with brief and gentle shaking. Aspirate H1 and repeat the rinse.
12. Preheat the collagenase/dispase solution at 37 °C.
13. Pipet 5 mL of the collagenase/dispase solution into the tube. Close the lid tightly and seal the lid with Parafilm®. Incubate at 37 °C with gentle shake for 5 min.
14. Aspirate and discard this first round of cell suspension (*see* **Note 24**).
15. Pipet 5 mL of the collagenase/dispase solution into the tube. Close the lid tightly and seal the lid with Parafilm®. Incubate at 37 °C with gentle shake for 10 min.
16. Collect the cell suspension and transfer to a new 50 mL conical tube containing 20 mL of Hanks/10% FBS on ice.
17. Repeat **steps 16** and **17** three more times. Eventually you will have collected about 20 mL cell suspension from four rounds

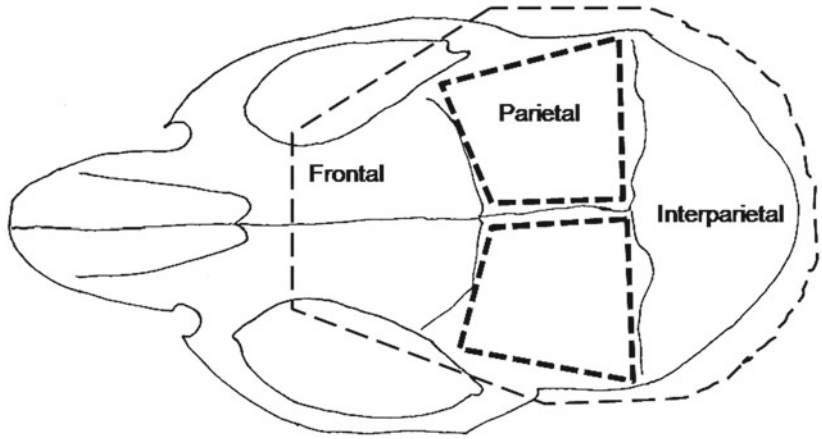

Fig. 6 Incision line to dissect parietal bones

of digestion with the collagenase/dispase solution in a 50 mL conical tube containing 20 mL of Hanks/10% FBS.

18. Filtrate the cell suspension using Falcon® 70 μm Cell Strainer. Collect the flow-through in a 50 mL conical tube.
19. Divide the cell suspension into four 15 mL conical tubes and centrifuge at 500 × *g* for 5 min at room temperature.
20. Discard the supernatant, taking care not to aspirate the cell pellets.
21. Resuspend the cells in 5 mL of αMEM/10% FBS. Centrifuge at 500 × *g* for 5 min at room temperature.
22. Discard the supernatant, taking care not to aspirate the cell pellets.
23. Resuspend the cells in 4 mL of αMEM/10% FBS and collect them into one 15 mL conical tube.
24. Count the number of cells using hemocytometer.
25. Spread the cell suspension on a 6 cm dish.
26. On the next day, change the medium (αMEM + 10% FBS) and continue the culture until ready for experiments.

4 Notes

1. Bend a paper clip using pliers.
2. The plastic tube may become fragile following freezing and therefore, the tube wall may crack because of impacts by the homogenizing bullet. It may be necessary to find durable, impact-resistant tubes that can be used at these temperatures. Note that if cracks do appear in the tube wall, the tissue samples can still be used for mRNA and protein extraction.

3. We have used TRIzol® (Thermo Fisher Scientific, Waltham, MA, USA) and NucleoSpin® RNA (Takara, Shiga, Japan) with successes.
4. Any trace of DEPC must be removed, as DEPC can interfere with enzymatic reactions. If the fruity smell of DEPC is detectable, additional autoclaving is recommended. We observed in our lab that Milli-Q® water can be used for RNA extraction without any sample degradation, but the usage of DEPC-treated water is at the researcher's discretion.
5. The periosteum induces cartilage formation and endochondral ossification. Removal of the periosteum affects bone regeneration solely by membranous ossification, providing a steady model for membranous bone formation.
6. A self-made ruler (Fig. 3) is useful to keep the positioning of the drill holes constant.
7. It is not recommended to skip the 0.8 mm pilot hole and go directly to a 1.2 mm hole, as it can cause fractures.
8. Wash the bone fragments thoroughly.
9. These are to facilitate permeation of fixation and decalcification solutions to the tissue. It is not necessary to remove all the muscle.
10. Do not cut in the center of the drill hole, as it can break the fragile regenerating tissue. The centering will be done during sectioning.
11. If the femur can not be smoothly cut with a razor blade, decalcify for three more days.
12. Each incubation time can be lengthened. An automatic tissue processor is convenient.
13. Heat the forceps for 2–3 s.
14. This must be done quickly before the paraffin solidifies. Commercially available embedding centers that can warm the embedding mold and forceps are convenient. If it is unavailable, and the researcher experiences difficulty handling the tissue and paraffin in the mold, it is recommended to pour melted paraffin in the mold, place the tissue in the paraffin, and leave them in the incubator at 60 °C for 1–2 h. Adjust the location and orientation of the femur in the incubator, using pre-warmed forceps. Once it is correctly adjusted, gently remove the mold from the incubator, taking care not to disturb the melted paraffin, and place the mold on a table to let it solidify at room temperature.
15. Trim the tissue until the desired sections appear. Check the sections under a microscope.

16. Immunostainability of cut sections diminishes even when sections are stored at –20 °C. Tissues embedded in paraffin block are more suitable for long-term preservation of stainability, although it still decreases over time. We found that the best strategy is to cut and stain the tissues as soon as possible.
17. Reagents for hematoxylin and eosin stain can be stored at room temperature and reused many times.
18. Shake several times to aid diffusion.
19. Do not allow running water directly on the glass slides, as it may cause the sections to detach.
20. Since bone sections can readily detach from glass slides, heat-mediated antigen retrieval in standard microwave ovens or autoclaves that boil water should not be used.
21. Antibodies raised in animals other than mice are recommended, although some antibodies raised in mice can stain mouse tissue exceptionally well. Optimal antibodies to detect specific proteins are to be empirically determined. In our experience, commercially available rabbit monoclonal antibodies are an appropriate choice. In addition, dilution factors should also be determined empirically; we recommend an initial dilution of 1:500.
22. Do not overstain because it may make observations difficult. In instances of weak immunohistochemical staining, it is recommended to omit counterstaining.
23. This procedure reduces the number of hematopoietic cells, resulting in relative enrichment of regenerating tissue. Insert the needle into the cut end of the bone marrow and inject normal saline. Do it gently, taking care not to dislodge the regenerating tissue.
24. This step removes fibroblasts and enriches for osteoblasts.

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Chapter 28

Design and Analysis of CCN Gene Activity Using CCN Knockout Mice Containing LacZ Reporters

Jie Jiang, Zhengshan Hu, and Karen M. Lyons

Abstract

Two developments have greatly facilitated the construction of CCN mutant mouse strains. The first is the availability of modified embryonic stem (ES) cells and mice developed through several large-scale government-sponsored research programs. The second is the advent of CRISPR/Cas9 technology. In this chapter, we describe the available mouse strains generated by gene targeting techniques and the CCN targeting vectors and genetically modified ES cells that are available for the generation of CCN mutant mice. Many of these mutant mouse lines and ES cells carry a β-galactosidase reporter that can be used to track CCN expression, facilitating phenotypic analysis and revealing new sites of CCN action. Therefore, we also describe a method for β-galactosidase staining.

Key words Gene targeting, Homologous recombination, Cre recombinase, LoxP, β-galactosidase, International Mouse Phenotyping Consortium (IMPC), Embryonic stem (ES) cells

1 Introduction

Advancements in recombinant DNA technology and embryology have made genetically modified animals among the most useful tools in biomedical research. These genetically engineered models include transgenic mice in which exogenous DNA is introduced into the genome through nonhomologous recombination; these have been utilized most commonly to overexpress or misexpress a gene of interest. Alternatively, gene replacement models, generated through homologous recombination, have been used most commonly to generate loss-of-function alleles, missense (point) mutations, insert a reporter sequence under the control of the targeted gene, or generate a modified allele whose activity can be controlled in a temporal and/or spatially specific manner. This chapter focuses on animal models generated by homologous recombination to target endogenous CCN alleles.

In the context of the CCN family of matricellular proteins, almost all genetically engineered animal models have been

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developed in the mouse. The mouse is comparable to human with respect to tissues, organ systems, physiological systems, and even many behavioral traits. Additionally, the mouse is the lowest phylogenetic model in which it is possible to study many of the developmental and disease processes in which CCNs are involved. However, it is important to bear in mind that there are significant differences in CCN function between mice and humans. A very clear demonstration of this concerns CCN6, where homozygosity for loss-of-function mutations in *CCN6* causes pseudorheumatoid dysplasia in humans [1]. In marked contrast, in the mouse, *Ccn6* is expression appears to be restricted to the testis, and *Ccn6* deficient mice exhibit no apparent phenotype [2]. Nonetheless, mouse models have been instrumental in uncovering CCN functions during development as well as in normal physiological and pathological processes in adults.

The currently available CCN mutant mouse strains have been produced using gene targeting technology. Specifically, predesigned DNA sequences, often containing a selectable marker, replace native coding sequences in the mouse genome using homologous recombination [3]. Mario Capecchi, Martin Evans, and Oliver Smithies were awarded the Nobel Prize in Medicine for developing techniques for genetic modifications in mouse embryonic stem (ES) cells [4]. Advancements in gene targeting technology have enabled the production of conditional knockout strains using the Cre-LoxP system. Several large-scale government-sponsored research programs that include: Knockout Mouse Project (KOMP; USA), EUCOMM (Europe), North American Conditional Mouse Mutagenesis Project (NorCOMM; Canada), and Texas A&M Institute for Genomic Medicine (TIGM; USA) have provided access to targeting vectors, ES cells or mice harboring a large number of mutated alleles. The alleles generated by these consortia are coordinated by the International Knockout Mouse Consortium (IKMC), which publishes on its website a searchable list of all available vectors, ES cell clones, and mice (http://www.knockoumouse.org). The IKMC is a component of the International Mouse Phenotyping Consortium (IMPC) (http://www.mousephenotype.org). The goal of the IMPC is to produce loss-of-function alleles for 20,000 known and predicted genes in the mouse genome. Some of these alleles have been created by gene targeting using vectors designed to target specific loci, and others have been generated through gene-trapping technologies. Modified ES cells obtained from these projects can greatly simplify and accelerate the process of generating a mutant mouse strain. Currently global knockouts of all 6 CCN members are available through these consortia, either as ES cells or mice (Table 1).

While global knockouts have been extremely useful to elucidating the roles of CCNs in vivo, there are some drawbacks. Specifically, loss of *Ccn1* or *Ccn2* results in prenatal or immediate

Table 1
Currently available CCN family knockout mice/ES cells

Gene	Available animals	Description	Reference
CCN1 (Cyr61)	Mouse	Global knockout	
		Gene targeting: exon 1 and part of exon 2 are replaced with a *LacZ-PGK-neo* cas	Mo et al. [5]
		Conditional knockout	
		1. Promoter through exon 2 are flanked by *loxP* sites	1. Kim et al. [16]
		2. Exon 2 is flanked by loxP sites	2. Liu et al. [17]
		Point mutation:	
		1. Exon 1 and half of exon 2 are replaced with Ccn1 mutant cDNA cassette where two alanine substitutions within the putative heparin-binding motif between aa 280–290 and aa 306–312. This mutation inhibits binding to integrin $\alpha_6\beta_1$	Chen et al. [29]
		2. Exon 1 and part of exon 2 are replaced with Ccn1 mutant cDNA cassette with D125A substitution in domain II. This mutation inhibits binding to integrin $\alpha_v\beta_3$	Jun et al. [30]
	ES cells	Global knockout:	
		Part of exon 1 through part of exon 5 is replaced with *LacZ*	KOMP (knockout mouse project) repository

(continued)

Table 1
(continued)

Gene	Available animals	Description	Reference
CCN2 (CTGF)	Mouse	Global knockout	
		Exon 1 is replaced by a *PGKneopA* Exon 3 through 5 is replaced by neo cassette	1. Ivkovic et al. (2003) [6] 2. Doherty et al. (2010) [31]
		Exon 3 through part of exon 5 is replaced by *LacZ-neo* cassette	3. Crawford, et al. (2009) [32]
		Conditional knockout	
		1. Exon 4 is flanked *loxP* site	1. Liu et al. [18]
		. 2. Promoter through exon 2 are flanked by *loxP* sites	2. Mangiavini et al. [20]
		3. A *COIN* comprises of *lox66_SA-Egpf-polyA_lox71* intron is inserted into the middle of exon 2. After Cre recombination inversion of the *COIN* element result in expression of exon 1, part of exon 2 and *COIN* (*Egfp*) element exon	3. Canalis et al. [19]
	ES cells	Global knockout	
		Exons 2 to 5 are replaced by a *LacZ-neo* cassette	KOMP repository
		Conditional knockout Exon 3 through 5 are flanked by *loxP* sites	KOMP repository
CCN3 (NOV)	Mouse	Global knockout Exons 1 through part of exon 3 were replaced with the *noe* cassette	Shimoyama et al. [24]
		Global knockdown: (*see* **Note 6**) Exon 3 is replaced with a *TKneo* cassette	Heath et al. [23]
CCN4 (WISP-1)	Mouse	Global knockout	
		1. An *PGK-neo* cassette is inserted in exon 2 that causes a frameshift 2. Exons 1 through 5 are replaced by a *LacZ-neo* cassette	1. Maeda et al. (2015) [33] 2. KOMP repository

CCN5 (WISP-2)	Mouse	Global knockout 1. Exons 2 through 5 are replaced with a *LacZ-neo* cassette	KOMP repository
	ES cells	Conditional knockout Part of exon3 is flanked by *loxP* sites (*see* Fig. 3)	KOMP repository
CCN6 (WISP-3)	Mouse	Global knockout Part of exon 2 through 5 are replaced by a *LacZ-neo* cassette	Kutz et al. (2005) [2]
	ES cells	Global knockout Exons 1 through 5 are replaced by a *LacZ-neo* cassette	KOMP repository

postnatal lethality. *Ccn1* global knockout mice exhibit early embryonic lethality with impaired placental angiogenesis [5]. *Ccn2* global knockouts are perinatal lethal due to defects in respiration resulting from severe malformations of the skeleton [6]. *Ccn2* knockouts also exhibit vascular remodeling defects [7]. Even though the early lethality of these models makes it challenging to assess CCN1 and CCN2 function in adults, analysis of mice heterozygous for these mutations has been informative. *Ccn1+/–* mice exhibit severe atrioventricular septal defects [8]. *Ccn2+/–* mice develop less severe fibrosis and kidney malfunction in STZ-induced diabetic nephropathy, but do not exhibit attenuated disease progression in a more severe kidney injury model [9, 10] (*see* **Note 1**). Secondly, even for global loss of function CCN strains that do not exhibit embryonic lethality (CCN 3–6), it can be challenging to ascertain particular cell types and stages of expression that are impacted to yield a specific phenotype.

This limitation has been overcome for several of the CCN family members through the generation of conditional mutant strains. Conditional gene inactivation was initially developed to achieve loss of function in a selected cell type [11, 12] using the Cre-LoxP system. In this system the target gene is modified by insertion of recombinase recognition (*loxP*) sites [12]. Conditional mutation is achieved by cell type-specific expression of the DNA recombinase Cre, resulting in the modification of the target gene harboring the *loxP* sites. This system can be used to generating mutated alleles in a cell-type-specific manner by expressing the Cre recombinase under the control of a tissue-specific promoter. Additional control can be exerted by regulating the activity of Cre recombinase. This is most commonly achieved using a Cre-ERT variant, a fusion protein comprised of Cre recombinase fused to a modified estrogen receptor; this fusion protein is excluded from the nucleus until the ER(T) module binds tamoxifen. Tamoxifen binding permits entry of the fusion protein into the nucleus, where Cre can catalyze recombination [13, 14]. Conditional knockout alleles have been developed for CCN1 and CCN2 (Table 1). The construction of conditional alleles for CCN 1–5 is in progress as a part of the IMPCT. The availability of conditional alleles for these CCN genes will be necessary to identify shared tissue-specific functions of CCN family members. The remaining chapter focuses on design considerations when developing CCN conditional mutant (floxed) mice. We also present a protocol for visualization of β-galactosidase (LacZ), as there are now mouse mutants or ES cells harboring LacZ insertions in each of the CCN genes. Analysis of patterns of LacZ expression in these models should reveal previously unknown sites of CCN action, and elucidate previously unknown cellular mechanisms of CCN action in known target tissues.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Store all reagents at room temperature unless otherwise indicated.

2.1 Preparation of Tissues and Embryos

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4. To prepare 1 L of 1× PBS pour 800 mL of double distilled water into a beaker. Add in order: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4. Adjust the pH to 7.4 with HCl (*see* **Note 2**). Add double distilled water to a total volume of 1 L.
2. Fixative: 0.2% glutaraldehyde, 0.02% NP-40, 0.5 mM EDTA, 2 mM $MgCl_2$; in PBS. To prepare 250 mL solution add 2 mL of 25% glutaraldehyde, 2.5 mL of 2% NP-40, 2.5 mL of 0.5 M EDTA, 0.5 mL of 1 M $MgCl_2$ in 244.5 mL of 1× PBS. Store at 4 °C. This reagent can be kept for several weeks.
3. 4% PFA: 4 g paraformaldehyde, add 1× PBS to a total of 100 mL. Dissolve by heating to 65 °C. Place on ice and use fresh. Fixative can be stored at −20 °C but freshly prepared is preferable.
4. Washing buffer: 2 mM $MgCl_2$; 0.01% 2 mL DOC; 10 mL of 0.02% NP-40; in PBS. To prepare 1 L of solution add: 2 mL of 1 M $MgCl_2$, 2 mL of 5% DOC, 10 mL of NP-40 to 986 mL of 1× PBS. Store at 4 °C. This reagent can be stored indefinitely at 4 °C (*see* **Note 3**).

2.2 X-Gal Staining Reagents

1. X-gal stock solution: 50 mg/mL X-gal. Prepare stock solution X- in dimethyl sulfoxide (DMSO) or dimethylformamide (DMF). Store at −20 °C (*see* **Note 4**).
2. X-Gal staining solution: 1 mg/mL X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 20 mM Tris (pH 7.4), in washing buffer. To make 200 mL add: 0.424 g potassium ferricyanide trihydrate, 0.328 g potassium ferricyanide and 4 mL of 1 M Tris (pH 7.4) in 192 mL of wash buffer. Add 4 mL of X-gal stock solution just prior to staining (*see* **Note 4**).

2.3 Sectioning Reagents

1. Graded ethanols: 50%, 75%, and 95% prepared using double-distilled H_2O.
2. 100% ethanol.
3. Xylene.
4. 1:1 xylene–paraffin wax, stored in an oven at 60 °C.
5. Paraffin wax (molten).
6. Nuclear fast red or eosin (optional).
7. Mounting medium .

2.4 Equipment

1. Dissection tools: forceps, scissors, tungsten dissecting needles. Plastic pipettes can be used for transferring tissues or embryos.
2. Containers for samples: Glass scintillation vials are ideal because their clarity makes it possible to visualize the progress of the staining protocol. However, smaller samples can be placed in 1.5 ml Eppendorf tubes or in individual wells of 24-well plates.
3. Aluminum foil or light-tight containers.
4. Rotary shaker.
5. 4 °C cold room.
6. 37 °C incubator or warm room.
7. Dissecting and light microscopes for imaging stained samples.
8. 60 °C oven for melting paraffin wax.
9. Microtome for sectioning paraffin-embedded tissues.
10. Cryostat for direct X-gal staining of sections.
11. Superfrost microscope slides.
12. Slide dryer.

3 Methods

3.1 Design of Vectors for Conditional Gene Targeting

The design of standard conditional knockout alleles involves two major considerations: (1) Inserting the *loxP* sites into regions of the target gene such that the encoded protein is inactivated through Cre-mediated excision; (2) The presence of the *loxP* sites should not disrupt the targeted gene prior to Cre-mediated recombination. There are several approaches to designing and obtaining conditional CCN alleles. The first approach involves self-designed vectors based on cloning or PCR amplification of the vector homology arms. The second approach relies on ready-made targeting vectors that are pre-designed to yield conditional alleles, available through IMPC. The third approach uses the new CRISPR/Cas9 system for direct genome modification; this system can be used to generate conditional alleles directly in zygotes without the need to generate ES through direct injection of CRISPR/Cas9 nucleases [15].

3.2 Self-Designed Conditional Alleles

Traditional gene targeting methods have been used to generate the majority of currently available CCN mutant mice. The basic design schematic for this method is shown in Fig. 1. The major drawbacks include time-consuming vector construction, labor-intensive intermediate steps to generate mutant mice, and high costs associated with mouse breeding steps to obtain germ line transmission of the targeted allele. With the advancement of the CRSPR/Cas9 system of genomic engineering, the traditional homologous recombination method to produce loss-of-function animals is starting to be phased out. Due to this shift in technology, this chapter does not

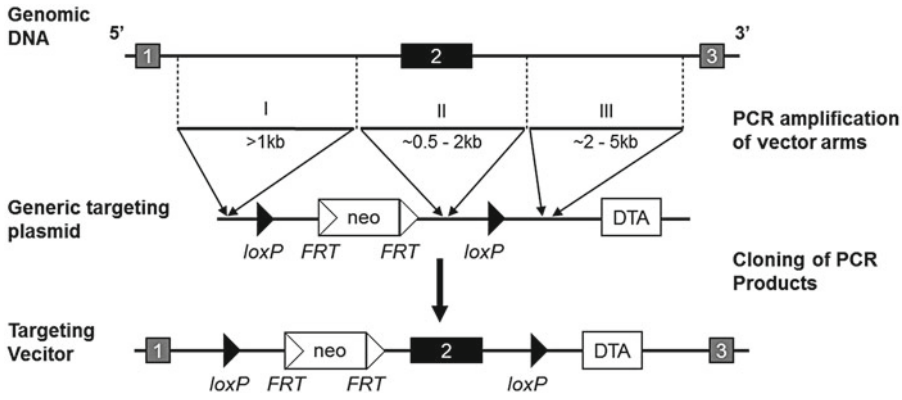

Fig. 1 The basic design schematic for PCR-based assembly of a vector for conditional gene targeting. A hypothetical target gene that contains three exons (*numbered rectangles*) is shown at the *top*. The targeting vector is assembled by PCR amplification of the genomic segments, (*I*) the 5′ homology region, (*II*) the *loxP*-flanked region, and (*III*) the 3′ homology region. *Thin dashed lines* connecting the targeting vector and genomic locus indicate the locations of insertion of the *loxP* sites and an *FRT*-flanked positive selection marker. The PCR primers must include restriction sites that are compatible to sites within the generic targeting plasmid. The final targeting vector contains a *FRT*-flanked neomycin resistance gene (neo) and the second exon is flanked by *loxP* sites. The diphtheria toxin expression cassette (DTA) or an HSV-thymidine kinase cassette allows for negative selection of ES cell clones carrying random integrations of the vector [3]

discuss in detail the older methods used to generate targeting vectors and mutant ES cells mentioned in this chapter. Detailed methods can be found in the following references [3].

3.3 Conditional Alleles Generated by Large-Scale Mutagenesis Programs

As discussed above, several large-scale research programs have been launched in the past decade in order to generate public libraries of mutated mouse ES cells for over 20,000 genes. These efforts are coordinated by the International Knockout Mouse Consortium (IKMC), which publishes on its website a searchable list of available vectors. Figure 2 shows typical IKMC vector design and targeting strategies for the generation of conditional alleles. The key design feature used by the EUCOMM/KOMP consortia for conditional alleles is the identification of the "critical exon," which will be targeted for Cre excision. The critical exon is established by four criteria, and these should be applied when self-designing vectors to generate CCN conditional alleles.

1. Cre-induced deletion results in a frameshift in the coding sequence, leading to a nonfunctional allele and/or nonsense mediated mRNA decay.
2. The exon is present in all transcript isoforms of the targeted gene.
3. The size of the exon is ideally below 1 kb to assure that the distance of the *loxP* sites is short enough for efficient Cre recombination.

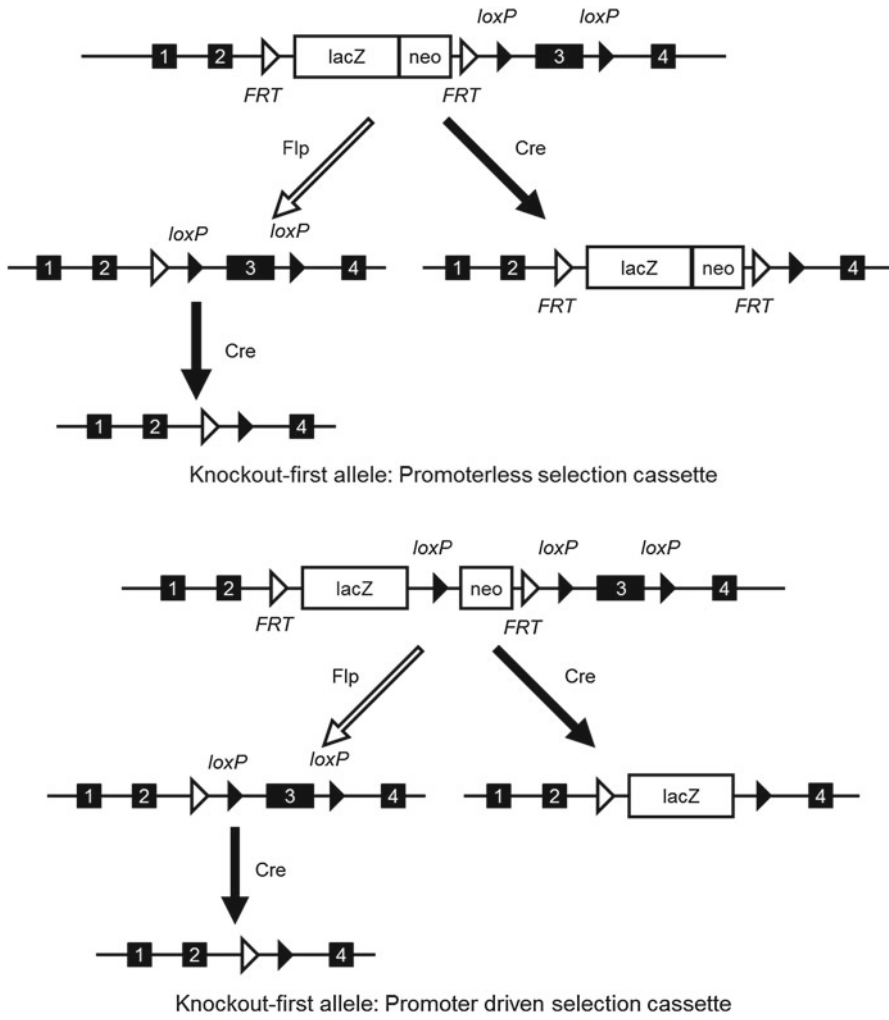

Fig. 2 Schematic of knockout-first alleles generated by IKMC. The targeting strategies used by IKMC rely on the identification of a "critical" exon. The generation of the knockout-first allele is flexible and can produce reporter knockouts, conditional knockouts, and null alleles following exposure to site-specific recombinases Cre and Flp [28]

4. The size of the flanking introns is at least 0.5 kb, so that *loxP* sites can be placed in non-conserved regions that are not required for endogenous splicing.

Figure 3 shows all of the vectors currently available from IKMC that target CCN genes (*see* **Note 5**). The generation of ES cells targeted with these vectors is in progress. The IKMC database is updated regularly to indicate the status of each project to generate specific conditional mutant strains. In addition to generating ES cells with the conditional vectors in Fig. 3, the IKMC program has generated constitutive knockout as well as BAC-mediated reporter mice.

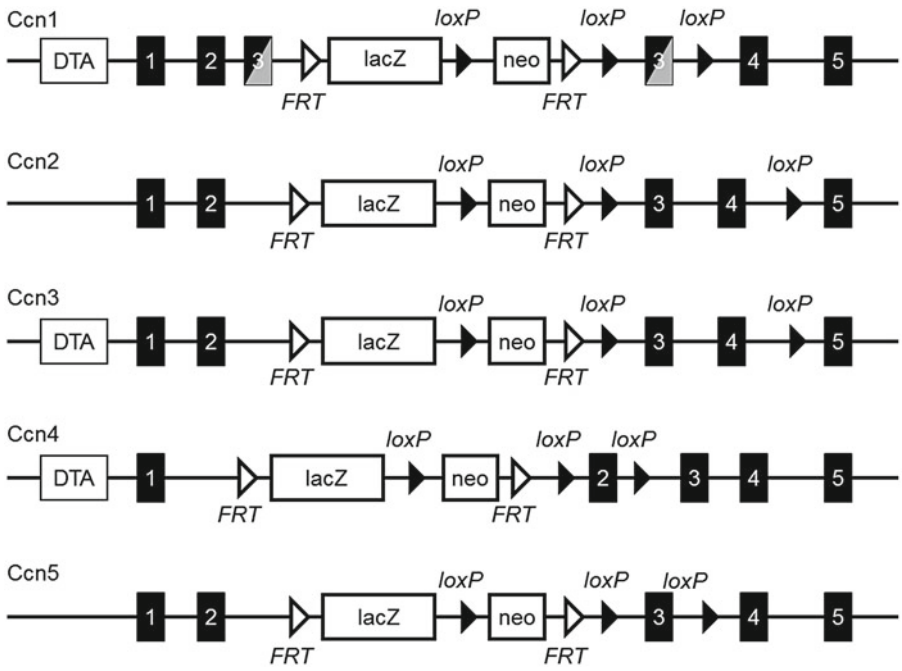

Fig. 3 IKMC-designed CCN knockout-first targeting vectors available to investigators. For *Ccn1*, An artificial intron is inserted into the middle of exon 3. A lacZ-LoxP-neo cassette flanked by two *FRT* sites is inserted into the artificial intron. The 3′ half of exon 3 and the neo are flanked by *loxP* sites. Flp-mediated recombination generates a conditional allele in which exon 3 is flanked by *loxP* sites (SD Int: Splice Donator Ifitm2 Intron; Int SA: Intron Splice Acceptor). For *Ccn2* and *Ccn3*, a lacZ-LoxP-neo cassette flanked by *FRT* sites is placed between exons 2 and 3. Exons 3 and 4 and neo are flanked by *loxP* sites. Flp-mediated recombination generates a conditional allele in which exons 3 and 4 are flanked by *loxP* sites. For *Ccn4*, a lacZ-LoxP-neo cassette flanked by two *FRT* sites is placed between exons 1 and 2. Exon 2 is flanked by *loxP* sites. Flp-mediated recombination generates a conditional allele in which exon 2 is flanked by *loxP* sites. For *Ccn5* (a), a lacZ-LoxP-neo cassette flanked by two *FRT* sites is placed between exons 2 and 3. Exon 3 is flanked by *loxP* sites (promoter-driven selection cassette). Flp-mediated recombination generates a conditional allele in which exon 3 is flanked by *loxP* sites. ES cells in which this vector has been integrated can be obtained through EUMMCR. For *Ccn5* (b), A lacZ-neo cassette flanked by two *FRT* sites is placed between exons 2 and 3. Exon 3 is flanked by *loxP* sites (promoterless selection cassette). Flp-mediated recombination generates a conditional allele in which exon 3 is flanked by *loxP* sites. For each *Ccn* allele, a diphtheria toxin cassette (DTA) is placed outside of the arms of homology to permit selection against randomly inserted vectors. For each *Ccn* allele, Cre-mediated recombination generates loss-of-function alleles containing lacZ reporters. These vector maps were obtained from IKMC website (http://www.mousephenotype.org/)

3.4 CRISPR/Cas9 Genomic Engineering

In the last 5 years the advancement of CRISPR/Cas9 technology has revolutionized genomic engineering. CRISPR/Cas9 is a genome editing technique in which the generation of 20-nt guide sequences determines the site of genomic editing. Error-prone DNA repair mechanisms can be exploited to introduce insertions or deletions. The technology can also be used to generate specific point mutations or conditional alleles. When inserting *loxP* sites, the modified nucleotide sequence used to generate conditional

alleles requires ~60 bp homology on each arm of the insert. In addition to the efficiency of the technique, CRISPR/Cas9 guide sequences can be injected into the zygote (rather than in ES cells) to directly generate mutant offspring. Thus gene-modified mice can be produced in as few as 4 weeks, compared with homologous recombination using ES cells, which typically requires 9–12 months [15]. Detailed methodology for generating mutant animals using CRISPR/Cas9 is evolving but can be found in Yang et al. [15]. Additionally, there are many core facilities and commercial groups that cost-effectively generate mouse models using CRISPR/Cas9 technology.

3.5 Specific Strategies for Knocking Out CCNs

Regardless of the method used to insert the *loxP* site into the genome, it is essential to consider the nature of the genetic alteration following Cre-recombination. As discussed above, IKMC has recommended identifying a "critical exon" and has outlined criteria for the ideal placement of *loxP* sites. Due to the unique structure of genes within the CCN family, these criteria often cannot all be satisfied. CCNs share a similar modular structure, and each exon generally encodes one of the modular domains of the protein. Additionally, most of the exons are in frame, such that deletion of one or multiple exons generally does not result in a frame shift in the remaining coding sequence, which can retain multiple functional modules (*see* **Note 6**). Lastly, introns within CCN genes are often small, which cannot satisfy the criteria of the *loxP* sites being be flanked by at least 0.5 kb of intronic sequence.

Thus far mice carrying floxed alleles have been generated for *Ccn1* and *Ccn2* (two for *Ccn1* and three for *Ccn2*). In the original floxed *Ccn1* strain, developed in the laboratory of Dr. Lester Lau, *loxP* sites flank the promoter regions through exon 2 [16]. A second floxed *Ccn1* allele was generated in the laboratory of Dr. Andrew Leask. In this allele, exon 2 was flanked by *loxP* sites [17]. In both cases, Cre-mediated excision leads to loss-of-function. Three conditional *Ccn2* alleles have been generated thus far. A conditional *Ccn2* allele was generated by flanking exon 4 of *Ccn2* [18] with *loxP* sites. A second *Ccn2* allele was reported in Canilis et al. [19]. This allele contains a *COIN* element in the middle of exon 2. The *COIN* element creates an introduced intron within exon 2, In this case, the *loxP* elements are oriented such that Cre-mediated recombination drives inversion of sequences flanked by the *loxP* sites rather than excision. Thus, after Cre recombination, inversion of the *COIN* element results in expression of a transcript containing exon 1 part of exon 2 and an inserted *Egfp* reporter [19]. A third conditional *Ccn2* allele was generated by inserting *loxP* sites flanking the promoter region through exon 2 [20] (*see* **Note 7**). Thus, two of the five floxed strains discussed above were generated by inserting *loxP* sites to flank the promoter region, transcription start site, and exons 1 and 2 [16, 20]. The insertion

of *loxP* sites in the proximal promoter region is generally not recommended unless it can be verified that the proposed alteration does not change native gene expression. On the other hand, this strategy led to complete loss-of-function at the transcriptional level, and thus there are no concerns that Cre-mediated recombination leads to a truncated CCN protein that possesses residual or novel activities in these strains. When inserting *loxP* sites into the promoter regions of any gene, the *loxP* sites should be sufficiently removed from any known enhancer sequences so as not to disrupt the normal expression of the gene. At a minimum, a multi-species sequence comparison should be performed to find an area for *loxP* insertion that shows no phylogenetic conservation.

3.6 Conditional Knockout and Reporter Mice

As mentioned in the Introduction, the inactivation of the target gene can be restricted to a selected cell type without temporal control, or can be induced at a chosen time point using *Cre-loxP* systems. In a simple conditional knockout, mice carrying the floxed *Ccn* allele are bred to mice transgenic for an expression cassette driving Cre recombinase under the control of a cell type specific promoter. To achieve temporal control, a fusion protein of Cre and a mutant estrogen receptor ligand-binding domain (*Cre-ERT*) can be expressed from a cell type-specific promoter. The recombinase activity can be induced in vivo by the administration of the small molecule inducer tamoxifen [13, 14].

For the generation of conditional *Ccn* mutants, two types of mouse strains are thus required. The floxed *Ccn* strain harboring the *loxP*-flanked gene segment and the strain providing the Cre recombinase gene expressed from a cell type-specific promoter region. The generation and design of Cre strains is beyond the scope of this chapter. Currently there are over 1500 Cre expressing mouse strains that enable conditional mutagenesis in a variety of cell types and tissues. A listing of all published Cre lines can be obtained through Jackson Mouse Genome Informatics website (www.informatics.jax.org).

For most of the published Cre lines, the recombinase expression profile has been well characterized. However, for any experiment involving conditional knockouts, Cre reporter strains are highly recommended to visualize the specific cell populations in which the target gene is knocked out. As Cre recombination is an irreversible event when using standard floxed *Ccn* alleles, the use of these strains tracks the cells in which the excision originally took place, and all targeted descendants, regardless of whether the descendant cells continue to express Cre recombinase. Cre reporter alleles are available for the detailed characterization of recombinase expression profiles. Reporter alleles usually contain a *loxP*-flanked DNA segment that initially prevents the expression of the reporter gene. The most widely used indicator strains are constructed as knockin alleles into the widely expressed Rosa26 locus. A list of

Table 2
R26 Cre reporter mice

Reporter name (Jackson)	Reporter prior recombination	Reporter upon recombination	References
R26-LacZ	None	β-galactosidase	Soriano [34]
ROSA26-EGFP	None	EGFP	Mao et al. [35]
Rosa-YFP	None	EYFP	Srinivas et al. [36]
ROSA26-Fluc	None	Luciferase	Safran et al. [37]
Rosa-RFP	none	tdRFP	Luche et al. [38]
mT/mG	tdTomato	GFP	Muzumdar et al. [39]
R26R-Confetti	None	CFP/GFP/RFP/YFP (one of the four colors is expressed after recombination)	Snippert et al. [40]
R26R-GR	None	EGFP (nucleus), mCherry (membrane)	Chen et al. [41]
ROSA$^{nT\text{-}nG}$	tdTomato (nucleus)	GFP (nucleus)	Prigge et al. [42]

most widely used reporter strains is presented in Table 2 and is available through the Jackson Laboratories website. One of the earliest reporter strains is the Rosa-LacZ strain. The expression of β-galactosidase reporter can be monitored in tissue sections using X-Gal stain. Moreover, many of the *Ccn* strains listed in Table 1, particular those available through KOMP, contain LacZ expression cassettes that enable visualization of *Ccn* gene expression. This feature enables the identification of sources of CCN expression that can greatly facilitate phenotypic analysis, and can uncover novel sites of CCN action (*see* **Note 8**). Given the availability of these stains and the importance of lineage tracing in inferring phenotype, we provide a protocol for the detection of β-galactosidase activity.

3.7 X Gal-Staining

The protocol described below is designed for visualizing β-galactosidase activity in limbs of adult mice. Detailed protocols are available for X-gal staining of embryos and some other adult tissues [21]

3.7.1 Tissue Preparation

1. Euthanize Mice.
2. Remove the skin using scissors, and dissect out the limbs with muscle still attached. Place the tissues in ice-cold PBS (*see* **Note 9**).
3. Place the limb in a glass scintillation vial and rinse with 1× PBS multiple times until the solution is clear of blood and tissue debris.

3.7.2 Whole Mount Staining

1. Fix limbs in Fixative for 1 h in room temperature. For alternative fixation strategies, *see* **Note 10**.
2. Decant or aspirate the fixative, and wash the limbs in washing buffer three times (20 min each) at 4 °C.
3. During the final wash step, add the X-gal Stock Solution to the X-gal staining solution. Discard the washing buffer, and add X-gal staining solution. Cover the vials with aluminum foil, and incubate overnight at 37 °C in the dark (*see* **Note 11**).
4. Monitor the progress of the staining reaction by examining the samples for blue stain using a dissecting microscope. If the stain is weak, incubation in fresh X-gal staining solution might improve staining intensity (*see* **Note 12**).
5. Decant or aspirate off the staining solution, and wash the samples in 1× PBS for 5 min. Follow with two washes in 1× PBS for 20 min each. Samples should be kept in foil or other dark conditions during this process because residual X-gal may be present.
6. Fix the stain in the samples by incubation in 4% PFA for 1 h at room temperature, or overnight at 4 °C on a shaker. Samples should be kept in foil or other dark conditions during this process.
7. Wash in 1× PBS for 30 min. Stain should be fixed at this point and samples can now be processed exposed to light.
8. When visualizing X-gal in hard tissues such as bone, the samples should be decalcified in 19% EDTA (pH 7.2) in 4 °C after fixation. Replace with fresh EDTA solution every 2–3 days. Adult limb samples should be decalcified within 2 weeks (*see* **Note 13**).
9. Samples can be imaged under a microscope or processed for sectioning as described below.

3.7.3 Sectioning of Paraffin-Embedded X-Gal-Stained Samples

10. Wash samples three times (20 min each) in 1× PBS at room temperature.
11. Serially dehydrate by incubating the samples in: 50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH, 100% EtOH; 1 h each at room temperature.
12. Incubate in 100% EtOH overnight at room temperature.
13. Clear samples by incubation in 1:1 EtOH–xylene for 1 h at room temperature (*see* **Note 14**).
14. Incubate twice in 100% xylene (perform each incubation for 1 h at room temperature) (*see* **Notes 14** and **15**).
15. Infiltrate the samples in paraffin by incubation at 60 °C for 1 h each in 1:1 xylene–paraffin, 100% paraffin, 100% paraffin.
16. Incubate in 100% paraffin overnight at 60 °C.

17. Embed in cassettes in the desired orientation and section samples at 5–10 μm with a microtome.
18. Mount onto slides.
19. Deparaffinize sections with 100% xylene two times for 5 min each
20. Counter-stain with Eosin or nuclear fast red (optional).
21. Coverslip.
22. Visualize under a microscope to examine X-gal staining in more detail (*see* **Note 16**).

3.7.4 X-Gal STAINING of Cryo-embedded Sections

For detailed analysis of cellular staining patterns, X-gal can be visualized directly on cryo-embedded sections without prior fixation.

1. After sectioning and mounting the tissues in the cryostat, let slides air-dry.
2. Wash with 1× PBS for 5 min at room temperature.
3. Fix with Fixative for 15 min at room temperature.
4. Wash with washing buffer three times, 15 min each at room temperature.
5. Place slides in a slide mailer and add X-gal Staining Solution to cover the slides.
6. Stain overnight at 37 °C in the dark (*see* **Note 11**).
7. Wash in 1× PBS for 5 min. Repeat twice. These steps should be performed in the dark as residual X-gal may be present.
8. Post fix in 4% PFA by incubation at room temperature for 10 min to 1 h.
9. Wash 3 times in PBS for 5 min each.
10. The samples can be counter-stained with eosin or nuclear fast red, or directly mounted for visualization.

4 Notes

1. Given that haploinsufficiency has been reported for multiple CCN loss-of-function mutants, any phenotypic analysis of *Ccn* mutant strains should include both WT (*Ccn*+/+) and heterozygous (*Ccn*+/–) controls.
2. pH 7.4 is optimal for bacterial β-galactosidase (LacZ) activity. A lower pH is likely to lead to increased detection of endogenous β-galactosidase activity.
3. Generally, we recommend using approximately seven times the volume of the tissue for fixing, washing and staining the tissue. We use approximately 1 ml solution for individual embryos up to E13.5; 1.5–2 ml solution for older or pooled embryos.

3–5 ml of each solution is used if washing/staining is carried out in a 10-ml scintillation vial.

4. This reagent is light-sensitive and should be stored at 4 °C in a container wrapped in aluminum foil.
5. Some available ES cell lines (which can be found by searching the Jackson Laboratories Mouse Genome informatics Database) contain modified *Ccn* alleles generated by gene-trapping techniques, where the design features in Subheading 3.3 were not taken into consideration. It is thus essential that careful analysis of levels of both mRNA and protein expression of the targeted CCN is performed, along with analysis of levels of potential truncated proteins that might retain some biological activities, or even exhibit new biological activities. Residual activity due to the presence of a truncated protein is not expected to be a complication for the *Ccn* alleles available in ES cells listed in Table 1 through the KOMP repository, as the majority of the *Ccn* coding sequences have been replaced by LacZ in these lines.
6. As discussed above, the members of the CCN family have unique protein and genomic structures that require special consideration and validation when designing a new knockout mouse [22]. As an example, Heath et al. designed a mutant allele whereby exon 3 of *Ccn3* is deleted [23]. Later evidence showed *Novdel3–/–* mice produce a mutant form of the CCN3 protein that is secreted and lacks the von Willebrand factor (VWC) domain, but contains other intact functional domains of CCN3. The phenotype observed for $Nov^{del3-/-}$, is quite distinct from *Ccn3* knockout mice produce later by Shimoyama et al., where 127 amino acids were deleted from the N-terminal end [24]. While the mouse produced by Shimoyama et al. still produces a protein, the resulting peptide is not secreted due to the absence of the secretory sequence.
7. The two floxed strains for *Ccn1* and the three strains for *Ccn2* appear to yield similar loss of-function phenotypes, but direct comparisons have not been made. It is thus of importance to characterize both the timing and efficiency of Cre-mediated excision. Small differences in the kinetics and/or efficiency of Cre-mediated excision in comparisons of different floxed strains for a single gene can lead to major differences in phenotype. This concern is most relevant to developmental phenotypes where differences in timing of excision can be critical.
8. Introduction of reporter cassettes into a gene can lead to lower levels of transcript expression from the modified allele. Thus, it is conceivable that the strains produced by the IMPC project may have hypomorphic phenotypes even in the absence of Cre. Thus, expression levels of the WT and modified alleles should be quantified in the tissue of interest using qRT-PCR to distinguish transcripts from each allele.

9. It is important to include a negative control, ideally a littermate that does not carry a LacZ insertion, because some tissues exhibit high levels of endogenous β-galactosidase activity. A positive control is very useful when examine the expression of a LacZ reporter allele for the first time. Positive controls can be any strain where the pattern of LacZ expression is already known.
10. The method of fixation for a particular tissue should be similar to that typically used for routine histological examination of the tissue. If a tissue is normally fixed through perfusion (e.g., brain) then this should also be done for X-gal visualization. After perfusion, tissues should be post-fixed at 4 °C overnight. Of all the fixatives, glutaraldehyde has been shown to have the least effect on β-galactosidase activity [25]. However, as glutaraldehyde can interfere with immunohistochemistry, paraformaldehyde (PFA) can be used to fix the tissue. However, exposure should be for the shortest amount of time needed to achieve fixation. Other protocols have suggested using 4% PFA at 4 °C for 1–4 h, but in our experience, this might lead to over-fixation. We recommend fixation with 0.4% PFA at 4 °C overnight when using PFA.
11. The staining reaction must be performed in the dark because X-gal is light-sensitive. Incubation must be at 37 °C because this is the temperature at which β-galactosidase exhibits its optimal activity. For maximum staining, the reaction should be carried out overnight.
12. A positive control strain is very helpful for ascertaining if a lack of staining or low intensity staining is due to the nature of the locus or to a technical issue with a reagent. Many well-characterized reporter strains are available from the Jackson Laboratory, and the appropriate choice will depend on the tissue of most interest.
13. The time required for decalcification depends on the amount of mineralized tissue present in the sample. Adequately decalcified bones are able to be bent by applying gentle pressure with forceps. After decalcification, samples can either be processed for whole mount staining or cryo-embedded for sectioning.
14. Xylene is toxic and volatile. Perform these incubations in a fume hood.
15. Orange oil based products can provide good clearing action with less toxicity than xylene. It is important to use a pure and stabilized product such as Histo-Clear, because orange oil breakdown products can interfere with staining procedures.
16. Special consideration is needed when visualizing X-gal staining of bone. Osteoclasts exhibit high levels of β-galactosidase activity and are naturally acidic. Thus, all X-gal staining protocols

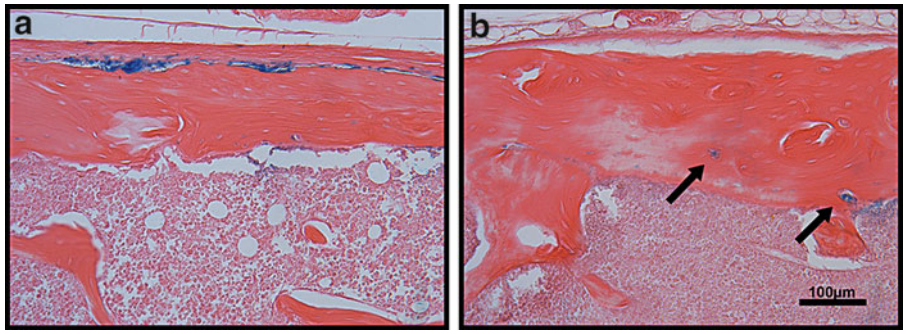

Fig. 4 Sample of X-gal staining for β-galactosidase. (**a**) *Ccn4+/LacZ* adult tibia showing X-gal positive cells in the periosteum. (**b**) Wild type *Ccn4+/+* adult tibia showing background X-gal staining in osteoclasts (*arrow*) due to high endogenous β-galactosidase activity in these cells

detect endogenous activity in these cells [26, 27]. The best control for endogenous β-galactosidase activity is comparison to a negative control sample that does not contain a LacZ cassette (Fig. 4).

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Chapter 29

Analysis of CCN4 Function in Osteogenic and Osteoclastic Cells Using Gain and Loss of Function Approaches

Azusa Maeda, Marian Young, and Mitsuaki Ono

Abstract

Analysis of CCN4 function in bone was assessed using both gain and loss of function approaches. In mice this was done by genetic engineering and homologous recombination to create mice with complete ablation of the protein. For human skeletal cells adenoviral gene transfer and shRNA were used for gain and loss of function respectively. Here we describe procedures used to make and then analyze osteogenic and osteoclastic cells with or without CCN4 to determine its role in osteogenic differentiation.

Key words CCN4, Gene knockout, Osteogenic differentiation, Osteoprogenitor cell

1 Introduction

The ability to manipulate gene expression either by total ablation or by over expression has had tremendous impact on understanding specific protein function. For the "loss of function" approach, genes are recombined in murine embryonic stem (ES) cells rendering them deficient in a specific protein's expression. ES cells injected into blastocysts can then incorporate into adult mice and when populated into germ cells can even be transferred to offspring making new mouse lines deficient in the protein of interest. Such genetically engineered mouse lines are often referred to as "knockout" (KO) mice [1]. These new strains of mice are then examined at the tissue and cellular levels to determine the role of the loss of the protein on skeletal function. Our lab generated mice deficient in CCN4 [2] and this chapter explains in detail how the cells from these mice were used to show how CCN4 affects bone-derived cell functions. We did this using: (1) bone marrow stromal cells (BMSCs) that form new bone and control osteoclast formation and (2) osteoclast progenitors that carry out bone resorption. We describe in this chapter how the osteogenic capabilities of mouse BMSCs are determined both in vitro and in vivo using induced differentiation assays and a transplant system [3] respectively.

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Different approaches were used to study CCN4 in osteogenic and osteoclastic human skeletal cells. For a loss of function studies BMSCs are transfected with lentivirus encoding shRNA (short hairpin RNA) that "knocks down" gene expression in transduced cells [4, 5]. For gain of function adenoviruses are used because they are particularly effective for human material giving transient but highly efficient and abundant protein production [5, 6]. The effectiveness of targeted knockdown or overproduction is determined by measuring mRNA and/or protein for CCN4 [4].

In summary, in this chapter, methods are described for isolating and analyzing osteoprogenitors from mouse and human sources to study the role of CCN4 in skeletal function.

2 Materials

Ccn4-KO mice, mouse bone marrow stromal cells (mBMSCs), primary osteoclasts, human bone marrow stromal cells (hBMSCs).

2.1 Genotyping

1. Tail biopsies from *WT* or *Ccn4*-KO mice.

2.1.1 DNA Extraction

2. Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA): Extraction solution, Tissue preparation solution, Neutralization Solution B.
3. Block incubator or PCR machine.

2.1.2 PCR

1. Oligonucleotides at 0.1 μg/μL corresponding to wild type and *Ccn4*-KO alleles [2].
2. GoTaq® Master Mix (Promega, Fitchburg, WI, USA).
3. DNA template (*see* Subheading 3.1.1).
4. Distilled DNase free water (DW).
5. PCR machine.

2.2 In Vitro Osteogenic Differentiation Assay Using Mouse Bone Marrow Stromal Cells (mBMSCs)

1. 6- to 8-week-old *WT* or *Ccn4*-KO mice.

2.2.1 mBMSCs Culture

2. mBMSCs culture media: α-MEM, 20% FBS (*see* **Note 1**), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 μM 2-mercaptoethanol.
3. Hank's balanced salt solution (HBSS).
4. 19, 23, and 26G needles.
5. 1 and 20 mL syringes.
6. Tissue culture treated dish.
7. Cell incubator (37 °C, 5% CO_2).

2.2.2 Plating mBMSCs for the Experiment

1. Cell preparation solution: 175 U/mL of Type IV collagenase, 1 U/mL of dispase, Hank's balanced salt solution (HBSS) (*see* **Note 2**).
2. HBBS.
3. 0.05% trypsin–EDTA.
4. mBMSCs culture media (*see* **Note 3**, Subheading 2.2.1).
5. Cell incubator (37 °C, 5% CO_2).

2.2.3 Osteogenic Differentiation of mBMSCs

1. mBMSCs (*see* Subheading 3.2.1).
2. Osteoblastic differentiation medium: mBMSC culture medium (*see* Subheading 2.2.1, **item 2**), 10^{-8} M dexamethasone, 100 μM ascorbic acid 2-phosphate, and 2 mM β-glycerophosphate.
3. 6-well culture plate.

2.2.4 Alizarin Red S Staining

1. BMSCs cultured until mineralized nodules are observed (*see* Subheadings 2.2.3 and 3.2.3).
2. Staining solution: 2% alizarin red S (pH 4.2; Sigma-Aldrich), DW.
3. 0.22 μm filter.
4. PBS, PH 7.4.
5. 60% isopropanol.

2.2.5 Quantification of the Alizarin Red S Staining

1. Eluting solution: 0.5% SDS, 0.5 N HCl.
2. 96-well plate.
3. Microplate reader (405 nm filter).

2.3 In Vivo Osteogenic Differentiation Assay

2.3.1 Transplants

1. mBSMCs (*see* Subheading 2.2.1).
2. Immunocompromised mice (*see* **Note 4**).
3. Gelfoam™ (Pfizer, New York City, NY, USA): cut in uniformed size of 5 × 5 × 7 mm.
4. Two sheets of sterilized filter paper.
5. Culture dishes.
6. Sharp-point tip straight sterile tweezers.
7. Sterile scissors.
8. Wound clip/tissue glue/suture.

2.3.2 Analysis

1. Micro-CT (*see* **Note 5**).
2. Histology (*see* **Note 6**).

2.4 In Vitro Bone Resorption Assay

2.4.1 Primary Osteoclasts Culture

1. 6- to 8-week-old *WT* or *Ccn4*-KO mice.
2. Primary osteoclast culture medium: α-MEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 U/mL amphotericin.

3. 19, 23, and 26G needles.
4. 1 and 20 mL syringes.
5. Tissue culture treated dishes or flasks.
6. 96-well plates.
7. 20 ng/mL of M-CSF (R&D Systems, Minneapolis, MN, USA).
8. 30 ng/mL of sRANKL (R&D Systems).

2.4.2 Bone Resorption Assay

1. Primary osteoclastic precursor cells (*see* Subheading 2.4.1).
2. OsteoAssay™ 96-well plates (Nunc, Rochester, NY, USA).
3. Primary osteoclast culture media (*see* Subheading 2.4.1, **item 2**).
4. 10% bleach solution (dilute sodium hypochlorite with double distilled water).

2.5 In Vitro Analysis Using Human Bone Marrow Stromal Cells (hBMSCs)

hBMSCs are isolated from human bone marrow which were obtained from healthy adult donors under sufficient informed consent or purchased from Lonza (Basel, Switzerland).

2.5.1 hBMSCs Culture

1. hBMSC culture medium: α-MEM, 20% lot-selected FBS, 2 mM glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin sulfate (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA).
2. Tissue culture treated dishes or flasks.
3. Humidified cell incubator (37 °C, 5% CO_2).

2.5.2 Gain of Function

1. Adenoviruses encoding human CCN4: generate using the BamHI/NotI fragment of full-length human CCN4 cDNA, cloned into the adenovirus vector VQ ad5 DMV K-NpA (Viraquest) (*see* **Note 7**).
2. hBMSC culture media (*see* Subheading 2.5.1, **item 1**).
3. Tissue culture treated dishes or flasks.
4. Humidified cell incubator (37 °C, 5% CO_2).

2.5.3 Loss of Function

1. shRNA lentivirus targeting human CCN4 or GFP control virus (OpenBioSystems; GE Dharmacon, Lafayette, CO, USA) (*see* **Note 8**).
2. 2 μg/mL of puromycin.
3. hBMSC culture medium (*see* Subheading 2.5.1, **item 1**).
4. Tissue culture treated dishes or flasks.
5. Humidified cell incubator (37 °C, 5% CO_2).

2.5.4 In Vitro Osteogenesis Assay

1. Osteogenic differentiation medium: hBMSC culture medium (*see* Subheading 2.5.1, **item 1**), 10^{-8} M dexamethasone, 100 μM ascorbic acid 2-phosphate, and 2 mM β-glycerophosphate.

2. Adenovirus-infected hBMSCs (*see* Subheading 2.5.2).
3. Tissue culture treated dishes or flasks.
4. Humidified cell incubator (37 °C, 5% CO_2).

2.5.5 In Vivo Osteogenesis Assay

1. Hydroxyapatite–tricalcium phosphate (HA/TCP).
2. 8-week-old immunocompromised mice (Athymic Nude-*Fox1nu*, Charles River).
3. Virus-infected hBMSCs (*see* Subheading 2.5.2).
4. Tissue culture treated dishes or flasks.
5. Humidified cell incubator (37 °C, 5% CO_2).

3 Methods

3.1 Genotyping

3.1.1 DNA Extraction

1. Cut tail (about 3–4 mm) and put into test tube.
2. Add 60 µL of extraction solution and 15 µL of Tissue preparation solution and mix well.
3. Incubate for 10 min at 28 °C, 10 min at 37 °C, 5 min at 98 °C, then cool down to 4 °C.
4. Add 60 µL of neutralization solution B to each DNA sample.

3.1.2 PCR

1. Mix Materials 1–4 (1 µL each of oligonucleotides, 10 µL of GoTaq® Master Mix, 1 µL of DNA template, 6 µL of DW; total 20 µL) to prepare PCR template.
2. Run PCR with "touch down" protocol (*see* below).

	94 °C	5 min
2 cycles	94 °C	30 s
	X°C	30 s
	72 °C	30 s
"Touch down": *X* = 68, 66, 64, 62, 60, 58, 56, 54, 52, 50		
20 cycles	94 °C	30 s
	48 °C	30 s
	72 °C	30 s
	72 °C	3 min
	4 °C	Fold (Fig. 1)

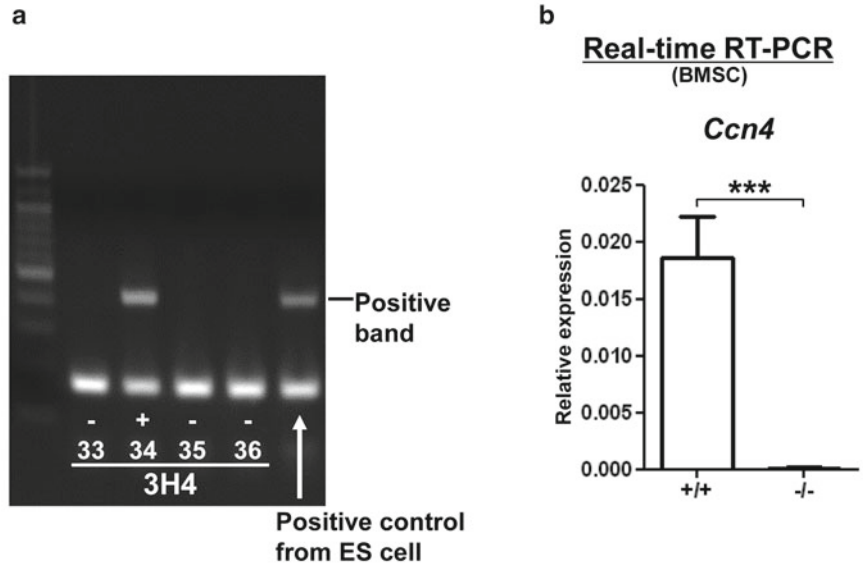

Fig. 1 Genotyping of *WT* and *Ccn4*-KO mice. (**a**) A small biopsy is removed from the tail and DNA extracted and subject to PCR using oligonucleotides that specifically amplify the recombined (targeted) *Ccn4* allele. A positively recombined ES cell clone (3H4) was used as control (*last lane*). One mouse, #34 contained the recombined allele while another (#36) did not. (**b**) BMSCs from mouse #34 were isolated and expanded in culture and mRNA extracted for real-time PCR analysis using oligonucleotides specific for *Ccn4*. ***$p < 0.001$. Mice with the successfully altered allele were devoid of *Ccn4* expression making them a true knockout (KO) mouse line

3.2 In Vitro Osteogenic Differentiation Assay Using Mouse Bone Marrow Stromal Cells (mBMSCs)

3.2.1 mBMSCs Culture

1. *Prepare the mouse*: Euthanize a mouse and keep it on ice. Soak the mouse in 70% ethanol for sterilization.
2. *Dissect bones*: Skin the mouse and dissect out the hind limb. It must be kept in HBBS (*see* **Note 3**) prior to starting the next step. After sanitizing the workspace, separate a femur from a tibia and remove muscles as much as possible. Cut off both ends of the femur and tibia and keep it in the culture media (*see* **Note 3**). Make sure that the marrow cavity is exposed.
3. *Flush marrow cells from the bone shaft*: Flush bone marrow into new 50 mL test tube from the bone shaft with culture medium using a 23 G needle. Flushing with culture media, move needle up and down to wash the inside of the bone shaft. Change to 16 G needle and suspend slowly to separate the cells in the 50 mL test tube.
4. *Spin down the cells*: at 277 × *g* (RCF) for 8 min.
5. *Cell seeding*: Seed cells into 1× T-75 cm^2 flasks (marrow from two femurs + two tibias). First medium change will be after 7 days.

3.2.2 Plating mBMSCs for the Experiment

1. Prepare cell preparation solution.
2. Wash the cells (it is best to use primary cells) with 15 mL of HBSS one time.

3. Incubate the cells with 10 mL of cell preparation solution for 1 h at 37 °C.
4. In the meantime, prepare ice and 15 mL of mBMSC culture medium in 50 mL test tube.
5. Collect the cell preparation solution into the Falcon tube and keep on ice.

Add 10 mL of 0.05% trypsin–EDTA into the flask and incubate for another 5–10 min at 37 °C.

6. Tap the flask several times, then add 5 mL of mBMSC culture medium and wash the inside the flask with the trypsin medium to get as many cells as possible. Place the trypsin medium into the Falcon tube kept on ice.

*Repeat **steps 4–5** if many cells remain on the culture flask.

7. Add 10 mL of mBMSC culture medium into the flask and wash, then transfer to the test tube.
8. Centrifuge at 277 × *g* (RCF) for 8 min. Aspirate most of the liquid without touching the pellet.
9. Add 10 mL of media and count the number of the cells.

3.2.3 Osteogenic Differentiation of mBMSCs

1. Culture mBMSCs in 6-well plate with mBMSC culture medium until 80% confluent.
2. Change medium with osteogenic differentiation medium 2–3 times a week for 3–6 weeks or more.
3. When the mineralized nodules are observed (phased bright), stain with alizarin red S (*see* Subheadings 2.2.4 and 3.2.4).
4. RNA and protein collection is at 1 or 2 weeks after induction (Fig. 2).

3.2.4 Alizarin Red Staining

1. Make the staining solution by dissolve 2 g of alizarin red S in 100 mL of distilled water.
2. Filtrate staining solution through 0.22 μm filter and store at RT.
3. Wash the BMSCs culture dish with PBS two times.
4. Fix the cells with 60% isopropanol for 1 min at RT.
5. Rehydrate with distilled water for 2–3 min
6. Stain with staining solution for 3 min at RT.
7. Wash the dish with distilled water several times.
8. Dry and observe under the microscope.

3.2.5 Quantification of the Alizarin Red S Staining

1. Make the eluting solution.
2. Add 500 μL/well (6-well plate) of eluting solution and wait 30 min at R/T.

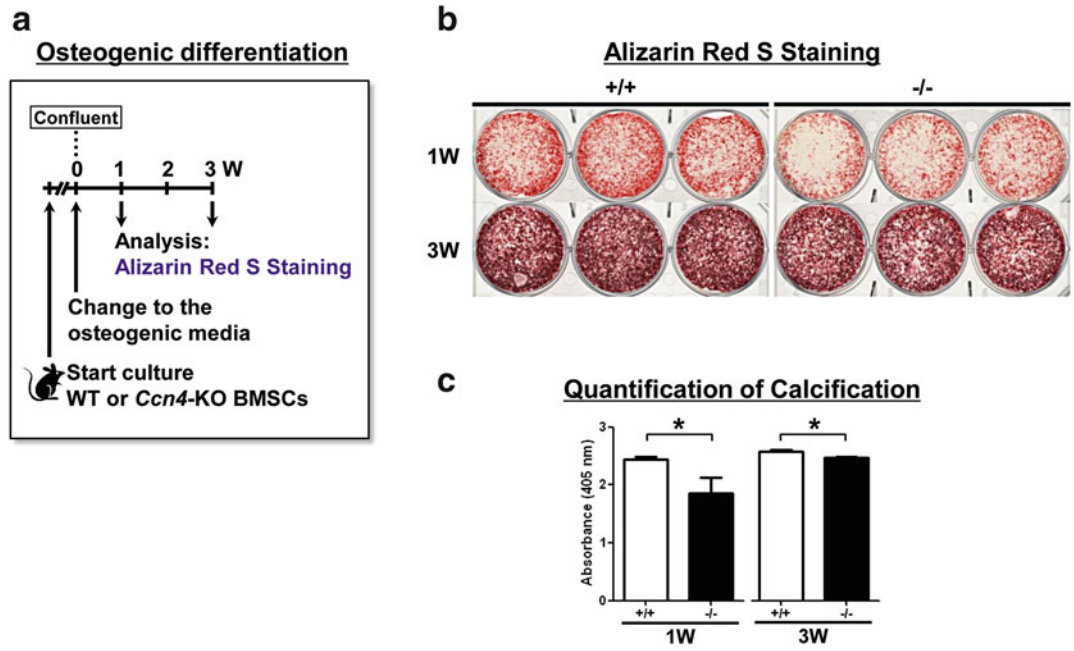

Fig. 2 (**a**) Outline of the temporal sequence of BMSC cell culture and analysis in weeks (W). (**b**) Representative alizarin red staining of BMSCs from *WT* (+/+) or *Ccn4*-KO (–/–) mice. (**c**) Quantitative assessment of the relative level of alizarin red staining in WT and *Ccn4*-KO at 1 and 3 weeks of culture. At both time points the *Ccn4*-KO cells accumulate less calcium that binds to alizarin red S. This is used as an indirect measure calcification and osteogenic differentiation. $*p < 0.05$

3. Transfer 150 μL of stained solution to 96-well plate. It is recommended to prepare triplicate for each sample to adjust for technical error.
4. Measure with microplate reader at 405 nm.

3.3 In Vivo Osteogenic Differentiation Assay

3.3.1 Transplants

1. *Preparation of cells*: Trypsinize cells and count them (*see* Subheadings 2.2.2 and 3.2.2). Resuspend to a concentration of 2.0×10^6 cells/mL and put a 1.0 mL aliquot into 1.5 mL test tube. Spin down the cells at $277 \times g$ (RCF) for 5 min, then aspirate the media down to about 400 μL (the volume between each tube needs to be equal).
2. *Preparation of the Gelfoam*™: Soak Gelfoam™ into the culture media (in the new culture dish) to take all air bubbles out. Fold sterilized paper in the middle and place it onto the new sterile 15 cm culture dish. Sandwich a piece of Gelfoam™ between sterilized paper to remove the culture media and air bubbles as much as possible.
3. Mix the cell suspension, and then gently put the Gelfoam™ into it to saturate it with the all the cells.

—Move to an animal procedure room—

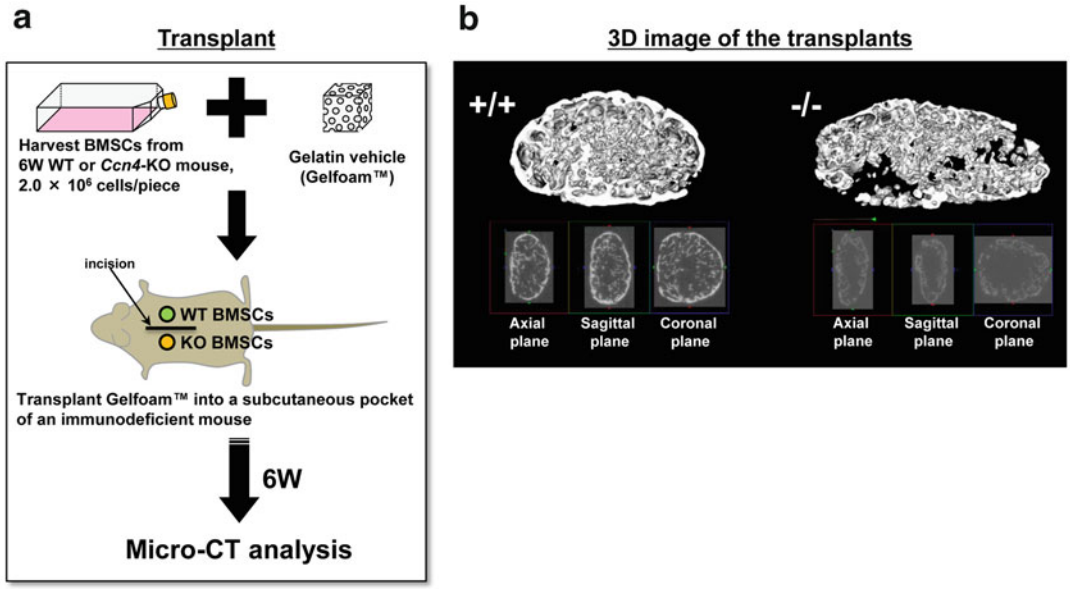

Fig. 3 (**a**) Diagram of the methods used to expand and analyze BMSC growth in vivo in subcutaneous transplants. (**b**) Micro-CT analysis of representative transplants from *WT* (+/+) and *Ccn4*-KO mice. *Top panel* shows a view of half the transplant and the bottom panels show axial, sagittal, and coronal views showing that in all cases the transplants formed from the *Ccn4*-KO cells have less radiodense bone formation (reproduced from [4] with permission from John Wiley and Sons)

4. Disinfect the skin of the back with povidone–iodine and alcohol.
5. Make a 1/2 to 1 in. incision and make a pocket by opening and closing scissors. Put a piece of Gelfoam™ into the subcutaneous tissue pocket on both sides of the upper/lower back, then close the wound with autoclips, suture, or tissue glue.
6. Wait for 6–8 weeks and then harvest.

   * The transplant will feel hard if it is mineralized (Fig. 3)

3.4 In Vitro Bone Resorption Assay

3.4.1 Primary Osteoclast Culture

1. Male mice 9–11 weeks old are euthanized via isofluorane (chloroform is not good for osteoclasts), the long bones [femur and tibia] are harvested and the marrow is flushed using α-MEM medium *with no serum* into 50 mL tubes. In order to break up all cell clusters and get a single cell suspension the cells are triturated using a syringe with a long 19 G needle followed by 23 and 26G needles.
2. Centrifuge the tube at 493 × *g* (RCF), RT for 10 min.
3. Remove the supernatant and resuspend in a volume of α-MEM medium containing 10% FBS that corresponds to the desired volume based on the size of the dish to be used (i.e., 10 mL per 10 cm dish).

4. Add the medium and add cells in treated culture dish. In general, use the same number of dishes as the number of mice.
5. After 3 h collect the supernatant and add it to a new treated culture dish and incubate overnight.
6. After the overnight incubation, collect the supernatant and put it into a 50 mL tube.
7. Centrifuge at $373 \times g$ (RCF), RT for 5 min.
8. Discard the supernatant and resuspend the cells according to the pellet size (usually 1 mL for cells pelleted from one animal).
9. Count the cells using a 1:20 dilution (add 25 μL of the cell suspension into 475 μL of α-MEM + 10% FBS medium).

3.4.2 Bone Resorption Assay

1. Plate 5.0×10^5 cells/cm^2 (1.65×10^5 cells per single well of 96-well plate) with 30 ng/mL of sRANKL and 20 ng/mL of M-CSF. To reduce an edge effect, fill surrounding experimental wells with liquid.
2. Change medium every other day until giant multinucleated cells (osteoclasts) appear (after ~3–5 days).
3. Add 100 μL/well of 10% bleach solution.
4. Incubate at RT for 5 min.
5. Aspirate bleach solution and wash wells with 150 μL/well of double distilled water twice.
6. Air-dry at RT for 3–5 h.
7. To visualize the resorbed area, stain with Von Kossa or 1% toluidine blue.

3.5 In Vitro Analysis Using Human Bone Marrow Stromal Cells (hBMSCs)

1. Culture cells with hBMSC culture media until 80% confluent.
2. Use hBMSCs from third to seventh passage because BMSCs are heterogeneous populations and gradually begin to lose the ability to differentiate after repeated and extensive passaging.

3.5.1 hBMSCs Culture

3.5.2 Gain of Function

1. Culture hBMSCs with culture medium until 80% confluent.
2. Change media to contain adenovirus (5×10^3/particles/cell) to allow for transduction of adenovirus into the BMSCs.
3. Cells can be used for analysis 72 h after transduction.

3.5.3 Loss of Function

1. Culture hBMSCs with culture medium until 80% confluent.
2. Change medium containing 2 μg/mL of puromycin for positive selection.
3. Add shRNA lentivirus targeting human *CCN4* or *GFP* control virus to the cells.
4. Cells were used for analysis 48 h after transduction.

3.5.4 In Vitro Osteogenesis Assay

1. Culture cells with hBMSC culture medium until confluent.
2. Switch medium to osteogenic differentiation medium and change twice a week for 3–6 weeks or more.
3. When the mineralized nodules are observed, stain with alizarin red S and visualize phase bright in an inverted microscope.
4. RNA and protein collection is at 1 or 2 weeks after induction (for details *see* ref. 4).

3.5.5 In Vivo Osteogenesis Assay

1. $2.0–3.0 \times 10^6$ of virus-infected hBMSCs were mixed with 40 mg of sterilized HA/TCP powder.
2. Incubate them at 37 °C for 90 min on a shaker.
3. Spin down $193 \times g$ (RCF) for 1 min.
4. Implant it under the skin on the backs of 8-week-old immunocompromised mice as described above.
5. After 6 weeks, harvest the transplants and fix with 4% PFA and embedded in paraffin.
6. 6-μm sections are stained with hematoxylin and eosin.
7. Quantification of de novo bone formation was performed using a microscope equipped with an analyzer (Fig. 4).

4 Notes

1. To use "lot selected FBS" is necessary for BMSCs culture.
2. Cell preparation solution must be protected from light and good through 1 month at 4 °C.
3. It is advised to keep the HBBS or culture media on ice.
4. The immunocompromised mice can also be purchased from Harlan and Taconic and using the strain: Nude-Fox1^{nu}.
5. The precise method for analyzing the transplants by micro-CT will depend on the type of machine you are using. After the transplants are fixed they can be scanned in the micro-CT and then processed for histology (*see* **Note 6**). Many parameters can be assessed using the including Bone Volume/Total Volume, trabecular number and trabecular spacing. Because of the potential variation in the experimental outcome it is suggested that comparisons between *WT* and *Ccn4*-KO be made within the same animal.
6. The BMSCs form a hard tissue within the transplant can be first fixed then subject to micro-CT scanning and then subsequently de-calcified prior to embedding and processing. One standard means of decalcifying is to soak the tissue in a solution of 10% EDTA in 1× PBS. The efficacy of demineralization is determined by X-ray: the decalcified tissues will have no

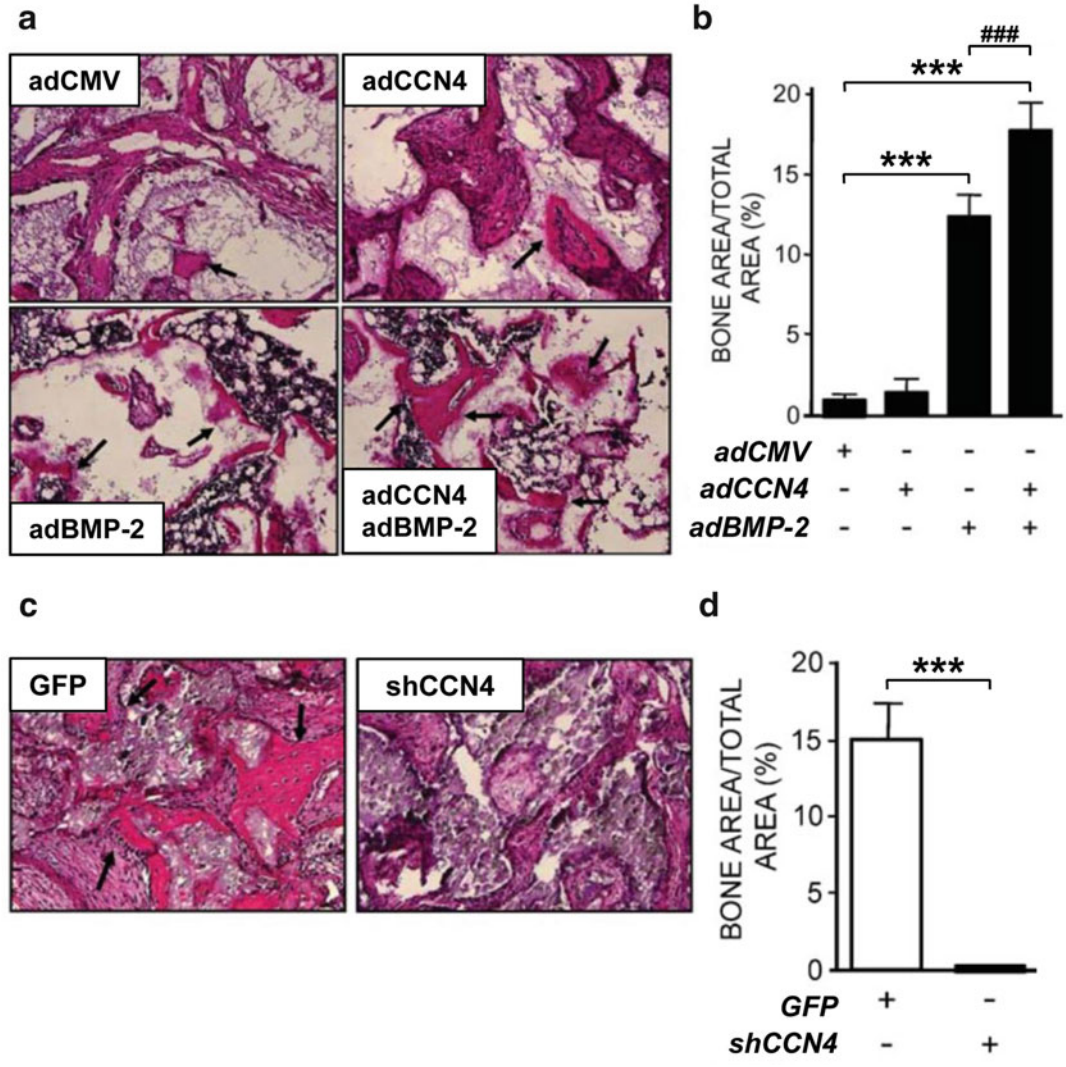

Fig. 4 (**a**) Transplants of hBMSCs transduced with control (adCMV) that does not contain an overexpressing transgene, ad*CCN4*, ad*BMP-2* that overexpress CCN4 and or BMP-2 respectively. (**b**) Quantitation of the bone area in the transplants shown in **a** $^{***}p < 0.001$ between control *adCMV* and *adCCN4* or *adBMP-2*, $^{\#\#\#***}p < 0.001$ between *adBMP-2* and *adCCN4* plus *adBMP-2*. (**c**) Transplants from hBMSC infected with control lentivirus (*GFP*) or lentivirus encoding sh*CCN4* RNA. (**d**) Quantitation of the bone area made by control lentivirus compared to sh*CCN4* lentivirus. $^{***}p < 0.001$ between control GFP and shCCN4 (reproduced from [4] with permission from John Wiley and Sons)

radiopaque (white) areas in the X-ray. Standard histology is performed after sectioning using H&E staining. The bone is characteristically pink and quantified by tracing the bone area/total area.

7. The concentration of virus used should be titrated for each experiment to determine the optimal dose to use. In our hands 5×10^3/

particles per cell were sufficient to give robust CCN4 expression in hBMSCs. Note that the adenovirus transduction provides a transient but efficient level of protein production. The maximum expression of a transgene after transduction will be from 3–5 days and then tapers off until 2 weeks post-transduction.

8. In general, several different clones of lentivirus should be tested to check for their efficacy. For CCN4 knockdown, five clones were tested and ranged in their ability to reduce CCN4 expression from ~30% of controls (the least effective) to 90% (the most effective). The efficiency of knockdown was determined by measuring the relative CCN4 mRNA expression/S29 (a ribosomal gene) in the infected BMSCs. Note also that the lentivirus provides a stable integration of the shRNA into the host cell genome.

Acknowledgements

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Chapter 30

Construction and Analysis of an Allelic Series of *Ccn1* Knockin Mice

Ricardo I. Monzon, Ki-Hyun Kim, and Lester F. Lau

Abstract

The embryonic lethality of mice with conventional global knockout of *Ccn1* (*Cyr61*) precludes analysis of *Ccn1* functions in late embryonic development or in adulthood. To circumvent this limitation, we have generated conditional knockout mice that allow cell type-specific deletion of *Ccn1*, and constructed an allelic series of *Ccn1* knockin mice that express CCN1 defective for binding specific integrins in lieu of the wild type protein. Here we describe the construction of these mice and discuss how analysis of these animals can provide unique insights into *Ccn1* functions mediated through specific integrin receptors. It is anticipated that future analysis of mice carrying specific mutations in genes of the *Ccn* family will be greatly facilitated by application of the CRISPR/Cas9 gene editing methodology.

Key words Knockin mice, Mutation, CCN1, *Cyr61*, *Cre*, *LoxP*

1 Introduction

Targeted deletion of genomic sequences in mammalian cells is an important approach for understanding the functions of specific genes, and by now all members of the *Ccn* family have been subjected to scrutiny by targeted gene knockout [1, 2]. *Ccn1*-null mice die in mid-gestation with placental insufficiency, impaired vascular integrity, and severe atrioventricular septal defects [3, 4]. Although these results have revealed important developmental roles of *Ccn1*, the embryonic lethality of *Ccn1*-null mice preludes functional analysis in late embryonic development or in adulthood. This obstacle can be circumvented by various strategies, including: (1) selective deletion of *Ccn1* in a cell type-specific manner by means of the *Cre-LoxP* system, or (2) genomic knockin of alleles that impairs specific CCN1 functions. Each of these approaches entails distinct advantages and drawbacks. General procedures for the generation of conditional knockout and allelic knockin mutants

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have been described elsewhere [5, 6]. Here we outline the design and construction of these mice as applied to *Ccn1*, and discuss issues pertaining to the genetic analysis of CCN matricellular protein functions.

The Cre recombinase from the bacteriophage P1 can mediate excision of sequences flanked by *LoxP* sites [7], allowing inactivation of a target (*floxed*) gene constructed with *LoxP* sites flanking crucial sequences. When *floxed* mice are crossed with mice expressing *Cre* under a cell type-specific promoter, deletion of the target gene occurs only in *Cre*-expressing cells. Prior identification of the relevant cell types that express the target gene is useful for the selection of appropriate *Cre*-expressing mouse lines for deletion. However, since CCN proteins are secreted and their functions are not necessarily cell-autonomous, deletion of *Ccn* genes in one cell type may affect the function of another, sometimes unexpectedly. For example, hepatocyte-specific deletion of *Ccn1* has a significant effect on the functions of hepatic stellate cells, illustrating the paracrine action of CCN1 [8]. Furthermore, in tissue microenvironments where multiple cell types may express a particular *Ccn* gene, deletion of the gene in one of several contributing cell types may not result in a clear phenotype. In some instances, these problems may be overcome by using a Cre recombinase expressed under a global promoter whose function is activated only in the presence of an inducer. For example, the Cre-ERT fusion protein coupling Cre to a tamoxifen-binding estrogen receptor is able to enter the nucleus where it catalyzes DNA recombination only upon administration of tamoxifen [9].

An alternative approach to analyze CCN functions is to construct knockin mice that carry specific mutations that alter CCN activities or structural features. As a matricellular protein, CCN1 acts primarily through direct binding to distinct integrin receptors in a cell type-specific manner to induce diverse cellular responses [1, 10]. Among the integrins that CCN1 binds are $\alpha_6\beta_1$ in fibroblastic cells, $\alpha_M\beta_2$ in monocytes, and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in vascular and epithelial cells. The noncanonical CCN1 binding sites for these integrins have been identified and specific CCN1 mutants that abolish binding to these integrins have been created, allowing analysis of integrin-specific functions [11–13]. Many of these integrin binding sites are well conserved among CCN family members, suggesting conservation of integrin-binding functions. To dissect the functions of CCN1-integrin interaction in vivo, we have constructed an allelic series of knockin mice, including: (1) $Ccn1_{D125A/D125A}$ mice in which *Ccn1* is replaced with the $_{D125A}$ allele encoding an Asp-125 to Ala substitution that abolishes CCN1 binding to $\alpha_v\beta_3/\alpha_v\beta_5$ [14]; and (2) *Ccn1DM/DM* mice in which *Ccn1* is replaced with the *DM* allele encoding an $\alpha_6\beta_1/\alpha_M\beta_2$-binding defective CCN1 [15]. Both *Ccn1D125A/D125A* and *Ccn1DM/DM* mice are viable and fertile, allowing analysis of these mice from development to adulthood.

The analysis of these *Ccn1* knockin mice offers several advantages over conditional deletions. First, the phenotypes observed can be attributed to the specific mutations, and in all cases analyzed to date the observed phenotypes can be ascribed to specific integrin-mediated functions in distinct cell types [14–18]. Coupled with in vitro studies targeting the cognate integrins, analysis of these mice provides compelling in vivo evidence for CCN1 functions through specific integrin pathways. Second, unlike *Cre*-mediated deletions, knockin mice can be analyzed without requiring an appropriate *Cre*-expressing mouse line.

To date, studies on *Ccn1* deletion using the *Cre-LoxP* system in hepatocytes, endothelial cells, and progesterone-responsive tissues have been reported [8, 19, 20], as has *Ccn1* deletion using a *Cre* recombinase whose activity is inducible with tamoxifen [20, 21]. Analyses of *Ccn1D125A/D125A* mice have led to dissection of $\alpha_v\beta_3/\alpha_v\beta_5$-mediated functions in biliary regeneration and in phagocytic clearance of apoptotic cells [14, 16], and studies on *Ccn1DM/DM* mice have uncovered $\alpha_6\beta_1$ and $\alpha_6\beta_1/\alpha_M\beta_2$-mediated CCN1 induction of cellular senescence [22] and apoptosis [15, 23], restriction of cutaneous and hepatic fibrosis [8, 22], promotion of intestinal mucosal healing [17], and suppression of hepatocellular carcinoma [18].

Since members of the CCN family are known to bind integrins and other cell surface receptors, growth factors, and extracellular matrix molecules [1], analyses of mice with mutations in members of the *Ccn* gene family altering the binding sites for these molecules will likely be informative. Recent advent of CRISPR/Cas9 gene editing technology has made the construction of mice with specific mutations significantly less time-consuming and labor-intensive, and detailed methodology for the application of this procedure has been described [24]. Thus, we anticipate that future studies using the CRISPR/Cas9 technology to construct and analyze mutant mice carrying specific mutations in *Ccn* genes, similar to the *Ccn1* mutations described herein, will advance our understanding of CCN functions in development and disease processes.

2 Materials

2.1 Plasmids and Mouse Strains

1. The targeting vector is constructed from the pLoxPNT vector. *Ccn1* genomic DNA is cloned from a 129Sv/J library.
2. *EIIa-Cre* deleter mouse strain (Jackson Laboratory). *Ccn1flox* and knockin mice are backcrossed at least 10 times into the C57BL/6 background.
3. Adenovirus vectors expressing *Cre* or *LacZ* under the CMV promoter.

2.2 ES and MEF Cell Culture and Selection Media

1. Mouse embryonic stem cell (ES) line J1 (129S4/SvJ background) (American Type Culture Collection).
2. ES cell growth media and supplements.
3. MEF Growth Medium (DME with 10% FBS, penicillin-streptomycin).
4. Fetal bovine serum (FBS), DMEM, and PBS.
5. Trypsin–EDTA solution: 0.25% trypsin, 1 mM EDTA.
6. Mitomycin C-treated and neomycin-resistant fibroblast feeder cells.
7. G418 and ganciclovir.
8. ES cell freeze medium: 20% heat denatured FBS, 5% DMSO, ES cell growth media.

2.3 DNA Isolation

1. Lysis buffer: 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, 1 mg/ml Proteinase K.
2. DNA isolation kit (e.g., EZNA Tissue DNA Kit, Omega Bio-tek).

2.4 PCR Reagents and Target Oligonucleotides

1. Oligonucleotide synthesis (e.g., Oligos Etc Inc. and Sigma Genosys). Refer to Table 1 for list of primers.
2. Taq polymerase (e.g., GoTaq polymerase, Promega).

2.5 DNA Genomic Probe

1. *Eco*RI-*Bam*HI restriction fragment, which is excluded from the targeting vectors, is obtained from the 3′ region of the *Ccn1* genomic sequence (Fig. 1a). Southern blotting using this probe will only hybridize to DNA fragments that originate from the *Ccn1* genomic locus.

Table 1
List of PCR primer sequences

Primer	Sequence
F1	5′-cgctaaacaactcaacgag-3′
F2	5′-caatacacttctcttggctaataaac-3′
F3	5′-tgttccttgccttctccac-3′
R1	5′-ttccgatcatattcaataaccc-3′
R2	5′-agcctctgttccacatacac-3′
F1′	5′-cggcttgttggttctgtgtcg-3′
R1′	5′- atttgcaggatggatcatcatg-3′
F2′	5′-caacggagccaggggag-3′
R2′	5′-agttttgctgcagtcctcg-3′

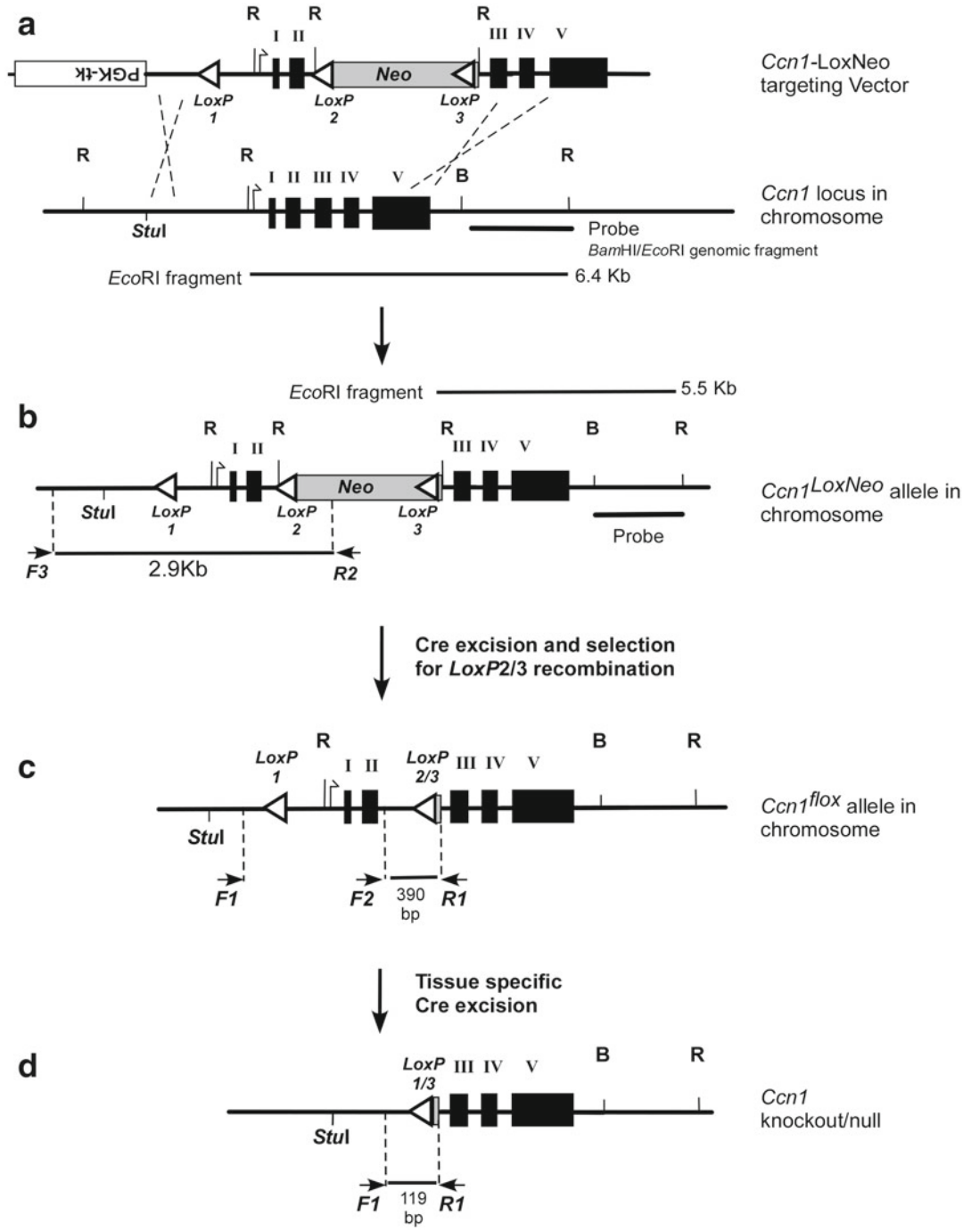

Fig. 1 Generation of *Ccn1-floxed* allele. (**a**) The *Ccn1-LoxNeo* targeting vector contains the *Ccn1* genomic sequence with a *LoxP1* site placed upstream of the *Ccn1* transcription start site. A *LoxP-Neo-LoxP* cassette has been inserted into intron II of *Ccn1*. (**b**) Double homologous recombination of the targeting vector with the ES cell *Ccn1* genomic sequence creates the *Ccn1LoxNeo* allele. ES cells with the *Ccn1LoxNeo* allele are selected and injected into blastocysts, and chimeric animals with germ line transmission are generated. (**c**) *Ccn1LoxNeo/+* mice are crossed with the *Ella-Cre* deleter strain, and excision of the neomycin resistance gene after recombination at *LoxP2* and *LoxP3* results in the *Ccn1flox* allele. Further mating generates *Ccn1flox/flox* mice ready for conditional *Ccn1* knockout. (**d**) Cre recombinase acting upon the *Ccn1flox* allele deletes the first two exons, leading to *Ccn1* knockout. R = *Eco*RI; B = *Bam*HI

2. DNA Random priming labeling kit.
3. P^{32} α-ATP radionucleotide.

3 Methods

3.1 Construction and Verification of Ccn1flox/flox Mice

1. To generate ES cells with the *Ccn1flox* allele, a *Ccn1-LoxNeo* targeting vector is constructed using pLoxPNT [25]. This targeting vector contains three *LoxP* sites: *LoxP1* is located 5′ of the *Ccn1* transcription start site, and the neomycin resistance gene flanked by *LoxP2* and *LoxP3* is within the second intron (Fig. 1a). This vector is transfected into J1 ES cells, and double homologous recombination between the vector and the ES cell genome generates the *Ccn1LoxNeo* allele (Fig. 1b). ES cells with the desired recombination events are enriched by selection for resistance to G418 due to the presence of the neomycin resistance gene. The targeting vector also contains a thymidine kinase (tk) gene driven by the phosphoglycerate kinase (PGK) promoter, which allows negative selection against ES cells with random integration of the targeting vector by treatment with ganciclovir. Allele replacement by homologous recombination would exclude the PGK-tk sequence (Fig. 1a, b), whereas cells with random integration of the targeting vector may express thymidine kinase, leading to inhibition of DNA synthesis in the presence of ganciclovir.
2. The *Ccn1LoxNeo* allele can be distinguished from wild type *Ccn1* by the introduction of an additional *Eco*RI restriction site adjacent to the *Neo* cassette, leading to the detection of a novel 5.5 kb *Eco*RI genomic fragment in lieu of the 6.4 kb fragment by Southern blotting (Fig. 1b and 2a). PCR using primer F3 that targets endogenous sequences in the 5′ region of the *Ccn1* genomic locus and R2 targeting the *Neo* gene generates an expected product of 2.9 kb if the *LoxNeo* cassette is integrated (Fig. 1b, Table 1). PCR assays are done with GoTaq DNA polymerase using standard protocols provided by the manufacturer.
3. ES cells confirmed to carry the *Ccn1LoxNeo* allele are injected into mouse blastocysts, and chimeric mice with germ line transmission of *Ccn1LoxNeo* are selected. However, the *Neo* cassette should be removed since it may affect the expression of *Ccn1*. Therefore, *Ccn1LoxNeo/+* mice are crossed with a deleter mouse strain carrying the *Ella-Cre* transgene; this can result in several distinct excision events from recombination between either *LoxP1/P2* or *LoxP1/P3* or *LoxP2/P3*. Recombination between *LoxP2* and *LoxP3* creates the desired *Ccn1flox* allele by removing the *Neo* gene but leaving all exons intact (Fig. 1c). The two remaining *LoxP* sites will allow subsequent *Cre*-mediated deletion of exons I and II, leading to knockout of *Ccn1* (Fig. 1d).

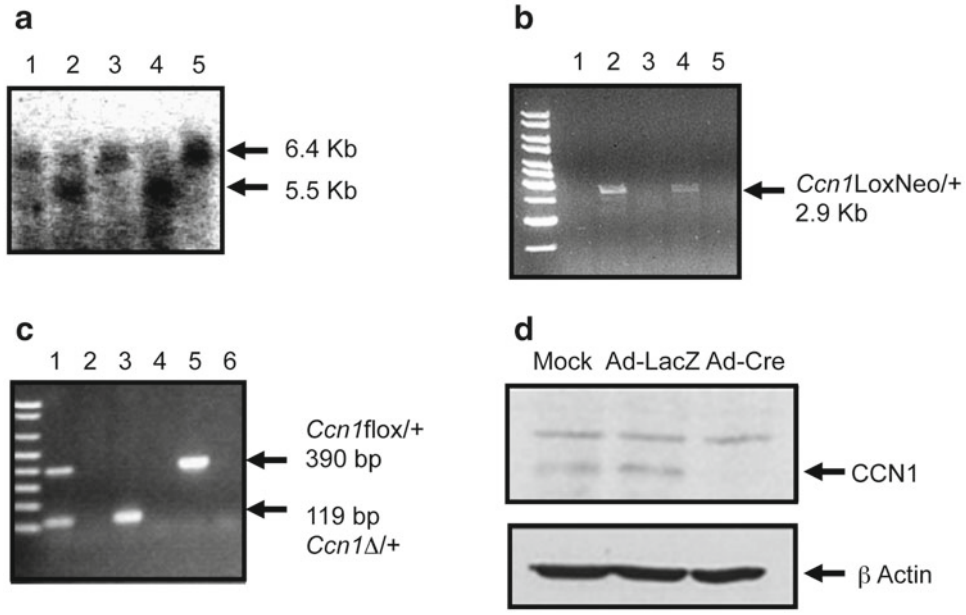

Fig. 2 Verification of the *Ccn1flox* allele. (**a**) Progeny from blastocyst injection of ES cells with the *Ccn1LoxNeo* allele was screened by Southern blotting of genomic DNA after *Eco*R1 digest. Hybridization to the 3′ *Bam*HI/*Eco*RI *Ccn1* genomic probe (Fig. 1b) yields a 6.4 kb band for wild type *Ccn1* (*lanes 1*, *3*, *5*) and a 5.5 kb band for the *Ccn1LoxNeo* allele (*lanes 2*, *4*). (**b**) PCR using primers F3 (targeting endogenous *Ccn1* sequence) and R2 (targeting sequences in *Neo*) yields a 2.9 kb product from *Ccn1LoxNeo/+* DNA (*lanes 2*, *4*), and no product from the wild type allele (*lanes 1*, *3*, *5*). (**c**) PCR analysis of progeny from mating of *Ccn1LoxNeo/+* and *Ella-Cre* mice using the F1, F2, and R1 primers. A 390 bp product results from recombination of *LoxP2/P3* (*lanes 1* and *5*), whereas the 119 bp product identifies recombination of *LoxP1/P3*, resulting in a *Ccn1*-null allele (*lanes 1*, *3*). (**d**) Isolated MEFs are either mock infected or infected with *Ad-LacZ*, or *Ad-Cre* viral vectors. Immunoblotting with anti-CCN1 antibodies shows that CCN1 expression is lost in cells infected with *Ad-Cre*

4. When *Ccn1LoxNeo/+* mice are crossed with *EIIa-Cre* transgenic mice, the progenies are identified using PCR primers designed to distinguish between possible recombinants: a forward primer F1 corresponds to the 5′ region located upstream of the *LoxP1* site, F2 targets a region within intron 2, and a reverse primer R1 that targets an exogenous sequence immediately downstream of the *LoxP3* site (Fig. 1c). The desired *Ccn1flox* allele generates a 390 bp PCR fragment with the F2 and R1 primers. Progenies of this mating may be mosaic since multiple excision events can occur, and thus further mating is necessary to segregate the correct *Ccn1flox* allele and also to remove the *Ella-Cre* transgene. *Ccn1flox/+* animals are backcrossed to the C57BL/6 background at least ten times and mated to derive *Ccn1flox/flox* mice suitable for cell type-specific deletion of *Ccn1*.
5. *Ccn1flox/flox* mice crossed with *Cre* expressing mice will result in deletion of exons I and II in cells with active Cre recombinase, thus inactivating the *Ccn1* gene (Fig. 1d). PCR primers F1, F2 and R1 can be used for screening animals in which recombina-

tion between *LoxP1* and *LoxP2/3* have occurred (Fig. 2c). Transgenic mouse strains with various cell type- or tissue-specific *Cre* expressions are available from researchers working with these mice and from sources such as the Jackson Laboratory. The *Gt(ROSA)26Sortm1(cre/ERT)Nat* mice or *Tg(UBC-cre/ERT2)1Ejb/J* mice (Jackson Laboratory) globally express a Cre recombinase fused to a mutant estrogen receptor (ER) under the *ROSA26* or ubiquitin C promoter, respectively [26]. The Cre-ER fusion protein is restricted to the cytoplasm and translocates to the nucleus only upon binding to tamoxifen, thus enabling Cre-mediated recombination in a tamoxifen-inducible manner.

3.2 Construction and Verification of Ccn1DM/DM Knockin Mice

1. The full length *Ccn1DM* cDNA encoding mutations in the CCN1 binding sites for integrins $\alpha_6\beta_1$ and $\alpha_M\beta_2$ is subcloned into a targeting vector with the neomycin resistance gene and *Ccn1* genomic DNA as depicted in Fig. 3a. When the targeting vector is transfected into ES cells, double homologous recombination can occur to integrate the *Ccn1DM-Neo* targeting cassette into the *Ccn1* locus in the ES cell genome. ES cells that incorporate the target construct are positively selected by treatment with G418, and random integrations are negatively selected against by exposure to ganciclovir.
2. ES cell clones obtained are injected into blastocysts, and the chimeric animals generated are analyzed by Southern blotting. *Eco*RI digested genomic DNA hybridized to a 3′ *Ccn1* genomic probe detects a 7.4 kb fragment from the *Ccn1DM-Neo* cassette, whereas the wild type allele produces a 6.4 kb fragment (Fig. 3b and 4a). PCR using primer F1′ (corresponding to *Ccn1* sequence between the transcriptional start site and exon 1) and R1′ (corresponding to sequences in exon 5) generates a 2.1 kb product from the *Ccn1* genomic sequence, whereas a 1.1 kb product is generated from the *Ccn1DM* knockin allele (Fig. 4b). A unique *Sph*I site in the *Ccn1DM* allele bisects the 1.1 kb PCR product into 0.9 and 0.2 kb fragments (Figs. 3b and 4b). *Ccn1DM/+* mice are backcrossed to the C57BL/6 background and mated to generate *Ccn1DM/DM* mice.

3.3 Construction of Ccn1D125A/D125A Knockin Mice

1. Construction of knockin mice with the *Ccn1D125A* allele [12], which encodes a mutant CCN1 unable to bind integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, is accomplished using a similar strategy as described above for *Ccn1DM*, except that a *Ccn1D125A* targeting vector is used (Fig. 3c).
2. Integration of the *Ccn1D125A* cassette is confirmed by Southern blotting using a genomic probe, yielding a 7.4 kb *Eco*RI fragment from the *Ccn1D125A* allele and 6.4 kb fragment from wild type *Ccn1* (Figs. 3c and 4c).

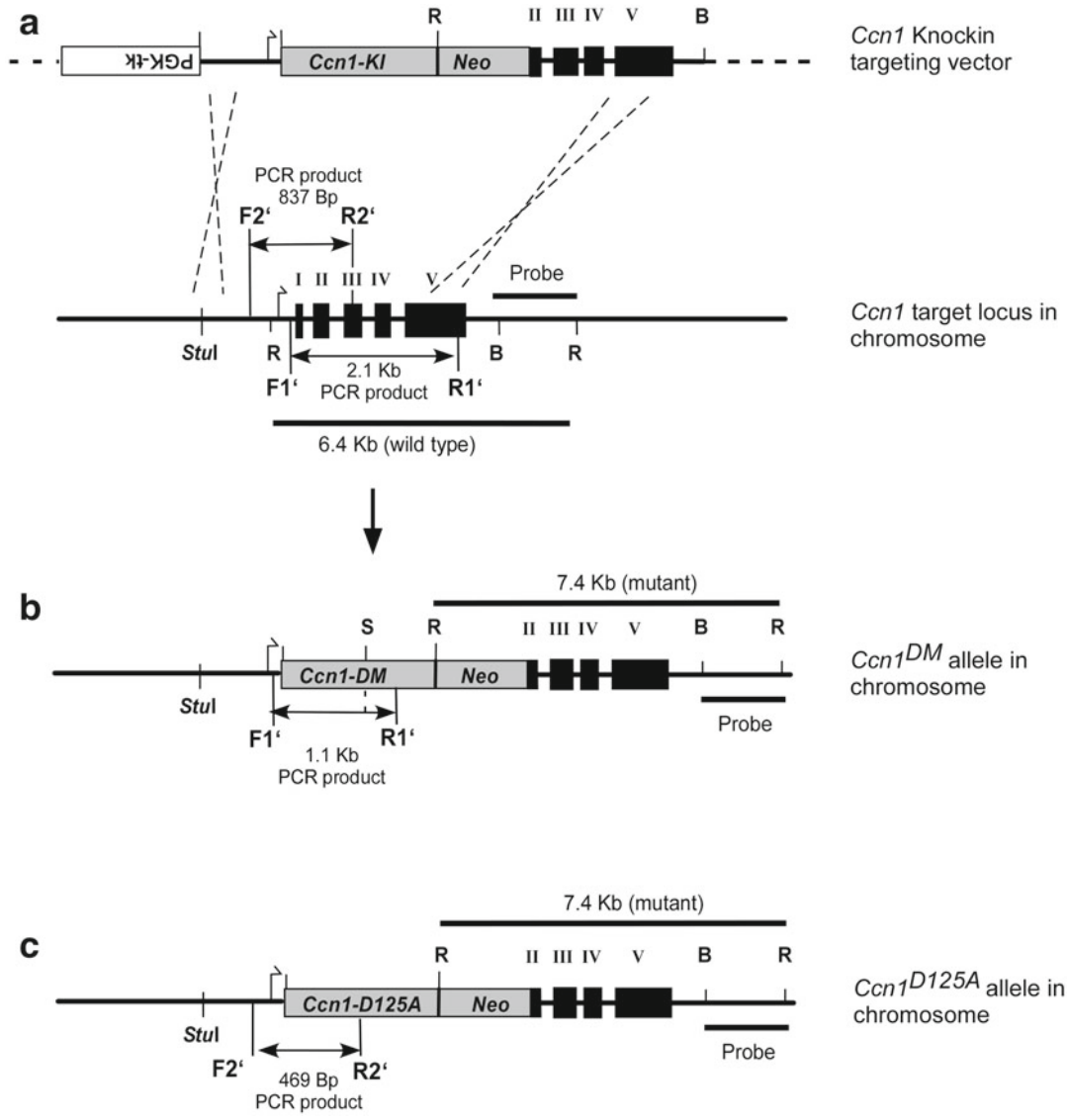

Fig. 3 Generation of *Ccn1* knockin alleles. (**a**) The targeting vector is comprised of the *Ccn1* 5′ genomic sequence, the allelic knockin cDNA (*Ccn1-KI*) followed by the neomycin resistance gene and the 3′ portion of the *Ccn1* genomic sequence. R = *Eco*RI; B = *Bam*HI; S = *Sph*I. (**b**) The *Ccn1DM* allele integrated into the genome after homologous recombination between the targeting vector and the ES cell *Ccn1* locus. A unique *Sph*I site is incorporated into the *Ccn1DM* cDNA. (**c**) The *Ccn1D125A* allele integrated into the genome

3. PCR using a primer F2′ (recognizes a sequence 300 bp upstream of the open reading frame) and R2′ (corresponds to sequences in exon 3) generates a product of 469 bp from *Ccn1D125A* and a product of 837 bp from wild type *Ccn1* as predicted (Figs. 3c and 4d). *Ccn1D125A* mice are backcrossed to the C57BL/6 background and mated to generate *Ccn1D125A/D125A* mice.

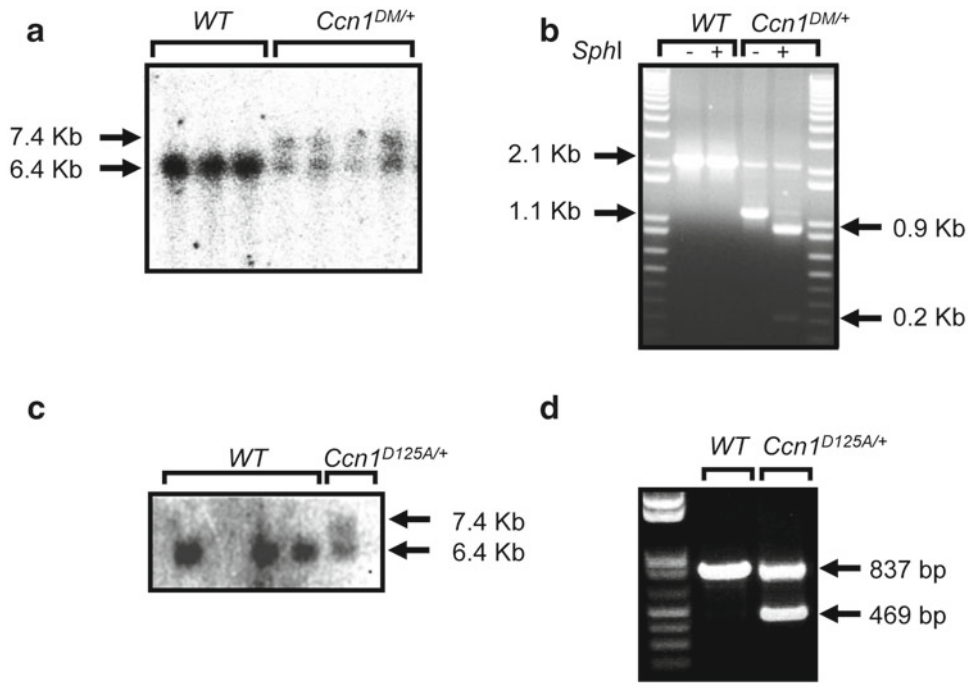

Fig. 4 Verification of *Ccn1* knockin alleles. (**a**) Chimeric animals derived from blastocyst injection of ES cells carrying the *Ccn1DM* allele are analyzed by Southern blotting of *Eco*RI digested genomic DNA using a 3′ *Bam*HI-*Eco*RI *Ccn1* genomic fragment as probe (Fig. 3b). A 7.4 kb fragment is detected from the *Ccn1DM* allele, whereas a 6.4 kb fragment is detected from wild type *Ccn1*. (**b**) Wild type and *Ccn1DM/+* mice can be distinguished by PCR analysis using the F1′ and R1′ primers, which generate a 2.1 kb fragment from wild type *Ccn1* and a 1.1 kb, *Sph*I-sensitive fragment from the $Ccn1^{DM}$ allele. (**c**) Southern blot analysis using a 3′ *Bam*HI-*Eco*RI *Ccn1* genomic fragment as probe (Fig. 3c) detects a 7.4 kb *Eco*RI fragment from the *Ccn1D125A* allele and 6.4 kb *Eco*RI fragment from wild type *Ccn1*. (**d**) Genotype of *Ccn1D125A/+* mice is verified by PCR analysis using the F2′ and R2′ primers (Fig. 3c), yielding a 837 bp product from wild type *Ccn1* and a 469 bp product from the *Ccn1D125A* allele

3.4 Verification of Ccn1 Knockin Alleles in Isolated Mouse Embryonic Fibroblasts

1. To test the functionality of the *Ccn1flox* allele, mouse embryonic fibroblasts (MEFs) are isolated from *Ccn1flox/flox* mice using established procedures described below. The ability of Cre recombinase to delete the *Ccn1* gene is tested by introduction of *Cre*-expressing vectors and loss of CCN1 protein expression is determined by immunoblot analysis (*see* **Note 1**). Immunoblot analysis of cell lysates shows that control MEFs (mock infected or infected with an adenovirus expressing *LacZ*) express CCN1 (Fig. 2d). However, CCN1 expression is lost when MEFs are infected with *Cre*-expressing adenovirus, providing support for *Cre*-mediated *Ccn1* knockout. MEFs can also be used for functional verification of knockin mutant alleles. For example, we show that the CCN1-DM mutant protein expressed in MEFs from *Ccn1DM/DM* mice have lost the ability to bind heparin, a characteristic of the DM protein [15]. Similar strategies can be used to verify the expression of other knockin mutants with distinguishing functional characteristics or epitope tag.

2. Mouse embryonic fibroblasts are prepared as described below. Briefly, mouse embryos (12–13 days) are dissected to remove placental and chorionic tissues under sterile conditions. The embryos are rinsed with PBS and placed on a sterile dish and minced using surgical scissors. Ensure that the tissue fragments are small enough to fit through the bore of a 5 or 10 ml pipette. The minced tissue is placed into a 50 ml conical tube with 5 ml of trypsin–EDTA solution and passed through a 5 or 10 ml pipette 2–4 times to break up the tissue (*see* **Note 2**). Incubate the cells on a rocking platform for 5–10 min at 37 °C, checking periodically to check for digestion of tissues.
3. Add an additional 5 ml of trypsin–EDTA solution and pass the cells through a pipette 1–2 times to break up large tissue particles. Incubate again for 5–10 min at 37 °C. If tissue fragments remain, pass the cell suspension through a pipette 1–2 times, and incubate for 5 additional minutes.
4. To stop trypsin activity, add an equal volume of growth medium (DME media with 10% FBS) to each tube and mix the contents using a 10 ml pipette. Using the same 10 ml pipette, pass the cell suspension in aliquots through a cell strainer into a fresh 50 ml conical tube. The cells are then pelleted on a clinical centrifuge at 500 × *g* for 5 min.
5. After the cells are pelleted, the supernatant is removed and the cell pellet resuspended in 10 ml of fresh growth medium. The cells can be plated on 1–2 10 cm plastic tissue culture plates containing 10–15 ml of growth media. Place in a humidified incubator set at 37 °C with 5–10% CO_2 and grow until 80–90% confluent, usually in 2–5 days.
6. Passage the cells by 1:5–1:10 on 10 cm plates with 10–15 ml of growth medium. Grow the cells at 37 °C and 5–10% CO_2 and prepare a portion of the cells for studies and the rest for storage in liquid nitrogen.

3.5 Functional Analysis Using a Ccn1 Allelic Series

To dissect the functions of CCN1 in vivo, we use the allelic series described above to study the role of CCN1 in tissue repair, including biliary injury repair in response to cholestasis, a common cause of liver transplants. For these studies, *Ccn1flox/flox* mice are crossed with *Albumin-Cre* mice, generating *Ccn1ΔHep* mice with hepatocytes-specific *Ccn1* deletion [8]. *Ccn1+/+*, *Ccn1ΔHep*, *Ccn1DM/DM*, and *Ccn1D125A/D125A* mice are subjected to bile duct ligation (BDL), a well-established procedure that induces cholestatic injury in the liver [27]. Briefly, under ketamine and xylazine anesthesia, a midline abdominal incision is made and the common bile duct is double-ligated using 4–0 silk suture. Sham-operated mice have their common bile duct exposed and manipulated but not ligated. Mice are closely monitored for 7 days after surgery and

euthanized, whereupon liver tissues are collected for histological and biochemical analyses. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are monitored for hepatocyte damage, and serum alkaline phosphatase (ALP) is measured to assess cholestatic damage. Surprisingly, *Ccn1ΔHep* mice do not show any defect in injury repair after BDL. All *Ccn1*+/+, *Ccn1ΔHep*, and *Ccn1DM/DM* mice survive the 7 day experimental period, and these mice undergo extensive biliary regeneration with cholangiocyte proliferation and expansion of bile ducts [16]. However, 75 % of *Ccn1D125A/D125A* mice perished within 7 days with massive hepatic necrosis with greatly elevated levels of serum ALT, AST, and ALP. Further analysis shows that *Ccn1D125A/D125A* mice are severely impaired in cholangiocytes proliferation and bile duct expansion, leading to the finding that CCN1 induces cholangiocyte proliferation by binding to integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$, triggering the activation of NFkB and transcription of *Jag1*, thereby resulting in Jag1/Notch signaling and cholangiocyte proliferation [16]. These studies illustrate how using this allelic series of *Ccn1* mice can provide compelling genetic evidence that CCN1 functions through $\alpha_v\beta_5/\alpha_v\beta_3$ are critical for cholangiocytes proliferation and biliary regeneration. However, *Ccn1* expression in hepatocytes is not required for biliary repair, and CCN1 functions through integrins $\alpha_6\beta_1/\alpha_M\beta_2$ are not essential for these processes.

3.6 ES Cell Culture and Selection

1. DNA is introduced into cultured mouse ES stem cells (J1 strain) by electroporation. ES cells from two 10 cm plates are trypsinized and resuspended in growth media to inhibit trypsin activity; after two washes in PBS 7.4, the ES cells are resuspended in 0.8 ml electroporation buffer (PBS) containing linearized targeting vector DNA (40 μg) and placed on ice. The cells are electroporated with a single pulse of 400 V and 25 μF, and allowed to rest on ice for 10 min and split into five 10 cm plates with mitomycin treated fibroblast feeder layers and 10 ml ES growth medium.
2. After 1 day, the cell medium is replaced with ES cell growth medium containing G418 (350 μg/ml) and ganciclovir (2 μM). Change the ES selection media every 2 days and check the appearance of the colonies to insure they retain the undifferentiated phenotype (*see* **Note 3**).
3. Individual colonies should be detectable microscopically after 3–5 days in selection. The individual colonies can be isolated and transferred to a 96-well plate containing PBS minus Mg^{++} and Ca^{++}. Exercise caution when isolating colonies that are close together to avoid cross contamination.
4. After colonies have been isolated, 50 μl of trypsin–EDTA solution is added to the 96-well plate to begin disaggregation of the ES cells (5 min, room temperature).

5. Using a pipette to gently mix the cells, half of the cell suspension is re-plated on new 96-well plates containing irradiated fibroblasts and 100 µl ES cell growth media for DNA isolation and analysis (*see* **Note 4**). The other half of the trypsinized ES cells remaining in the original 96-well plate are combined with 50 µl ES cell freeze media. The plates are sealed with paraffin wrap and the lids are placed over the paraffin to produce a snug fit on the plates. The plates are then put through a stepwise freezing procedure and finally stored in an ultralow freezer.

3.7 Preparation of Positive ES Cell Clones

1. Positive recombinant ES clones identified by Southern blotting analysis are thawed from 96-well plates previously stored in an ultralow freezer. Prepare a 48-well dish with fibroblasts feeder layers containing 400 µl of ES growth media.
2. After thawing the ES cell clones in the 48-well dishes, monitor each well for growth and retention of non-differentiated state. Not every selected clone will grow and some clones may have acquired a differentiated state. The growth state of colonies may also vary so it is crucial to monitor each well and be prepared to passage cells at different times.
3. After determining which clones require passaging, remove the growth media of the marked wells and rinse with 500 µl of PBS. Remove the PBS and add 100 µl trypsin–EDTA solution and let incubate in a 37 °C CO_2 incubator for 5 min. Add 200 µl of ES growth media to stop the reaction and disaggregate the cells by pipetting.
4. Add half of the disaggregated cell sample to a cryotube containing an equal volume of freeze media and mix prior to capping. Seal the tubes in a Styrofoam container and freeze overnight at −70 °C before transferring the frozen tubes to liquid nitrogen storage.
5. For the remaining cells in the 48-well dish, add 400 µl of ES growth media, and allow cells to grow prior to isolation of DNA.

3.8 Generation and Selection of Ccn1flox and Knockin Mice

1. Blastocyst injection and implantation can be accomplished by an experienced transgenic/gene targeting service (*see* **Note 5**). The Research Resources Center at UIC conducted these procedures for the construction of *Ccn1* knockin mice.
2. Appropriate ES cell clones are thawed and propagated as described above. Cells are collected and resuspended in 2 ml of injection buffer and placed in a new 3 cm gelatin coated dish to begin sorting the ES cells from the fibroblast feeder cells.
3. Monitor the dish on an inverted phase contrast microscope and observe the larger fibroblast feeder cells that settle to the bottom while the ES cells remain in suspension. Remove the cell suspension with the majority of ES cells and place into a

new 3 cm gelatin coated dish, being careful not to disturb the fibroblast cells that settle on the dish. On the second dish, look for additional fibroblast cells to settle before removing the supernatant containing the ES cells. Reducing the number of contaminating fibroblasts insures a higher percentage of ES cells in the sample prior to blastocyst injection.

4. Successful injection of ES cells into blastocysts and implantation into pseudopregnant mice will produce chimeric animals, which are backcrossed into the C57BL/6 strain to determine germ line transmission of the ES cell lines. All offspring of subsequent matings are genotyped by PCR assay of genomic DNA obtained from tail snips (*see* **Note 6**).

4 Notes

1. In order for the Cre-mediated knockout to work properly, it is imperative that the *LoxP* sites in the *Ccn1flox* allele are properly maintained (Fig. 1c). In designing PCR target primers, we use a combination of unique and endogenous *Ccn1* sequences to confirm recombination events. We employ unique sequences in the targeting vector adjacent to the *LoxP3* site that are not part of the endogenous gene (Primer R1) to confirm the maintenance of the *LoxP3* site in the *Ccn1LoxNeo* and *Ccn1flox* alleles (Fig. 1b–d). We also verify retention of the *LoxP1* site using a hybrid reverse primer (targeting the LoxP1 site) in combination with the F3 primer that targets endogenous sequences 5′ of the integration site.
2. Procedures for the isolation of mouse embryonic fibroblasts were adapted from established protocols [28]. We prefer using pipettes for breaking up the minced tissues rather than passing the tissues through needles, which may decrease cell recovery. The trypsin activity and incubation time will also vary based on the source of trypsin reagent used, so it is important to monitor the reaction by periodically checking for tissue disaggregation by gently agitating the tube and noting the "cloudy" appearance of the media over time.
3. The effective concentration of G418 used in selection can vary with supplier used and ES cell conditions, and should be titrated by performing a "kill" curve in which the lowest concentration of G418 that effectively kills all ES cells within 3–4 days is determined. We use a concentration of 350 μg/ml for ES cell selection; after stable ES lines are isolated lower concentrations of G418 (<200 μg/ml) can be used to maintain the cells.

4. Southern blot or PCR analysis can be used for the first round of detection in ES cells [28] using DNA isolated with either the alkaline lysis or column-based DNA isolation method. ES cells used for DNA isolation can be grown to high density to promote high yield of DNA.
5. In consultation with a transgenic service, determine the number of ES cell clones needed to be amplified prior to blastocysts injection. More than one ES cell line is typically used for injection to increase the chances of a successful transfer into mouse blastocysts. Transgenic services should be able to provide an estimate of the efficiency of the injection and implantation procedure based on their experience.
6. Tail snip DNA can be extracted using a DNA isolation kit according to manufacturer's instructions (e.g., from Omega Bio-Tek or Qiagen). A variety of DNA isolation kits can be used; however, a column-based isolation kit is recommended for obtaining the necessary DNA quality for performing PCR analysis.

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Chapter 31

Production and Analysis of Conditional KO Mice of CCN2 in Kidney

Naohiro Toda, Hideki Yokoi, Kiyoshi Mori, and Masashi Mukoyama

Abstract

CCN2 has been shown to be closely involved in the progression of renal fibrosis, indicating the potential of CCN2 inhibition as a therapeutic target. Although the examination of the phenotypes of adult CCN2 knockout mice with renal diseases has yielded valuable scientific insights, perinatal death has limited studies of CCN2 in vivo. Conditional knockout technology has become widely used for the deletion of genes in the desired cell populations and time points through the use of cell-specific Cre recombinase-expressing mice. Accordingly, several lines of CCN2 floxed mice have been developed for the assessment of the functional role of CCN2 in adult mice.

CCN2 levels are increased in renal fibrosis and proliferative glomerulonephritis, which represent good disease models for evaluating the effects of CCN2 deletion on the kidney. Of these, anti-glomerular basement membrane antibody glomerulonephritis has become the most widely used model for evaluating the effect of increased renal CCN2 expression. Herein, we describe the construction of CCN2 floxed mice and inducible systemic CCN2 conditional knockout mice and methods for the induction of anti-glomerular basement membrane antibody glomerulonephritis.

Key words Cre-loxP system, Anti-glomerular basement membrane (GBM) nephritis

1 Introduction

In the kidney, CCN2 (CTGF) is expressed by podocytes and parietal epithelial cells under normal conditions in humans and rats [1, 2]. CCN2 is strongly upregulated in mesangial proliferative lesions and crescents, formed as a result of parietal glomerular cell proliferation, in crescentic glomerulonephritis including anti-glomerular basement membrane (GBM) antibody glomerulonephritis [1, 3]. CCN2 is closely associated renal fibrosis progression [4], indicating an important role for CCN2 in the progression of crescentic glomerulonephritis. Previous reports, including by our groups, have demonstrated that the inhibition of CCN2 by antisense ODN ameliorates renal fibrosis and diabetic nephropathy [5, 6]. A neutralizing antibody against CTGF also demonstrated

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therapeutic potential for diabetic nephropathy in humans [7]. However, heterologous CCN2 knockout (KO) mice did not demonstrate any improvement in renal fibrosis elicited by unilateral ureteral obstruction [8], questioning the degree of fibrogenesis inhibition conferred by heterozygous deletion of CCN2 may not be enough to counteract the fibrogenesis. Complete ablation of CCN2 is the desired experimental approach for evaluating the contribution of CCN2 to the development of renal diseases. CCN2 KO mice, however, die shortly after birth from respiratory distress caused by skeletal defects [9]. These results prompted us and other groups to attempt to generate conditional CCN2 KO mice. Several lines of CCN2 conditional KO mice have been established, including at our institution. The Cre/loxP system involves flanking of an essential exon of the target gene with two loxP sites for Cre recombinase-driven recombination [10]. Mice carrying loxP sites are designated as "floxed" mice. Cell-specific Cre recombinase-expressing mice have been used to generate cell-specific conditional KO mice. A list of reported conditional CCN2 knockout mice is presented in Table 1.

Inducible Cre-recombinase mice have been developed to allow the deletion of genes of interest at the desired time points such as through the combination of Cre recombinase with mutant human estrogen receptor [11]. The CreER recombinase is inactive under basal conditions, but can be activated by 4-hydroxytamoxifen (4-OHT) or tamoxifen. Active CreER translocate to the nucleus and mediates the deletion of the target gene flanked by loxP sites. $CreER^{T2}$ recombinase is the most frequently used and most efficient CreER recombinase constructs [12]. Type I collagen alpha 2 chain promoter/enhancer-driven CCN2 KO mice has been shown to be protected from skin fibrosis induced by bleomycin [13]. Systemic inducible CCN2 KO mice have demonstrated reduced biliary fibrosis severity [14] and impairment of corneal wound reepithelialization [15], indicating a role for CCN2 in the accumulation of the extracellular matrix protein. However, the deletion of

Table 1
CCN2 floxed mouse and Cre recombinase mouse

Deleted allele of CCN2 floxed mouse	Cre recombinase mouse	Reference
Unknown	Amhr2-Cre, Pgr-Cre	Nagashima et al. [19]
Exon 4	Ubc-CreERT2	Gibson et al. [15]
	Ubc-CreERT2	Pi et al. [14]
Exon 4	Col1a2-$CreER^{T2}$	Liu et al. [13, 16, 20, 21]
	Rosa-$CreER^{T2}$	Fontes et al. [17]

CCN2 in skin fibroblasts was not found to impair cutaneous tissue repair [16]. In addition, a recent study reported no significant difference in cardiac fibrosis induced by transverse aortic constriction in an inducible systemic CCN2 KO mouse [17]. These results indicate that CCN2 may be a modulator of fibrosis on the surrounding factors rather than a potent inducer of fibrosis.

To examine the role of CCN2 in renal disease, we generated inducible systemic CCN2 KO mice through crossing of CCN2-floxed mice with Rosa-CreER^{T2} mice. To assess the role of CCN2, we induced anti-GBM nephritis in mice carrying homozygous CCN2 floxed alleles and CreER^{T2} gene inserted into Rosa26 locus (Rosa CTGF cKO mice) (*see* **Note 1**). Anti-GBM nephritis results in characteristic glomerular injury including podocyte injury and macrophage recruitment into a glomerulus, providing an appropriate model of ECM accumulation and inflammatory responses [18]. Herein we provide protocols for the generation of CTGF conditional KO mice and the induction of anti-GBM nephritis.

2 Materials

2.1 Generation of CCN2 Conditional Knockout Mice

2.1.1 Generation of CCN2 Floxed Mice

1. CCN2 floxed mice carrying a loxP-flanked sites at both ends of CCN2 gene coding region.

2.1.2 Cre Mice

1. C57BL/6-*Gt(ROSA)26Sortm9(Cre/ESR1)Arte*mice (RosaCreER^{T2} mice; Taconic, NY, USA).

2.1.3 Induction of Recombination

1. 4-hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO, USA).
2. Tamoxifen (Sigma-Aldrich).
3. Ethanol.
4. Sunflower seed oil (e.g., Sigma-Aldrich).
5. Homogenizer.
6. Insulin syringe 1 ml 30G.
7. Stomach tube.

2.1.4 Preparation of mRNA for Analysis of Recombination

1. RNA extraction kit (e.g., RNeasy mini kit, QIAGEN, Valencia, CA, USA).
2. 2-mercaptoethanol.
3. Ethanol.
4. PCR system.

2.1.5 Reverse Transcription for Analysis of Recombination

1. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
2. PCR system.

2.1.6 Real-Time PCR for Analysis of Recombination

1. Taq polymerase (e.g., TAKARA premix Ex Taq, TAKARA bio, Kusatsu, Japan).
2. 96-well plate.
3. Adhesive film.
4. Real-time PCR system.
5. Primers and probe for mouse CCN2 (Sigma-Aldrich).

2.2 Analysis of CCN2 cKO Mice in Anti-GBM Nephritis

1. JW Rabbit.
2. ddY mouse (Japan SLC, Hamamatsu, Japan).
3. Complete Freund's adjuvant (BD Bioscience).
4. Homogenizer.

2.2.1 Preparation of Anti-GBM Antibody

2.2.2 Induction of Anti-GBM Nephritis

1. Rabbit IgG Cappel 55944 (MP Biomedicals, Santa Ana, CA, USA).
2. Complete Freund's adjuvant Difco 0638-60-7 (BD Bioscience).
3. Normal saline.
4. 21G needle.
5. Anti-GBM antibody (Rabbit serum).
6. Insulin syringe 1 ml 30G.

2.2.3 Measurement of Urinary Protein

1. Metabolic cage (e.g., Shinano Manufacturing, Tokyo, Japan).

2.2.4 Tissue Preparation and Embedding for Histological Analysis

See Chapter 36.

2.2.5 Double Immunofluorescent Study for CCN2 and Podocin

1. Goat anti-human CCN2(CTGF)antibody (sc-14939; Santa Cruz Biotechnology, Sant Cruz, CA, USA).
2. FITC-conjugated donkey anti goat antibody (Jackson ImmunoResearch, West Grove, PA, USA).
3. HiLyte Fluor 555 labeling kit-NH_2 (Dojindo, Kumamoto, Japan).
4. Anti-rabbit podocin antibody (Sigma-Aldrich).
5. DMSO.
6. Normal donkey serum.
7. Aqueous Mounting Medium PermaFluor (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.6 Periodic Acid-Schiff (PAS) Staining

1. Xylene.
2. Ethanol.
3. 0.5% periodic acid solution (periodic acid dihydrate 1 g, distilled water 200 ml).
4. Schiff reagent.
5. Sulfurous acid solution.

2.2.7 Computer-Aided Histological Analysis)

1. Light microscopy.
2. Computer-aided manipulator (e.g., MetaMorph software; Molecular Devices Sunnyvale, CA, USA).

2.2.8 MAC2 Staining

1. Rat anti-Mac2 antibody (CL8942AP; CEDARLANE, Ontario, Canada).
2. Peroxidase-conjugated donkey anti-rat antibody (Jackson ImmunoResearch).
3. 0.3% H_2O_2.
4. Xylene.
5. Ethanol.
6. Normal donkey serum.
7. Triton X.

3 Methods

3.1 Generation of CCN2 Conditional Knockout Mice

3.1.1 Generation of CCN2 Floxed Mice

Floxed mice are produced by standard technique including construction of targeting vector, chromosome engineering in ES cells derived from C57BL/6J background, selection of positive clones, analysis of DNA from ES cells by Southern blotting, and introduction of altered ES cells into blastocysts. The targeting vector was designed to conditional deletion of entire coding region of the mouse *CCN2* gene. pBluescript II SK(+) vector (Addgene, Cambridge, MA, USA) was subcloned into the entire mouse *CCN2* gene with 6112 bp long arm and 2695 bp short arm (Fig. 1). Mouse *CCN2* gene was amplified by using BAC cone RP23-257E12 and flanked by two loxP sites, which are inserted at 12 bp before the start codon and at immediately after stop codon. The neomycin cassette was flanked by two flippase recognition target (FRT) sites (Fig. 1).

Plasmids are linearized before electroporation to trigger recombination in C57BL/6 ES cells. After 24 h incubation of electroporated ES cells, the ES medium is replaced with medium containing G418 to select positive clones. Genomic DNA obtained from 14 day-cultured ES cells are used for PCR analysis to identify positive clones. To identify loxP insertion near start codon, PCR is performed using two primers located at long arm and at exon as follows: forward primer, 5′-TGTAGGACTCCATTCAGTTCT TTG-3′ and reverse primer, 5′-GTACATGTCAAGGTGACGA GCTA-3′. Wild type ES cells yield 848 bp and mutant ES cells containing loxP yield 882 bp. Subsequently, Southern blot is performed using 5′ probe (392 bp) or 3′ probe (398 bp) after *Spe*I digestion because *Spe*I site is inserted between neomycin and long arm (Fig. 2). Probes are constructed by PCR-based genomic amplification as follows: forward (5′ probe), 5′-TCTGGAG

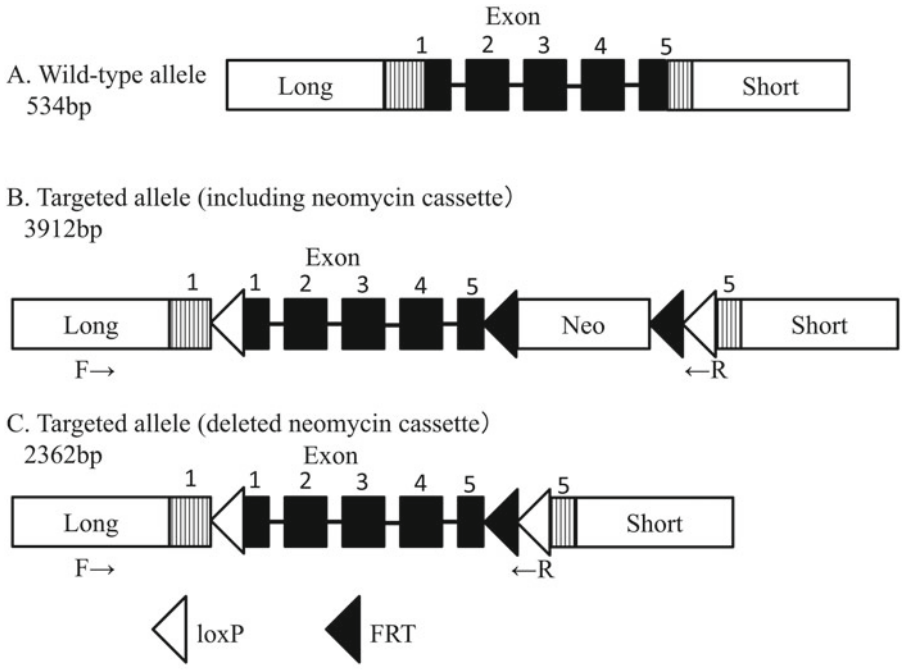

Figure 1. Toda et al.

Fig. 1 Schematic representation of a vector to rearrange a specific targeted locus. (**a**) Wild-type allele of mouse CCN2 gene. *Hatched boxes* indicate untranslated regions, and *solid boxes* represent coding regions. (**b**) A targeting vector for conditional removal of the exon of CCN2 has been generated. (**c**) A floxed allele of CCN2 gene. Flippase induces recombination to excise the neo cassette. PCR analysis of chromosomal rearrangements. The *arrows* represent PCR primers

TCCACTTGAGATzTAAGAC-3′ and reverse (5′ probe), 5′ GGACTGGTTTCATCACATTTTTAAC-3′; forward (3′ probe), 5′-AGCTGATAGGAGATTCAGTGAGAGA-3′ and reverse (3′ probe),5′GTGTAGGAAAGAGAGATGAACAAzGC-3′. Neomycin sequence is also identified by Southern blotting using neomycin probe which is amplified by two primers; forward, 5′-GAACAAGATGGATTGCACGCAGGTTCTCCG-3′ and reverse, 5′-CGCCAAGCTCTTCAGCAATA-3′.

The injection of positive C57BL/6 ES cells into Balb/c hosts gives rise to a chimeric mice judged by coat color. The ICR strain is used for pseudopregnant surrogate and for lactating foster mothers and for vasectomized males. Chimeric mice are further crossed with C57BL/6 mice to obtain F1 mice. PCR analysis is performed using genomic DNA from F1 mice with two primer sets which recognize neomycin sequences and loxP insertion near the start codon. Primers for neomycin sequences are 5′-CGGAGAACCTGCGTGCAATCCATCTTGTTC-3′ (forward) and 5′-TCTGGTCCTAGAATGAATAAACACA-3′ (reverse) which

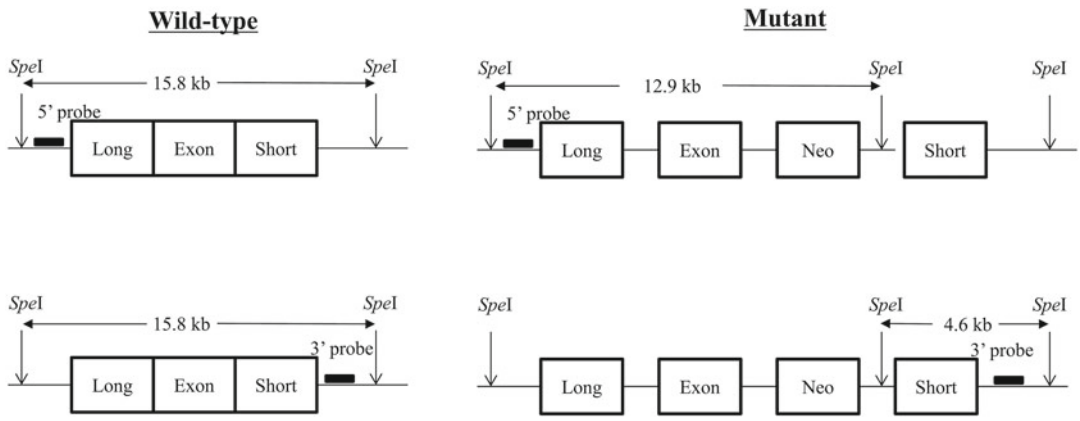

Fig. 2 Southern blot analysis of chromosomal rearrangements. Southern blot screening for insertion of loxP

yield 4579 bp for mice carrying neomycin cassette. Mutant mice are then crossed with flippase (FLP)-transgenic mice to eliminate neomycin by FLP-FRT recombination. To identify the removal of neomycin cassette, we used two primers located at long arm and at loxP-short arm junction; forward, 5′-TGTAGGACTCCATTCAGTTCTTTG-3′ and reverse, 5′-TTCCGATCATATTCAATAACCCTTA-3′, which yield 2362 bp for successful CCN2 floxed mice.

3.1.2 Generation of Inducible Systemic CCN2 Conditional Knockout Mice

Inducible-systemic CCN2 knockout mice (Rosa-CCN2 cKO) are generated by mating of *CCN2fl/fl* mice and Rosa-CreER^{T2} mice that systemically expresses CreER^{T2} recombinase under the control of Rosa26 promoter. CreER^{T2} is a fusion protein of Cre recombinase and mutant human estrogen receptor. Administration of 4-hydroxytamoxifen or tamoxifen induces Cre recombinase activity and the target gene flanked with loxP sequence throughout the body can be deleted.

3.1.3 Preparation and Administration of 4-Hydroxytamoxifen (4-OHT) for Recombination

1. Suspend 25 mg of 4-hydroxytamoxifen (4-OHT) in 0.5 ml of ethanol in a 50 ml tube.
2. Add 9.5 ml of autoclaved sunflower oil.
3. Mix the suspension sonicate for 30 min on ice (*see* **Note 2**).
4. 4-OHT stock solution (5 mg/ml) stored at −20 °C in a light-shielding tube.
5. Before use, thaw the 4-OHT solution at 37 °C (*see* **Note 3**).
6. Three-week-old RosaCreER^{T2}/CCN2 floxed mice are intraperitoneally injected with 0.05 mg/gBW 4-OHT for 3 consecutive days.

3.1.4 Preparation and Administration of Tamoxifen for Recombination

1. Suspend 100 mg of tamoxifen in 0.5 ml of ethanol in a 50 ml tube.
2. Add 4.5 ml of autoclaved sunflower oil.

3. Mix the suspension sonicate for 5 min on ice.
4. Tamoxifen stock solution (20 mg/ml) is stored at –20 °C in a light-shielding tube.
5. Before use, thaw the tamoxifen solution at 37 °C.
6. RosaCreER^{T2}/CCN2 floxed mice were intraperitoneally injected or administered per os with 0.2 mg/gBW and 0.1 mg/gBW tamoxifen for 3 consecutive days both at 3 weeks and 6 weeks of age, respectively.

3.1.5 Preparation of RNA for Analysis of Recombination

1. Extraction of mRNA of glomeruli is performed using RNeasy Mini kit.
2. Add 350 μl Buffer RLT and β-mercaptoethanol 3.5 μl to sample and mix well.
3. Add 350 μl ethanol and mix by pipetting.
4. Transfer the 700 μl sample to RNeasy column, and centrifuge for 1 min at 36,220×*g*, discard flow-through.
5. Add 350 μl Buffer RW1 to RNeasy column, and centrifuge for 1 min at 36,220×*g*.
6. Add 10 μl DNaseI incubation mix to 70 μl Buffer RDD and mix gently.
7. Add DNase mix to RNeasy column and incubate for 15 min at room temperature.
8. Add 350 μl Buffer RW1 to RNeasy column, and centrifuge for 1 min at 36,220×*g*, discard flow-through.
9. Add 500 μl Buffer RPE to RNeasy column, and centrifuge for 1 min at 36,220×*g*, discard flow-through, for two times.
10. Place the RNeasy spin column in a new 1.5 ml tube, add 30 μl of RNase-free water to RNeasy column, and centrifuge for 1 min at 36,220×*g* to elute the RNA.
11. RNA is stored at –80 °C.

3.1.6 Reverse Transcription

1. To reverse-transcribe RNA of glomeruli, we use High Capacity cDNA Reverse Transcription Kit.
2. Prepare 20 μl PCRs in sterile thin-walled PCR tube. Add reagents in the order listed. Mix gently.

Distilled water	*X*μl to a final volume of 20 μl
10× RT buffer	2 μl
25× dNTP mix	0.8 μl
10× RT primer	2 μl
Reverse transcriptase	1 μl
RNA	100–1000 ng

3. Perform the reaction under the following conditions.

25 °C	10 min
37 °C	120 min
85 °C	5 s
4 °C	∞

4. cDNA is diluted 1 ng/ml by distilled water.

3.1.7 Real-Time RT-PCR

1. Mouse CCN2 primers and probe for Taqman PCR are as follws: (Forward) 5′-ttcccgagaagggtcaagct-3′, (Reverse) 5′-tccttgggctcgtcacaca-3′, (Probe) 5′-FAM-cctgggaaa tgctgcaaggagtgg-TAMRA-3′.
2. Prepare 96-well plate. Add reagents without cDNA in the order listed to prepare premix reagent. Mix gently.

Distilled water	0.2 μl
Premix Ex Taq (×2)	5 μl
Forward primer	0.2 μl
Reverse primer	0.2 μl
Taqman probe	0.2 μl
ROX reference dye	0.2 μl
cDNA (1 ng/μl)	4 μl

3. Apply premix reagent 6 μl per well, and add cDNA at 4 μl each well. Seal 96-well plate by adhesive film.
4. Perform real-time PCR under the following conditions.

Initial denaturation	95 °C, 1 s
40 cycles	95 °C, 5 s
	60 °C, 31 s

3.2 Analysis of CCN2 cKO Mice in Anti-GBM Nephritis

3.2.1 Preparation of Anti-GBM Antibody

1. Glomeruli are isolated by differential sieving from the ddY mouse renal cortex.
2. Disrupt by sonication.
3. The GBM is collected by centrifugation, and emulsified with complete Freund adjuvant.
4. Rabbits are immunized by a subcutaneous injection with mouse GBM once a week for 4 consecutive weeks.
5. Rabbit serum including anti-mouse GBM antibody is collected 1 week after last injection.

3.2.2 Induction of Anti-GBM Nephritis

1. An acceleration form of anti-GBM nephritis is induced in CCN2 cKO mice.
2. Mice are immunized by an intraperitoneal injection of 0.5 mg/20 gBW of normal rabbit IgG emulsified with complete Freund's adjuvant at 8-weeks of age.
3. An anti-GBM antibody or control normal rabbit serum is injected via femoral vein, 5 days later.

3.2.3 Measurement of Urinary Protein

Urinary protein levels and body weights are measured at days 0, 3, 7, 14, and 28 after induction of anti-GBM nephritis. For urinary measurements, each animal is housed separately in a metabolic cage. Urinary protein, urinary creatinine, and serum creatinine are measured by the enzymatic method.

3.2.4 Tissue Preparation and Embedding (See Chapter 36)

1. Place a tissue in 4% PFA or Doboscq-Brazil fixative overnight at room temperature (*see* **Note 4**).
2. Place a tissue biopsy in 70% EtOH or 4% formalin.
3. Tissue is embedded in paraffin.
4. Cut 1–4 μm sections of the paraffin-embedded tissue on microtome.

3.2.5 Double Immunofluorescent Study for CCN2 and Podocin (Fig. 3)

Podocin protein predominantly expressed by podocytes and constitutes the slit diaphragm between the footprocesses of podocytes. Podocin staining is useful for detecting the podocytes. We use the double immunofluorescent staining for identifying the cellular localization of CCN2.

3.2.6 Preparation of HiLyte Flour 555-Labeled Rabbit Podocin Antibody

The excitation and emission wavelengths of the HiLyte Fluor 555-labeled protein are 555 and 570 nm.

1. Add 100 μl WS buffer and anti-podocin antibody (50–200 μg) to a filtration tube and mix the solution with pipetting, and centrifuge at 8000 × *g* for 10 min.
   Add 10 μl DMSO to NH_2-Reactive HiLyte Fluor 555, and dissolve with pipetting.
2. Add 100 μl reaction buffer to the filtration tube, and then add 8 μl NH_2-reactive HiLyte solution to the filtration tube and pipette the mix.
3. Incubate at 37 °C for 10 min.
4. Add WS buffer to the filtration tube and centrifuge at 8000 × *g* for 10 min for three times.
5. Add 200 μl WS buffer. And pipette about ten times to recover the conjugate.
6. Store at 4 °C in light-shielding tube.

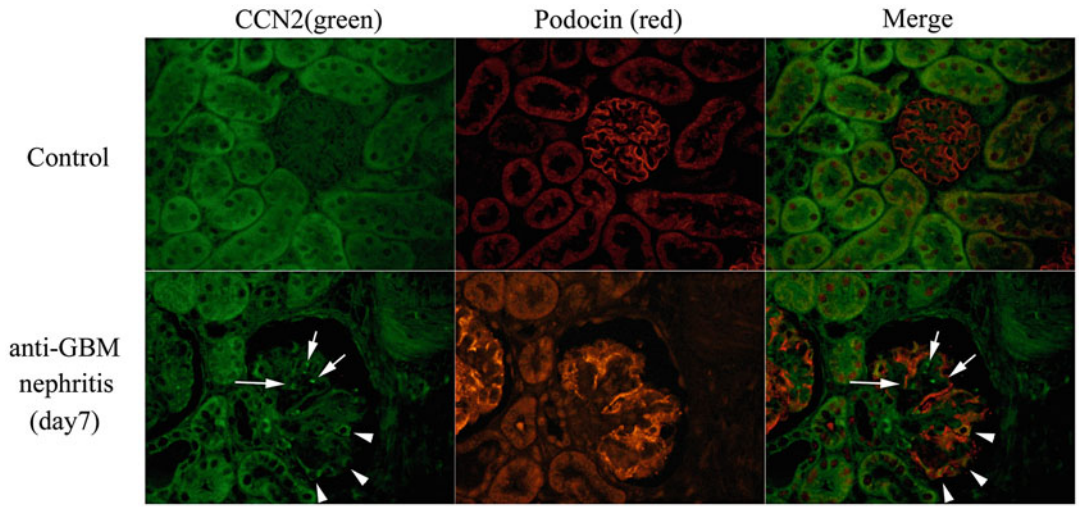

Fig. 3 Double immunofluorescent study for CCN2 and podocin. Podocin (*red*) predominantly expressed by podocytes and CCN2 (*green*) is localized at tubular cells under control conditions. At 7 days after anti-GBM nephritis induction, CCN2 expression was increased in podocytes (*arrowheads*) and mesangial cells (*arrows*)

3.2.7 Double Immunofluorescent Staining for CCN2 and Podocin (see ***Note 5****)*

1. Treat with xylene three times for 5 min.
2. Dehydrate tissue through graded alcohols (100% ethanol three times, 90% once, 80% once and 70% ethanol once).
3. Place antigen retrieval in the pressure cooker. Heat the pressure cooker without slides until boiling.
4. Do not secure the lid of the pressure cooker at this time.
5. When boiled, place slides within a slide basket in a pressure cooker.
6. Rinse two times with 1× PBS for 3 min each.
7. Boil for 10 min.
8. Rinse two times with 1× PBS for 3 min each.
9. Outline sections with a hydrophobic wax pen to avoid antibody spreading and possible drying.
10. Incubate slides with 10% donkey serum in 1× PBS at room temperature for 30 min.
11. Incubate with primary antibody (goat anti-CCN2 antibody 1:50 + HiLyte Flour 555-labeled rabbit anti-podocin antibody 1:50) in 1× PBS at room temperature for 1 h.
12. Rinse two times with 1× PBS for 3 min each.
13. Incubate with secondary antibody (FITC-donkey anti-goat antibody 1:100) in 1× PBS at room temperature for 1 h.
14. Rinse two times with 1× PBS for 3 min each.
15. Coverslip using PermaFlour.

3.2.8 Periodic Acid-Schiff (PAS) Staining

1. Treat with xylene three times for 5 min.
2. Dehydrate tissue through graded alcohols (100% ethanol three times, 90% once, 80% once and 70% ethanol once).
3. Wash with water.
4. Treat with 0.5% periodic scid solution for 10 min.
5. Treat with Schiff reagent for 20 min.
6. Treat with sulfurous acid solution for 3 min three times.
7. Wash in distilled water.
8. Dehydrate in graded ethanol.
9. Clear in xylene three times for 5 min.
10. Coverslip using mounting medium.

3.2.9 Histological Analysis

Kidney sections stained with periodic acid-Schiff are examined by light microscopy.

The ratio of the number of glomeruli containing crescents to total glomeruli is calculated as the crescent formation ratio. The mesangial areas in ten superficial glomeruli are measured quantitatively using a computer-aided manipulator.

3.2.10 MAC2 Staining

MAC2 antigen is expressed in macrophages (*see* **Note 6**).

1. Treat with xylene three times for 5 min.
2. Dehydrate tissue through graded alcohols.
3. Wash two times in 1× PBS for 5 min.
4. 0.3% H_2O_2 15 min for blocking of endogenous peroxidase activity.
5. Wash two times in 1× PBS for 5 min.
6. Three times microwave for 5 min for antigen retrieval.
7. Wash two times in 1× PBS for 5 min.
8. Incubate slides with 10% donkey serum in 1× PBS at room temperature for 10 min.
9. Incubate with primary antibody (Rat anti-MAC2 antibody 1:100) in 0.3% Triton X diluted in 1× PBS at room temperature for 1 h.
10. Wash three times in 1× PBS for 5 min.
11. Incubate with secondary antibody (peroxidase-conjugated donkey anti-rat antibody 1:100) in 1× PBS at room temperature for 1 h.
12. Wash three times in 1× PBS for 5 min.
13. Cover sections with the 3,3′-diaminobenzidinetetrahydrochloride (DAB) for 10 min at room temperature.
14. Wash with tap water.
15. Stain with Weigert's iron hematoxylin for 1 min.

16. Wash in tap water, 3 min.
17. Dehydrate through ethanol.
18. Clear in xylene.
19. Coverslip using mounting medium.

3.2.11 Macrophage Infiltration Analysis

More than 20 consecutive glomerular sections in each mouse are examined, and the mean number of MAC2-positive cells per glomerular cross section is calculated (*see* **Note 7**).

4 Notes

1. The mouse CCN2 coding region (Exon 1–5) is flanked by the loxP recognition sequences, and Cre recombinase is used to delete all CCN2 coding sequences (Fig. 1).
2. It takes long time to dissolve 4-OHT by sonication. As sonication causes production of heat, sonication must be done on ice.
3. Hydroxytamoxifen stock solution may have precipitated particles at the bottom of the stock tube. Before use, thaw the 4-OHT solution at 37 °C to dissolve 4-OHT completely.
4. Immunofluorescent staining for CCN2 may be weak in the case of the frozen specimen.
5. We performed double immunofluorescent study to determine the localization of CCN2.
6. For MAC2 and CCN2 immunofluorescence staining, Duboscq-Brazil-fixed samples may be stained better than 4% PFA-fixed samples.
7. For mesangial area measurement and MAC2-positive cell counting, glomeruli including vascular pole are to be analyzed.

Acknowledgments

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Chapter 32

Generation and Analysis of Cartilage-Specific CCN2 Overexpression in Transgenic Mice

Takako Hattori, Shinsuke Itoh, and Masaharu Takigawa

Abstract

Recent progress in gene-editing technology has provided a strong impact for improved our understanding of molecular functions in living organisms. Here we describe our method to generate transgene-overexpressing mouse models, which method involves the use of tissue-specific promoters for analyzing a certain molecule (s) in special tissues. The protocol described in this chapter uses the *Col2a1* promoter-enhancer, which is known for driving specific and strong transgene expression in cartilage and is based on several of our studies showing a positive role of the connective tissue growth factor (CCN2) in cartilage-bone development and maintenance of articular cartilage. These mice show strongly accelerated endochondral ossification resulting in enhanced bone elongation, as well as resistance to age-related articular degeneration. This protocol also describes how to analyze the molecular mechanisms of these phenomena by use of chondrocytes isolated from CCN2-overexpressing cartilage.

Key words Transgenic, *Col2a1* promoter-enhancer, Cartilage, Endochondral ossification, Development, IGF, Skeletal preparation, Histochemistry, LacZ staining, Aging

1 Introduction

CCN2, also known as connective tissue growth factor (CTGF), has multiple growth-supporting functions in connective tissues and is strongly expressed in developing chondrocytes. Its role in several endochondral ossification steps including the proliferation, maturation, and hypertrophy of chondrocytes [1], the proliferation and differentiation of osteoblasts [2], as well as the proliferation, migration, and tube formation of vascular endothelial cells [3] has been demonstrated in in vitro culture systems and in vivo by use of CCN2-deficient mice [4]. Mice lacking CCN2 show abnormal bone shape and lethality just after birth. Additional valuable information on the role of CCN2 in cartilage formation and skeletal development in vivo has also been obtained by overexpression of CCN2 in cartilage. Here we describe the specific overexpression of transgenes in cartilage under the strong promoter and enhancer

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DOI 10.1007/978-1-4939-6430-7_32,

of the type II collagen gene (*Col2a1*), which has been proven a powerful tool in numerous studies [5–10]. Type II collagen is a major cartilage-specific matrix component; and it is expressed in all cartilaginous tissues including hyaline, elastic, and fibrous cartilages in all stages of development, starting with early cartilage blastemas in the limb and in the somite sclerotome up to mature articular cartilage and nasal, tracheal, and auricular cartilages. In early embryonic stages type II collagen is also expressed transiently at low levels in non-cartilaginous tissues, including the heart, epidermis, and brain [11]; although the functions of this collagen in these tissues has not yet been clearly defined.

The *cis*-acting regulatory elements of the *Col2a1* gene, which are essential for cartilage-specific expression of *Col2a1*, have been well characterized [12–17]. Constructs containing a region 3 kb upstream of the transcription start site and including 3 kb of intron 1 linked with exon 1 of the *Col2a1* gene and 70 bp 5′ proximal of intron 1 show strong promoter activity in cartilage, as monitored by reporter gene activity [13]. Therefore this construct has been widely used in mouse models for specific overexpression of many genes in cartilage [5–10]. Overexpression of Cre recombinase directed by the *Col2a1* promoter-enhancer is used for deletion of cartilage-specific *lox*-P-flanked genes [18]. A tamoxifen-inducible conditional gene deletion, *Col2a1-CreER*^{T2} is also used as a powerful method to determine the function of genes that are either embryonic lethal or associated with marked abnormalities in morphology and tissue architecture [19, 20]. Alternatively, expression of transgenes under the *aggrecan* (*Agc*) promoter [21] permits expression only in cartilaginous tissues.

For assessment of cre-mediated recombination in vivo, either *Col2a1*-Cre or *Col2a1*-Cre ER^{T2} transgenic mice are crossed with *Rosa26R* reporter mice (*R26R* strain) [22, 23]. The *Rosa26R* mice have the lacZ or other reporter gene inserted into the ubiquitously expressed Rosa locus that is preceded by a transcriptional stop cassette flanked by *lox*P sites. Since *Col2a1*-Cre *R26R* transgenic mice show reporter gene activity in chondrocytes, the cells which had been expressed *Col2a1* gene have reporter gene activity and are tracked their cell fates. Recent works have revealed transdifferentiation of chondrocytes into osteoblasts [24, 25].

CCN2 enhances *ccn2* expression in autocrine manner, and strong induction of *ccn2* expression driven by the *Col2a1* promoter-enhancer would be expected to further enhance its expression. CCN2 also stimulates *Col2a1* expression in in vitro culture; therefore, enhanced expression of *ccn2* in cartilage would be expected to continue from embryonic stages to growth and maturation stages of cartilage (Fig. 1).

Below we present protocols for generating a DNA construct for *Col2a1* promoter-enhancer-driven *ccn2* overexpression in transgenic mice, which construct can be used for verification of CCN2 expression from the targeting construct, analysis of transgene expression in animals, and analysis of skeletal phenotypes.

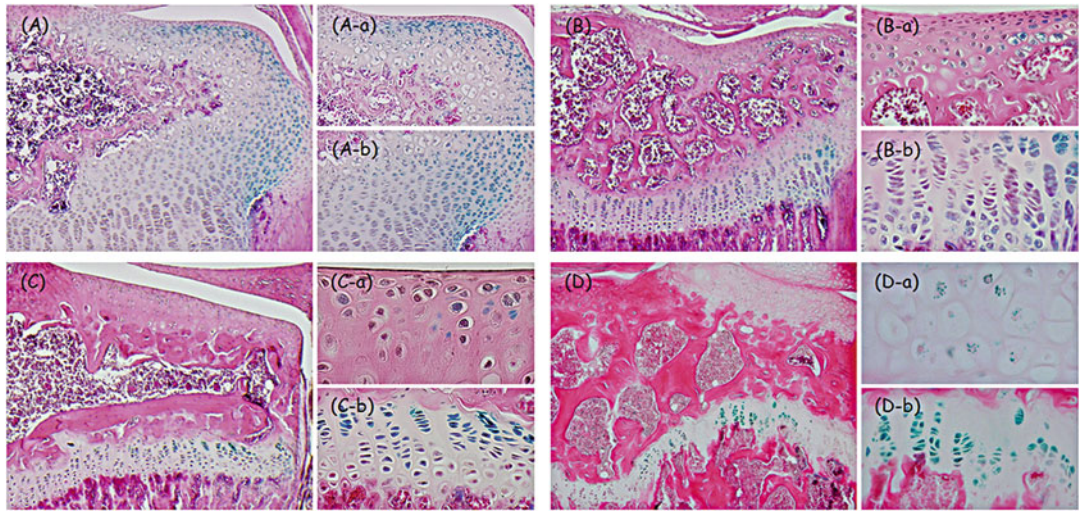

Fig. 1 Analyzing LacZ reporter gene expression in tibial articular (a) and growth plate (b) cartilage from *CCN2COL2A1*^{tg} mice (**A**) 14, (**B**) 40, (**C**) 60, and (**D**) 150 days old. Transgene expression derived from *Col2a1* promoter in not only the growth plate but also in the articular cartilage was active in 150-day-old tibia

2 Materials

2.1 Preparation of HA-Tagged CCN2 Expression Vector

1. Mouse primary chondrocytes.
2. Any total RNA isolation kit (e.g., RNeasy mini kit, Qiagen).
3. pFLAG-CMV2 vector (SIGMA).
4. Primers for amplification of full length mouse ctgf cDNA:
   mCTGF5′: 5′-ccgcgaattcaatgctcgcctccgtcgcaggtcccatc-3′
   mCTGF3′: 5′-cccgggatcctctagattaagatctcgccatgtctccgtaca tcttcct-3′
5. Taq DNA polymerase (e.g., Takara).
6. T4 DNA ligase.
7. Restriction enzymes: *EcoR*I, *Xho*I, *Bgl*II.
8. HA tag linker: Anneal 2 oligonucleotides: 5′HA 5′-gatcttac cca tac gat gtt cca gat tac gctg-3′ and 3′ HA 5′-gatccagcgta-atctggaacatcgtatgggtaa-3′ as follows:
   a. Mix 500 pmol of 5′ HA and 500 pmol of 3′ HA and 1 μl of 5 M NaCl (final 50 mM NaCl) and TE to adjust to 100 μl, and boil at 100 °C for 10 min, and cool down slowly by turning off the heater to room temperature. Phosphorylate the linker using T4 polynucleotide kinase (e.g., Takara) with 1 mM ATP.
9. Any *E. coli* competent cells.
10. Sequencing primers: pFLAG-F 5′-ctc cac ccc att gac gtc aat ggg ag-3′ and pFLAG-R 5′-ggt tcc caa tag acc ccg cag gcc ct-3′.

11. COS7 cells.
12. Dulbecco's modified Eagle's medium containing 10% FCS.
13. Fugene 6.
14. Reagents for Western blot: anti-CTGF antibody (8–86, *see* **Note 1**) anti-Flag M2 antibody (SIGMA), anti-HA antibody (Covance).

2.2 Preparation of DNA Constructs for Generation of Transgenic Mice

1. *Xho*I-*Hind*III fragment of *Col2a1* 3 kb–3 kb promoter from 3 kb–3 kb *Col2a1*/pBS M2.
   *Xba*I-*Xho*I fragment of *IRES-LacZ* from *IRES-LacZ-SV40*pA/pBS.
   *Hind*III-*Xba*I fragment of mCTGF-3HA from mCTGF-3HA/pFlag-CMV2.
2. Any DNA ligation kit (for example DNA ligation kit ver.2 (e.g., Takara)).
3. Sequencing primers:

   mCTGF918-942 5′-ccccgatggcgagatcatgaaaaag-3′.
   mCTGF106-82 5′-ctgcgcactgacattgcgcgctgca-3′.
   CL2-long 5′-atggtgcgggctcttcagtccctggc-3′.
   M13 rev 5′-cagga aacag ctatg ac-3′.
4. Restriction enzymes: *Xho*I, *Hind*III, *Xba*I, *Pme*I, *Swa*I.
5. Gel extraction kit (e.g., Qiagen).

2.3 Isolation of Mouse Primary Chondro-cytes from Rib Cartilage and Verification of CCN2 Overexpression

1. Embryonic stage 18.5 days to postnatal 2 days mouse.
2. Trypsin solution: 0.25% trypsin, 0.02% EDTA in Hank's Balanced Salt Solution (HBSS).
3. Collagenase solution: 3 mg/ml collagenase A (e.g., Roche) in DMEM.
4. Glutaraldehyde.
5. X-gal solution containing 0.2% X-gal (Wako), 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 5 mM $K_3Fe(CN)_6$ in PBS.

2.4 Analyzing LacZ Reporter Gene Expression in the Whole Mount Animals In Situ

1. Cold PBS.
2. Fixative: 0.1 M Na phosphate buffer (pH 7.3), 0.2% glutaraldehyde, 0.8% formaldehyde, 5 mM EGTA (pH 8), 2 mM $MgCl_2$.
3. Rinse solution: 0.1 M Na phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% Na deoxycholate, and 0.02% NP-40.
4. Staining solution: 0.1 M Na phosphate buffer (pH 7.3), 20 mM Tris–HCl (pH 7.3), 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.01% Na deoxycholate, 0.02% NP-40, and 1 mg/ml X-gal.
5. 100% ethanol.
6. Acetone.
7. 1% KOH.
8. Glycerol.

2.5 Determine Genotypes

1. Total genomic DNA isolation kit (e.g., Tissue and blood DNA extraction kit, Qiagen).
2. Any Taq DNA polymerase (e.g.,Quick Taq HS DyeMix, Toyobo).

2.6 Skeleton Preparation

1. 95 % ethanol.
2. Alcialn blue solution: 0.45 mg/ml alcian blue 8GX, 20 % acetic acid, 76 % ethanol, and 4 % distilled water.
3. 2 %KOH.
4. Alizarin red solution: 0.05 mg/ml alizarin red sodium sulfate (SIGMA) in 1 % KOH.
5. Glycerol.
6. Filter paper.

3 Methods

In the following protocols, we describe the preparation of a CCN2-HA fragment (*see* Subheading 3.1), preparation of a DNA construct for generation of transgenic mice (*see* Subheading 3.2), a method for verification of CCN2 expression from the construct in vitro (*see* Subheading 3.3), analysis of reporter gene expression in animals (subheading 3.4), genotype analysis (subheading 3.5), and tools to analyze the skeletal phenotype in whole-mount preparations (*see* Subheading 3.6).

3.1 Preparation of HA-Tagged CCN2 Expression Vector

1. Isolate mouse chondrocyte RNA (for isolation of primary mouse chondrocytes in primary culture, *see* Subheading 3.3 below) by use of any commercially available kit.
2. Amplify mouse *ccn2* cDNA by use of mCTGF-5′ and mCTGF-3′ primers that contain restriction sites at their 5′ ends, and any Taq DNA polymerase with high fidelity. Treat 5′ and 3′ ends of the amplified *ccn2* fragments and pFLAG-CMV2 vector with *Eco*RI and *Xho*I, ligate them using T4 DNA ligase, and transform *E. coli* with them. Pick several clones and analyze their sequences with pFLAG-F and pFLAG-R primers. Select one clone with minimal misamplification with no codon changes.
3. Plate COS7 cells in 6-well plates at a density of 5×10^5 cells/2 ml; and after 24 h incubation, transfect them with 1 μg of CCN2-expression vector. Then collect the cells and estimate the gene expression by performing Western blot analysis using anti-CCN2 monoclonal antibody 8–86 (1:5000), anti-HA antibody (1:1000), and anti-Flag antibody (1:1000; Fig. 2).
4. For distinguishing overexpressed CCN2 from the endogenous molecule, add an HA-epitope tag at the C-terminus before the terminal codon. The HA epitope linker, which is annealed to

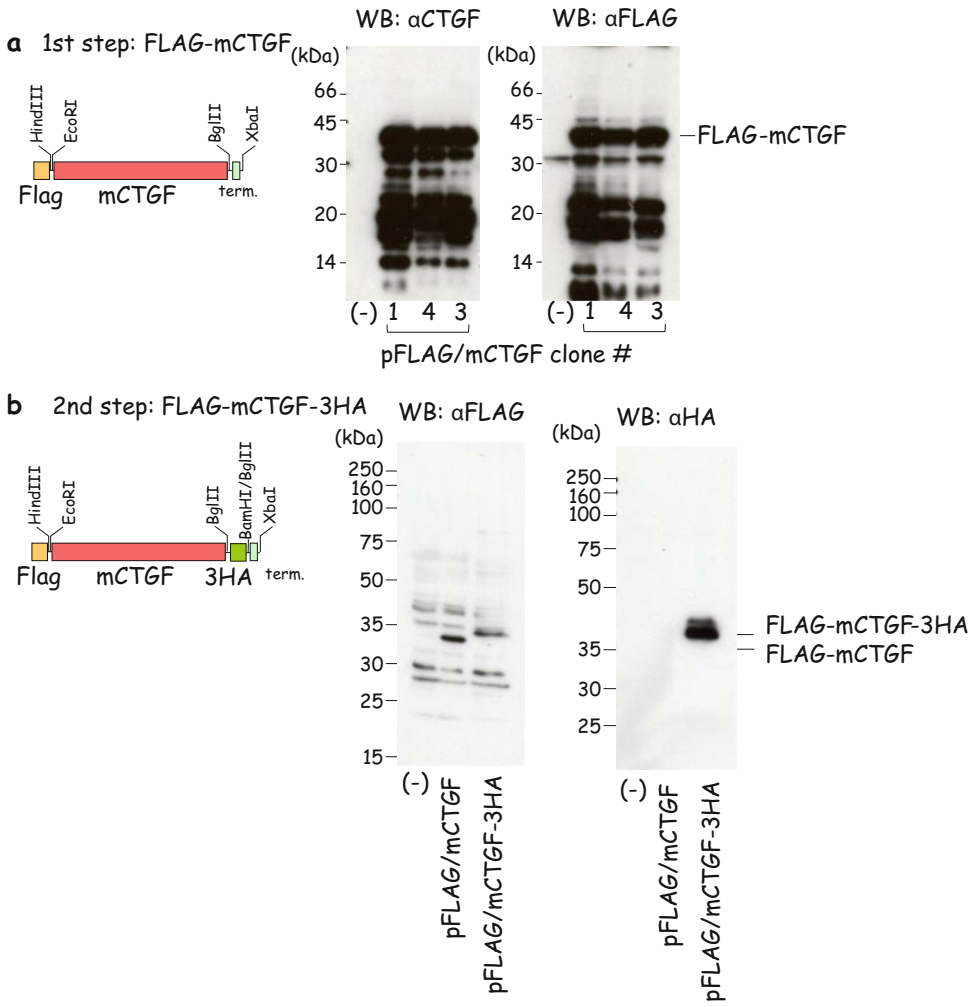

Fig. 2 Preparation of pFLAG-CMV2/mCTGF (**a**) and of pFLAG-CMV2/mCTGF-3HA (**b**) vectors and verification of their expression in COS7 cells. (**a**) mCTGF cDNA was amplified from mouse chondrocyte RNA by using mGTGF-5′ and mCTGF-3′ primers, and inserted into pFLAG-CMV expression vector. Several clones were picked up, and expression of CTGF protein was examined using anti-CTGF antibody and FLAG antibody. (**b**) Three times multimeric HA epitope was inserted at the 3′ end of mCTGF cDNA in frame. The expression of CTGF-3HA protein in COS7 cells was monitored by Western blot analysis using anti-FLAG and anti-HA antibody

5′HA and 3′HA oligonucleotides, has *Bgl*II and *Bam*HI restriction sites on its 5′ and 3′ ends, respectively; and since the linker has phosphate on the 5′ end of both strands, the linker can be inserted into the vector at *Bgl*II site. Direction of linker insertion can be monitored by PCR amplification using 5′HA and pFLAG-R oligonucleotides. To prepare multimeric epitopes, continue to digest the pFLAG-mCTGF-HA vector with *Bgl*II and insert the HA linker.

5. Confirm expression of FLAG-mCTGF-HA vector(s) in mammalian cells as above (Fig. 2). An anti-HA antibody (1:2000) can be also used for detection of FLAG-mCTGF-3HA.

3.2 Preparation of DNA Constructs for Generation of Transgenic Mice

1. Ligate *Xho*I-*Hind*III fragment of the *Col2a1* 3 kb–3 kb promoter, *Xba*I-*Xho*I fragment of *IRES-LacZ*, and *Hind*III-*Xba*I fragment of *mCTGF*-3HA, as shown in Fig. 3.
2. Analyze connections between these fragments. Primers used: CL2-long and mCTGF106-82 for border between *Col2a1* 3 kb–3 kb promoter and *mCTGF*-3HA; mCTGF918-942 for border between *mCTGF*-3HA and *IRES-LacZ*, and M13rev for border between *Col2a1* 3 kb–3 kb promoter and *IRES-LacZ* (Fig. 4)
3. Confirm CCN2 expression in the mouse chondrocytes in primary culture by transfecting them with the targeting vector, as described in subheading 3.3 (Fig. 5).
4. Linearize the targeting vector by use of *PmeI* and *SwaI*. The pBluescript vector (~3 kbps) needs be removed by loading it on a gel. Excising the col2a1-mCTGF-IRES-LacZ band (~11.9 kbps) from the gel, and eluting it from the gel by using any commercially available kit.
5. Determine DNA concentration by electrophoresis by comparing the band intensity with that of DNA standards.

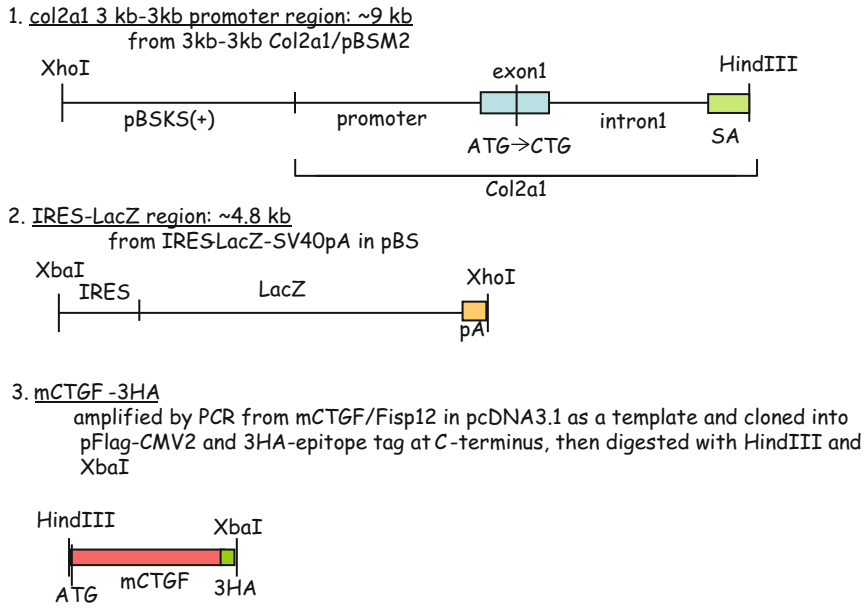

Fig. 3 Schematic representation of DNA fragments used to generate transgenic mice overexpressing CCN2 exclusively in cartilage. (*1*) Approximately 3 kb of *Col2a1* promoter sequence, 237 bp of exon 1 and approximately 3 kb of intron 1 (3 kb–3 kb *Col2a1*) followed by a splice acceptor (SA) sequences were cloned in pBluescriptKS(+) vector. The *Col2a1* translation initiation codon (ATG) was changed to CTG. (*2*) *Escherichia coli* β-galactosidase (*LacZ*) sequences was preceded by the internal ribosomal entry site (*IRES*) and followed by the bovine growth hormone gene polyadenylation signal (pA). (*3*) mCTGF-3HA fragment was excised from pFLAG-CMV2/*mCTGF*-3HA vector. All of the fragments were prepared using restriction enzymes as indicated

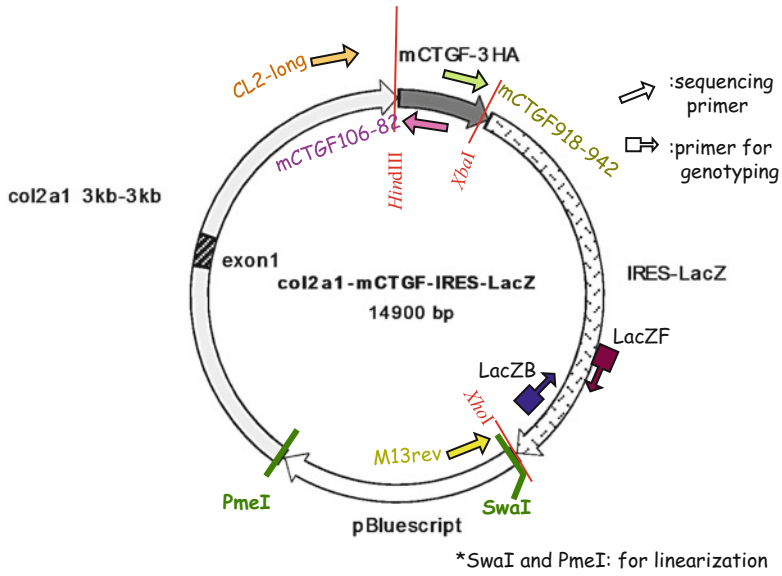

Fig. 4 Schematic representation of construct used to generate CCN2-overexpressing transgenic mice exclusively in cartilage. DNA fragments as shown in Fig. 2 were linked, and connection sequences were analyzed using primers as indicated by →. LacZB and LacZF primers were used to analyze the genotypes. For linearization, *PmeI* and *SwaI* restriction enzymes were used

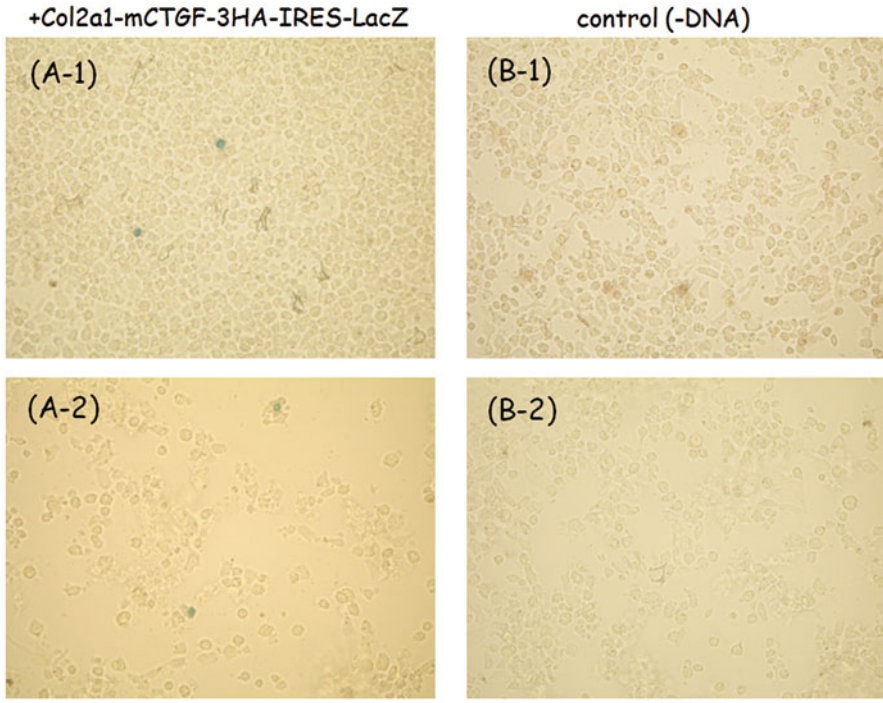

Fig. 5 Use of mouse primary chondrocytes in primary culture established from rib cage for analyzing *LacZ* reporter gene expression from DNA construct used to generate transgenic mice overexpressing CCN2. Twelve hours ahead of transfection, the cells were seeded. After 24 h the DNA transfection β-gal activity was detected with X-gal as a substrate. (*A-1,2*) Mouse primary chondrocytes transfected with *col2a1-mCTGF-3HA-IRES-LacZ* vector and (*B-1,2*) without DNA. 1 and 2 are photographs from different fields

6. For DNA injection into mouse oocytes, about 10–15 ng purified DNA per transgenic line is required. Inject DNA into pronuclei of one-cell mouse embryos isolated from C57BL/6CrSlc females and implant these embryos into ICR pseudopregnant foster mothers (*see* **Note 2**).

3.3 Isolation of Mouse Chondrocytes in Primary Culture Derived from Rib Cartilage and Verification of CCN2 Overexpression (See Note 3)

1. Use mice from 18.5-day-old embryos to newborns at 2 days after birth as the source of cartilage.
2. Clean the epiphyses of all four legs and rib cartilage by observation under a stereomicroscope, removing all soft tissues and bones and placing them on a sterile piece of autoclaved filter paper onto which the soft connective tissue will stick. Collect separately epiphyseal cartilage and rib cartilage.
3. Incubate the cartilage for 5 min in a solution of 0.25% of (sterile filtered) trypsin and 0.02% EDTA (fresh) in HBSS with gentle shaking at 37 °C.
4. Stop trypsin digestion by adding DMEM containing 10% FCS. Decant the trypsin and wash the cartilage with DMEM containing 10% FCS several times to remove soft tissue-derived cells.
5. Add collagenase solution and incubate under gentle shaking/agitation at 37 °C until the cartilage is almost completely digested (about 1–2 h). The remaining tissue fragment may be dissociated completely by repeated pipetting (*see* **Note 4**).
6. Remove undissociated tissue remains by filtering through a 20-µm nylon mesh.
7. To maintain a high expression level of chondrocytic marker genes, especially the *Col2a1* gene, seed chondrocytes at a high cell density, e.g., at $0.5–1 \times 10^5$ cells /well in 6-well plates with wells containing 2 ml of DMEM/ 10% FCS.
8. Incubate cells for 12 h at 37 °C with 5% CO_2.
9. Transfect chondrocytes with the *col2a1*-m*CTGF-IRES-LacZ* construct by the use of any commercially available transfection reagent. DNA linearization is not necessary for transgene expression. We use Fugene 6 and follow the procedure recommended by the supplier.
10. Incubate the cells for 24 h at 37 °C with 5% CO_2 and then wash them twice with PBS. Remove all of the final wash buffer.
11. Fix the cells by adding 2 ml of 0.25% glutaraldehyde in PBS and incubating them for 15 min at room temperature.
12. Remove the glutaraldehyde solution and wash the cells gently three times with PBS.
13. Add 1 ml of X-gal solution and incubate the cells at 37 °C for 1–16 h until the blue color of the cells becomes visible (Fig. 5).

3.4 Analyzing LacZ Reporter Gene Expression In Situ in Whole-Mount Animals

3.4.1 Dissection

1. For the analysis of whole embryos, kill the pregnant mother quickly by diethylether aspiration, remove whole uterus with embryos, and wash the embryos with cold PBS. Separate individual embryos, remove all membranes covering them, wash them briefly in PBS, and soak them in fixative. Remove the skin from embryos older than 15.5 days before fixation.

   For the analysis of postnatal stages, it is necessary to remove all skin and intestines before fixation.
2. For genotyping, keep small pieces of organs; or in some cases, placentas can also be a possible source for genotyping.

3.4.2 Staining

All steps should be performed at room temperature and refer all the solutions to Subheading 2.4.

1. Rock embryos in fixative for 15–30 min if embryos are younger than 13.5 days and for 30–60 min if they are older or if adult tissues are to be examined.
2. Rock embryos three times for 15–30 min in rinse solution.
3. Rock embryos in staining solution for a few hours to 36 h avoiding the light (*see* **Note 5**).

3.4.3 Clarification of Embryos/Newborn to Adult Tissue

For clarification, change the solution as follows:

1. Overnight/1 week in 100% ethanol.
2. 30 min/2 days in acetone.
3. Overnight/overnight in 1% KOH.
4. 1 week/1 week in 4:1 of 1% KOH–glycerol.
5. 1 week/1 week in 3:1 of 1% KOH–glycerol.
6. 1 week/1 week in 2:1 of 1% KOH–glycerol.
7. 1 week/1 week in 1:1 of 1% KOH–glycerol.
8. Store in 80% glycerol–water.

3.5 Genotype Analysis

1. Collect small piece (less than 1 mm^3) of tissues from any organs. If it is not possible to collect tissues from embryos, it is possible to collect the yolk sac or amniotic membrane. Placenta is also possible source for genotyping if the mother is wild type.
2. Isolate genomic DNA by using any commercial available kit and measure the DNA concentration with an absorption spectrometer. Adjust the DNA concentration to the minimum one (*see* **Note 6**).
3. Perform PCR using any Taq DNA polymerase kit commercially available with primer sets, for example, LacZF and LacZB; CL2-long and mCTGF106-82. The conditions for thermal cycling depend on the Taq provider. For example, for the Quick Taq HS DyeMix, these conditions are the following:

94 °C for 2 min, 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min (35 cycles), ending with 68 °C for 7 min. Positive and negative genomic DNA should be included as control reactions (*see* **Note** 7).

3.6 Skeleton Preparation

1. Remove skin and internal organs, and if possible, remove big muscles from shoulders, back, and limbs.
2. Fix remaining carcass overnight in 95 % ethanol.
3. Stain cartilage overnight in alcian blue solution.
4. Rinse in 95 % ethanol for at least 3 h.
5. Treat with 2 % KOH for 24 h, or longer for adult mice. Do not move samples since the junction of bone parts becomes very fragile.
6. Carefully remove the 2 % KOH solution. If the tissues become too soft, it is not necessary to remove the solution completely.
7. Stain bone overnight in alizarin red solution.
8. Clear skeleton in 2 % KOH overnight.
9. Change the medium to 1 % KOH–20 % glycerol.
10. Store the skeleton in 80 % glycerol.

* Staining solution should be filtered.

4 Notes

1. For anti-CCN2 monoclonal antibody (8–86), please *see* [26].
2. DNA injection to mouse oocytes and implantation into pseudo-pregnant female mice can be referred to somewhere else [27].
3. One can isolate chondrocytes from transgenic mice by using the same methods as above and analyze the effects of CCN2 overexpression in vitro.
4. Alternatively, 0.01 % collagenase in DMEM/10 % FCS may be used for 12 h at 37 °C.
5. Cartilage stained for LacZ activity can be analyzed histologically. In this case, long bones which are stained need to be washed with PBS, dehydrated by passage through a series of increasing concentrations of ethanol substituted to xylene, and embedded in paraffin. We have not been successful in monitoring LacZ activity on paraffin-embedded sections.
6. We also use 200 μl of 0.4 M NaOH at 100 °C for 10 min incubation with vigorous vortexing for elution of genomic DNA from tissues, followed by neutralization with 40 μl of 1 M Tris–HCl (pH 8.0). Amplification of transgene fragments is done by using a KOD Fx neo taq polymerase kit and LacZF and LacZB as primer sets.

7. Copy number of transgenes can be analyzed by using real-time PCR. After purification of genomic DNA by use of any commercially available kit, real-time PCR is performed with E. coli LacZ real F and E. coli LacZ real R primers, and any Sybr Green PCR mixture. The concentration of genomic DNA can be standardized with any genes of haploid complement. We use genome 5′ and genome 3′ primer sets for standardizing the genomic DNA concentration. *E. coli* LacZ real F, 5′-ggttacgatgcgcccatcta-3′; *E. coli* LacZ real R, 5′-acggcggattgaccgtaat-3′; genome 5′, 5′-cccctgtgtgtctgaggttt-3′; genome 3′, 5′-gtccccaa gtcagcagagag-3′.

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Chapter 33

Analysis of Transcytosis of CCN2 by Chondrocytes

Kazumi Kawata, Satoshi Kubota, and Masaharu Takigawa

Abstract

Transcytosis is a mechanism for the transcellular transport of biomolecules. Analysis of transcytosis is frequently performed in cells with distinct polarity, such as brain endothelial cells. However, in cells without evident polarity, analysis of transcytosis has not been performed. Here, we describe a method for analyzing transcytosis of a CCN family protein through chondrocytic cells having no apparent polarity.

Key words Analysis of transcytosis, Transwell, Cells without polarity, Chondrocytes, FLAG-tagged recombinant CCN2 protein

1 Introduction

Transcytosis indicates the phenomenon by which a cell encloses extracellular material in an invagination of the cell membrane to form a vesicle (endocytosis) and then moves the vesicle across the cell to eject the material through the opposite cell membrane by the reverse process (exocytosis [1]). In brain endothelial cells forming the blood–brain barrier that prevents free entry of blood-derived substances, transcytosis is known to allow the restricted entry of biomolecules through this physical barrier [2]. Analysis of transcytosis is widely performed to investigate this process in brain endothelial cells with an apical-basal polarity [3].

On the other hand, in growth-plate cartilage, such an investigation has not been performed in general. However, we noticed that CCN2 molecules were distributed in a region different from that containing *Ccn2* mRNA-positive cells [4]. Moreover, in cartilage, low-density lipoprotein receptor-related protein (LRP) 1, one of the CCN2 receptors [5], is also expressed [6]. One of the LRP1 functions is to act as a transporter for transcytosis through brain cells [7]. Therefore, we hypothesized that the difference in the distribution of CCN2 producer cells and CCN2 protein in vivo was caused by transcytosis [8]. However, in cells without polarity, such as chondrocytes, analysis of transcytosis had not been

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established. Accordingly, we attempted to construct a method for assaying transcytosis in chondrocytes by using FLAG/His-tagged recombinant CCN2 protein [9].

To visualize the amount of transcytosis, we performed sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting [8].

This chapter reviews these methods in detail.

2 Materials

Prepare all solution using ultrapure water (prepared by purifying deionized water to attain a conductivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to the reagents.

2.1 Transcytosis Assay Components

1. Transwell chambers: Pore size of 0.4 μM.
2. Type I collagen solution for coating Transwell chambers.
3. Dilute hydrochloric acid: Approximately 10^{-3} M, pH 3.0.
4. Serum free Dulbecco's modification of minimum essential medium (D-MEM) (*see* **Note 1**).
5. HCS-2/8 cells: A human chondrocytic cell line (*see* **Note 2**).
6. D-MEM containing 10% fetal bovine serum (FBS) (*see* **Note 3**).
7. 1× phosphate buffered saline (PBS) (*see* **Note 4**).
8. Full-length recombinant human CCN2 (rhCCN2) derived from *Escherichia coli* (*E. coli*): The expressed CCN2 carries a FLAG tag at its N-terminus and a 6× His tag at its C-terminus (*see* **Notes 5** and **6**).
9. RhCCN2 derived from HeLa cells stably overexpressing CCN2 (*see* **Notes 7** and **8**): The rhCCN2 derived from HeLa cells was biotin-labeled (*see* **Note 9**).
10. Recombinant LRP-associated protein 1 (LRPAP1) (*see* **Note 10**).
11. Serum-free D-MEM containing 2 μg/ml recombinant CCN2 protein.
12. Anti-FLAG affinity gel.
13. Ni-NTA (Ni2 + -nitrilotriacetate)-agarose gel.
14. Centrifugal ultrafiltration membrane for protein concentration: For example, Amicon® Ultra 0.5 ml Centrifugal Filters (Merck Millipore Corporation, Darmstadt, Germany) (*see* **Note 11**). This Amicon Ultra 0.5 ml centrifugal filter enables the concentration of proteins with a membrane nominal molecular weight limit (NMWL) of 30 kDa (*see* **Note 12**).

2.2 SDS-PAGE and Immunoblotting Components

1. Blocking buffer (Diluent solution): 3% dry nonfat milk in PBS (*see* **Note 13**). Store at 4 °C.
2. Roller bottle (*see* **Note 14**).

3 Methods

For the transcytosis assay of CCN2, carry out procedures at 4 °C unless otherwise specified. However, for SDS-PAGE and immunoblot analysis, carry out procedures at room temperature unless otherwise specified.

3.1 Transcytosis Assay of CCN2

1. Coat Transwell chambers with type I collagen solution (*see* **Note 15**). Dilute Cellmatrix Type I-P tenfold with dilute hydrochloric acid. Add Cellmatrix to the chamber, and then incubate it at room temperature for 30 min. After incubation, aspirate the Cellmatrix away. Dry the chamber and then wash it twice with serum-free D-MEM.
2. Seed HCS-2/8 cells in each Transwell chamber, which is then inserted into 6-well culture plates (density: 6×10^5 cells/well) containing D-MEM supplemented with 10% FBS and incubate the cells at 37 °C for 1 week.
3. Wash the cells three times on ice with cold PBS (*see* **Note 16**).
4. Allow HCS-2/8 cells to associate with tagged/labeled CCN2 at 37 °C for 1 h in serum-free D-MEM containing 2 μg/ml recombinant CCN2 protein (Fig. 1; *see* **Notes 17** and **18**). As a control, prior to the addition of CCN2, preincubate HCS-2/8 cells for 15 min at 37 °C in medium lacking FBS and containing LRPAP1 (*see* **Note 19**).
5. Harvest the medium in the lower chamber. Add protease inhibitor to the medium.
6. For the samples to which the FLAG/His-tagged protein was applied, add anti-FLAG affinity gel freezer-safe or Ni-NTA–agarose gel to the medium. Subsequently, incubate the mixture for 2 h with gentle rotation.
7. After the removal of the supernatant, add 1x SDS sample buffer diluted in PBS to elute the proteins bound to the gel.
8. For samples to which the biotin-labeled protein was applied, concentrate the medium by ultrafiltration by using an Amicon® Ultra 0.5 ml Centrifugal Filter.
9. After the concentration, add 1× SDS sample buffer diluted in PBS to the sample.

3.2 SDS-PAGE and Immunoblot Analysis

1. Prepare 12% polyacrylamide gel in a gel cassette.
2. Assemble the electrophoresis apparatus and fill the anode and cathode chambers facing the gel with SDS-PAGE running buffer.

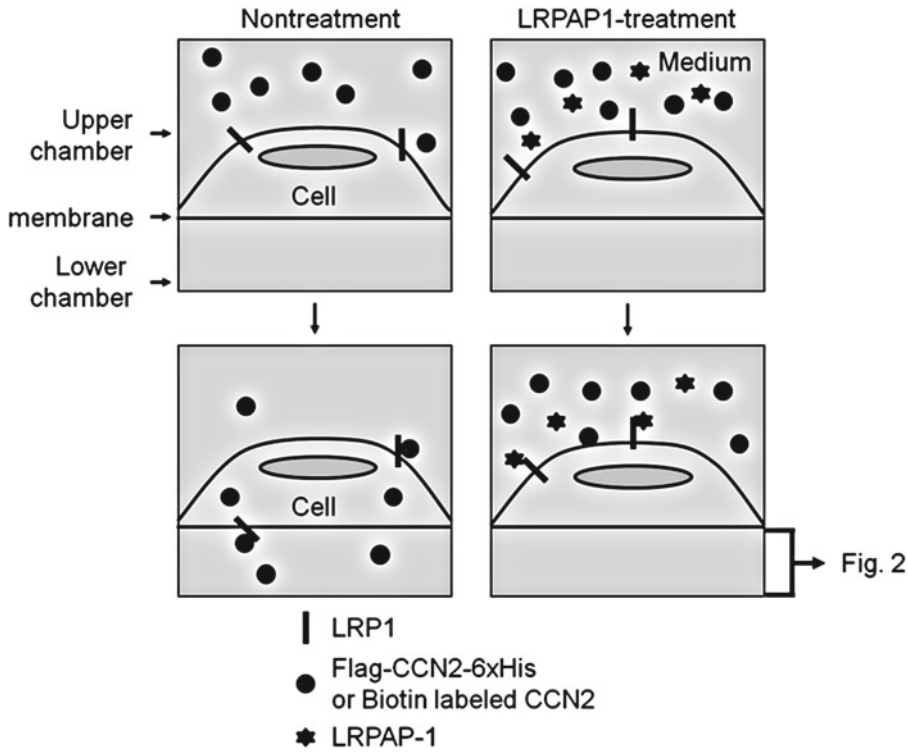

Fig. 1 Schematic representation of the sampling strategy is shown. CCN2 recombinant protein is added to untreated (*left panel*) or LRPAP1-treated (*right panel*) HCS-2/8 cells in the upper chamber of a Transwell, and the medium in the upper chamber is removed after 1 h, while the medium in the lower chamber (Fig. 2) is collected as illustrated

3. Heat the sample at 95 °C for 3 min. Centrifuge the heated samples to bring down the condensate. Load the samples. Add molecular weight markers to the left-end lane (*see* **Note 20**).
4. Electrophorese at 20 mA till the tracking dye reaches the bottom of the gel.
5. Following electrophoresis, separate the gel plates by use of tweezers. Keep the gel on one of the gel plates.
6. Cut a polyvinylidene difluoride (PVDF) membrane and six filter papers to the size of the gel.
7. Immerse the membrane in methanol. Then, rinse it once in distilled water and transfer buffer.
8. Immerse the six filter papers in the transfer buffer (*see* **Note 21**).
9. Lay the gel carefully on a PVDF membrane in Western blot transfer buffer (*see* **Note 22**).
10. Place three of the prewet filter papers on a blotting apparatus.
11. Place gel–membrane sandwich on the filter papers. Place membrane side down and the exposed gel side up.

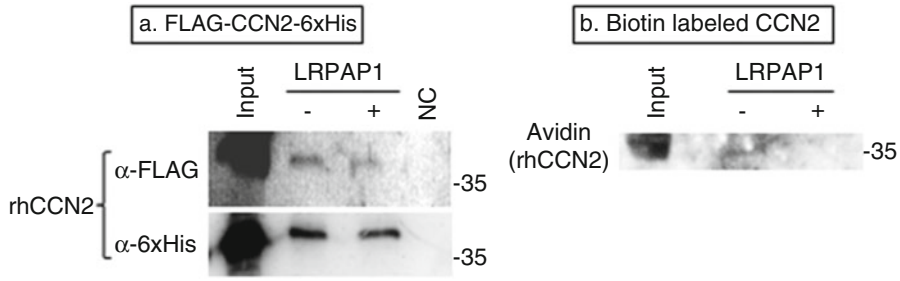

Fig. 2 (**a**) Immunoblotting was performed by using anti-FLAG or His tag antibody. The results obtained by immunoblotting with anti-His tag antibody recognizing a 6× His tag fused to the CCN2 C-terminus was similar to that obtained by using the anti-FLAG tag antibody. These signals were decreased in intensity by LRPAP1. (**b**) Comparable results were obtained with HeLa cell-derived biotinylated recombinant CCN2 detected by the Streptavidin conjugate. These results clearly indicate that CCN2 is transcytosed in chondrocytes mediated by LRP1. Positions of molecular weight markers (35 kDa) are shown at the right of the images. NC, the mixture of anti-FLAG® M2 affinity gel or Ni-NTA-agarose gel and serum-free D-MEM without FLAG or His-fusion protein as a negative control

12. Place the other three filter papers on top of the gel (*see* **Note 23**).
13. Transfer the proteins from the gel to the membrane for 1 h at 80 mA.
14. Incubate for 1 h in the blocking buffer.
15. Incubate overnight with anti-FLAG, anti-6× His, or streptavidin horseradish peroxidase (HRP) conjugate in the diluent buffer by using a roller bottle.
16. Wash five times with PBS.
17. Incubate for 2 h with HRP-conjugated secondary antibody in the diluent buffer by using the roller bottle.
18. Wash five times with PBS.
19. Detect immunoreactive proteins by using an ECL Western Blotting Detection System (Fig. 2).

4 Notes

1. D-MEM powder (4.75 g) is dissolved in distilled water, making 500 ml of solution. Then, it is sterilized by autoclaving at 121 °C for 15 min. After sterilization, the proper amount of 10% $NaHCO_3$ previously sterilized by autoclaving is added to the culture medium after having been cooled to room temperature. In this case, 10% $NaHCO_3$ is added until the color of the D-MEM becomes orange. Thereafter, 0.3 g of filter-sterilized l-glutamine is added to the D-MEM.
2. We commonly use human chondrocytic cell line HCS-2/8 cells established from a human chondrosarcoma [10, 11]. We have not tried using other chondrocytic cell lines or

chondrocytes in primary culture. However, it should be possible to use them in this type of experiment.

3. A 50-ml volume of serum suitable for HCS-2/8 cell cultures is added to 450 ml of serum-free D-MEM. Among different sera tested, the one yielding the highest expression level of chondrocytic matrix component genes is chosen.
4. PBS is cooled to 4 °C.
5. To clone hCCN2, we amplified the cDNA fragment encoding hCCN2 without a signal peptide by PCR using a 5′ primer (5′-GGC CGA ATT CCC AGA ACT GCA GCG GGC CGT GCC GGT GCC CG-3′) containing an EcoRI site and a 3′ primer (5′-ACG GAG ATC TTT AAT GAT GAT GAT GAT GAT GTG CCA TGT CTC CGT ACA TCT TCC TGT AGT-3′), which encodes a 6× His tag (underlined) at the C-terminus of CCN2 and contains a BglII site after the stop codon. The PCR fragments were cloned into the pT7-flag-1 vector at EcoRI/BglII sites. The recombinant plasmid was sequenced to ensure the absence of mutations [9].
6. For expression of rhCCN2, *E. coli* Rosetta™2 (DE3) pLysS cells (Novagen, Queensland Australia) are transformed with pT7-flag-1/hccn2, and cultured at 25 °C for 4 h before adding 0.05 mM IPTG (isopropyl β-d-thiogalactopyranoside). After another 18 h in culture, the bacteria are harvested by centrifugation and then frozen and thawed once to disrupt the cells. The cells are then resuspended in lysis buffer [0.5 M NaCl, 0.05 M Tris–HCl (pH 8.0), 1% Triton, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μM pepstatin A], sonicated, and centrifuged to remove cell debris. For purification of the rhCCN2 protein, 1 ml of Ni-NTA-agarose gel is added to the supernatant to adsorb the His-tagged proteins. After gentle shaking for 1 h at 4 °C, the agarose beads are washed with lysis buffer and then with buffer [20 mM Tris–HCl (pH 8.0), 10 mM KCl, 0.5 mM PMSF, and 1 μM pepstatinA]. The beads are subsequently loaded onto a column and washed, after which the protein is eluted with 20 ml of 500 mM imidazole elution buffer [500 mM imidazole, 20 mM Tris–HCl (pH 8.0), 10 mM KCl, 0.5 mM PMSF, and 1 μM pepstatin A]. The purity and quantity of the rhCCN2 protein is checked by Coomassie Brilliant Blue staining [9].
7. For construction of the CCN2 expression vector, a DNA fragment of CCN2 that contains the whole coding region of CCN2 cDNA is cloned into the pcDNA3.1(−) vector, and CCN2 is expressed under the control of the CMV promoter [12].
8. The CCN2 expression vector is introduced into HeLa cells by electroporation, and stable transformants that are resistant to G418 are selected. rCCN2 is purified from the conditioned

medium of stable transformants by heparin affinity chromatography (HiTrap Heparin column, Pharmacia Biotech, Uppsala, Sweden). Partially purified rCCN2 eluted from this chromatography step is further purified by affinity chromatography using an anti-CCN2 antibody. The purity of rCCN2 is analyzed by SDS-PAGE, with silver staining [12]. For details, refer to Chapter 10.

9. Biotinylation is performed with a commercially available kit, following the manufacturer's instructions (Biotin Labeling Kit-NH2; Dojindo Molecular Technologies, Inc., Kamimashiki-Gun, Japan).
10. LRPAP1 is also known as receptor-associated protein (RAP), and it is an antagonist of low-density lipoprotein receptor-related protein (LRP) 1 [13].
11. We have not tried ultrafiltration membranes other than Amicon® Ultra 0.5 ml Centrifugal Filters. However, one may be able to use other filters besides this one.
12. The molecular weight of CCN2 is 36 kDa [14]. Accordingly, the protein is concentrated by a membrane with an NMWL of 30 kDa.
13. Put 1.5 g of skim milk powder into a 50-ml conical tube, and bring the volume up to 50 ml with PBS. Then, put a lid tightly on the tube and shake it until the powder has completely dissolved.
14. The 110-, 200-, or 500-ml bottle supplied in the QIAGEN Plasmid kit can be used as a substitute for the roller bottle after they are cleaned. The QIAGEN bottle will prevent leaking of the diluent buffer even better than the roller bottle.
15. HCS-2/8 cells in culture tend to detach from the surface of Transwell chambers easily. Accordingly, collagen-coating is always necessary for long-term cultures as used in this type of experiment.
16. Complete confluency of HCS-2/8 cells is confirmed by using a microscope before washing. Apply PBS along the walls of Transwell chambers carefully so that the HCS-2/8 cells do not become detached. Moreover, confluent cultures are also confirmed by using a microscope after the PBS wash.
17. Distinguish exogenous protein from endogenous protein by tagging or biotinylation.
18. In order to rule out the possibility of contamination by *E. coli* components therein, the same experiment is repeated with recombinant CCN2 prepared from HeLa cells.
19. This experiment targets transcytosis by LRP1. Therefore, to prove that transcytosis occurs in chondrocytes, LRPAP1 (an antagonist of LRP1) is used. This step is always necessary to rule

out the possibility of major leakage through the uncovered part of the membrane. Inhibitors or antagonists should be changed based on the kind of molecules mediating transcytosis.

20. Always load the marker into the same lane for orientation.
21. Divide six filter papers into two sets. Then, immerse the sets in transfer buffer. This is the easiest way to prepare the filter paper–PVDF membrane–gel–filter paper sandwich on blotting apparatus. Setting the six filter papers one by one is troublesome and a time-consuming process.
22. Put a gel on the PVDF membrane in transfer buffer. It is easier to set the gel on the PVDF membrane when the latter is submerged in the buffer.
23. A 10-ml pipette is used to roll out the air bubbles from the filter paper–PVDF membrane–gel–filter paper sandwich on the blotting apparatus.

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Part III

Pathophysiology

Chapter 34

CCN2 in Skin Fibrosis

Andrew Leask

Abstract

CCN2, formerly known as connective tissue growth factor, was discovered in 1991. Soon after its discovery, CCN2 was shown to be upregulated in response to the profibrotic cytokine transforming growth factor (TGF) β and to be constitutively overexpressed in fibrotic conditions. These early observations led to the hypothesis that CCN2 was a key regulator of fibrosis. However, only recently have data been generated that directly demonstrate this hypothesis. This review summarizes these observations and suggests a mechanism of action for CCN2.

Key words CCN2, Adhesion, Contraction, Conditional knockout, Type I collagen, CTGF, CCN family

1 CCN2 Is Overexpressed in Skin Fibrosis

Approximately 20 years ago, the matricellular protein CCN2 (initially referred to as connective tissue growth factor) was shown to be overexpressed in a variety of fibrotic disorders of the skin. Takehara and colleagues investigated CCN2 mRNA expression in tissue sections from patients with localized scleroderma, keloid and other sclerotic skin disorders (eosinophilic fasciitis, nodular fasciitis, and Dupuytren's contracture) and found that CCN2 mRNA, although essentially absent from healthy or clinically unaffected skin, was found in fibroblasts scattered throughout the sclerotic lesions [1]. CCN2 mRNA did not show preferential distribution around inflammatory cells or around the vasculature [1]. Similar results were obtained when CCN2 mRNA was examined in hypertrophic scars [2].

In skin fibrosis, increased expression of CCN2 mRNA is paralleled by overexpression of CCN2 protein; for example, CCN2 protein is found in diffuse scleroderma (systemic sclerosis, SSc) fibroblasts both in vivo and when isolated by culturing [3]. Elevated levels of CCN2 are found in serum from patients with SSc that correlate with the extent of skin sclerosis and the severity of pulmonary fibrosis; patients with disease duration of 1–3 years have

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significantly elevated levels of CCN2 compared with dSSc patients with less than 1 year or more than three duration, suggesting CCN2 expression correlated with actively progressing disease [4]. More specifically, N-terminal cleavage products of CCN2 are elevated in plasma and dermal interstitial fluid of SSc patients, compared to healthy controls, and this correlates with severity of skin disease [5]. These data are consistent with more recent data showing a correlation between CCN2 levels and the degree of skin fibrosis as visualized histologically or by Rodnan's skin score [6].

2 The Mechanism Underlying CCN2 Overexpression in Skin Fibrosis

TGFbeta induces CCN2 expression in fibroblasts via canonical Smad3 pathways; yet overexpression in SSc fibroblasts is independent of these pathways [7] and is instead dependent on pathways including ERK and Sp1 [8, 9]. In some SSc patients, CCN2 overexpression has been linked to a functional polymorphism that results in elevated Sp1 promoter binding: a G-945C substitution represses CCN2 transcription, but the -945G allele is significantly associated with susceptibility to systemic sclerosis [10, 11]. In addition, overexpression of CCN2 has been lined to constitutively active ALK1 (caALK1) acting through endoglin via activation of Smad1 [12].

CCN2 upregulation in SSc is also due to other cell types than fibroblasts; SSc epidermal cells show an elevation of IL-1alpha production that, in a three-dimensional coculture system, cause an increase in endothelin-1 and transforming growth factor-beta expression culminating in elevated CCN2 expression [13]. Thus CCN2 overexpression in SSc may result from a complex interaction among multiple cell types.

3 The Role of CCN2 in Myofibroblast Differentiation and Recruitment in Fibrosis

CCN2 protein expression is largely absent in the dermis of control mice. However, upon exposure to bleomycin, CCN2 is induced in alpha-SMA-expressing myofibroblasts [14]. In cell culture activated myofibroblasts, CCN2 is expressed. In such cells, endogenous CCN2 directly binds fibronectin and the fibronectin receptors integrins alpha4 beta1 and alpha5 and syndecan 4; loss of endogenous CCN2 results in impaired spreading on fibronectin, delayed alpha-smooth muscle actin stress fiber formation, and reduced ERK and focal adhesion kinase phosphorylation in response to fibronectin [15]. Moreover CCN2-deficient myofibroblasts show impaired activation of adhesive signaling in response to TGFbeta [16]. These results are consistent with in vivo data showing that whereas TGF-beta alone induces transient fibrosis but coapplication of CCN2 causes the fibrotic response to TGFbeta in skin to persist [17].

In SSc fibroblasts, CCN2 RNA interference inhibits type I and III collagen expression [18]. Moreover, in a mouse model of skin fibrosis anti-CCN2 antibodies significantly reduce skin fibrosis including collagen deposition compared with control groups [19]. Although treatment of wounds with antisense oligonucleotides to CCN2 have no measurable effect wound closure, antisense therapy significantly limits subsequent a model of hypertrophic scarring in rabbits correlating with reduced numbers of myofibroblasts in scars and decreased transcription of TIMP-1 and types I and III collagen [20]. Indeed, loss of CCN2 by fibroblasts, although not affecting cutaneous tissue repair [21], results in resistance to fibrosis caused by either bleomycin or by the loss of PTEN expression (i.e., due to the enhancement of adhesive signalling) [22, 23]. Thus CCN2 appears specifically to mediate pathological fibrosis in vivo.

4 CCN2 Is Required for the Differentiation of Dermal Progenitor Cells into Myofibroblasts

An increasing body of evidence links pericyte-like progenitor cells (PC) to fibrosis including in SSc; for example, fibrotic lesions accumulate myofibroblasts which express pericyte and PC markers such as NG2 and Sox2 [24–28]. Isolated, cultured pericytes express alpha-SMA and CCN2 and acquire expression of fibroblast markers [29]. In response to bleomycin-induced fibrosis, cells derived from Sox2 expressing cells are recruited to the fibrotic lesions in a CCN2-dependent fashion [28]. Intriguingly, although only a small number of myofibroblasts derived from Sox2-expressing cells in cutaneous repair, recruitment and differentiation of these cells is also CCN2-dependent [30]. Cultured so-called skin progenitor cells (SKPs) spread and differentiate into myofibroblasts in response to serum in a CCN2-dependent fashion [29]. Thus (a) skin fibrosis appears to occur through the ability of dermal PCs to differentiate into myofibroblasts and (b) CCN2 appears to be required for this process.

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Chapter 35

Studying the CCN Proteins in Fibrosis

James Hutchenreuther, Andrew Leask, and Katherine Thompson

Abstract

CCN2 is a profibrotic matricellular protein. CCN2 directly promotes cell adhesion and indirectly promotes fibrosis by activating adhesive signaling in response to growth factors, cytokines, and extracellular matrix. The following protocols will allow the direct assessment of other CCN family members in these processes.

Key words Matricellular proteins, Adhesion, Contraction, Conditional knockout, CCN2, CCN3, CTGF, Nov, cyr61, CCN1

1 Introduction

The CCN family of proteins is named for the three prototypical members (*C*yr61, *C*TGF, and *N*ov), which were first discovered almost 25 years ago [1]. Initially identified as growth factors, they are now known to be matricellular proteins, a class of non-structural secreted extracellular matrix-associated protein that modulate cellular responses to their surrounding microenvironment [2, 3]. CCN proteins are structurally very similar with all but CCN5 consisting of four functional domains (an insulin-like growth factor binding protein-like module, a von Willebrand factor type C repeat module, a thrombospondin type-1 repeat module, and a cysteine-knot containing module) attached to a N-terminal secretory signal peptide [1, 2].

The CCN proteins signal through integrins and heparin-sulfate-containing proteoglycans (HSPGs) [4], and have had a wide variety of functions ascribed to them. They have been shown to be involved in angiogenesis, adhesion, extracellular matrix remodeling, skeletal development, and wound repair, as well as affecting proliferation and migration of various cell types [2–4]. CCN1 and CCN2 have been shown to have similar functions in vitro, with both of them promoting adhesion in fibroblasts through integrin α6β1 and HSPGs, increasing proliferation through sustained activation of p42/p44 MAPK, and acting as chemotactic agents in Boyden chamber assays [5]. Through this

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chemotactic action and stimulation of angiogenic integrins CCN1 and CCN2, along with CCN3, are able to promote angiogenesis by directing endothelial cell migration, adhesion, proliferation, and tubule formation [2–5]. In vivo CCN1 and CCN2 have been shown to promote cell proliferation, and show coinduction in the granulation tissue during wound repair [5]. Conversely, CCN3 has been shown to act as an antiproliferative agent, and frequently has expression patterns opposite to CCN1 and CCN2 in wound healing, with CCN3 expression being greatly reduced in the first 3 days after wounding, then increasing between days 5–7 [3].

CCN proteins are of particular interest with respect to fibrotic conditions. Fibrosis is a process by which the tissue repair and remodeling systems regulated by the CCN proteins are disturbed, resulting in constitutive production and deposition of extracellular matrix components, such as type I collagen. While it is still unknown how this process initiates, it is becoming clear that CCN2 is central to the progression of fibrotic diseases, as it seems to foster an environment which allows other pro-fibrotic signals to generate a persistent response [2, 4]. It has been shown repeatedly that CCN2 expression in fibroblasts is required to generate a fibrotic response [2, 6–9]. Unsurprisingly, given their similar in vitro functions, CCN1, CCN4, and CCN6 have been shown to have profibrotic effects in several organs as well [10–13]. Interestingly, despite CCN1 and CCN2 being strongly expressed during early wound repair, loss of both proteins from fibroblasts does not appear to impair wound healing [14]. Supporting the idea that a balanced system of CCN proteins prevents tissue remodeling from becoming unregulated, there is evidence that the addition of CCN3 and CCN5 attenuate the constitutive overexpression of CCN2, restoring fibrotic tissue to a healthy state [9, 15].

It has been difficult to develop in vitro assays of fibrosis, simply because, other than adhesion assays, there is no reliable in vitro assay that faithfully reproduces any fibrotic activity. This is because CCN proteins modify cellular responses to growth factors and cytokines in a context-dependent fashion. Thus in vivo assays are required. In vivo study of the CCN proteins has proven itself to be a challenge, as CCN1-null mice are embryonic lethal due to improper vascular development, and the CCN2-null mice show perinatal lethal due to skeletal defects [2]. Because the cause of death in both cases is developmental abnormalities, a conditional gene knockout system has been developed to remove the genes from specific tissues in adult mice using a tissue-specific promoter driven Cre-LoxP system. In this system mice have been bred to possess a sequence (LoxP) that is recognized by a Cre recombinase protein on either side of the gene of interest [16]. The mice are also bred to be hemizygous for a tamoxifen-dependent form of Cre recombinase, Cre-ER(T), which is not activated by endogenous steroid hormones and remains dormant until activation upon the administration of tamoxifen, allowing it to remove the gene flanked

by LoxP sites (or floxed) [16]. The tissue specificity of this system relies on the Cre-ER(T) being driven by a promoter that is only active in the tissue of interest. Fibroblasts are the effecter cell of fibrosis. One commonly used fibroblast-specific promoter that is used in this system is proα2(I)Collagen, located -350 bp from the *Col1a2* promoter, which has been shown to produce a fibroblast specific knockout when tamoxifen is administered to 3-week-old mice [16–18]. The resultant tissue can be examined histologically and biochemically for collagen and matrix deposition.

2 Materials

2.1 Cre-ER(T) Mouse Protocol

1. Three-week-old transgenic mouse with gene of interest floxed and Cre-ER(T) driven by appropriate promoter.
2. Tamoxifen (Sigma).
3. 100% ethanol.
4. Corn oil.
5. 1 mL syringe.
6. Hypodermic needle, 23–26.

2.2 H&E Examination of Skin Tissue

1. Skin sections embedded in paraffin wax, cut at 5 μm thick on glass slides (*see* **Note 1**).
2. Xylenes.
3. Ethanol ranging in percentage, 100, 95, 85, and 70%, diluted with H_2O
4. Hematoxylin Stain—Harris Formula (Modified)—Mercury Free (Ricca Chemical Company).
5. Eosin Y 5%wt. in water (Sigma).
6. Eukitt® Quick-Hardening Mounting Media (Fluka Analytical).

2.3 Soluble vs Insoluble Collagen Assay of Skin Tissue

1. Tissue samples.
2. QuickZyme Total Collagen Assay Kit.
3. 0.5M acetic acid.
4. 12 M HCl.

2.4 Adhesion Assay of Fibroblasts

1. Cultured fibroblasts.
2. Collagen or fibronectin.
3. BSA.
4. 0.5 mM EDTA.
5. DMEM media: 1 g/L d-glucose, l-glutamine, 110 mg/L sodium pyruvate
6. Vitality assay. Presto Blue (Life Technologies).

3 Methods

3.1 Cre-ER(T) Tamoxifen Inducible Knockout

1. Weigh 5 mg tamoxifen per mouse to be treated.
2. Dissolve tamoxifen in 50 μL 100% ethanol per mouse to be treated by incubating at 50 °C.
3. Add 450 μL of corn oil per mouse to be treated and mix thoroughly. Store at 4°C.
4. For 5 consecutive days, inject 0.1 cm^3 tamoxifen (or corn oil for vehicle control) via IP injection once per day (*see* **Note 2**).
5. Two weeks after injection perform genotyping to verify effectiveness of knockout.

3.2 H&E Examination of Skin Tissue

1. De-wax tissue sections; wash slides in xylenes once for 7 min, followed by two 5 min washes.
2. Rehydrate sections by washing with ethanol 5 min each: 100, 95, 85, then 70.
3. Rinse with dH_2O for 5 min (*see* **Note 3**).
4. Stain with hematoxylin solution for 1 min, followed by a rinse with cold running tap water for 5 min.
5. Stain with eosin-y stain (5%) for 7 min, followed by a rinse in cold running tap water for 5 min.
6. Dehydrate in ethanol by dipping slides 10 times into: 70, 80, 95, then 100%.
7. Dip into xylene ten times and mount using xylene based mounting media.

3.3 Soluble vs Insoluble Collagen Assay of Skin Tissue (See Note 4)

1. Cut up 10 mg of skin tissue and add 100 μL of 0.5M acetic acid, mix overnight at 4°C (*see* **Note 5**).
2. Centrifuge at 15,000 × *g* for 60 min. Keep supernatant.
3. Add supernatant 1:1 with 12 M HCl (final molarity is 6 M HCl), in screw-capped tube.
4. Take another 10 mg sample from same skin tissue and add in 100 μL 12 M HCl, into screw-capped tube.
5. Make standard; add 125 μL standard (1200 μg/ml) with 125 μL 12 M HCl into screw-capped tube (*see* **Note 7**).
6. Take the tubes with soluble collagen, total skin sample and standard; ensure tubes are tightly sealed and boil at 95°C for 20 h.
7. Cool to room temperature and centrifuge for 10 min at 13,000 × *g*.
8. Dilute samples 10× in 4 M HCl (*see* **Note 6**).
9. Dilute Standards:

Standard label	Sample from	4 M HCl	Conc (μg/ml)
S1	125 μl stock	125 μl	300
S2	120 μl S1	60 μl	200
S3	90 μl S2	90 μl	100
S4	90 μl S3	90 μl	50
S5	90 μl S4	90 μl	25
S6	90 μl S5	90 μl	12.5
S7	90 μl S6	90 μl	6.25
S8	0 μl	90 μl	0

10. Pipette 35 μL of each standard dilution and 35 μL of the diluted tissue samples into wells of a 96-well plate.
11. Add 75 μL of assay buffer to each well (*see* **Note 7**); cover the plate with adhesive seal and incubate at room temperature, while shaking, for 20 min.
12. Mix reagents A and B, will need L A and L B per well (*see* **Note 7**). Prepare enough of this detecting reagent mixture for each sample; then add 75 μL to each well, cover the plate with adhesive seal and ensure contents are mixed well and incubate for 60 min at 60°C.
13. Cool to room temperature and use microplate reader to read the plate at 570 nm.
14. Use standard curve to determine the amount of collagen in tissue. Subtract amount of soluble collagen found from total collagen to determine insoluble collagen amount.

3.4 Adhesion Assay of Fibroblasts

1. Coat 96-well plates with either Collagen or Fibronectin. Dilute in PBS at 10 μg/mL, enough to cover bottom of well and leave overnight at 4 °C.
2. Wash 96-well plate with PBS (*see* **Note 8**). Block the coatings in the 96-well plate for 1 h at room temperature with 10% BSA in PBS.
3. Remove cultured fibroblasts from plate with 0.5 mM EDTA at 37°C (*see* **Note 9**). Centrifuge cells and wash twice with DMEM low glucose media containing 1% BSA (*see* **Note 10**).
4. Count cells and calculate how to make 6000 cells/100 μL. Make this mixture in DMEM low glucose media containing 1% BSA.
5. After 1 h blocking, wash 96-well plate with PBS. Add 100 μL solution containing 6000 cells to each well in 1% BSA DMEM low glucose media. Incubate for 0, 30, 60, 90, and 120 min.

6. At each time point remove media carefully and wash twice with PBS (*see* **Note 11**), and add 90 µL 1% BSA DMEM low glucose media to each well.
7. When ready to evaluate add 10 µL of presto blue to each well, incubate at 37°C for 1.5 h and read using a plate reader. Fluorescence, Excitation: 560 nm and Emission: 590 nm.

4 Notes

1. Skin sections were previously fixed in 4% paraformaldehyde overnight and sent for tissue processing prior to embedding in paraffin.
2. Tamoxifen is a potential carcinogen, mutagen and teratogen that may be present and aerosolized in bedding and excrement of mice during and after treatment. Mice should be kept in filter top cages during treatment, and for 3–5 days afterwards to prevent exposure.
3. Check to ensure paraffin has been removed around the tissue.
4. This protocol has been modified from a protocol from QuickZyme Total Collagen Assay Kit [19].
5. Acetic acid will dissolve the soluble collagen (non-crosslinked). Ensure that tissue is cut up enough for an efficient extraction.
6. This dilution is for skin tissue, depending on the tissue type the dilution factor will vary, dependent on collagen content.
7. Collagen standard (1200 µg/mL), screw-capped tubes, assay buffer, reagent A and B are found in Quickzyme Collagen kit.
8. Only quick wash needed. Collagen matrix will have formed to coat bottom of wells, this wash is just to wash away any excess collagen left in well.
9. This step can take about 30 min. It is important to use EDTA as it does not disrupt adhering proteins.
10. 860 × *g* for 5 min.
11. Ensure to wash very carefully. Do not to disturb any cells that are adhering.

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Chapter 36

Analysis of Pathological Activities of CCN Proteins in Fibrotic Diseases: Kidney Fibrosis

Hideki Yokoi and Masashi Mukoyama

Abstract

Renal fibrosis is characterized by glomerulosclerosis and tubulointerstitial fibrosis. Transforming growth factor-β (TGF-β) is postulated to play a central role in the development of both fibrotic processes. Extracellular matrix proteins, particularly type I collagen and fibronectin, accumulate in the tissue during renal fibrogenesis. CCN2, also known as connective tissue growth factor (CTGF), is increased in the setting of fibrosis and modulates a number of downstream signaling pathways involved in the fibrogenic properties of TGF-β. Unilateral ureteral obstruction is one of the most widely used models of renal tubulointerstitial fibrosis. Herein, we describe unilateral ureteral obstruction in mice as an animal model of renal fibrosis and methods for immunohistochemical analyses of extracellular matrix proteins and CCN2. In addition, we describe the construction of podocyte-specific CCN2-transgenic mice for analyzing mesangial matrix expansion and glomerulosclerosis.

Key words CCN2, Connective tissue growth factor, TGF-β, Renal fibrosis, Transgenic mice, Diabetic nephropathy, Podocytes

1 Introduction

Organ fibrosis is characterized by the accumulation of extracellular matrix (ECM) [1]. Renal fibrosis, in particular, is represented by glomerulosclerosis and tubulointerstitial fibrosis [2, 3]. Although the mechanisms underlying renal fibrogenesis have yet to be fully elucidated, a number of cytokines and growth factors are reportedly involved in the associated fibrogenic and inflammatory processes. Of these, transforming growth factor-β (TGF-β) has been shown to play a central role in development of renal fibrosis [3]. TGF-β increases the expression of ECM proteins such as collagen type I and III, fibronectin, and laminin, as well as their downstream mediators [4]. A previous study demonstrated that the inhibition of TGF-β by antisense TGF-β1 oligonucleotides ameliorates fibrotic changes resulting from unilateral ureteral obstruction (UUO) in rats, indicating that TGF-β1 could be an essential

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mediator of renal fibrogenesis [5]. The binding of TGF-β to the TGF-β receptor type 2 stimulates the activation of serine/threonine kinases of the TGF-β receptor type 1, finally leading to the signal transduction by Smad proteins [3].

Several methods are reported for quantitative analyses of renal fibrosis, including Masson's trichrome staining, in which the areas of blue staining are considered fibrotic areas (Fig. 1) [6], immunohistochemical analysis of collagen type I and fibronectin, both of which are strongly induced in renal fibrosis [4, 7], and the hydroxyproline assay for quantification of collagen content [7].

CCN2, also known as connective tissue growth factor (CTGF), is considered to be a mediator of the fibrogenic properties of TGF-β [8]. Recombinant CCN2 potently induces the production of ECM proteins, including type I collagen and fibronectin [9]. In turn, blockade of CCN2 has been shown to attenuate organ fibrosis, including renal fibrosis [10–12]. We previously demonstrated that antisense oligonucleotides against CCN2 can ameliorate renal fibrosis without affecting renal TGF-β1 mRNA expression [13]. Several reports have shown that CCN2 binds to TGF-β, so that it enhances the affinity of TGF-β for its receptor binding, thus amplifying profibrotic signals [14, 15]. These findings together indicate that CCN2 could represent a promising therapeutic target against renal fibrosis by suppressing the TGF-β signaling pathway.

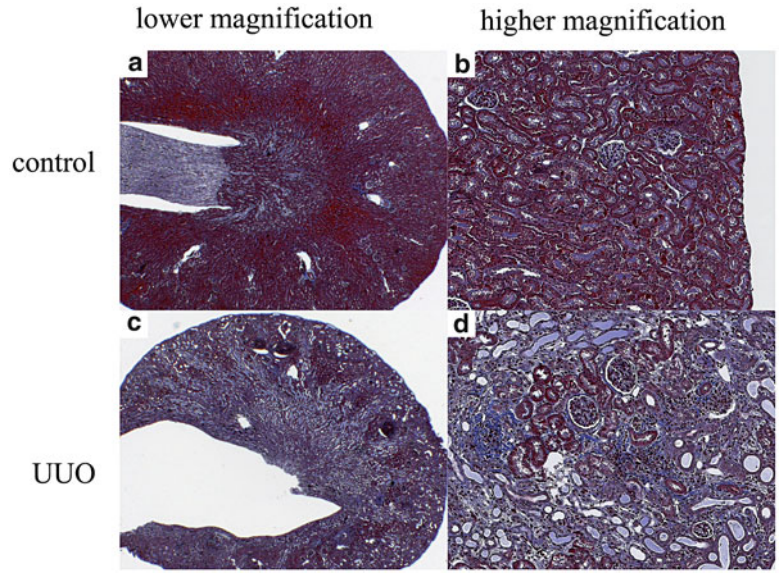

Fig. 1 Renal histology of normal (**a**, **b**) and obstructed (**c**, **d**) kidney. Masson's trichrome sections from sham-operated kidney (**a**, **b**). After UUO, the obstructed kidney at day 7 showed massive tubulointerstitial fibrosis stained by *blue* (**c**, **d**). (**a**, **c**) Lower magnification, (**d**, **e**) higher magnification

UUO is one of the most widely used models of renal fibrosis [16]. During early stages, the intra-tubular pressure is increased because of urinary flow obstruction [16]. Elevated intra-tubular pressure results in the activation of the tissue renin–angiotensin system, tubular apoptosis, and tubular atrophy, thereby leading to tubulointerstitial fibrosis [16]. Angiotensin-converting enzyme (ACE) inhibitors or angiotensin II type 1a (AT1a) receptor blockers (ARBs) have been shown to ameliorate renal fibrosis, indicating the importance of angiotensin II in renal fibrogenesis [17, 18]. Tubular obstruction reduces the renal plasma flow and glomerular filtration rate. The subsequent interstitial inflammatory process is initiated by macrophage infiltration [19]. Macrophages secrete a number of cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which collectively accelerate fibrotic processes [19]. Myofibroblasts, which contribute to renal fibrosis, are predominantly positive for α-smooth muscle actin (α-SMA) [20]. Although the majority of myofibroblasts are thought to be derived from resident fibroblasts, the origin of myofibroblasts remains controversial [21]. Several reports have suggested that myofibroblast precursor cells are derived from circulating bone marrow cells [22], or through transition of either epithelial or endothelial cells [23, 24]. Fibrocytes are defined as collagen-producing cells of hematopoietic origin [25]. Several lines of evidence support the notion that fibrocytes contribute to renal fibrosis [26, 27]. Some reports have suggested that epithelial and endothelial cells are in fact minor contributors to the development of renal fibrosis [22]. Another report has shown that resident renal fibroblasts, which are positive for platelet-derived growth factor receptor-β (PDGFR-β), are the major contributors to renal fibrosis [28]. CCN2 is also expressed by fibrocytes as well as fibroblasts [29].

To examine the role of CCN2 in ECM production by podocytes, we generated podocyte-specific CCN2-transgenic mice [30]. These mice do not demonstrate the accumulation of ECM proteins under the basal condition. Similarly, other reports have shown that overexpression of CCN2 in other tissues—such as in liver and heart—does not induce tissue fibrosis under basal conditions [31, 32]. In contrast, lung-specific CCN2-transgenic mice can develop organ fibrosis without stimulation [33]. These results suggest that other factors in addition to CCN2 overexpression might be necessary for the induction of tissue fibrosis. In the kidney, diabetic podocyte-specific CCN2-transgenic mice induced by streptozotocin demonstrate mesangial matrix expansion [30], suggesting that factors induced by the diabetic milieu could be indispensable for ECM accumulation. Herein we provide protocol details for the construction of the CCN2-transgenic mice.

2 Materials

2.1 Paraffin-Embedded Sections for Immunohistochemistry

2.1.1 Tissue Preparation and Embedding

1. 4% Paraformaldehyde (PFA): Add 4 g of PFA to 100 mL phosphate-buffered saline (1× PBS; pH 7.4: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4), heat to a maximum of 60 °C with stirring.
2. Phosphate-buffered saline (1× PBS), pH 7.4: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4.
3. Ethanol.
4. Chloroform.
5. Paraffin wax.
6. Stainless steel embedding molds.
7. Duboscq-Brasil fluid: Add 1 g of picric acid to 120 mL of 100% ethanol and 60 mL of 40% formalin, 15 mL of 100% acetic acid, and 30 mL of dH_2O.

2.1.2 Slide Preparation

1. Glass microscope slides, Superfrost/Plus (Matsunami glass, Osaka, Japan).
2. Plastic or metal slide racks.
3. Acetone.

2.1.3 Pretreatment of Tissue Sections

1. Ethanol.
2. 1× PBS.
3. Antigen retrieval solution 0.01 M citrate buffer pH 6.0.
4. Stainless steel pressure cooker.

2.1.4 Masson's Trichrome Staining

1. Weigert's iron hematoxylin stock solution (e.g., #40341, 40351; Muto Pure Chemicals, Tokyo Japan).
2. Masson's trichrome solution (acid fuchsin, xylidine Ponceau, glacial acetic acid, azophloxine, and distilled water, e.g., #40251, Muto Pure Chemicals).
3. Aniline blue (e.g., #40201, Muto Pure Chemicals).
4. Phosphotungstic/phosphomolybdic acid solution (e.g., #40181, Muto Pure Chemicals).
5. Mordant 1 (e.g., #40061, Muto Pure Chemicals).
6. Mordant 2 (e.g., #81411, Muto Pure Chemicals).
7. 3% Acetic acid.

2.1.5 Immunohistochemical Study for Type 1 Collagen, Fibronectin, α-SMA, and CCN2

1. Normal donkey serum (*see* **Note 1**).
2. Goat anti-human type I collagen antibody (SouthernBiotech #1310-01; Birmingham, AL, USA), rabbit anti-human fibronectin antibody (Sigma Aldrich #F3648; St Louis, MO, USA),

rabbit anti-human α-SMA antibody (Abcam #ab5694; Cambridge, UK), and goat anti-human CTGF antibody (Santa Cruz Biotechnology #sc-14939; Dallas, TX, USA) (*see* **Note 2**).

3. Donkey horseradish peroxidase-conjugated anti-goat and anti-rabbit antibodies (Jackson ImmunoResearch #705-035-147 and #711-035-152) and donkey fluorescein-conjugated anti-goat IgG antibody (Jackson ImmunoResearch #705-095-147).
4. Diaminobenzidine (DAB) chromogen substrate ImmPACT™ DAB (Vector Laboratories #SK-4105, Burlingame, CA, USA): When used, add one drop of ImmPACT DAB chromogen concentrate to 1 mL ImmPACT™ DAB Diluent.
5. Hydrogen peroxide.
6. 1× PBS.
7. Xylenes.
8. Ethanol.
9. Hematoxylin.
10. Mounting media "Mount-Quick" (Daido Sangyo, Tokyo, Japan).

2.2 OCT-Embedded Sections for Immunofluorescence Study

1. OCT compound (#4583, Sakura Finetek USA, Torrance, CA, USA).
2. Tissue-Tek Cryomold intermediate.

2.2.1 Tissue Preparation and Embedding

2.2.2 Slide Preparation

1. Glass microscope slides, Superfrost/Plus.
2. Plastic or metal slide racks.
3. Acetone.

2.2.3 Immunofluorescence Study for PDGFR-β

1. Normal donkey serum (*see* **Note 1**).
2. Rat anti-mouse PDGFR-β (CD140a) antibody (BioLegend, San Diego, CA, USA).
3. Donkey fluorescein-conjugated anti-goat IgG antibody (Jackson ImmunoResearch #705-095-147).
4. 1× PBS.
5. Aqueous Mounting Medium PermaFluor (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 Unilateral Ureteral Obstruction

1. C57BL/6J mice weighing 20–30 g.
2. Surgical materials (i.e., 5-0 silk, 4-0 nylon).
3. Anesthetics (i.e., pentobarbital, ketamine).
4. Surgical equipment (tissue forceps, scissors).

2.4 Transgenic Mouse Construction

1. Restriction enzymes.
2. DNA ligation kit ver 2.1. (TAKARA Bio, Kusatsu, Japan).
3. *Escherichia coli* DH5™.
4. 2× TY medium.
5. LB plates.
6. Ampicillin.
7. DNA purification kit.
8. Primers (for mouse CTGF full-length cDNA: 5′-tcctaccgc-gtcccgatcat-3′ and 5′-gctttacgccatgtctccgt-3′).
9. A plasmid carrying rabbit β-globin intron and polyadenylation site [34].
10. *Pfu* turbo (Agilent Technologies, Santa Clara, CA, USA).

3 Methods

3.1 Masson's Trichrome Staining and Immunohistochemical Study

3.1.1 Immersion Fixation

1. Cut the kidney along the minor axis, 1 mm thick, using brain slicer (Zivic Instruments, Pittsburgh, PA, USA).
2. Immerse tissue in 4% PFA or Duboscq-Brasil fluid, 10× the volume of tissue (*see* **Note 3**).
3. Store at 4 °C for 8–16 h, depending on tissue.

3.1.2 Tissue Embedding in Paraffin Wax and Sectioning

1. Dehydrate tissue through graded alcohols and clear in two changes of chloroform.
2. Infiltrate tissue with two changes of molten paraffin wax (approximately 56 °C) for a total of 4–6 h.
3. Orientate and embed tissue in fresh wax using warm molds and embedding cassettes.
4. Place tissue and molds in a –20 °C freezer for a minimum of 2 h before separating the block from the mold.
5. Four-micron-thick paraffin sections of each sample are cut on a microtome.

3.1.3 Masson's Trichrome Staining

1. Dewax sections and bring to 70% ethanol.
2. Mordant 1 for 15 min.
3. Wash in tap water, for 5 min.
4. Stain with Weigert's iron hematoxylin for 5 min.
5. Wash in tap water, shortly.
6. Mordant 2 for 45 s to 1 min.
7. Wash in tap water, for 3 min.

8. 3% Acetic acid solution for 1 min.
9. Stain in Masson's trichrome solution for 20 min.
10. 3% Acetic acid solution for 1 min.
11. Stain in 2.5% phosphotungstic/phosphomolybdic acid solution for 5 min.
12. 3% Acetic acid solution for 1 min twice.
13. Aniline blue for 2 min.
14. 3% Acetic acid shortly twice.
15. Isopropyl alcohol once.
16. Dehydrate through ethanol.
17. Clear in xylene.
18. Cover slip using mounting medium (Fig. 1).

3.1.4 Immunohistochemical Study for Type I Collagen, Fibronectin, α-SMA, and CCN2

1. Dewax sections and bring to 90% ethanol.
2. Wash in dH_2O for 5 min.
3. Place antigen retrieval in the pressure cooker (*see* **Note 4**). Heat the pressure cooker without slides until boiling. Do not secure the lid of the pressure cooker at this time.
4. When boiled, place slides within a slide basket in a pressure cooker.
5. Boil for 10 min.
6. Cool the pressure cooker with tap water until room temperature.
7. Wash in 1× PBS for 1 min.
8. Incubate with 1.5% hydroxyl peroxide in methanol at room temperature for 15 min.
9. Rinse three times with 1× PBS for 1 min each.
10. Outline sections with a hydrophobic wax pen to avoid antibody spreading and possible drying.
11. Incubate slides with 10% donkey serum in 1× PBS at room temperature for 10 min (*see* **Note 1**).
12. Incubate with a primary antibody at appropriate dilution in 1× PBS (e.g., for type I collagen, 1:20; for fibronectin, 1:100; for α-SMA 1:100; for CCN2 1:50) at room temperature for 1 h or overnight at 4 °C (*see* **Note 5**).
13. Rinse three times with 1× PBS for 1 min each.
14. Incubate with an appropriate secondary antibody at appropriate dilution in 1× PBS (e.g., for type I collagen, 1:50; for fibronectin, 1:100; for α-SMA 1:100; for CCN2 1:50) at room temperature for 1 h (for CCN2 immunofluorescent staining, *see* **Note 6**).
15. Rinse three times with 1× PBS for 1 min each.

16. Cover sections with the 3,3′-diaminobenzidinetetrahydrochloride (DAB) for 2–30 min at room temperature.
17. Rinse with dH_2O.
18. Counterstain in hematoxylin, dehydrate, and cover slip with mounting medium.

3.2 Immunofluorescence Study for PDGFR-β

3.2.1 Tissue Embedding in OCT Compound and Sectioning

1. Cut the kidney along the minor axis, 1 mm thick, using brain slicer.
2. Freeze in OCT using cold acetone supplemented with dry ice.
3. Four-micrometer-thick sections are cut on a cryostat.
4. Dry cryostat sections using cool air dryer for 20 min.

3.2.2 Immunofluorescence Study for PDGFR-β

1. Rinse three times with 1× PBS for 1 min each.
2. Incubate with 4% PFA for 5 min.
3. Rinse three times with 1× PBS for 1 min each.
4. Outline sections with a hydrophobic wax pen to avoid antibody spreading and possible drying.
5. Incubate slides with 10% donkey serum in 1× PBS at room temperature for 30 min (*see* **Note 1**).
6. Incubate with primary antibody (rat anti-mouse PDGFR-β antibody, 1:25) in 1× PBS overnight at 4 °C.
7. Rinse three times with 1× PBS for 1 min each.
8. Incubate with secondary antibody (rhodamine-conjugated donkey anti-rat IgG; Jackson ImmunoResearch #712-025-153, 1:200) in 1× PBS at room temperature for 1 h.
9. Rinse three times with 1× PBS for 1 min each.
10. Cover slip using PermaFluor.

3.3 Unilateral Ureteral Obstruction

UUO is one of the most popular models of chronic kidney disease, which elicits tubulointerstitial fibrosis in a short time. UUO is induced by ligation of the ureter of one kidney. The obstruction of the urinary tract causes an increase in pressure within the tubules and leads to dilatation of the renal pelvis. Many have reported that the activation of the renin-angiotensin system is involved in the progression of tubulointerstitial fibrosis. In 1 or 2 weeks, severe fibrosis is established (Fig. 1).

1. Anesthetize mice via intraperitoneal injection of pentobarbital (50 mg/kg).
2. Mice are kept warm (37 °C) using a heating pad.
3. Shave the hair around the abdomen and disinfect with ethanol.
4. Make a midline incision from the bladder to the xiphoid process.

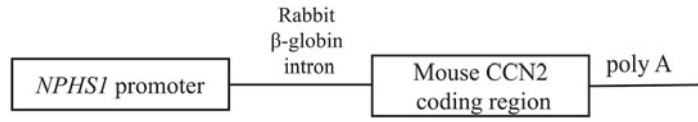

Fig. 2 Construction of podocyte-specific CCN2 transgenic mice. Schematic structure of the transgene. The transgene construct carries *NPHS1* promoter, rabbit β-globin intron, mouse CCN2-coding sequences, and rabbit β-globin polyA

5. Set the retractor to facilitate the view of the left ureter by moving the digestive tract.
6. Prepare the ureter from the surrounding tissue.
7. Place the ligation using 5-0 silk suture.
8. After ligation, close the incision. Muscles and skin are closed layer by layer.
9. Monitor mice until mice recover from anesthesia.

3.4 Transgenic Mouse Construction

Podocyte-specific CCN2 transgenic mice are constructed using a plasmid which contains β-globin intron and the polyadenylation site (Fig. 2) [30]. Transgenic mice carrying rabbit β-globin intron have reported to induce the augmented expression of the transgene [35].

3.4.1 Isolation of Mouse CCN2 cDNA

Initial denaturation	95 °C, 1 min
30 Cycles	95 °C, 30 s 58 °C, 30 s 72 °C, 30 s
1 Cycle	72 °C, 5 min

1. Full-length mouse CCN2 cDNA was PCR amplified from C57BL/6 J mouse kidney cDNA. Two specific primers are used: 5′-tcctaccgcgtcccgatcat-3′ and 5′-gctttacgccatgtctc-cgt-3′. Prepare 25 μL PCRs in sterile thin-walled PCR tube. Add reagents in the order listed. Mix gently.
2. Perform PCR

Distilled water	X μL to a final volume of 25 μL
5× Pfu turbo buffer	25 μL
dNTP mix (25 mM each dNTP)	0.25 μL
DNA template (mouse kidney cDNA)	50 ng cDNA
Forward primer	20 ng
Reverse primer	20 ng
Pfu turbo	1 μL

3. Add 1 μL of rTaq to reacted PCR tube to add terminal nontemplated adenosine to PCR products for TA cloning and incubate at 72 °C for 10 min.
4. PCR products are analyzed by gel electrophoresis. Isolate the 1.2 kb CCN2 fragment with the QIAGEN Gel Extraction kit and determine the concentration of DNA by Nanodrop.
5. Set up a 10 μL ligation reaction by mixing 25 ng pGEM-T-Easy vector and 25 ng CCN2 fragments with 1× ligation buffer and 1 μL ligase. Incubate overnight at 16 °C.
6. Use 5 μL of the ligation reaction for transformation of 50 μL competent *E. coli* cells, like DH5a, and plate cells on LB agar plates containing ampicillin (100 μg/mL). Incubate the plates overnight at 37 °C.
7. Pick up six colonies grown on the plates and shake each one in 5 mL 2× TY medium with ampicillin (100 μg/mL) overnight at 37 °C. Isolate plasmid DNA using Promega DNA plasmid wizard kit. Using restriction enzyme *Eco*RI, bands of CCN2 are detected in agarose gel electrophoresis.
8. Sequence one positive clone.

3.4.2 Isolation of Human Nephrin (NPHS1) Promoter DNA

1. Full-length human nephrin promoter (*NPHS1*) was PCR amplified from human genomic DNA. Two specific primers are used: 5′-ctgaggcagatggatcacctgagg-3′ and 5′-tcacaggtccccctact-gtgaccc-3′. PCR is performed according to Subheading 3.4.1. Cloning vector pSTBlue-1 AccepTor is used.

3.4.3 Construction of CCN2 Expression Vectors

1. *Hin*d III and *Eco*RI sites of the plasmid are replaced with *Xho*I and *Eco*RI sites, respectively.
2. The plasmid is digested with *Xho*I and extracted using phenol, chloroform, and isopropanol solutions.
3. Human nephrin promoter DNA is extracted from the cloning vector by digestion with *Xho*I and purified with QIAGEN Gel Extraction kit.
4. To eliminate self-ligated empty vector, digested vector is incubated with BAP enzyme at 65 °C for 1 h. After BAP reaction, vector is recovered from the agar gel electrophoresis.
5. Set up a 10 μL ligation reaction by mixing 25 ng vector and 25 ng human nephrin promoter DNA with 1× ligation buffer and 1 μL ligase. Incubate overnight at 16 °C.
6. Pick up clones and purify the plasmid. Isolate positive clones according to Subheading 3.4.1.
7. Next, mouse CCN2 is inserted into the plasmid carrying human nephrin promoter. The plasmid is digested with *Eco*RI and extracted using phenol, chloroform, and isopropanol solutions.

8. Mouse CCN2 cDNA is extracted from the cloning vector by digestion with NotI and purified with QIAGEN Gel Extraction kit.
9. Perform ligation, pick up, plasmid purification, and isolation of positive clones.

3.4.4 Generation of Transgenic Mice

1. Transgenic mice are produced by standard pronuclear injection with linearized DNA. Injected eggs are subsequently returned to a surrogate mother to complete gestation.
2. Several transgenic positive founders should be characterized by real-time PCR copy number assay (*see* **Note** 7) or Southern blotting.
3. Several medium- and high-copy transgenic lines should be selected to generate G1 offspring.
4. Podocyte-specific expression is examined by collecting glomeruli by graded sieving method. Localization is examined by immunohistochemical study for CCN2.

4 Notes

1. For blocking in immunohistochemical study, use serum from the species that the secondary antibody was generated in. Serum from the secondary antibody species would prevent nonspecific binding of the secondary antibodies.
2. These antibodies can cross-react with mice and rats.
3. For Masson's trichrome and CCN2 staining, a Duboscq-Brasil-fixed sample might be stained better than a formalin-fixed sample.
4. There are a number of commonly used enzyme and antigen retrieval methods, such as protease digestion, trypsin digestion, acid treatment, and heat-induced epitope retrieval. Microwave oven as well as pressure cooker are often used in heat-induced epitope retrieval. Staining of fibronectin may be better with microwave oven.
5. To decrease background staining, a temperature of 4 °C is used in combination with overnight or longer incubations. Slide incubated for extended periods should be placed in a humidity chamber to prevent evaporation and drying of tissue section.
6. FITC-labeled donkey anti-goat IgG antibody (Jackson ImmunoResearch #705-095-147) can detect goat anti-CCN2 antibody.
7. TaqMan Copy Number Assays are designed to measure copy number variation. TaqMan Copy Number Reference Assay, Mouse, Tert, or Tfrc is used as control. The real-time reaction reveals the copy number of the interested genes.

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Chapter 37

Analysis of Pathological Activities of CCN Proteins in Fibrotic Diseases: Liver Fibrosis

Li Chen and David R. Brigstock

Abstract

Hepatic fibrosis is a complex pathology arising from chronic injury. Pathological features are dominated by the excessive production of extracellular matrix proteins, particularly collagens which are deposited as insoluble scar material that can compromise tissue function. Fibrosis in the liver can often be assessed by staining for collagen in tissue sections and this is an approach that is widely used for grading of fibrosis in human biopsies. However, the recognition of the molecular components that drive fibrosis, including CCN proteins, and the involvement of hepatic stellate cells (HSC) as the principal collagen-producing cells in fibrosing liver, has resulted in a wide variety of molecular and cellular approaches to study the pathogenesis of fibrosis both in vivo and in vitro.

Key words Immunohistochemistry, In situ hybridization, RT-PCR, Immunoprecipitation, ELISA, Chromatin immunoprecipitation, Electrophoretic mobility shift assay

1 Introduction

Hepatic fibrosis is a debilitating pathology of the liver that is characterized by deposition of collagenous material during chronic injury, leading to tissue dysfunction and increasing the risk of developing cirrhosis, cancer, or end-stage organ disease [1]. Collagen is produced principally by hepatic stellate cells (HSC) following their injury-induced activation from quiescent cells to myofibroblasts—in which pathways of fibrogenesis are highly stimulated [2]. While fibrosis may be diminished upon removal of the primary insult, it persists in a large number of individuals for whom novel fibrosis therapies are desperately needed [3]. Knowledge of the molecular mechanisms that drive fibrosis, especially in HSC, has resulted in new leads for potential anti-fibrotic therapies [4]. For example, CCN2 is produced in fibrotic liver at high levels that reflect its central role as an activation-induced pro-fibrogenic molecule in HSC. Through its autocrine and paracrine pathways of action in activated HSC, CCN2 drives collagen production

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downstream of transforming growth factor [5]. Antagonists of CCN2 are effective at inhibiting fibrogenic pathways in cultured HSC as well as fibrosis in experimental animal models [6]. Since CCN2 is present at only very low levels in quiescent HSC, knowledge of the mechanisms that naturally suppress CCN2 expression may help to define novel therapeutic options. Low levels of CCN2 expression in quiescent HSC arise, at least in part, from the binding of miR-214 to the CCN2 3′-UTR, causing CCN2 expression to be inhibited [7]. In turn, miR-214 is transcriptionally stimulated by Twist1 which is expressed at high levels in quiescent HSC but at low levels in activated HSC [8]. The ability to assess key markers of liver fibrosis and of HSC function is an important element in understanding fibrotic pathology and the efficacy of therapeutic interventions.

2 Materials

Whenever possible, reagents and drugs administered to animals should be of pharmaceutical grade. Controlled substances should be locked securely with access restricted only to authorized individuals. Meticulous record-keeping for all drugs is essential. Animal protocols require institutional approval.

2.1 Experimental Animal Model

1. Carbon tetrachloride (CCl_4).
2. Thioacetic acid (TAA).

2.2 Colorimetric Staining

1. Pico-Sirius red stain: Sirius Red, 0.1% Sirius Red in saturated picric acid.
2. Bouins solution: 75 ml Saturated aqueous picric acid, 25 ml 37–40% formaldehyde, 5 ml glacial acetic acid.
3. Biebrich Scarlet-Acid Fuchsin solution: 360 ml 1% Biebrich Scarlet, 40 ml 1% Acid Fuchsin, 4 ml glacial acetic acid.
4. Phosphomolybdic-phosphotungstic acid: 25 g Phosphomolybdic acid, 25 g phosphotungstic acid. Make to 1000 ml with distilled water.
5. Aniline blue solution: 25 g Aniline blue, 20 ml glacial acetic acid. Make to 1000 ml with distilled water.

2.3 Immunohistochemistry

1. Blocking reagent: Skim milk, 1× PBS. Dissolve 3 g skim milk in 100 ml PBS (1×).
2. PBS-T: PBS, 0.1% Tween 20.
3. Mounting medium, e.g., Vectashield (Vector Laboratories, Burlingame, CA, USA) or equivalent containing 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain Store at 4 °C.

2.4 In Situ Hybridization

1. Locked nucleic acid (LNA™) microRNA probes pre-labeled with double-digoxigenin (DIG) (Exiqon Inc., Woburn, MA, USA). Store at –20 °C.
2. Hybridization kit: miRCURY LNA™ microRNA in situ hybridization (ISH) optimization kit (Exiqon Inc.). Store at 4 °C.
3. MicroRNA ISH Buffer (e.g., microRNA ISH Buffer, Exiqon Inc.). Store at 4 °C.
4. Saline-sodium citrate (SSC) buffer (20×): 175.3 g NaCl, 88.2 g trisodium citrate. Make to 1000 ml with distilled water (pH 7.0).
5. KTBT buffer: 7.9 g Tris–HCl (50 mM) pH 6.8, 8.7 g NaCl (150 mM), and 0.75 g KCl (10 mM). Make to 1000 ml with distilled water.
6. ISH block solution (e.g., ISH block solution, Roche, Indianapolis, IN, USA).
7. Nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolylphosphate (BCIP). Store at 4 °C.
8. Eukitt® Mounting Medium (VWR., Radnor, PA, USA), Prolong Gold Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies., Carlsbad, CA). Store at 4 °C.

2.5 Quantitative RT-PCR

1. MicroRNeasy mini kit (Qiagen, Valencia, CA, USA), miScript II RT kit (Qiagen). Store at 4 °C.
2. Primers (Table 1).

2.6 Western Blotting

1. Radioimmunoprecipitation (RIPA) buffer: 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (pH 7.4). Make to 100 ml with distilled water. Store at 4 °C.
2. Bicinchoninic acid (BCA) dye-binding assay (Bio-Rad, Hercules, CA, USA).
3. Loading buffer (2×) for sodium dodecyl sulfate gel electrophoresis (SDS-PAGE): 8 M Urea, 70 mM Tris–HCl pH 6.8, 3% SDS, 0.005% bromophenol blue, 5% β-mercaptoethanol. Make in distilled water. Store at –20 °C.
4. Western blot transfer buffer: 25 mM Tris–HCl pH 8.3, 150 mM glycine, 20% (v/v) methanol. Make in distilled water.
5. Enhanced chemiluminescence kit (Thermo Scientific, Pierce, USA). Store at 4 °C.
6. Image analysis software.

2.7 Hepatic Collagen Assay

1. Sircol™ assay (Biocolor Ltd., Carrickfergus, UK).

Table 1
Primers used for RT-PCR

Gene	GenBank accession number	Primers		Product size (bp)
		Sense	Antisense	
CCN2	NM_010217	5′ CACTCTGCCAGTGGAGTTCA 3′	5′ AAGATGTCATTGTCCCCAGG 3′	111
miR-214	NR_029796	5′ ACAGCAGGCACAGACAGGCA 3′	Universal anti-sense	20
Collagen 1(α1)	NM_007742	5′ GCCCGAACCCCAAGGAAAAGAAGC 3′	5′ CTGGGAGGCCTCGGTGGACATTAG 3′	148
αSMA	NM_007392	5′GGCTCTGGGCTCTGTAAGG3′	5′CTCTTGCTCTGGGCTTCATC3′	148
GAPDH	NM_002046	5′ TGCACCACCAACTGCTTAGC 3′	5′ GGCATGGACTGTGGTCATGAG 3′	66

2.8 Serum Hyaluronic Acid (HA) Assay

1. TECO® Hyaluronic Acid kit (QUIDEL® Corporation, San Diego, CA, USA).

2.9 HSC Isolation

1. Perfusion solution: 1× PBS, pronase, collagenase IV. Add pronase (1.33 mg/ml) and collagenase IV (0.5 mg/ml) into PBS (1×) before use.
2. Digest solution: Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12), pronase, DNase I. Dissolve pronase (1 mg/ml) and DNase I (1 mg/ml) into DMEM/F-12 medium before use.
3. Gradient solution: OptiPrep (Life Technology, Carlsbad, CA, USA). Dilute OptiPrep to 15% or 11% in Hanks' Buffered Salt Solution (HBSS) before use.
4. Culture medium: DMEM, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin. Add 10% FBS, 4.5 g/l L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin into DMEM medium. Store at 4 °C.

2.10 Human LX-2 Cell Culture

1. LX-2 human HSC from Millipore (Temecula, CA, USA).

2.11 Radioimmunoprecipitation Assay

1. Cysteine- and methionine-free DMEM (GIBCO-Invitrogen Carlsbad, CA, USA).
2. [^{35}S] methionine/cysteine (MP Biomedicals, Irvine, CA, USA).
3. Agarose beads.
4. X-ray film.

2.12 CCN2 Enzyme-Linked Immunosorbent Assay

1. CCN2 enzyme-linked immunosorbent assay (ELISA) kit (Antigenix America, Huntington Station, NY, USA).

2.13 CCN2 Promoter Activity Assay

1. Phospha-Light kit (Applied Biosystems, Forster City, CA, USA).
2. Galacto-star kit (Applied Biosystems).
3. Luminometer, e.g., LMax II 384 (Molecular Devices, Sunnyvale, CA, USA) or equivalent.

2.14 SiRNA Suppression of TGF-β1 or CCN2 Expression Assay

1. Human TGF-β1 or CCN2 GIPZ lentiviral shRNAmir plasmids, negative scramble siRNA (Open Biosystem, Huntsville, AL, USA).
2. pRNAT-CMV3.1/Neo vector (GenScript Corporation, Piscataway, NJ, USA).
3. Nucleofector Kit (Lonza, Koln, Germany).

2.15 Electrophoresis Mobility Assay (EMSA)

1. EMSA lysis buffer.
2. EMSA binding buffer: 50% Glycerol, 1% NP-40, 100 mM $MgCl_2$, 200 mM EDTA, 1× binding buffer, 1 M KCl, 1 μg/μl Poly(dI:dC).
3. Tris/borate/EDTA (TBE) buffer (5×) for EMSA: 54 g Tris base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0). Make to 1000 ml with distilled water.
4. Biotin 3′ end labeling kit, deoxynucleotidyl transferase (Thermo Scientific).
5. Chemiluminescent Nucleic Acid Detection module kit (Thermo Scientific).
6. Streptavidin-horseradish peroxidase conjugate (Thermo Scientific).

2.16 Chromatin Immunoprecipitation

1. EpiTect chromatin immunoprecipitation (ChIP) kit (Qiagen).
2. Magnetic protein A beads (e.g., Sure Beads™, BioRad).

2.17 Antibodies

We commonly use monoclonal anti-digoxigenin-alkaline phosphatase antibody (Roche, Indianapolis, IN), monoclonal anti-mouse αSMA antibody (Dako Cytomation, Denmark), polyclonal anti-rabbit collagen 1(α1), polyclonal anti-rabbit Twist1 (Millipore, Temecula, CA, USA), CD9 antibody (SBI System Biosciences, Mountain View, CA, USA), Alexa Fluor®488 goat-anti chicken IgY, Alexa Fluor® 568 goat-anti-rabbit IgG, Alexa Fluor® 647 goat anti-mouse IgG (Life Technologies), secondary antibody (e.g., anti-rabbit IgG, or anti-sheep IgY, anti-mouse IgM) conjugated to horseradish peroxidase (Sigma-Aldrich).

3 Methods

3.1 Experimental Liver Fibrosis in Animal Models (See Notes 1–3)

3.1.1 CCl_4-Induced Liver Fibrosis

1. Inject male mice (6–8 weeks of age) with CCl_4 (175 μl in 1325 μl corn oil/kg, i.p or i.m.) three times per week for at least 5 weeks.
2. Control mice receive the same frequency and route of administration of corn oil (1500 μl/kg).
3. Sacrifice mice 1–3 days after the last injection.

3.1.2 TAA-Induced Liver Fibrosis

1. Inject male mice (6–8 weeks of age) with TAA (100 mg/kg, i.p.) in saline three times per week for at least 5 weeks.
2. Control mice receive an equivalent volume of saline (i.p.).
3. Sacrifice mice 1–3 days after the last injection.

3.1.3 Bile Duct Ligation (BLD)-Induced Liver Fibrosis

1. Anesthetize male mice (6–8 weeks of age) with isoflurane in 3% oxygen.
2. Using a scalpel, cut the abdomen through a median line.

3. Open the abdominal cavity, turn down the liver lobules, and carefully exteriorize the intestines to uncover the bile duct.
4. Pull out the extrahepatic common bile duct, fix with forceps, and ligate it with a piece of 6-0 silk. A second ligature is placed within 2 mm of the first.
5. Replace the intestines and livers into their correct positions within the abdominal cavity.
6. Close the musculature and subcutaneous tissues with absorbable suture.
7. Close the skin with non-absorbable suture.
8. Sham-operated mice are treated in the same manner except that the bile duct is not ligated.
9. Sacrifice the animals on postoperative days 7, 14, 21, 28, 35, or 42.

3.2 Detection of Hepatic CCN2, αSMA, or Collagen 1(α1) in Tissue Sections

1. Anesthetize mice with ketamine (66 mg/kg) and xylazine (14 mg/kg) and ensure a very deep plane of anesthesia by strong toe pinch. Optionally, blood (0.5–0.7 ml) is collected by cardiac puncture using a 25-gauge needle (*see* **Note 4**).

3.2.1 Tissue Preparation

2. Place the animal on its back and spray the abdomen with 70% alcohol. Make a V-cut through the skin and abdominal wall about 1 cm caudal to the last rib. Make a small incision in the diaphragm to equalize the heart chamber and external pressures, lift the sternum upwards, cut the rib cage laterally about 1 cm in each direction to access the heart chamber, insert a 23–25-gauge butterfly needle into the left ventricle, and clip the aorta with scissors.
3. Improved staining of sections may be obtained by perfusion of the liver to remove blood components. Perfuse animals with 20–30 ml 4% paraformaldehyde for fixation. Alternatively tie off one lobe of the liver with suture material (for later RNA extraction) prior to perfusion of the remainder of the liver with 4% paraformaldehyde.
4. Resect the liver and place overnight in 4% paraformaldehyde at 4 °C.
5. Wash fixed livers three times with 70% ethanol before embedding in paraffin blocks.
6. Cut sections of 5–6 μm and mount on slides. Deparaffinize sections with xylene and ethanol.

3.2.2 Hematoxylin and Eosin Staining (H&E)

1. Place sections in distilled water and stain with hematoxylin until the tissue becomes red.
2. Rinse slides in running tap water and destain background with 0.3% acetic acid in 70% ethanol.

3. Rinse with water and stain with eosin for 2 min.
4. Dehydrate the sections through 70% alcohol, 95% alcohol, and 100% alcohol at room temperature, and mount the slides with Vectashield Mounting Medium.
5. Examine using light microscopy.

3.2.3 Colorimetric Staining of Hepatic Collagen Using Sirius Red

1. Heat slides and deparaffinize.
2. Rinse well in several changes of tap water for 10 min.
3. Place slides in 0.2% phosphomolybdic acid for 1–5 min.
4. Rinse well in distilled water.
5. Place slides in the 0.1% Picrosirius Red Solution for 90 min.
6. Wash in 0.01 N HCl for 2 min.
7. Rinse in 70% alcohol for 45 s.
8. Dehydrate in graded alcohols and cover slip.
9. Collagen is red (Fig. 1). Under polarized light, type 1 collagen appears yellow and type 3 collagen appears green.

3.2.4 Colorimetric Staining of Hepatic Collagen Using Trichrome Staining (See Note 5)

1. Deparaffinize in xylene and hydrate to water using graded alcohols. Ten dips in each reagent: xylene (three changes), 100% ethanol (two changes), 95% ethanol (two changes), 70% ethanol, and distilled water.

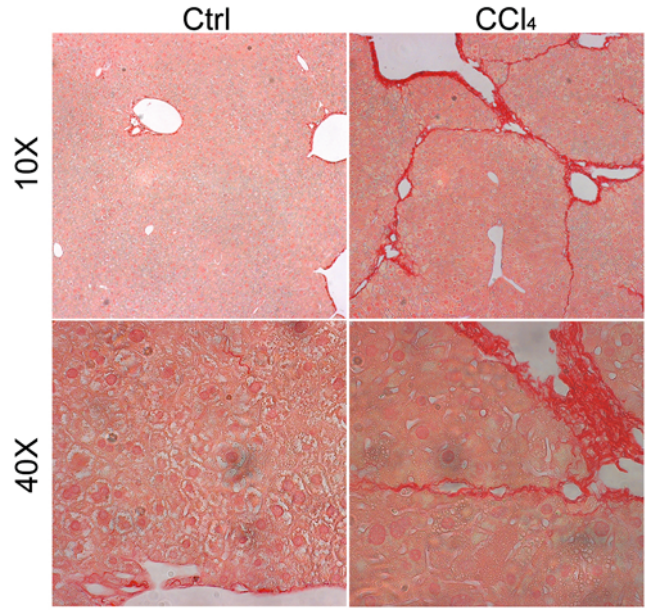

Fig. 1 Sirius red staining. Sections of mouse livers from control mice or mice treated for 5 weeks with CCl_4 to induce hepatic fibrosis were incubated with Sirius red stain. Collagen fibers in the interstitial spaces stain red

2. Rinse well in distilled water.
3. Mordant sections in Bouin's solution over night at room temperature.
4. Remove slides and wash in running water until yellow color disappears.
5. Rinse in distilled water.
6. Stain sections in Weigert Hematoxylin for 10 min.
7. Wash in running tap water for 10 min.
8. Rinse in distilled water.
9. Stain sections in Biebrich Scarlet-Acid Fuchsin for 2 min.
10. Rinse in distilled water.
11. Place slides in phosphomolybdic-phosphotungstic acid solution for 15 min. Discard the solution after use.
12. Stain in aniline blue for 5 min.
13. Rinse in distilled water.
14. Place slides in 1% acetic acid for 5 min. Discard solution after use.
15. Dehydrate with 95% ethanol and 100% ethanol, ten dips for two changes each.
16. Clear with two or three changes of xylene or Americlear (ten dips each).
17. Coverslip with Permaslip.
18. Nuclei stain back, cytoplasm, keratin, and muscle stain red, collagen, and mucus stain blue.

3.2.5 Evaluation of Colorimetric Staining of Hepatic Collagen

1. Code slides for a blinded assessment of pathology and type and degree of fibrosis.
2. Use a standardized grading system to document the type and extent of fibrosis:
   0. No detectable fibrosis.
   1. Portal or early reticulin fibrosis.
   2. Portal fibrosis with sinusoidal or early bridging fibrosis.
   3. Established bridging fibrosis.
   4. Cirrhosis.
3. Evaluate at least five sections from each animal.

3.2.6 Immunohistochemistry

1. Block liver sections with PBS containing 3% skim milk at 37 °C for 1 h.
2. Rinse and incubate the sections with antibodies: e.g., anti-CCN2 (5 μg/ml), anti-αSMA (1:1000), or anti-collagen 1(α1) (1:250) overnight at 4 °C under agitation.
3. Rinse the sections four times with PBS-T, 15 min each time.

4. Add, respectively, Alexa Fluor®568 goat-anti chicken IgY, Alexa Fluor® 488 goat-anti mouse IgG, or Alexa Fluor® 647 goat anti-rabbit IgG (all at 1:1000) for 1 h at room temperature.
5. Rinse the sections three times in PBS-T for 15 min and one time in PBS for 15 min at room temperature with agitation.
6. Mount the slides with Vectashield Mounting Medium containing DAPI nuclear stain, and examine by fluorescence or confocal microscopy (Fig. 2).

3.2.7 In Situ Hybridization

1. De-paraffinize the liver sections through xylene, 100% alcohol, 95% alcohol, and 70% alcohol at room temperature with a final transfer into PBS.
2. Incubate with proteinase-K for 10 min at 37 °C and wash twice with PBS (*see* **Note 6**).
3. Dehydrate the sections by sequential rinsing with 70, 96, and 99.9% ethanol (for each step, dip ten times and then immerse for 1 min) and air-dry the slides on clean paper towels for 15 min.
4. Place slides on a flat surface and hybridize the sections with a probe to the mRNA of interest (e.g., CCN2) or scramble sequence probes.
5. Apply sterile and RNase-free cover glasses onto the sections and seal along all four edges with Fixogum for 60 min at 55 °C.
6. Remove Fixogum using tweezers and carefully detach cover glasses and place the slides into 5× SSC solution. Wash the slides with graded concentrations of SSC buffer (5×, once for 5 min; 1×, twice for 5 min each; and 0.2× SSC, three times for 5 min each) at 55 °C.

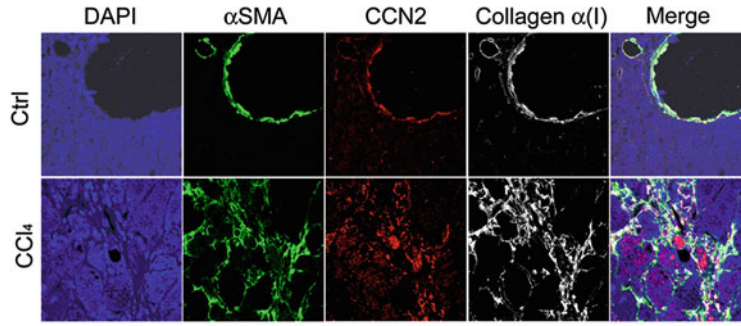

Fig. 2 Immunohistochemistry. Sections of mouse livers from control mice or mice treated for 5 weeks with CCl_4 to induce hepatic fibrosis were stained with DAPI for nuclear localization and processed for CCN2, αSMA, or collagen 1(α1) immunohistochemistry. Images were merged to determine areas of co-localization of CCN2 with αSMA or collagen α(I) (i.e., activated HSC)

7. Incubate the slides with a monoclonal anti-digoxigenin-alkaline phosphatase antibody (1:800) for 60 min after blocking the sections with block solution.
8. Wash the slides with PBS-T and apply NBT/BCIP substrates at 30 °C for 2 h. Stop the reaction with KTBT buffer and wash with water twice.
9. Counterstain the slides with Nuclear Fast Red™, dehydrate slides in graded ethanol solutions as in **step 6**, mount with Eukitt® Medium, and examine by confocal microscopy.

3.3 Evaluation of RNA or Protein in Liver Extracts

3.3.1 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

1. Extract total RNA (including microRNAs) from liver tissues using a microRNeasy mini kit according to the manufacturers' protocols.
2. Reverse transcribe RNAs into cDNAs using a miScript II RT kit according to the manufacturers' protocols.
3. Use qRT-PCR to analyze expression of relevant transcripts, such as those for miR-214, CCN2, αSMA, or collagen 1(α1) (Table 1). Run each reaction in triplicate, and normalize to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Negative controls are reactions lacking reverse transcriptase or sample (template).

3.3.2 Western Blot Analysis

1. Homogenize 100 mg liver in 1 ml RIPA buffer.
2. Protein assay: Measure tissue protein using a BCA dye-binding assay with bovine serum albumin as a standard. Prepare samples for electrophoresis by boiling them in sample loading buffer (2×) for 5 min.
3. Subject clarified tissue supernatant containing 20 μg of total protein to 12 or 18% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 70–120 V.
4. Electro-transfer the separated proteins in the gel on to nitrocellulose membranes in transfer buffer at 300 mA for 1.5 h or at 27 V overnight at 4 °C.
5. Block the membrane in PBS-containing 3% skim milk at 37 °C for 1 h.
6. Incubate the membrane with appropriate antibodies such as anti-CCN2 (5 μg/ml) anti-collagen 1(α1) (1:400), or anti-αSMA (1:1000) overnight at 4 °C under agitation.
7. Wash the membranes four times with PBS-T, 15 min each time, at room temperature.
8. Incubate blots with an appropriate secondary antibody (e.g., anti-rabbit IgG, or anti-chicken IgY, anti-mouse IgM) conjugated to horseradish peroxidase (1:5000) for 1 h at room temperature with agitation.

9. Rinse the membranes and develop with an enhanced chemiluminescence detection kit.
10. Quantify the intensities of immunoreactive bands using image analysis software. Use the migration position of pre-stained standards to assess M_r of immunoreactive proteins (*see* **Note** 7).

3.4 Other Tests for Fibrotic Liver Markers

3.4.1 Hepatic Collagen Assay

1. Add 1 ml 0.5 M acetic acid to 0.5–1 g liver tissue, and homogenize the liver tissue on ice. Centrifuge the tube at 14,000 × *g* for 10 min.
2. Test an aliquot of the supernatant for acid-soluble hepatic collagen using a Sircol™ assay according to the manufacturer's directions.

3.4.2 Serum Hyaluronic Acid (HA) (See Note 8)

1. Using a TECO® HA Assay Kit, dilute standards, controls, and serum samples 1:50 with sample diluent, and pipette 100 µl into a microtiter plate. Incubate the plate for 2 h at room temperature with agitation.
2. Rinse the plate three times with 350 µl diluted wash buffer per well, and pipette 100 µl of HA-binding protein-HRP conjugate into each well. Incubate the plate for 30 min at room temperature and rinse the plate five times with the wash buffer.
3. Add 100 µl of TMB substrate solution into each well, and incubate the plate for 30 min at room temperature in the dark.
4. Stop the reaction by adding 100 µl of stop solution. Measure the color reaction at 450 nm.

3.5 Hepatic Fibrogenic Pathways In Vitro

HSC can be successfully isolated from the liver of a wide variety of strains of mice (*see* **Note** 9).

3.5.1 Isolation of Primary Mouse HSC

1. Follow the procedure in Subheading 3.2.1, **step 2**, except that the surgical approach is to access the liver through an abdominal incision. Introduce a 22GA needle (1.00IN, 0.9 × 25 mm) into the infrahepatic vena cava and snip the portal vein with scissors. Initiate systemic perfusion of 20–30 ml PBS (results in immediate death) followed by 10 ml PBS containing 1.33 mg/ml pronase and 0.5 mg/ml collagenase IV.
2. Remove perfused livers from five mice, mince with scissors, and digest in 1 mg/ml pronase and 1 mg/ml DNase I in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium.
3. Stop the digestion with 3 ml serum and centrifuge at 300 × *g* for 10 min (4 °C). Rinse the pellets and centrifuge them again under the same conditions.
4. Filter the resulting cell suspension through a metal sieve (120 µm) and centrifuge at 300 × *g* for 10 min (4 °C) and then remove the supernatant.
5. Prepare OptiPrep to 15% or 11% in Hanks' Buffered Salt Solution (HBSS).

6. Resuspend the cell pellet in 15% OptiPrep and place in a centrifuge tube. Over the top of this solution, layer the 11% OptiPrep solution, followed by HBSS on top of that. Centrifuge the resulting cell suspension at 1400×*g* for 30 min without braking. HSC separate into a hazy band just above the interface of the gradient in the aqueous buffer. Harvest the band.
7. Wash the cells and resuspend them at 10^5 cells/ml in DMEM containing 10% fetal bovine serum, 4.5 g/l L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.
8. Plate the cells into flasks or multi-well plates as needed.
9. Verify HSC identity and purity by buoyant density, phase-contrast microscopy, oil red staining, and immunostaining for glial fibrillary acidic protein (GFAP) or desmin (*see* **Note 10**). Immunostaining can also be performed for additional markers such as αSMA, CCN2, or collagen 1(α1) which are induced in the cells as they become autonomously activated in culture.

3.5.2 Culture of Established HSC Lines: LX-2 Cells

1. Grow LX-2 cells in DMEM/10% FBS medium and split them (1:3) every 5 days. Use until passage 10.
2. To assess the effect of potential stimulants of HSC function, replace the growth medium with low- or serum-free medium for 24 h before assay.

3.5.3 Immunocytochemistry for CCN2, αSMA, or Collagen 1(α1)

1. Fix cultured HSC with 4% paraformaldehyde for 1 h at room temperature and then rinse the cells three times with PBS-T.
2. Perform ICC as described for IHC in Subheading 3.2.6.

3.5.4 CCN2 Radioimmunoprecipitation Assay

1. Culture HSC in cysteine- and methionine-free DMEM for 12–24 h to deplete the cellular cysteine or methionine levels.
2. Incubate cells in cysteine- and methionine-free DMEM containing 100 μCi/ml [^{35}S]methionine/cysteine in the presence or absence of CCN2 stimulants or inhibitors.
3. At 6–48 h, remove medium and centrifuge to remove cell debris. Collect the supernatant.
4. Lyse the cells in 0.5–1 ml RIPA buffer. Centrifuge the lysate and collect the supernatant.
5. Incubate supernatants at 4 °C for 12 h with anti-CCN2 IgY (190 μg/ml) (*see* **Note 11**).
6. Add IgY-precipitating agarose beads and mix for 2 h at for 2 h at room temperature.
7. Centrifuge the tubes and aspirate the medium. Resuspend the beads at the bottom of the tube with 1 ml RIPA, mix, and centrifuge again. Repeat 2–3 times.
8. Aspirate all the medium from each tube, taking care not to disrupt the beads at the bottom of the tube. Add 25 μl 2× SDS-PAGE loading buffer directly to the beads.

9. Mix well, place in boiling water bath for 2 min, mix, and then centrifuge for 2 min. Load 20 μl of supernatant on to 18% SDS-PAGE gels and subject to electrophoresis.
10. Dry the gel and expose to X-ray film.
11. Use migration of radioactive markers to estimate Mr. of immunoreactive proteins (*see* **Note 12**).

3.5.5 CCN2 Enzyme-Linked Immunosorbent Assay (ELISA)

1. Culture mouse HSC or LX-2 cells in 6-well plates with DMEM/10% FBS for 24 h.
2. Replace medium with serum-free medium for the next 24 h.
3. Treat cultures with stimulants or inhibitors for 24–28 h.
4. Remove conditioned medium from cells and save.
5. Lyse cells in RIPA buffer.
6. Clarify conditioned medium or cell lysates by centrifugation.
7. Test the supernatants using a CCN2 ELISA kit.

3.5.6 Stimulation of CCN2, αSMA, or Collagen 1(α1) Expression in HSC

1. Culture mouse HSC or LX-2 cells in 6-well plates with DMEM/10% FBS for 24 h.
2. Replace medium with serum-free medium for the next 24 h.
3. Incubate triplicate wells of cells for up to 48 h in the presence of the stimulant, tested over an appropriate dose range, e.g., 0–25 mM ethanol, 0–20 ng/ml TGF-β1, or 0–100 μM acetaldehyde (*see* **Notes 13** and **14**).
4. Isolate RNA and evaluate cells for CCN2, αSMA, or collagen 1(α1) mRNA expression by RT-PCR.
5. Evaluate cells for CCN2, αSMA, or collagen 1(α1) protein production by Western blot, immunocytochemistry, or ELISA (see above).

3.5.7 Transfection of HSC with CCN2 Promoter Reporter Constructs

1. Cultivate HSC for 24 h in 12-well plates in DMEM with 10% FBS medium at 37 °C.
2. Transfect cells with plasmids (3 μg/well) containing a secreted alkaline phosphatise (SEAP) reporter gene fused to the wild-type CCN2 promoter (nucleotides −805 to +17), deletion mutant: −805 to −166 or −805 to −86, or individual point mutants targeting the Smad site or the basal control element (BCE-1) (*see* **Note 15**).
3. Starve the cells for 18 h prior to addition of stimulants (e.g., 0–50 mM ethanol, 0–20 ng/ml TGF-β) for 0–240 min.
4. Measure CCN2 promoter activity with Phospha-Light kit to detect SEAP reporter expression. Determine β-galactosidase expression by Galacto-star kit. Measure SEAP levels using a luminometer.

5. Calculate SEAP reporter activity after adjustment for differences among samples in transfection efficiency as determined by co-transfection with a cytomegalovirus (CMV) promoter-β-galactosidase (CMV-β-gal) reporter gene.

3.5.8 SiRNA Suppression of TGF-β1 or CCN2 Expression in HSC

1. Purchase human TGF-β1 or CCN2 GIPZ lentiviral shRNAmir plasmids or negative scramble siRNA from Open Biosystem.
2. Clone mouse CCN2 siRNA into pRNAT-CMV3.1/Neo vector from GenScript Corporation (mouse CCN2 siRNA target sequences are 5′-CGCAAGATCGGAGTGTGCTTCAAGAGAGCACACTCCGATCTTGCGGTT-3′ (sense) and 3′-GGCGTTCTAGCCTCACACGAAGTTCTCTCGTGTGAGGCTAGAACGCCAA-5′ (antisense).
3. Transfect cells with 4 μg plasmid or negative scramble siRNA using electroporation with a Nucleofector Kit (*see* **Note 16**).
4. Incubate the transfected cells in medium containing 10% FBS for 12 h.
5. Change to serum-free medium, with or without test agents, for 24–48 h.
6. Determine transfection efficiency as assessed by GFP that is co-expressed from the pRNAT-CMV3.1/Neo vector (*see* **Note 17**).

3.5.9 Electrophoretic Mobility Shift Assay

1. Lyse 5×10^7 mouse HSC in electrophoretic mobility shift assay (EMSA) lysis buffer according to the manufacturer's recommendation.
2. Centrifuge the lysate (10,000 × *g*, 1 min).
3. Collect the nuclear pellet and resuspend it in extraction buffer.
4. Centrifuge the mixture (14,000 × *g*, 5 min, 4 °C), and collect the supernatant (contains solubilized nuclear proteins).
5. Label wild-type or mutant DNM3os promoters at their 3′ end using biotin (0.5 μM) and terminal deoxynucleotidyl transferase (0.2 U/μl) (*see* **Notes 18** and **19**).
6. Purify the labeled promoters using chloroform:isoamyl alcohol (1:1).
7. Incubate HSC nuclear protein (1 μg) for 25 min at room temperature with labeled oligonucleotides (20 fmol/reaction assay) in binding buffer. For some groups add an antibody against the transcription factor being tested, to assess supershift of immunoreactive complexes (e.g., 0.5 μg/ml anti-Twist1 antibody (1:500)) (*see* **Note 20**).
8. Mix samples with 5× loading buffer and electrophorese on a 5% DNA retardation gel in 0.5× TBE buffer.

9. Transfer complexes to a nylon membrane and incubate with streptavidin-horseradish peroxidase conjugate (1:300) at 37 °C for 30 min.
10. Analyze by chemiluminescence.

3.5.10 Chromatin Immunoprecipitation Assay

1. Treat 2×10^6 HSC with 1% formaldehyde for 10 min at room temperature.
2. Terminate the reaction with 0.125 M glycine.
3. Isolate the cells and sonicate on ice to generate DNA shear fragments of ~200–1000 bp.
4. Pellet, pre-clear, and immunoprecipitate with a transcription factor antibody (10 μg/ml) or control IgG.
5. Rotate overnight at 4 °C with magnetic protein A beads.
6. Collect the beads and elute the immune complexes with ChIP-grade proteinase K.
7. Destroy cross-links by heating the samples at 45 °C for 30 min.
8. Perform ChIP-PCR on the recovered DNA using primers for the target sequence (e.g., DNM3os) according to the manufacturers' instructions.

4 Notes

1. Many *in vivo* models have been described to establish experimental liver fibrosis in rodents. Commonly used approaches are repeated administration of toxins or ligation of the common bile duct: these procedures are reliable, and reproducible, within the expertise of most labs, and result in rapid onset and progression of fibrosis (fibrosis is well developed within 5–6 weeks) [9, 10]. Other methods involve administration of special diets or expression of hepatitis antigens that will induce specific disease states (steatosis, non-alcoholic steatohepatitis) or other pathologies (e.g., hepatocyte injury) but not all of these approaches will necessarily result in significant fibrosis [11–13].
2. Whatever method of fibrosis induction is used, different strains of mice have different sensitivities to the agents used, as well as different susceptibilities to develop fibrosis in response to them [14].
3. Whenever possible, verify data using human clinical samples.
4. Cardiac function will be compromised but the heart will still beat slowly.
5. Three dyes are used for trichrome staining. Sections are first stained with basic Weigert Hematoxylin to stain basophilic components (DNA) in the nuclei. Sections are then stained with Biebrich scarlet which binds all acidophilic tissues such as

cytoplasm, muscle, and collagen. Subsequent treatment with phosphotungstic-phoshomolybdic acids cause the Biebrich scarlet to diffuse out of the collagen but not out of the cytoplasm. The acid groups in phosphotungstic phosphomolybdic acids then serve as a link between the decolorized collagen and the aniline blue. The pH of the phosphotungstic-phosphomolybdic acid solution increases selective collagen staining and aids in removing the Biebrich scarlet.

6. The Proteinase-K concentration range must be optimized for individual tissues. It is recommended to test with 2.5 μg/ml in mouse.
7. Proteins on the blot can be alternatively visualized by staining them with Ponceau red (0.2% Ponceau red and 3% TCA in distilled water) for 10 s at room temperature, and then rinsing with distilled water. Unstained molecular mass standards can be used with this approach.
8. Many serum molecules have been implicated in grading liver fibrosis. Some tests of individual serum markers have been combined into panels for more accurate staging, e.g., PGA index, FIB-4 index, Fibrometer, Fibrotest, Fibrosure, Act-test, SAFE, hepascore, FibroQ, AAR, APRI, CDS, API, Pohls score, and Loks model.
9. Typically, HSC are collected and pooled from five male mice (4–6 weeks of age, approx. 25–30 g) of the same strain and then cultured in vitro.
10. Freshly isolated HSCs contain cytoplasmic lipid droplets which are visualized by uptake of oil red O stain. Fix the cells in ice-cold 4% paraformaldehyde in PBS for 20 min at room temperature prior to incubation for 10 min in a saturated solution of oil red O (Polysciences, Warrington, PA) in isopropanol (Sigma-Aldrich).
11. This can be preceded by a pre-clearing step in which the immunoprecipitating beads are added and mixed with the sample and then removed by centrifugation, allowing the supernatant to be further processed. This will reduce background signal due to nonspecific binding of radioactive components to the beads, prior to addition of the antibody.
12. Non-radioactive approaches involve a final Western blot step using antibodies to the protein(s) of interest. Ensure that the Ig that is running in the gel from the IP step is not subsequently detected, or can at least be discriminated, on the Western blot. To avoid the problem, use an immunoprecipitating antibody from a different species than is used for the primary antibody on the Western blot and then using a secondary antibody for the Western blot that does not cross-react between the two species.
13. Acetaldehyde evaporates very rapidly. Use fresh acetaldehyde.

14. Pathways of action can be mechanistically tested by co-treating cells with pathway inhibitors, e.g., 2 mM 4-methylpyrazole (an inhibitor of alcohol dehydrogenase I, which is required for oxidative ethanol metabolism), or 1 mM *N*-acetyl-L-cysteine (a scavenger of reactive oxygen species).
15. Activation of CCN2 gene transcription by TGF-β1 or ethanol involves Smad and Ets-1 elements in the CCN2 promoter [15, 16].
16. To gain the highest transfection efficiency or gene expression activity, the use of an electroporation system for transfection is recommended.
17. Mouse HSC transfection efficiency is ~40%
18. DNM3os is an opposite strand transcript of the dynamin 3 (DNM3) gene that encodes miR-214 and miR-199a, both of which are direct regulators of the 3′ untranslated region of CCN2.
19. Design mutations to target the binding site for the transcription factor being tested (e.g., an E-box motif that lies upstream of miR-214/199a is a binding site for Twist1 transcription factor).
20. Use 4 pmol unlabeled oligonucleotide in some tubes to competitively inhibit formation of shifted complexes. Omit nuclear protein from some tubes to verify its requirement for complex formation.

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Chapter 38

Cellular or Exosomal microRNAs Associated with CCN Gene Expression in Liver Fibrosis

Li Chen and David R. Brigstock

Abstract

Liver fibrosis occurs during chronic injury and represents, in large part, an exaggerated matrigenic output by hepatic stellate cells (HSCs) which become activated as a result of injury-induced signaling pathways in parenchymal and inflammatory cells (hepatocytes, macrophages, etc.). The molecular components in these pathways (e.g., CCN proteins) are modulated by transcription factors as well as by factors such as microRNAs (miRs) that act posttranscriptionally. MiRs are small (~23 nt) noncoding RNAs that regulate gene expression by specifically interacting with the 3′ untranslated region (UTR) of target gene mRNA to repress translation or enhance mRNA cleavage. As well as acting in their cells of production, miRs (and other cellular constituents such as mRNAs and proteins) can be liberated from their cells of origin in nanovesicular membrane exosomes, which traverse the intercellular spaces, and can be delivered to neighboring cells into which they release their molecular payload, causing alterations in gene expression in the target cells. Here we summarize some of the experimental approaches for studying miR action and exosomal trafficking between hepatic cells. Insights into the mechanisms involved will yield new information about how hepatic fibrosis is regulated and, further, may identify new points of therapeutic intervention.

Key words Immunohistochemistry, In situ hybridization, RT-PCR, Immunoprecipitation, Co-culture, Exosome

1 Introduction

Fibrosis arises due to the overt deposition of insoluble collagenous material during chronic organ injury. In the liver, fibrogenic cascades are triggered principally within hepatic stellate cells (HSCs), which are ordinarily quiescent and store vitamin A in fat droplets but which undergo a process of activation during injury. HSC activation is characterized by a phenotypic transition of the cells into myofibroblasts which unrelentingly deposit collagen into the interstitial spaces during chronic injury [1–3]. Pathways of fibrosis in the liver are initiated downstream of inflammation and wound repair and are regulated by a broad variety of cytokines, growth factors, and matricellular proteins that include transforming growth

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factor-beta (TGF-β) as well as CCN1 and CCN2 [4–8]. CCN2 is directly pro-fibrotic and often acts downstream of TGF-β to drive fibrosis. Whereas quiescent HSCs do not produce CCN2 or its receptors, CCN2 promoter activity is highly stimulated in HSC undergoing activation in vivo or in vitro, resulting in high levels of CCN2 mRNA expression and protein production [6].

Recent evidence has shown that microRNAs (miRs) exert important regulatory actions in liver fibrosis. MiRs are small (~23 nt) noncoding RNAs that regulate gene expression by specifically interacting with the 3′ untranslated region (UTR) of target gene mRNA to repress translation or enhance mRNA cleavage [9]. We have recently identified a binding site for miR-214 in the CCN2 3′-UTR [10]. High levels of miR-214 in non-injured liver or quiescent HSC engage the CCN2 3′-UTR and inhibit CCN2, thus contributing to suppressed expression of CCN2 under resting (non-pathological) conditions. In contrast the levels of miR-214 are highly diminished in fibrotic liver or activated HSC, allowing CCN2 expression to be manifested [11].

A very new area of research relates to the manner in which fibrosis is regulated by exosomes. Exosomes are 50–150 nm membranous vesicles that arise by inward budding from the limiting membranes of multivesicular bodies (MBV). Upon fusion of MVBs with the plasma membrane, exosomes are liberated from the cells, and traverse intercellular spaces, and may be taken up by neighboring cells [12–14]. Exosomes contain a complex mixture of miRs, mRNAs, and proteins and therefore their uptake by target cells represents a novel communication pathway through which molecular information is exchanged between donor and recipient cells. MiR-214 is exosomally shuttled between neighboring HSCs and can exert epigenetic effects on the CCN2-UTR in recipient cells [11, 15].

2 Materials

Whenever possible, reagents and drugs administered to animals are to be of pharmaceutical grade. Controlled substances should be locked securely with access restricted only to authorized individuals. Meticulous record-keeping for all drugs is essential. Animal protocols require institutional approval.

2.1 HSC Isolation Components

1. Perfusion solution: 1× PBS, pronase, collagenase IV. Add pronase (1.33 mg/ml) and collagenase IV (0.5 mg/ml) into PBS (1×) before use.
2. Digest solution: Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12), pronase, DNase I. Dissolve pronase (1 mg/ml) and DNase I (1 mg/ml) into DMEM/F-12 medium before use.

3. Gradient solution: OptiPrep (Life Technology, Carlsbad, CA, USA). Dilute OptiPrep to 15% or 11% in Hanks' Buffered Salt Solution (HBSS) before use.
4. Culture medium: DMEM, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin. Add 10% FBS, 4.5 g/l L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin into DMEM medium. Store at 4 °C.

2.2 In Situ Hybridization Components

1. Locked nucleic acid (LNA™) microRNA probes pre-labeled with double-DIG or pre-labeled with double-FITC (Exiqon Inc., Woburn, MA, USA). Store at –20 °C.
2. Hybridization kit (e.g., miRCURY LNA™ microRNA in situ hybridization (ISH) optimization kit, Exiqon Inc.). Store at 4 °C.
3. MicroRNA ISH Buffer (e.g., microRNA ISH Buffer, Exiqon Inc.). Store at 4 °C.
4. Saline-sodium citrate (SSC) buffer (20×): 175.3 g NaCl, 88.2 g trisodium citrate. Make to 1000 ml with distilled water (pH 7.0).
5. KTBT buffer: 7.9 g Tris–HCl (50 mM) pH 6.8, 8.7 g NaCl (150 mM), and 0.75 g KCl (10 mM). Make to 1000 ml with distilled water.
6. ISH block solution (e.g., ISH block solution, Roche, Indianapolis, IN, USA).
7. Nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyl phosphate (BCIP). Store at 4 °C.
8. Eukitt® mounting Medium (VWR, Radnor, PA, USA), Prolong Gold Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA). Store at 4 °C.

2.3 Immunofluorescence Components

1. Blocking reagent: Skim milk, 1× PBS. Dissolve 3 g skim milk in 100 ml PBS (1×).
2. PBS-T: PBS, 0.1% Tween 20.
3. Mounting medium, e.g., Vectashield (Vector Laboratories, Burlingame, CA, USA) or equivalent, containing 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. Store at 4 °C.

2.4 Quantitative RT-PCR Components

1. MicroRNeasy mini kit (Qiagen, Valencia, CA, USA), miScript II RT kit (Qiagen). Store at 4 °C.
2. Primers (Table 1).

2.5 CCN2 3′-UTR Construction and miR-214 Targeting Assay Components

1. Fire-Ctx sensor lentivector (SBI, Mountain View, CA, USA). Store at –20 °C.
2. Electroporation kit (Nulceofector, Lonza, Houston, TX, USA). Store at 4 °C.
3. Pre-mir-214 (Life Technologies). Store at –20 °C.

Table 1
Primers used for RT-PCR

Gene	GenBank accession number	Primers		Product size (bp)
		Sense	Antisense	
CTGF	NM_010217	5′ CACTCTGCCAGTGGAGTTCA 3′	5′ AAGATGTCATTGTCCCCAGG 3′	111
miR-214	NR_029796	5′ ACAGCAGGCACAGACAGGCA 3′	Universal antisense	20
Collagen 1(α1)	NM_007742	5′ GCCCGAACCCCAAGGAAAAGAAGC 3′	5′ CTGGGAGGCCTCGGTGGACATTAG 3′	148
αSMA	NM_007392	5′GGCTCTGGGCTCTGTAAGG3′	5′CTCTTGCTCTGGGCTTCATC3′	148
GAPDH	NM_002046	5′ TGCACCACCAACTGCTTAGC 3′	5′ GGCATGGACTGTGGTCATGAG 3′	66

4. pRL-CMV vector, E1910 Dual Luciferase Reporter Assay System (Promega, Madison WI, USA). Store at –20 °C.
5. Cytotoxic reagent (CTX) (Clontech, Mountain View CA, USA). Store at –20 °C.
6. CytoSelect™ assay kit (Cell Biolabs Inc., San Diego, CA, USA). Store at 4 °C.

2.6 Exosome Assay Components

1. PureExo Exosome Isolation Kits (101Bio, Palo Alto, CA, USA).
2. Dynamic light scattering instrument, e.g., BI 200SM (Research Goniometer System, Brookhaven Instruments Inc., Holtville NY, USA) or equivalent.
3. Zeta Potential Analayzer, e.g., ZetaPALS (Brookhaven Instruments Inc., Holtsville, NY, USA) or equivalent.
4. Carbon-coated 400 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA, USA).
5. Electron microscope for transmission electron microscopy (TEM), e.g., H-7650 (Hitachi High Technologies America, Pleasanton, CA, USA) or equivalent.
6. Electron microscope for cryogenic TEM, e.g., Tecnai G2 F20 microscope (FEI, Hillsboro, Oregon, USA) or equivalent.
7. Radioimmunoprecipitation (RIPA) buffer: 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (pH 7.4). Make to 100 ml with distilled water. Store at 4 °C.
8. Loading buffer (2×) for sodium dodecyl sulfate gel electrophoresis (SDS-PAGE): 8 M Urea, 70 mM Tris–HCl pH 6.8, 3% SDS, 0.005% bromophenol blue, 5% β-mercaptoethanol. Make in distilled water. Store at –20 °C.
9. Western blot transfer buffer: 25 mM Tris–HCl pH 8.3, 150 mM glycine, 20% (v/v) methanol. Make in distilled water.
10. Enhanced chemiluminescence kit. Store at 4 °C.
11. PKH26 fluorescent dye. Store at 4 °C.
12. Culture-Insert μ-Dish 35 mm system (Ibidi Inc., Verona, WI, USA).

2.7 Antibodies

We commonly use monoclonal anti-digoxigenin-alkaline phosphatase antibody (Roche, Indianapolis, IN), monoclonal anti-mouse αSMA antibody (Dako Cytomation, Denmark), polyclonal anti-rabbit collagen 1(α1) (Abcam, Cambridge, MA), CD9 antibody (SBI System Biosciences, Mountain View, CA, USA), Alexa Fluor®488 goat-anti-chicken IgY, Alexa Fluor® 568 goat-anti-rabbit IgG, Alexa Fluor® 647 goat anti-mouse IgG (Life Technologies).

3 Methods

3.1 Isolation of Mouse HSC

1. HSC can be successfully isolated from the liver of a wide variety of strains of mice. Typically, HSCs are collected and pooled from five male mice (4–6 weeks of age, approx. 25–30 g) of the same strain and then cultured in vitro.
2. Anesthetize mice with ketamine (66 mg/kg) and xylazine (14 mg/kg) and ensure a very deep plane of anesthesia by strong toe pinch. Optionally, blood (0.5–0.7 ml) is collected by cardiac puncture using a 25-gauge needle. Cardiac function will be compromised but the heart will still beat slowly. Place animal on its back and spray the abdomen with 70% alcohol. Access the liver through an abdominal incision. Introduce a 22GA needle (1.00IN, 0.9×25 mm) into the infra-hepatic vena cava and snip the portal vein with scissors. Initiate systemic perfusion of 20–30 ml PBS (results in immediate death) followed by the perfusion solution.
3. Remove perfused livers from five mice, mince with scissors, and digest in the digest solution.
4. Stop the digestion with 3 ml serum and centrifuge at 300×*g* for 10 min (4 °C). Rinse the pellets and centrifuge them again under the same conditions.
5. Filter the resulting cell suspension through a metal sieve (120 μm) and centrifuge at 300×*g* for 10 min (4 °C) and then remove the supernatant.
6. Prepare the gradient solutions.
7. Resuspend the cell pellet in 15% OptiPrep gradient solution and place in a centrifuge tube. Over the top of this solution, layer the 11% OptiPrep gradient solution, followed by HBSS on top of that. Centrifuge the resulting cell suspension at 1400×*g* for 30 min without braking. HSCs separate into a hazy band just above the interface of the gradient in the aqueous buffer. Harvest the band.
8. Wash the band of cells and resuspend them at 10^5 cells/ml in the culture medium.
9. Plate the cells into flasks or multi-well plates as needed.
10. Verify HSC identity and purity by buoyant density, phase-contrast microscopy, oil red staining, and immunostaining for glial fibrillary acidic protein (GFAP) or desmin (*see* **Note 1**). Immunostaining can also be performed for additional markers such as alpha smooth muscle actin (αSMA), connective tissue growth factor (CCN2), or collagen 1(α1) which are induced in the cells as they become autonomously activated in culture.

3.2 Detection of microRNA in Hepatic Tissue by In Situ Hybridization

1. Tissue preparation: Superior results are obtained by perfusion of the liver to remove blood components. Follow the procedure in Subheading 3.1, **step 2**, except that the surgical approach is to make a V-cut through the skin and abdominal wall about 1 cm caudal to the last rib. Make a small incision in the diaphragm to equalize the heart chamber and external pressures, lift the sternum upwards, cut the rib cage laterally about 1 cm in each direction to access the heart chamber, insert a 23–25-gauge butterfly needle into the left ventricle, and clip the aorta with scissors.
2. Perfuse animals with 20–30 ml 4% paraformaldehyde for fixation. Resect the liver and place in 4% paraformaldehyde overnight at 4 °C. Wash fixed livers three times with 70% ethanol before embedding in paraffin blocks. Cut sections of 6 μm and mount on slides (*see* **Note 2**). As an alternative approach, one lobe of the livers is tied off with suture material after PBS perfusion (for later RNA extraction) prior to perfusion of the remainder of the liver with 4% paraformaldehyde. The various lobes are then processed for histology as above or frozen for RNA extraction (*see* Subheading 3.5).
3. Hybridization is performed as described in the instructions for the miRCURY LNA™ microRNA ISH optimization kit.
4. De-paraffinize the liver sections with xylene and ethanol solutions at room temperature by placing with sections in a slide rack, and then move among jars according to Table 2, ending up in PBS.
5. Incubate with proteinase-K for 10 min at 37 °C (*see* **Note 3**) and wash twice with PBS. Dehydrate the sections in graded alcohol (Table 3) and air-dry the slides on clean paper towels for 15 min.
6. Place the slides on a flat surface and hybridize the sections with a probe in microRNA ISH buffer for the microRNA of interest (e.g., miR-214 for CCN2) or scramble sequence probes (*see* **Note 4**).
7. Apply sterile and RNase-free glass cover slips onto the sections and seal along all four edges with Fixogum for 60 min at 55 °C (*see* **Note 5**).
8. Remove Fixogum using tweezers and carefully detach cover slips. Place the slides in 5× SSC solution. Wash the slides with varying concentrations of SSC buffer at 55 °C as shown in Table 4 (*see* **Note 5**).
9. Incubate the slides with anti-digoxigenin-alkaline phosphatase antibody (1:800) for 60 min after blocking the sections with block solution.

Table 2
De-paraffinization steps for ISH

Step	Solvent	Duration
1	Xylene	Immerse 5 min
2	Xylene	Immerse 5 min
3	Xylene	Immerse 5 min
4	99.9% Ethanol	Dip 10 times
5	99.9% Ethanol	Dip 10 times
6	99.9% Ethanol	Immerse 5 min
7	96% Ethanol	Dip 10 times
8	96% Ethanol	Immerse 5 min
9	70% Ethanol	Dip 10 times
10	70% Ethanol	Immerse 5 min
11	PBS	Immerse 2–5 min

Table 3
Dehydration steps for ISH

Step	Solvent	Duration
1	70% Ethanol	Dip 10 times
2	70% Ethanol	Immerse for 1 min
3	96% Ethanol	Dip 10 times
4	96% Ethanol	Immerse for 1 min
5	99.9% Ethanol	Dip 10 times
6	99.9% Ethanol	Immerse for 1 min

Table 4
Post-hybridization SSC wash steps for ISH

Step	Buffer	Duration	Temperature
1	5× SSC	5 min	Hyb temp
2	1× SSC	5 min	Hyb temp
3	1× SSC	5 min	Hyb temp
4	0.2× SSC	5 min	Hyb temp
5	0.2× SSC	5 min	Hyb temp
6	0.2× SSC	5 min	Room temp

10. Wash the slides with PBS-T and apply NBT/BCIP substrates at 30 °C for 2 h. Stop the reaction with KTBT buffer and wash with water twice.
11. Counterstain the slides with Nuclear Fast Red™, dehydrate the slides in graded ethanol solutions according to Table 3, mount with Eukitt® Medium, and examine by confocal microscopy.

3.3 Detection of microRNA in Mouse Primary HSC by ISH

1. Fix HSC with 2.5% glutaraldehyde for 30 min, wash with 4.5% sucrose in PBS, and then permeabilize the cells with 0.3% Triton X-100 in PBS for 15 min, followed by PBS-T for 5 min. All steps are at room temperature.
2. Incubate HSC with proteinase-K for 10 min at 37 °C (*see* **Note 6**) and fix the cells with 4% paraformaldehyde for 5 min at room temperature. Wash HSC with PBS-T and treat the cells with 50% formamide/2× SSC before hybridization.
3. Hybridize HSCs with probe(s) to the microRNA of interest (e.g., miR-214 for CCN2) or with a scrambled sequence (*see* **Note 7**) for 30 min at 58 °C in microRNA ISH buffer followed by washes with graded concentrations of SSC buffer (Table 4) at 58 °C. Mount cells in Prolong Gold Mounting Medium with DAPI and examine by confocal microscopy.

3.4 Immunofluorescent Staining of CCN2, αSMA, or Collagen 1(α1) in Liver Sections or Mouse HSC

1. De-paraffinize liver sections with xylene and ethanol as described above (Table 2). Fix cultured HSC with 4% paraformaldehyde for 1 h at room temperature and then rinse the cells three times with PBS.
2. Incubate liver sections or mouse HSC with blocking reagent for 1 h at room temperature.
3. Rinse and apply primary antibodies against CCN2 (5 μg/ml), αSMA (1:100), or collagen 1(α1) (1:250) overnight at 4 °C with mixing.
4. Rinse the sections four times with PBS-T, 15 min each time.
5. Add, respectively, Alexa Fluor®488 goat-anti-chicken IgY, Alexa Fluor® 568 goat-anti-rabbit IgG, or Alexa Fluor® 647 goat anti-mouse IgG (all at 1:1000) for 1 h at room temperature.
6. Rinse the sections three times in PBS-T for 15 min and one time in PBS for 15 min at room temperature with agitation.
7. Mount the slides with Vectashield Mounting Medium and examine by fluorescence or confocal microscopy.

3.5 Extraction and Quantitative RT-PCR (qRT-PCR) of Cellular RNAs

1. Extract total RNA (including microRNAs) from frozen tissues or cultured HSC using a microRNeasy mini kit according to the manufacturers' protocols.
2. Reverse transcribe RNAs into cDNAs using a miScript II RT kit according to the manufacturers' protocols.

3. Use qRT-PCR to analyze expression of relevant transcripts, such as those for miR-214, CCN2, αSMA, or collagen 1(α1) (Table 1). Run each reaction in triplicate, and normalize to GAPDH. Negative controls are reactions lacking reverse transcriptase or sample (template).

3.6 miR-Selection Fire-Ctx Lentivector-CCN2 3′-UTR Construction

1. Identify potential binding sites for microRNAs that directly regulate CCN2 (e.g., miR-214) by performing a BLAST search of the CCN2 3′-UTR (mouse, human).
2. For mouse, amplify the full-length 997 bp 3′-UTR of CCN2 (Genbank SEQ ID: BC006783.1) by PCR from primary mouse HSC genomic DNA using forward primer 5′-GAGGGATCCGTCACACTCTCAACAAATAAAC TGCCC-3′ and reverse primer 5′-GAGGAATTCAGCC AGGAAGTAAGGGAACCGAACTCA-3′ (Fig. 1a).
3. Digest the PCR fragment with BamH I and EcoR I and then subclone CCN2 3′-UTR into Fire-Ctx sensor lentivector, downstream of the *Firefly* luciferase reporter and cytotoxin (*CTX*) drug sensor genes. Verify the construction by DNA sequencing (Fig. 1b).
4. Incorporate a 5-base mutation into the CCN2 3′-UTR at the predicted binding site for the miR of interest (e.g., GTCCG→ACAAT for miR-214; Fig. 1a). Amplify the mutated form of CCN2 3′-UTR from the wild-type UTR using relevant primers (e.g., forward primer

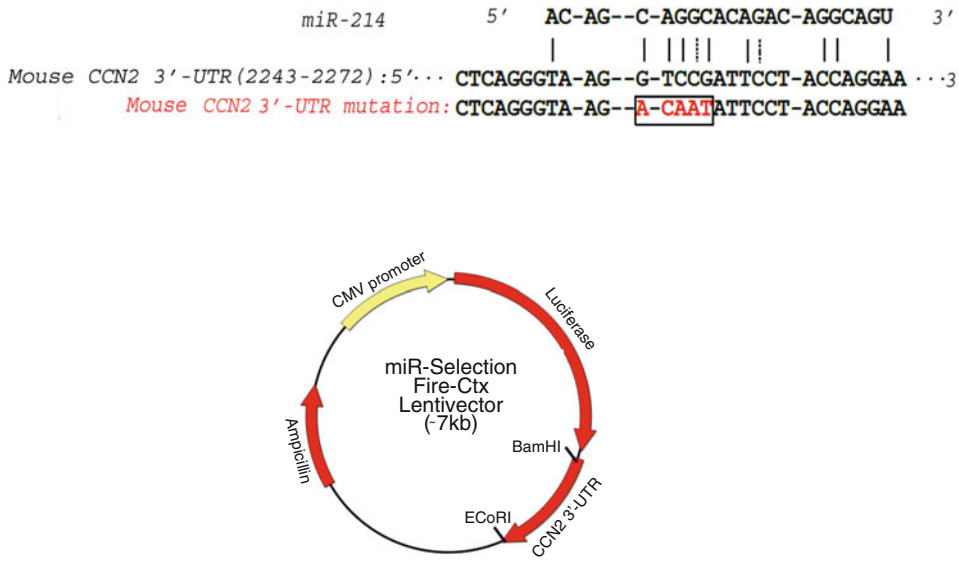

Fig. 1 Characterization of miR-214—CCN2 3′-UTR interactions. (**a**) Alignment of miR-214 with its binding site in the mouse CCN2 3′-UTR (Genbank SEQ ID: BC006783.1). The *red box* shows the mutated version of the UTR. (**b**) Organization of the miR-Selection Fire-Ctx lentivector containing CCN2 3′-UTR

5′-CTGGCTCAGGGTAAGACAATATTCCTACC AGGAAG-3′ and reverse primer 5′-CTTCCTGGTAGGAAT ATTGTCTTACCCTGAGCCAG-3′ for the miR-214-binding mutant), and verify by DNA sequencing.

3.7 Demonstration That miR-214 Directly Targets the CCN2 3′-UTR

1. Co-transfect mouse HSC by electroporation (*see* **Note 8**) with 100 nM of the hairpin precursor of miR-214 (pre-mir-214) and 3 μg Fire-Ctx sensor lentivectors containing wild-type or mutant CCN2 3′-UTR, or vector alone. To control for transfection efficiency, cells are also transfected with 0.8 μg pRL-CMV vector containing *Renilla* luciferase reporter gene.
2. Measure luciferase activity in triplicate using an E1910 Dual Luciferase Reporter Assay System after 24 h. Use *Renilla* luciferase activity for normalization, and compare firefly luciferase activity in pre-mir-214 transfected cells to that in mock-transfected cells.
3. Alternatively, 24 h after transfection, treat the cells with CTX reagent (1:1000) for 3–4 days. Assess the cell viability using a CytoSelect™ assay.

3.8 Exosome Isolation and Characterization

1. Harvest exosomes from HSC cultures by subjecting HSC-conditioned medium to a low-speed centrifugation step (e.g., 10,000 × *g* for 20 min at 4 °C) from which the supernatant is then subjected to ultracentrifugation at 100,000 × *g* for 90 min at 4 °C. The supernatant is removed and pellet is resuspended in 1 ml of cold (4 °C) PBS and then subsequently ultracentrifuged at 100,000 × *g* for 90 min at 4 °C. Resuspend the pellet in 250–500 μl PBS.
2. Harvest circulating exosomes from serum using PureExo Exosome Isolation Kit.
3. Perform dynamic light scattering (632.8 nm laser, 90° detection angle) using a BI 200SM Research Goniometer System to establish the size range of the exosomes.
4. Use a ZetaPALS analyzer to establish zeta potential (net charge) of the exosomes.
5. Perform transmission electron microscopy (TEM) to establish exosome size and morphological characteristics. Coat exosomes on to carbon-coated 400 mesh copper grids, stain with 2 % uranyl acetate, air-dry, and image by TEM with an H-7650 microscope. Alternatively, to preserve membrane integrity and morphology, coat exosomes on to carbon-coated grids, blot the surface to dry it, and then plunge-freeze in liquid nitrogen-cooled ethane slush. Cryogenic TEM is performed using a Tecnai G2 F20 microscope (Fig. 2) (*see* **Note 9**).
6. Perform SDS-PAGE and Western blot analysis to verify the presence of well-characterized exosome markers (e.g., CD9,

CD81). Resuspend the exosome-rich pellet in loading buffer, separate on a polyacrylamide gel, and transfer to a nitrocellulose membrane using transfer buffer. Block the membrane with blocking reagent and incubate first with a CD9 antibody overnight at 4 °C, followed by goat anti-rabbit HRP secondary antibody (1:2000) for 1 h at room temperature. Detect the immunoreactive CD9 protein band by enhanced chemiluminescence.

3.9 Determination of Exosomal microRNA or Protein Content

1. Transfect P6 primary mouse HSC with pre-mir-214 (100 nM) by electroporation, and incubate the cells in fresh serum-free DMEM/F12 medium in T-75 culture flasks for 36 h. Controls include non-treated or mock-transfected cells.
2. Harvest and purify exosomes released into the conditioned medium. Isolate total RNA from 200 μg exosomes using miRNeasy mini kits as described above. Run triplicate RT-PCR reactions for the miRs of interest. Normalize the data to an unchanged constituent, e.g., let-7a (*see* **Note 10**).
3. Evaluate exosomal proteins by Western blot as described in Subheading 3.8, **step 6**.

3.10 Assessment of Exosomal Uptake and Action in Target Cells

1. Exosome components can be fluorescently tagged to allow visualization after addition to target cells (*see* **Note 11**). Either (a) mix exosome-enriched cell medium with fluorescent dye PKH26 (4 mM) for 1 h and then purify the stained exosomes as in Subheading 3.8, **step 1**, or (b) transfect mouse HSC with 4 μg CD9-GFP or miR-214 RFP plasmids by electroporation, incubate the transfected cells in fresh serum-free DMEM/F-12 medium in T-75 culture flasks for 48 h, and then collect exosomes from the medium as in Subheading 3.8, **step 1**.
2. Add aliquots of exosomes to mouse HSC cultures for 2–48 h and examine for PKH26, GFP, or RFP fluorescence using con-

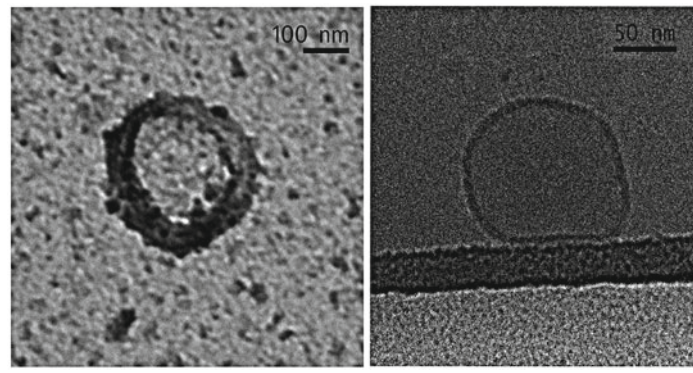

Fig. 2 Transmission electron microscopy of HSC-derived exosomes. Exosomes were isolated by sequential centrifugation of conditioned medium from P6 mouse HSC. TEM (*left*) and cryogenic TEM (*right*)

focal microscopy (Fig. 3). Alternatively, wash the recipient cells with PBS, lyse the cells in RIPA buffer, and measure fluorescent intensity of cell lysates.

3. Exosome effects in recipient cells are assessed by determining changes in levels of key target molecules (e.g., CCN2, αSMA, collagen 1(α1) at the RNA or protein level). Alternatively, the effects can be measured in terms of altered activity of relevant gene reporters that were transfected into the recipient prior to exosome treatment.

3.11 Demonstration of Cell–Cell Exosomal Communication Using a Coculture System

The Culture-Insert is made from biocompatible silicone material and is divided into two wells by a central silicone wall that can be cut away and removed from the system (Fig. 4) (*see* **Note 12**).

1. Seed cells in coculture system: *Exosome donor cells*: Freshly isolated donor mouse HSC (<48 h of culture) contain high endogenous levels of miR-214 (*see* **Note 13**). Prepare cell suspension and add 70 μl of HSC (approx. 10^4 cells) into one well. Some wells also receive GW4849 to inhibit exosome production (*see* **Note 14**). HSC donor cells can also be fluorescently stained using the methods described in Subheading 3.10. Incubate the cells at 37 °C and 5% CO_2 as usual for 12 h. *Exosome recipient cells*: Recipient HSC contain the parental miR-Selection Fire-Ctx lentivector or the same vector containing either wild-type or mutant CCN2 3′-UTR. Prepare cell suspension and add 70 μl of HSC (approx. 10^4 cells) into the other well. Incubate at 37 °C and 5% CO_2 for 12 h.

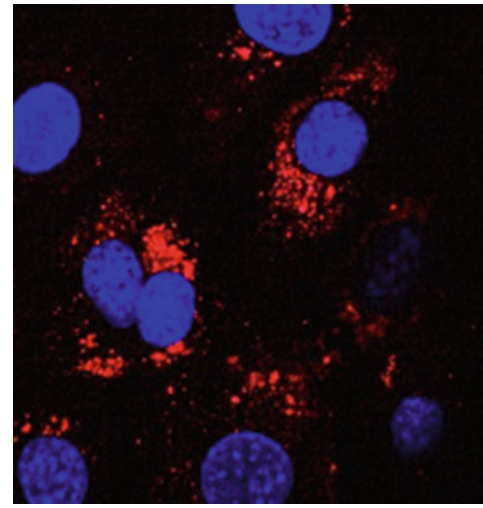

Fig. 3 Uptake of HSC-derived exosomes by other HSC. HSCs were incubated for 12 h with exosomes purified from HSC-conditioned medium that had been stained with PKH26. Recipient HSCs were visualized for exosome fluorescence (*red*) and DAPI immunofluorescence (*blue*) by confocal microscopy

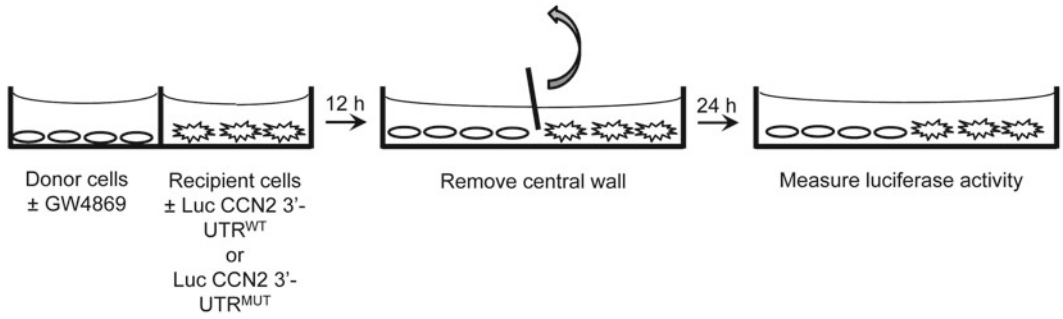

Fig. 4 HSC coculture for assessing exosomal release and uptake of miR-214. The coculture device comprises two silicone micro-culture wells (0.22 cm^2 growth area per well) separated by a shared silicone wall. Approximately 10^4 donor HSCs are placed in one well in 70 μl medium for 12 h, with or without GW4869. Meanwhile, ~10^4 recipient HSCs transfected with wild-type or mutant CCN2 3′-UTR miR-Selection Fire-Ctx lentivector or the parental vector control are placed in the other well in 70 μl medium for 12 h. The central divider is then removed and the cells are cocultured for a further 24 h prior to analysis by luciferase or cytotoxic assays. *Luc* luciferase, *WT* wild-type, *MUT* mutant

2. Carefully excise the silicone wall between to the two cell cultures and allow direct communication between the cells for the next 24 h (*see* **Note 15**).
3. If donor cells were stained (e.g., using PKH26, GFP, RFP, SYTO RNA Select Green), assess transfer of the stain to recipient cells by direct fluorescence microscopy (*see* **Note 16**).
4. Measure the luciferase activity in triplicate in recipient cells using the dual luciferase reporter assay system. Firefly luciferase activity in miR-214-transfected cells is compared to that in non-transfected cells, with Renilla luciferase activity used for normalization. Luciferase activity is decreased upon exposure of the recipient cells to the donor cells. Proof that donor exosomes are mediating this effect will be seen by failure of CCN2-UTR activity to be regulated in the presence of GW4869-treated donor cells. Proof that exosomally delivered miR-214 directly targets CCN2-UTR is shown by failure of luciferase activity to be regulated in recipient cells transfected with the mutant CCN2-UTR.

4 Notes

1. Freshly isolated HSCs contain cytoplasmic lipid droplets which are visualized by uptake of oil red O stain. Fix the cells in ice-cold 4% paraformaldehyde in PBS for 20 min at room temperature prior to incubation for 10 min in a saturated solution of oil red O (Polysciences, Warrington, PA) in isopropanol (Sigma-Aldrich).
2. Prepare all solutions using RNase-free water.

3. The Proteinase-K concentration range must be optimized for individual tissues. It is recommended to test with 2.5 μg/ml in mouse.
4. For identifying the optimal probe concentration, it is recommended to test the double-DIG-labeled LNA™ microRNA probe and the LNA™ scrambled microRNA probe at 1–200 nM. Denature the LNA™ probes at 90 °C for 4 min before using.
5. For identifying the optimal hybridization temperature, it is recommended to test at 55 °C. At low temperature the LNA™ probes give higher signal, but the risk of cross-hybridization to highly similar sequences is increased. However, the LNA™ probes give weaker signal at high temperature.
6. The Proteinase-K concentration range must be optimized for individual cells. It is recommended to test with 1 μg/ml.
7. For identifying the optimal probe concentration, it is recommended to test the double-DIG-labeled LNA™ microRNA probe and the LNA™ scrambled microRNA probe at 1–200 nM. Denature the LNA™ probes at 90 °C for 4 min before using.
8. To gain the highest transfection efficiency or gene expression activity, it is recommended to use electroporation system for transfection.
9. Cryogenic TEM preserves the structure of exosomes and allows visualization of exosomes as translucent particles with an outer bilayer membrane [16].
10. To identify optimal reference gene(s), it is recommended to test stable microRNAs or small RNAs (e.g., 5S rRNA, U6 snRNA) as controls [17–19].
11. Alternatively, stain the cells with SYTO RNASelect green (Thermofisher). This selectively stains RNA, resulting in a fluorescent signal in exosomes that can be subsequently detected in the target cells, indicative of exosomally delivered RNA.
12. Use specified immersion oils (Immersol 518F and Immersol W2010 from Zeiss, or Immersion liquid from Leica) when applying to oil immersion objectives. The use of a non-recommended oil could lead to the damage of the plastic material and the objective.
13. If endogenous levels of the miR of interest are low in the donor cells, then they can be transfected with the appropriate miR mimic, with or without a fluorescent tag.
14. GW4869 blocks neutral sphingomyelinase2 (nSMase2) which is required for the biosynthesis of ceramide on which exosome production is dependent. Alternatively, use siRNA to block nSMase 2 or components of exosome biogenic pathways (e.g., Rab proteins).

15. Control experiments include exposure of recipient HSC to cell-free donor micro-wells containing pre-mir-214 in the medium at the same concentration as used for donor HSC transfection.
16. For ease of identification, the recipient cells can be pre-stained with a differently fluorescing vital dye such as PKH67.

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Chapter 39

TGF-β1- and CCN2-Stimulated Sirius Red Assay for Collagen Accumulation in Cultured Cells

Philip C. Trackman, Debashree Saxena, and Manish V. Bais

Abstract

A simple method for the determination of relative levels of insoluble collagen accumulation in fibroblast cultures is presented. Confluent cell cultures are provided with sodium ascorbate which is then permissive for collagen deposition. At intervals, cultures are fixed and stained successively with sirius red and then crystal Violet to, respectively, assess for relative changes in collagen accumulation in response to factors such as TGF-β1 or matricellular CCN2 and changes in DNA content as an index of changes in cell density.

Key words Collagen deposition, Collagen accumulation, Sirius Red, Fibrosis, Fibroblast, Cell culture

1 Introduction

Collagen biosynthesis is complex and involves synthesis, intracellular posttranslational modifications, secretion, and extracellular modifications to finally produce an insoluble and functional extracellular matrix [1]. Although measurements of collagen synthesis are useful to understand changes in early stages of collagen matrix production, there is often a need to determine whether or not corresponding changes in insoluble collagen deposition occur. This is because posttranslational steps in collagen biosynthesis can be regulated independently [1, 2]. In addition, a small change in collagen synthesis over time can result in very significant changes in collagen accumulation [3].

The most definitive measure of collagen accumulation is to determine the content of hydroxyproline after acid hydrolysis of cell layers by amino acid analysis, or by colorimetric methods [3, 4]. These approaches, however, are laborious and time consuming. Sirius Red is a dye which binds to collagens and has been used both as a histologic stain and for quantitation of deposited collagens in cell culture. Here we describe a convenient step-by-step procedure for measuring collagen accumulation in fibroblast cultures. This

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assay has been successfully employed to measure changes in collagen deposition by human gingival fibroblasts in response to TGF-β1 and the matricellular pro-fibrotic protein CCN2 [5], and is adapted from a protocol developed to measure collagen accumulation by cultured osteoblasts [6].

2 Materials

1. Spectrophotometer or plate reader.
2. Mammalian cell culture capable of depositing an extracellular matrix under specified conditions in 6-well cell culture plates (*see* **Notes 1–3**).
3. Digital camera to photograph stained plates.
4. Sodium ascorbate.
5. Recombinant human TGF-β1, 50–200 ng/ml CCN2, or another fibrogenic factor.
6. Bouin's solution: 75 ml aqueous saturated picric acid, 25 ml formalin, 5 ml glacial acetic acid. This solution is also available pre-made from Sigma.
7. PBS: 8.2 g NaCl (140 mM NaCl), 186 mg KCl (2.5 mM KCl), 0.218 mg KH_2PO_4 (1.6 mM KH_2PO_4), 2.15 g Na_2HPO_4 (15 mM Na_2HPO_4) in 1 l final volume.
8. Sirius Red: Dissolve 50 mg Sirius Red F3BA (Chroma, Germany) in 50 ml of water-saturated picric acid (*see* **Notes 4** and **5**).
9. Sirius Red wash buffer: 0.01 M HCl.
10. Sirius Red elution buffer: 0.1 M NaOH.
11. Crystal Violet stain: 0.1% Crystal violet in water.
12. Crystal Violet elution: 100% Methanol.

3 Methods

1. Culture fibroblasts under specified experimental conditions in at least triplicate wells of 6-well plates (*see* **Note 3**). For an example see the caption to Fig. 1. Collagen accumulation is initiated in confluent cultures by supplementation of growth medium with 50 μg/ml sodium ascorbate on day 0 of the experimental period. The length of time of the initial culture and of the experimental periods should be optimized for different types of fibroblasts. For primary human gingival fibroblasts, a 7-day experimental period was sufficient to observe significant levels of cell layer collagen accumulation. The positive control should be one group of cells grown in the constant presence of a fibrogenic factor such as 10 ng/ml TGF-β1, and

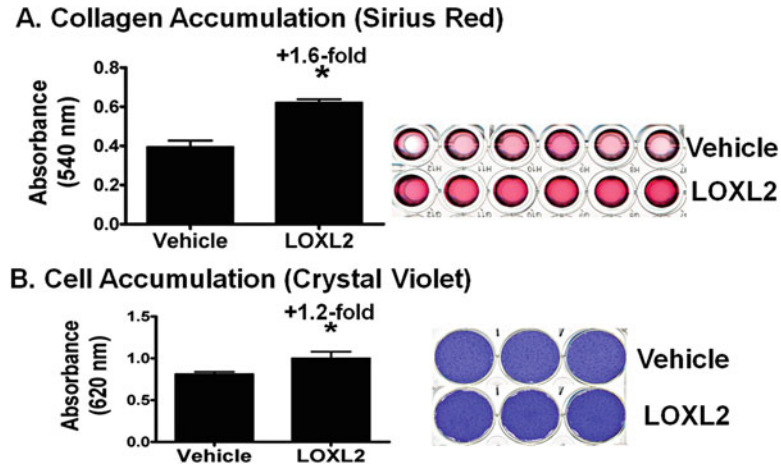

Fig. 1 Collagen accumulation in fibroblast cultures stimulated by recombinant human lysyl oxidase like-2. Primary human gingival fibroblasts were cultured to confluence in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum, 0.1 mM nonessential amino acids, and 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C until confluent in two six-well cell culture plates. Medium was refreshed every 3 days. Cells were maintained at confluence for 7 days. Collagen accumulation was then initiated by the addition of 50 μg/ml sodium ascorbate in the presence or absence (vehicle) or 10 μg/ml of active human lysyl oxidase like-2 (R&D Systems) on day 0, and cultured for an additional 7 days with a media change on day 4. The Sirius Red (**a**) and Crystal Violet (**b**) staining protocol was then performed as described. In (**a**), aliquots of the Sirius Red elutions were placed in a multiwell plate for microplate absorbance determinations and photography. In B, cell layers stained with Crystal Violet from one of the two 6-well plates is shown. Bar graphs show fold changes in data calculated from absorbance measurements made from respective elutions. Data are means +/−SD from one experiment with six replicate samples; $*p < 0.05$

50 μg/ml sodium ascorbate in cell culture medium. The negative control is a blank (empty) 6-well plate with which to carry out all steps below.

2. At the end of the experimental treatment period, rinse cells three times with ice-cold PBS.
3. Fix cells by adding 3 ml Bouin's solution in each well for 1 h at room temperature.
4. Remove Bouin's solution, and rinse plates under cold running tap water until the yellow stain is no longer visible on the plates.
5. Air-dry plates in a fume hood overnight.
6. Add 2 ml of Sirius Red dye solution for 1 h at room temperature.
7. Remove and discard the dye solution, and then wash each well four times with 5 ml 0.01 M HCl each to remove unbound dye.

8. Photograph the plates for a qualitative record.
9. Elute the bound dye with 1 ml of 0.1 M NaOH by mixing on an orbital shaker for 30 min.
10. Recover the eluted dye, and measure the absorbance at 550 nm with a spectrophotometer against a blank of 0.1 M NaOH.
11. Subtract the value obtained from the empty multiwell plate from all other readings.
12. Determine total absorbance recovered per well. Average data from all wells for each experimental condition, and apply descriptive statistics as required by the experimental design (*see* **Note 6**).
13. To assess for obvious differences in cell density between experimental groups, stain the remaining cell layer with the DNA-binding dye Crystal Violet. Following elution of Sirius Red, rinse each well with 5 ml PBS three times.
14. Add 3 ml of Crystal Violet solution to each well and incubate for 30 min at room temperature. Rinse wells with running tap water until the rinse water is no longer blue. Air-dry overnight.
15. Photograph the plates for a qualitative record.
16. Elute the dye with pure methanol (5 ml/well), measure the optical density of and volume of the eluate at 540 nm either with a spectrophotometer or transfer aliquots to micro-titer plate, and measure absorbance with a plate reader. Subtract the value obtained from the empty multiwell plate from all other readings.
17. Calculate the total eluted absorbance (*see* **Note 6**).

4 Notes

1. The time of culture after reaching confluence is a variable that should be optimized for each fibroblast source. For example, 7 days has been found to be adequate for measuring collagen accumulation in primary human gingival fibroblasts. Most immortalized cell lines do not deposit much collagen.
2. For agents which may induce relatively small changes in collagen accumulation such as CCN2, six replicates may be required per experiment to observe significant data within an experiment. At least three independent experiments ($n = 3$) should be performed, and then averages of the independently obtained means (+/− SEM) reported, and then statistics should be applied such as ANOVA or *Students t-test.*
3. The assay can also be performed in 10 cm^2 cell culture plates, or in 24-well plates, depending on the cells and the amount of collagen that they deposit.

4. Sirius Red F3BA powder should be purchased from Chroma (Germany). Sirius Red from other vendors may not work. There are structural differences between Sirius Red preparations.
5. Picric acid-containing solutions should be stored for no more than 1 year. Sirius Red dye solution is best made fresh for each experiment.
6. In determining the optical density of the eluted Sirius Red dye in **step 10** and Crystal Violet in **step 16**, if the absorbance exceeds the accurate range of the spectrophotometer, accurately dilute the eluate as needed to bring the absorbance in range, and calculate the total absorbance accordingly. Most spectrophotometers are accurate to an absorbance value of about 1.5 optical density. Some plate readers may have a wider accurate range. Data is expressed as optical density/ml × volume of eluate = total absorbance. Fold change can be calculated if appropriate. *See* Fig. 1 for an example of data obtained from a typical experiment.

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Chapter 40

CCN Detection of Cancer Tissues by Immunohistochemistry Staining

Cheng-Chi Chang, Yue-Ju Li, and Min-Liang Kuo

Abstract

CCN family members are involved in many physiologic and pathological functions, and be detected in many different cancer types. Immunohistochemistry (IHC) is an important diagnostic pathology tool to demonstrate protein expression in clinical and research fields. Here, we explain the preparation of sample slides, staining procedure, and the problems that might be met during the time. The differential staining of CCN proteins is shown in breast cancer, oral cancer, lung cancer, colorectal cancer, and gastric cancer.

Key words CCN, Immunohistochemistry, Bovine serum albumin, Antigen retrieval, Polymer-horseradish peroxidase

1 Introduction

CCN family includes six members, CCN1 to CCN6, which are secreted protein and involved in many physiologic functions and also correlated with cancer progression in many different cancer types, such as breast cancer, oral cancer, lung cancer, colorectal cancer, and gastric cancer [1–7]. In order to detect the protein expression of tissue, immunohistochemistry (IHC) has become an easy and convenient tool and also it is an essential diagnostic pathology tool in clinical diagnoses. For this particular method, the tissue preparation is extraordinarily important [8]. IHC requires appropriate tissue collection, fixation, and sectioning. Preparation of the samples is critical to protect tissue architecture, cell morphology, and antigenicity of target epitopes. Formalin-fixed paraffin-embedded tissue sections offer well-preserved tissue architecture and morphology for staining [9]. For paraffin-embedded tissues, antigen retrieval is often necessary, and applies by heating the sections [10]. The major problems of IHC staining include noise background staining, weak antigen staining, and autofluorescence. Reporter enzymes or endogenous biotin or antibodies cross-reactivity are common causes of noise background

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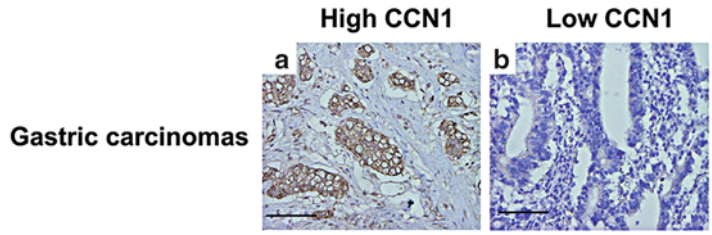

Fig. 1 CCN1 immunohistochemical staining of human gastric carcinomas. (**a**) High CCN1 expression is shown in invasive and advanced gastric tumors (200×). (**b**) Low CCN1 protein expression is noted in a noninvasive gastric tumor specimen (200×) [7]

staining. Weak staining might be resulted in poor enzyme activity or primary antibody effect [11]. Moreover, autofluorescence might be caused by the fixation method or the nature of the tissue.

We have detected CCN proteins in many types of cancer tissues and adjacent normal tissues (Figs. 1 and 2) which retrieved antigens by heating and blocked by bovine serum albumin. Horseradish peroxidase helps to level the protein expression as low or high.

2 Materials

2.1 Preparation of Tissue Slides

1. Gloves.
2. Formalin.
3. Alcohol.
4. Xylene.
5. Paraffin.
6. Adhesive glass slides.
7. Floatation water bath.
8. Staining racks.
9. Oven.
10. Dissection needle and brush.
11. Microtome.
12. Autoclave
13. Humidified chamber.
14. Parafilm.
15. Embedding cassettes.

2.2 Procession of Tissue

1. Neutral buffered formalin: 4% Formalin.
2. Dehydration buffer: 70, 80, and 95% alcohol.
3. Xylene.

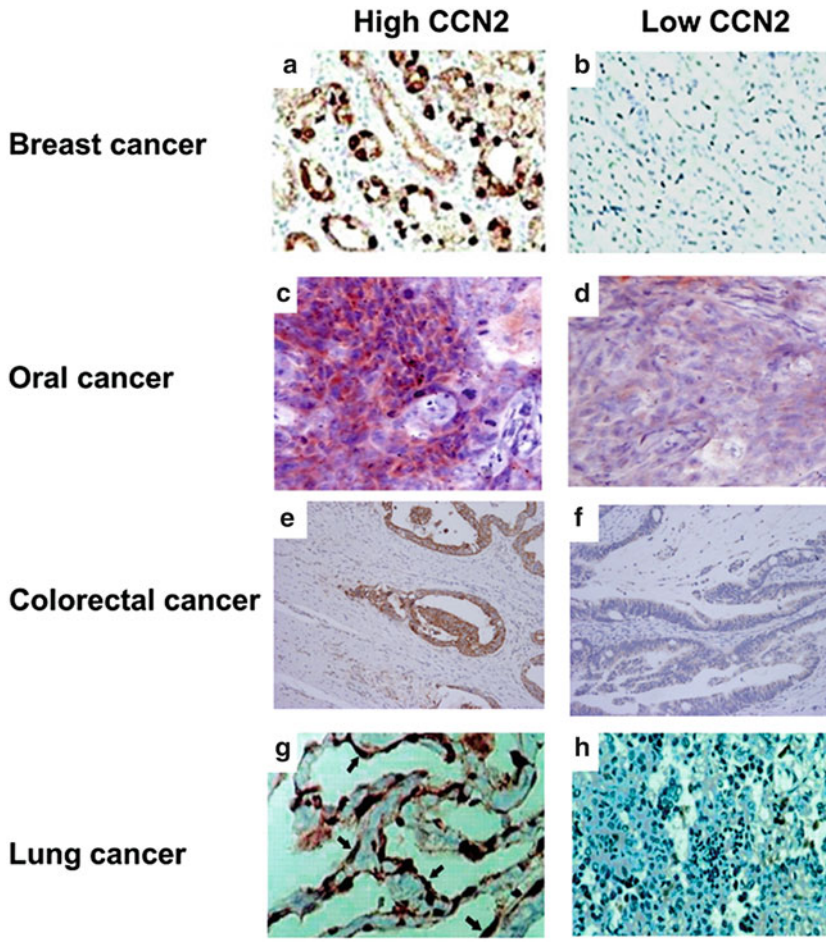

Fig. 2 Detection of CCN2 in formalin-fixed paraffin-embedded samples of human cancer tissues. High CCN2 expression staining is shown in **a**, **c**, **e**, and **g**. Low CCN2 protein expression staining is shown in **b**, **d**, **f**, and **h** [3–6]

4. Phosphate-buffered saline (PBS).
5. Tris-buffered saline tween-20 (TBST): pH 7 TBS with 0.1% tween-20.
6. Nonspecific binding blocking solution: 3% Bovine serum albumin (BSA) in PBS.
7. Antigen retrieval solution: Commercial solution (AR-10, Biogenex, Fremont, California, USA).
8. Test antibodies: Rabbit polyclonal anti-CCN1 and goat polyclonal anti-CCN2.
9. Secondary antibody.
10. Ready-to-use IHC/ICC kit (Biotin free), one-step horseradish peroxidase (HRP) polymer anti-mouse, rat and rabbit IgG (H + L) with DAB (BioVision, Milpitas, CA, USA).

3 Methods

3.1 Preparation of Tissue Blocks and Slides

1. Add 4% paraformaldehyde to cover freshly dissected tissue (<3 mm thick) from 2 h to overnight at room temperature (*see* **Note 1**).
2. Dehydrate the tissue through 70, 80, and 95% alcohol, following 10 min of each step.
3. Place samples in embedding cassettes.
4. Infiltrate the tissue with paraffin.
5. Section the paraffin tissue block at 4 μm thickness on a microtome and float in a water bath (*see* **Note 2**).
6. Transfer the sections onto adhesive glass slides for immunohistochemistry and dry the slides for overnight and store slides at room temperature.

3.2 Immunostaining of CCN

1. Incubate the slides in an oven at 52 °C for 15 min.
2. Deparaffinize slides in xylene for 30 times, and then shack slides in xylene for another 5 min (*see* **Note 3**).
3. Transfer slides to 95% alcohol for 10 min, and then transfer through 95, 75, and 50% alcohols, respectively, and shack for 5 min each.
4. Wash with PBS for 5 min in 100 rpm.
5. Unmask the antigenic epitope by antigen retrieval processing. Antigen retrieval solution covers the slides in a container and autoclave the slides at 120 °C for 30 min. Remove the container to cool down to room temperature (*see* **Note 4**).
6. Wash slides with PBS three times, 5 min each at speeding 100 rpm.
7. Apply 100 μl nonspecific binding blocking solution, cover with parafilm (*see* **Note 5**) onto the sections of the slides, and incubate it in a humidified chamber at room temperature for 2 h (*see* **Note 6**).
8. Drain off the nonspecific binding blocking solution from the slides.
9. Add 100 μl diluted primary antibody (dilute primary antibody with PBS containing 1% BSA in ratio 1:200) onto the sections of the slides, cover with parafilm, and then incubate in a humidified chamber at 4 °C temperature overnight.
10. Wash the slides with TBST three times, 5 min each at speeding 100 rpm.
11. Apply the ready-to-use reagent or diluted secondary antibody to the sections on the slides, cover with parafilm, and then incubate slides in a humidified chamber at room temperature for 1 h.

12. Wash slides with TBST for six times, 10 min each at speeding 100 rpm.
13. Apply 100 μl polymer-HRP reagent to the sections on the slides with covering parafilm and incubate in a humidified chamber at room temperature for 20 min (*see* **Note 7**).
14. Wash slides with TBST three times, 10 min each at speeding 100 rpm.
15. Apply 100 μl DAB substrate solution (*see* **Note 8**) to the sections and cover with parafilm. Allow the color development for less than 10 min until the desired color intensity is reached (*see* **Note 9**).
16. Wash the slides in running tap water for 10 min.
17. Counterstain slides by immersing sides in hematoxylin for 1–2 min.
18. Wash the slides in running tap water for 10 min.
19. Air-dry the slides.
20. Cover slides with mounting solution and cover slip. The mounted slides can be stored at room temperature permanently (*see* **Note 10**).
21. Observe the staining slides of tissue sections under microscopy.

4 Notes

1. When handling fresh tissue, it must be always with care and wear gloves, lab coat, and eye protection.
2. The tissue sections should be cut as thin as possible and adhere on the charged slides. Immunohistochemistry staining assay treat section in heat or cold buffers for hours or days. Therefore, the suitable adhesive slides can provide a better condition to avoid section float off.
3. Volatile material, xylene, can be only used in chemical hood.
4. Antigen retrieval solution should cover the tissue sections on the slides, and must avoid the sections to dry during all the time. Dry section may happen when the sections are transferred from the heated buffer; it will result in poor staining or even loss of staining.
5. As always, the interaction of sections and solutions must be consistent; applying parafilm might help to equal the activity on the whole section and also to avoid the section from drying.
6. Although antibodies show affinity for specific epitopes, they might weakly or partially bind to nonspecific proteins. Nonspecific binding leads to high noise background staining

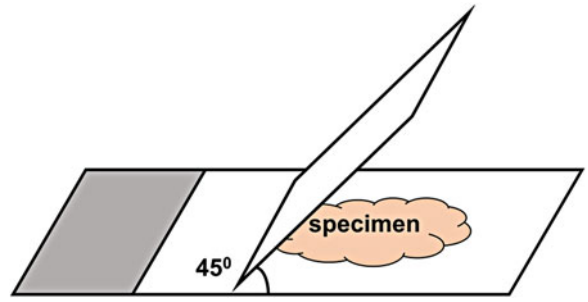

Fig. 3 Preparing a readable and storable slide

to garble the target antigen observation. To decrease background staining, samples are incubated with a blocking buffer, which avoids non-specific primary and secondary antibodies binding.

7. HRP is a kind of enzyme, which is sensitive to light; therefore, it must be processed in dark place during HRP staining procedure.
8. Freshly made DAB substrate needs to be prepared just before use in a ratio of one drop DAB chromogen in 1 mL stable DAB buffer.
9. DAB is a suspect carcinogen. Therefore, always handle it with gloves, lab coat, and eye protection.
10. Hold the cover slide at a 45° angle to the adhesive slide, and touch the slide gently (Fig. 3).

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Chapter 41

Detection of CCN1 and CCN5 mRNA in Human Cancer Samples Using a Modified In Situ Hybridization Technique

Priyanka Ghosh, Snigdha Banerjee, Gargi Maity, Archana De, and Sushanta K. Banerjee

Abstract

In situ hybridization is an ideal tool for the detection and localization of mRNA expression of specific gene(s) in tissue sections and cell lines for prognosis, predictive markers, and highlighted potential therapeutic targets. Given the importance of CCN1 and CCN5 in breast and pancreatic cancer progression, these two secretory proteins could be novel therapeutic targets. Thus, evaluating the distribution of mRNA of these targets using in situ hybridization could be important preclinical tools. This chapter describes a detailed in situ hybridization technique for the detection of CCN1 and CCN5 in formalin-fixed, paraffin-embedded patient samples of breast and pancreatic cancers.

Key words In situ hybridization, RNA isolation, Reverse-transcriptase polymerase chain reaction, Probe preparation, CCN1, CCN5

1 Introduction

Cancer is a mixed bag of cells with collections of diseases. Thus, tumor heterogeneity (TH) has important implications for the progression, metastasis, and drug resistance [1]. Although our knowledge is rudimentary about how TH dictates the disease progression and drug resistance, epithelial to mesenchymal transition (EMT) within a tumor cell population is one of the important hallmarks in cancer scenarios [2]. EMT is associated with drug resistance and migration, invasion, and metastasis to a distant organ. Initially, EMT process was not considered as an important event in neoplasia due to lack of convincing evidence [3]. However, over the past several years, it has become clear that EMT is essential for the cancer progression in a tightly regulated manner [2, 4].

Several reports considered involvement of CCN family of matricellular proteins in EMT process [5–8]. In general, the CCN [Cyr61 (cysteine-rich 61), CTGF (connective tissue growth factor), Nov (nephroblastoma-overexpressed gene)] family consists of

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six members that are present in extracellular matrix (ECM) as secretory proteins [6]. CCN proteins share conserved multimodular domains with diverse biological functions, including angiogenesis, stem cell differentiation, and carcinogenesis [5, 6, 9, 10]. Six members of the CCN family: cysteine-rich protein 61 (Cyr61 or CCN1), connective tissue growth factor (CTGF or CCN2), nephroblastoma-overexpressed protein (Nov or CCN3), Wnt-inducible secreted protein-1 (WISP-1 or CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) are characterized by having four conserved, cysteine-rich domains which contain the insulin-like growth factor-binding domain (IGFBP), the von Willebrand factor type C domain (VWC), the thrombospondin type 1 repeat (TSR), and a C-terminal domain (CT) with a cysteine knot motif. Among these six members of CCN family proteins, CCN5 does not exhibit CT domain [5, 11]. CCN5 has both growth-promoting and growth-arresting properties, depending on the cell types and the microenvironment of the cells. On the support of this statement, studies from our group precisely reported that overproduction of CCN5 by EGF or IGF-1 is required for the mitogenic action in estrogen-receptor-positive breast tumor cells, while the mitogenic action of CCN5 is undetected in the absence of ER-α rather it exhibits some inhibitory effect [5]. Consistent with this finding, it was documented that CCN5 acts as a growth arrest-specific (gas) gene in vascular smooth muscle cells and prostate cancer cell [12, 13]. Moreover, CCN5 prevents the progression of breast and pancreatic cancer by reprogramming the mesenchymal to epithelial transition (MET) [14, 15].

Interestingly, when CCN5 gained importance as a tumor-suppressor gene, CCN1 became famous as a tumor-promoting gene, at least in pancreatic cancer. CCN1 is recognized as a secretory, multifunctional, growth factor-inducible, and an immediate early response gene [16]. It is believed that CCN1 is either localized intracellularly or associated with the cell surface and extracellular matrix [17]. CCN1 is involved in the adhesion, proliferation, migration, differentiation, and angiogenesis during normal and pathophysiological processes [6]. The studies demonstrated that CCN1 expression is markedly increased in different human cancers including pancreatic ductal adenocarcinoma (PDAC) [18–20]. CCN1 was also reported to exert positive effects on tumorigenicity in the case of human breast carcinomas, which are characterized by loss of normal cell proliferation, resulting in epithelial hyperplasia. After progression to carcinoma, numerous cellular alterations can be identified, including increased expression of oncogenes, such as c-myc, decreased expression of tumor-suppressor genes (e.g., p53, Rb), alteration in cell structure such as an increase in vimentin expression, loss of cell adhesion that may involve E-cadherin and integrins, and increased expression of angiogenic factors (e.g., VEGF, FGF) [21]. However, this is not always the case as CCN1 downregulation was found in lung

cancers, leiomyomas [22], and liver carcinogenesis [23]. Our earlier studies documented that CCN1, when overexpressed in PDAC and its precursor lesions, promotes proliferation, EMT, and migration of pancreatic cancer cells and, possibly, regulates stemness of these cells through the regulation of stemness regulatory genes and microRNAs [24]. To track how the CCN1 system becomes rewired at the molecular and cellular levels for promoting PDAC growth, we found that CCN1 impacts both sonic hedgehog (SHh) and Notch pathways through integrins in PDAC cells [25]. Furthermore, Maity et al. [26] indicated that pancreatic tumor cell-secreted CCN1 promotes endothelial cell migration in recruiting aberrant blood vessel formation/tumor angiogenesis, and SHh plays a vital role in this event. Collectively, multiple studies suggest that the two CCN proteins, CCN1 and CCN5, appear to exert distinct and opposite effects.

Given the importance of the functional dichotomy of CCN1 and CCN5 in the progression of various cancers, it is critical to understand the transcriptional distribution of these two important genes for translational and clinical research and therapies. Therefore, this chapter outlines a detailed in situ hybridization technique for the detection of CCN1 and CCN5 mRNAs in formalin-fixed, paraffin-embedded human breast and pancreatic cancer tissue samples.

2 Materials

2.1 Human Tissue Samples and Cell Lines

1. Formalin-fixed, paraffin-embedded breast and pancreatic cancer tissue samples were obtained from University of Kansas Medical Center Core Facility, and Kansas City VA Hospital.

Human pancreatic cancer cell line, i.e., Panc-1 and human breast adenocarcinoma cell line, i.e., MCF-7 was purchased from American Type Culture Collection (ATCC, Manassas, VA).

2.2 In Situ Hybridization

1. *Probe*: Probe for hybridization is prepared using ULYSIS kits (Universal Linkage System Alexa Flour 488 Nucleic Acid Labelling Kit) (*see* **Note 1**).
2. *EZ-DeWax buffer:* This buffer is required for the deparaffinization of the tissue sections (*see* **Note 1**).
3. *Proteinase K:* Proteinase K solution is used in this study for RNA-targeting probes.
4. *Wash solution A:* 2× Sodium citrate saline (2× SSC).
5. *Wash solution B:* 0.5× Sodium citrate saline (0.5× SSC).
6. *Peroxide block:* 3% Hydrogen peroxide is dissolved in sterile distilled water and is used as peroxide blocker.
7. *Power block:* One vial of Power Block solution was provided with the kits (*see* **Note 1**).

8. *Liquid diaminobenzidine (DAB) chromogen:* DAB chromogen, which offers a great sensitivity as an HRP colorimetric chromogen was provided with the kits (*see* **Note 2**).
9. *Stable DAB substrate buffer:* This component was used only with DAB chromogen and comprises Tris buffer containing the peroxide and stabilizers (*see* **Note 1**).
10. *Hematoxylin:* The sections are counterstained with hematoxylin. One vial of counterstain hematoxylin was provided with kits (*see* **Note 2**).
11. *Dehydrating and clearing reagent:* Dehydration of the tissue sections is carried out using 50, 70, 90, and 100% alcohol grade. Xylene is used as clearing reagent.
12. *Permount reagent:* Permount reagent is used to permanently mount the slide.

2.3 Antibodies and Conjugates

1. *Anti-fluorescein antibody:* Mouse anti-fluorescein antibody in PBS with carrier protein and 0.09% sodium azide is required as a primary antibody.
2. *Polymer-HRP:* Anti-mouse IgG labeled with enzyme polymer in phosphate-buffered saline with stabilizers and ProClin™ 300 is required as secondary antibody.

3 Methods

3.1 Cell Lines and Cell Culture

1. The cell lines are cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin in a 37 °C incubator in the presence of 5% CO_2. About 70% confluent cells are used for RNA extraction and probe preparation.

3.2 Probe Preparation for In Situ Hybridization

3.2.1 RNA Isolation

1. RNA is isolated using standard Trizol method [14]. Briefly, after removing the cell culture media, add 1 ml Trizol reagent into the supernatant to lyse the cells.
2. Add chloroform (200 µl) to the lysate and centrifuge at 12,500 × *g* for 15 min at 4 °C and separate the aqueous layer.
3. Add isopropanol (500 µl) into the aqueous layer and then centrifuge at 12,500 × *g* for 10 min at 4 °C for the precipitation of total RNA.
4. To wash the precipitated RNA, add 75% ethanol (1 ml) and centrifuge at 7500 × *g* for 5 min at 4 °C.
5. Followed by precipitation, semidry the RNA by keeping the samples at room temperature for 5 min, then add 20 µl dH_2O to dissolve the RNA, and quantify by spectrophotometric measurement.

6. Determine the ratio of OD values separately measured at 260 and 280 nm, respectively, to check the purity of the sample. The qualities of the RNA isolated can also be verified by visual estimation of the intensity of the 28 and 18 s rRNA bands in ethidium bromide-stained UV-illuminated 1 % agarose gels.

3.2.2 cDNA Synthesis and RT-PCR

1. One μg of total RNA is reverse transcribed into cDNA using GeneAmp cDNA synthesis kit (Applied Biosystem, New Jersey). All cDNA samples are amplified using CCN1/Cyr61- or CCN5/WISP-2-specific primers for probe preparation (Fig. 1).
2. The sequences of the primers are as follows: CCN1/Cyr61 (Probe): forward primer CAGCTGACCAGGACTGTGAA and reverse primer TGTAGAAGGGAAACGCTGCT. CCN5/WISP-2 (Probe): Forward primer CCTACACACACAGC CTATAT and reverse primer CCTTCTCTTCATCCTACCC.
3. PCR reaction is carried out in 100 μl final volume. Polymerase activation for CCN1 is done at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s and final extension was at 72 °C for 10 min. The annealing temperature for CCN5 is 60 °C for 30 s. Amplicon size is determined using 2 % agarose gel electrophoresis (Fig. 1).
4. Low DNA mass ladder

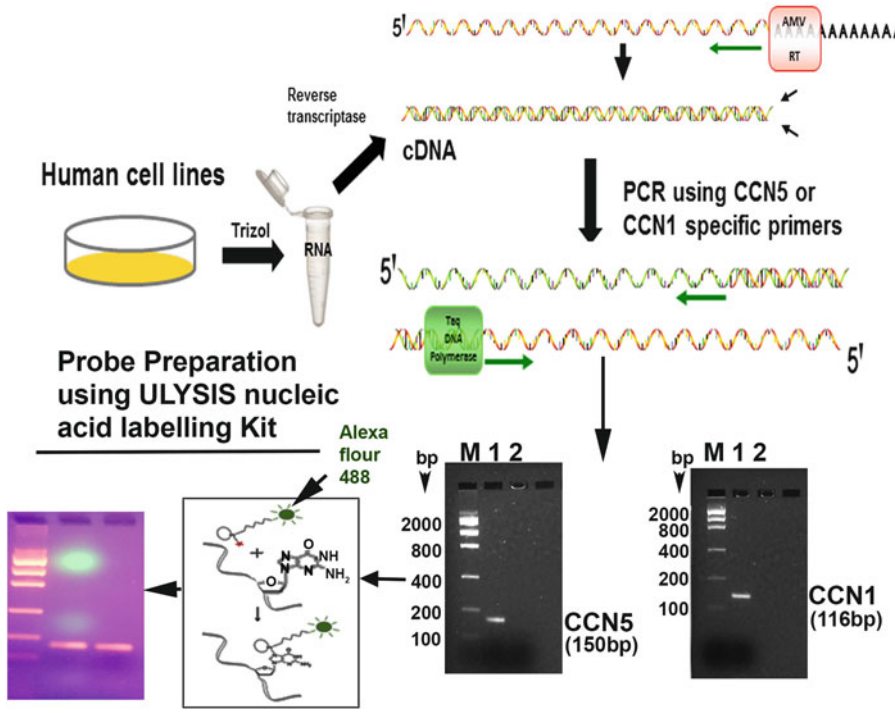

Fig. 1 Schematic illustration of ULYSIS-labeled probe preparation for in situ hybridization

5. (Invitrogen, Carlsbad, CA) is used for the determination of the concentration of the PCR products using CareStream software followed by agarose gel electrophoresis (*see* **Note 3**).

3.2.3 Probe Preparation

1. Incubate PCR products at 95 °C for 5 min to denature the DNA followed by snap chilled on ice.
2. To label the DNA (PCR products), add 1 μl ULS labeling reagent and 1 μg DNA in a reaction tube and make the final volume to 25 μl with labeling buffer (5 mM Tris-1 mM EDTA, pH 8.0) provided with kits. Incubate samples at 80 °C for 15 min (*see* **Note 4**). The quality of the probe are confirmed by agarose gel electrophoresis (Fig. 1).
3. The concentration of the labeled DNA is determined using the following formula depicted in the ULYSIS Nucleic Acid Labeling Kit manual:

   Nucleic acid concentration:

   $$\text{mg/ml} = (\text{A base} \times \text{MW base})/(€\ \text{base} \times \text{path length})$$

   $$\text{A base} = \text{A260} - (\text{A dye} \times \text{CF260})$$

3.2.4 In Situ Hybridization

In situ hybridization is carried out using supersensitive one-step polymer HRP ISH detection system (BioGenex ISH kit, Fremont, CA) with brief modifications.

1. Deparaffinize the formalin-fixed, paraffin-embedded tissue sections (5 μm) by incubating the slides in EZ-DeWax™ buffer for 5 min each for three times (*see* **Note 5**).
2. Rinse the slides thoroughly with deionized (DI) water three times.
3. Incubate the sections with proteinase K solution (20 mg/ml) for 20 min followed by washing with DI water six times.
4. Denature the ULS-labeled probe by incubating at 90 °C for 10 min using PCR machine.
5. Add denatured probes immediately on the section at a concentration of 1–1.5 ng probe/μl hybridization buffer/section.
6. Incubate the slides at 37 °C in a humidified chamber overnight.
7. Following incubation, wash slides with wash solution A and then solution B.
8. Incubate section with peroxide blocker for 10 min at room temperature to block endogenous peroxidase and then incubate with power blocker for 10 min at room temperature to block endogenous avidin and biotin. The blockers are provided with kits (BioGenex ISH kit).
9. Incubate the sections with anti-fluorescein antibody (ready-to-use reagent provided in the kits) for 30 min and then wash with DI water three times.

10. Incubate the sections with polymer-HRP secondary antibody (ready-to-use reagent) for 30 min and wash with DI water three times.
11. Incubate the sections with DAB (3,3′-diaminobenzidine) solution for a period sufficient to yield dark brown color, which is usually taken 10 min, followed by washing in tap water for 5 min.
12. Incubate section with hematoxylin for 3 min as a counterstain.
13. Rinse the slides in running tap water for 5 min and then dehydrate the sections by incubating in up-graded alcohols and mount by paramount for the examination of mRNA expressions of CCN1 and CCN5 in different human cancer tissue samples using a Leica photomicroscope with total magnification of 200× (*see* **Note 6**).

4 Notes

1. These reagents and solutions cannot be obtained from other suppliers. ULYSIS is available from Life Technologies (Carlsbad, CA; Cat No. U-21650). EZ-DeWax™ buffer is available from BioGenex (Fremont, CA; Cat No. HK585-5K). Power Block is available with the kits and can also be obtained separately (Cat No. HK083-05K, BioGenex, Fremont, CA).
2. These regents can be obtained from other suppliers and equally good for the detection of HRP.
3. The concentration of the PCR products can be measured by other software that have capability to measure the DNA/RNA concentrations.
4. If 1 μl ULS labeling reagent and 1 μg DNA make the final volume of the reaction to 25 μl then no labeling buffer is required to add into the reaction tube.
5. Tissue sections can be deparaffinized by incubating the slides in xylene for 15 min followed by xylene/alcohol (50% each) for 15 min at room temperature.
6. Given the current challenge of utilizing CCN-family proteins as therapeutic targets, there is a pressing need for gold-standard tools that help in identifying CCN-producing cells. The present modified in situ hybridization method has proven instrumental in the tracking of at least CCN5 and CCN1 transcripts in human breast and pancreatic cancer tissue samples (Fig. 2). We found that both CCN5 and CCN1 genes are transcribed exclusively in normal and cancer epithelial cells of breast and pancreas. The mRNA expression of CCN5 was detected in the

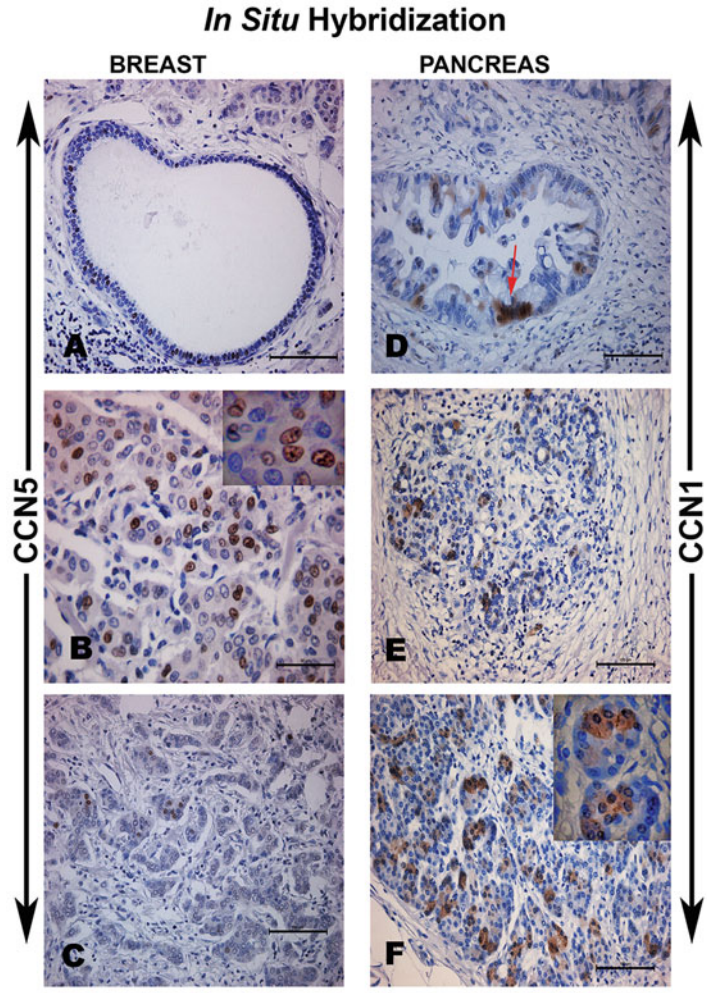

Fig. 2 Detection of CCN1 and CCN5 mRNAs in formalin-fixed paraffin-embedded human breast and pancreatic cancer tissue sections by in situ hybridization using ULYSIS Nucleic Acid Labeling probes. The *brown* color indicates the expression of CCN1 and CCN5 mRNAs. Sections were counterstained with hematoxylin. (**a**) Adjacent normal breast duct showing very few DAB-positive (*brown* color; mRNA expression) nuclei. (**b**) ER-positive breast cancer tissue section illustrating the DAB-positive (mRNA expression) cancer cells. The inset area illustrates the higher magnification of DAB-positive cells in tissue sections. Note that CCN5 mRNA is exclusively localized in the nuclei. (**c**) ER-negative invasive TNBC ductal carcinoma with minimal or no positive staining for CCN5 mRNA. (**d**) Pancreatic ductal adenocarcinoma (PanIN lesion) expressing the CCN1 mRNA. *Arrow* indicates the positive cells that are present inside the duct. (**e**) The invasive area of pancreas showing the *brown* color positive cells. Note that there are no positive cells in the stroma. (**f**) The adjacent normal acini are expressing CCN1 mRNA (DAB positive). The inset area shows both nucleus and cytoplasm that are DAB positive. Scale bar = 100 μm

nucleus of the normal ducts and lobules and estrogen-receptor-positive highly differentiated breast cancer samples (Fig. 2a, b), while its expression was undetected or minimally detected in poorly differentiated triple-negative breast cancer samples (Fig. 2c). These findings are consistent with our previous studies [27]. CCN1 mRNA was highly expressed in pancreatic cancer samples and correlates with poor prognosis. The CCN1 mRNA was localized in both nucleus and cytoplasm (Fig. 2d–f). Both CCN1 and CCN5 mRNA expression was undetected in stromal cells. Since previous studies have suggested that both CCN1 and CCN5 have pathological role in regulation of breast and pancreatic cancers via regulation of microenvironments, these studies strongly suggest that CCN1 and CCN5 could be drug targets for these cancers.

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Chapter 42

Analysis of Pathological Activities of CCN Proteins in Bone Metastasis

Tsuyoshi Shimo, Norie Yoshioka, Masaharu Takigawa, and Akira Sasaki

Abstract

Bone metastasis is a common occurrence in human malignancies, including breast, prostate, and lung cancer, and is associated with a high morbidity rate because of intractable bone pain, pathological fractures, hypercalcemia, and nerve compression. Animal models of bone metastasis are important tools to investigate the pathogenesis and develop treatment strategies. However, there are few models of spontaneous bone metastasis despite the fact that animals often spontaneously develop cancer. Here, we describe methods for developing a mouse model of breast cancer bone metastasis achieved by injection of MDA-MB-231 breast cancer cells into the heart. This assay can be applied to studies on roles of CCN proteins in tumor metastasis and development of treatment strategies targeting CCN proteins.

Key words CCN2, CTGF, CCN proteins, Tumor, Metastasis, Bone

1 Introduction

Several in vivo bone metastasis models, including left ventricle injection, have been established to study the events involved in metastasis [1]. The procedure can be adapted to many types of cancer cells including the highly bone-metastatic human breast cancer cell line MDA-MB-231, which is a highly bone-selective clone originally derived by Yoneda's group [2]. The left ventricle injection is the most typical method for achieving bone metastasis [1]. The bone metastasis animal model is a useful tool for evaluation and clarification of the mechanisms underlying the effects of neutralizing antibodies [3] and small-molecule inhibitors [4, 5] as novel targets for the development of therapies.

With the exception being ovarian and lung cancers, *CCN2* expression is found to be elevated in various types of tumors. These malignancies include breast cancer, prostate cancer, glioma, pancreatic cancer, colon cancer, thyroid carcinoma, chondrosarcoma, gallbladder carcinoma, melanoma, and leukemia [6, 7]. In those tumors with enhanced *CCN2* expression, the most prominent

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characteristics commonly observed are enhanced tumor angiogenesis and metastasis. We previously reported that neutralizing anti-CCN2 antibody inhibits bone metastasis of breast cancer in a mouse model [3]. This result suggests that CCN2 protein is a possible target for bone metastasis therapy. In addition to CCN2 protein, other CCN family proteins have been reported to be associated with malignancies in the case of a wide variety of tumors. Moreover, some CCN proteins, such as CCN3, are known to be tumor suppressive [6–8]. Therefore, it is important to investigate the roles of those CCN proteins in tumor metastasis and to develop treatment strategies targeting these proteins [6–8].

In this chapter, we describe a method for assaying bone metastasis that can be used for elucidating the function of the CCN2 protein and for assessing the effects of anti-CCN2 neutralizing antibody. This assay can be applied to similar studies on other CCN proteins as well.

2 Materials

1. Tumor cell bone metastatic breast cancer cell line MDA-MB-231.
2. Dulbecco's modified Eagle's medium (DMEM).
3. Phosphate-buffered saline (PBS)
4. Animal: 4–5-week-old female BALB/c nu/nu mice.
5. Clean bench.
6. 1 ml Disposable syringe (Tuberculin Syringe) and 27G × 1/2—needle.
7. Pentobarbital sodium solution (50 mg/ml, Dynamo).
8. Sterilized surgical gloves.
9. Scotch tapes (3 M).
10. 70% Ethanol on a gauze.
11. Cabinet type soft X-ray imaging system (type SRO-M50, SOFRON Co., Ltd, Tokyo, Japan).
12. X-ray film (industrial ENVELOPAK-FR, Tokyo, Japan) or an equivalent.
13. HE staining.
14. TRAP staining.
15. Microscopy.
16. Image analyzing software.
17. Neutralizing CCN2 mAb, JMAb 31 (*see* **Note 1**), or other test samples (*see* **Note 2**).
18. Control IgG or other corresponding controls.

3 Methods

3.1 Cell Preparation

1. Culture the highly metastatic variant of the estrogen-independent human breast cancer cell line MDA-MB-231/MDA231 [1, 9] in DMEM (Invitrogen, Grand Island, NY) containing 10% FCS (JRH Bioscience, Lenexa, KS) and 1% penicillin/streptomycin solution (Invitrogen) in a 37 °C atmosphere of 5% CO_2/air.
2. Passage the cells at 80–90% confluence and replate them at a tenfold dilution in a T150 flask.
3. Change the medium to fresh DMDM containing 10% FCS on the day before cell preparation (*see* **Note 3**).
4. Trypsinize the cells with 0.05% trypsin-0.02% EDTA-PBS without calcium and magnesium (*see* **Note 4**).
5. Remove the detached cells from the flask immediately with 20 ml of DMEM containing 10% FBS. Centrifuge the suspended cells in a 50 ml conical tube (Falcon) at 1000 rpm for 5 min.
6. Resuspend the pellet in 20 ml of PBS without calcium and magnesium, centrifuge for 5 min, repeat twice, and count the cell number.
7. Resuspend the cells at 10^6 cells/ml in PBS.

3.2 Heart Injection

1. Dilute the stock solution of pentobarbital sodium solution (50 mg/ml) with sterile PBS at a ratio of 1:6.
2. Inject mice intraperitoneally (i. p.) with the diluted anesthetic 0.1–0.15 ml (0.05 mg/g mouse) by using a 27G×1/2 needle (*see* **Note 5**).
3. Position each mouse on its back with chest facing up, and fix both forelegs at the epigastrium by using scotch tape (*see* **Note 6**).
4. Swab the chest twice with 70% ethanol on gauze.
5. Mark the injection point on the chest located midway between the sternal notch and top of the xyphoid process and slightly left of the sternum by using an oily felt pen (Fig. 1; *see* **Note 7**).
6. Draw up suspended cancer cells into a 1 ml disposable syringe.
7. Hold syringe pen grasp, keep needle upright, and insert the needle to a 3–5 mm depth without stopping (*see* **Note 8**).
8. Confirm the injection point (*see* **Note 9**).
9. Depress the plunger of the syringe (10^5 cells/100 μl volume) at a regular speed (*see* **Note 10**).
10. Pull the needle out of the heart ventricle without injuring the tissue (*see* **Note 11**).

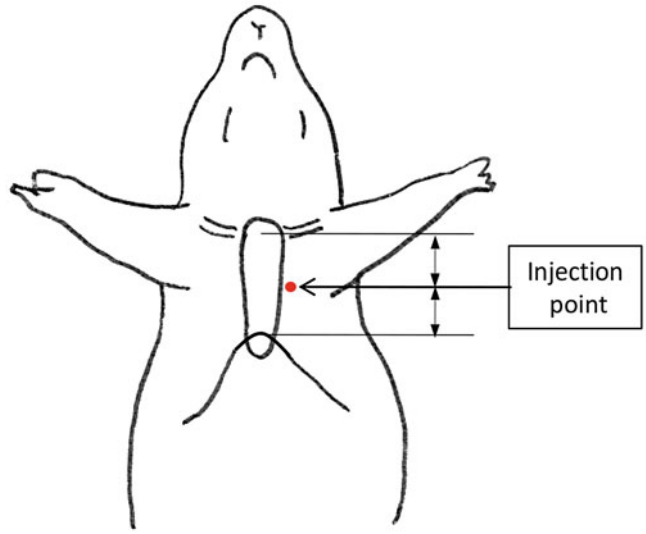

Fig. 1 Scheme for point of injection of the mouse left ventricle. The needle is inserted slightly to the left of the sternum between the sternal notch and xyphoid process

3.3 Treatment with Anti-CCN2 Antibody

1. Divide the mice inoculated with tumor cells through the left cardiac ventricle into three treatment groups ($n = 10$/group, *see* **Note 12**). These three groups comprise mice injected with neutralizing CCN2 mAb (JMAb 31) or other test sample, those given control IgG or corresponding control, and those left uninjected.
2. Administer the antibodies intraperitoneally at a dose of 100 μg in a volume of 100 μl per mouse twice per week (*see* **Note 13**).

3.4 Evaluation of Bone Metastasis

1. MDA-MB-231 cell-injected mice with bone metastasis usually gain body weight time dependently for 3 weeks, subsequently lose weight quickly, and die of cachexia at 4–5 weeks.
2. Take X-rays starting at 2 weeks to confirm the osteolytic lesion, which will be visible in the distal ends of femur, humerus, tibia, and fibula (Fig. 2).
3. Place the mice laterally in the prone position against the X-ray film (22 × 27 cm; Fuji Industrial Film) and expose them to soft X-rays at 35 kV for 15 s by using a SOFRON apparatus to assess the osteolytic bone metastasis radiographically [10].
4. Observe the radiolucent bone lesions in the hind limbs microscopically (1 × 81, Olympus Corporation), and quantify the lesion areas with Lumina Vision/OL (Mitani Corporation, Tokyo Japan; Fig. 3a; *see* **Note 14**).

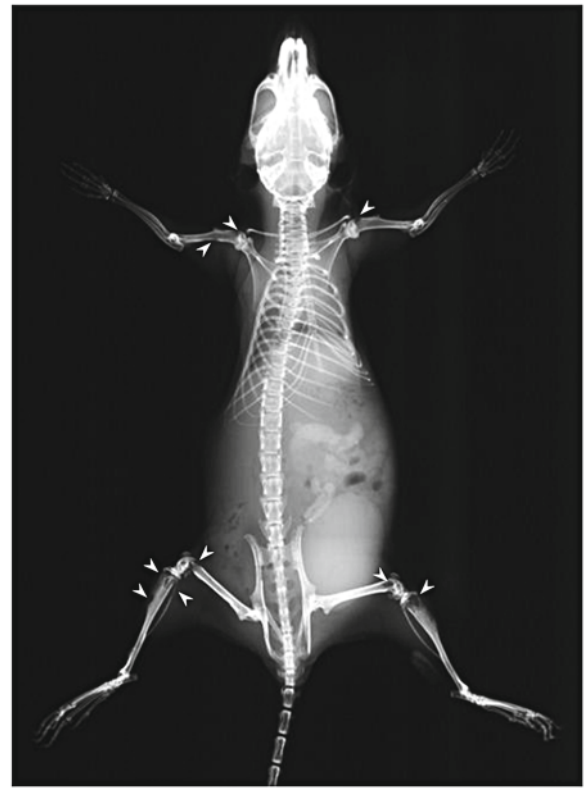

Fig. 2 Radiographic analysis of bone from a mouse bearing MDA-MB-231 cells. Representative radiographs of hind limbs taken 25 days after tumor inoculation. The *arrowheads* indicate osteolytic lesions

3.5 Histochemical and Histomorphometric Analysis

1. Excise the hind limb long bones of mice with bone metastasis, fix them in 10% neutral-buffered formalin, decalcify them, and embed them in paraffin.
2. Cut serial sections (5 mm) longitudinally, and stain the sections with Mayer's hematoxylin-eosin (HE) solution.
3. Photograph the images of the growth plate and proximal tibia by using an Olympus IX81 microscope (Fig. 3b).
4. Stain the sections for TRAP activity for the measurement of osteoclast number.
5. Evaluate the tumor area and osteoclast number/mm of tumor/bone interface in midsections of the tibiae and femora without knowledge of the experimental groups by using Lumina Vision/OL-analyzing software.

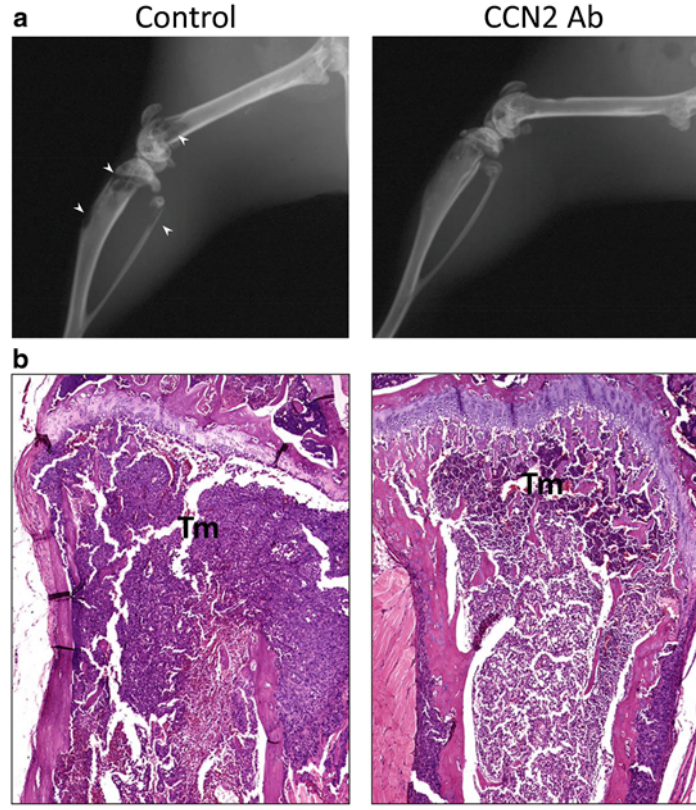

Fig. 3 Radiographic and histomorphometric analysis of bone from mice bearing MDA231 cells and treated with either control IgG or neutralizing CCN2 mAb ($n = 10$/group). (**a**) Representative radiographs of hind limbs from mice 25 days after tumor inoculation. The *arrowheads* indicate osteolytic lesions. (**b**) Bone histology of the mid-tibial metaphysis. Tumor (Tm) cells have filled the marrow cavity and replaced normal cellular elements in mice treated with control IgG. Significant loss of trabecular and cortical bone is observed in this group. In contrast, only small foci of tumor cells are present in mice treated with anti-CCN2, and bone destruction is not evident. The data are from Shimo et al. [3]

4 Notes

1. This anti-CCN2 monoclonal antibody, JMAb 31, was prepared and humanized based on an established methodology, using a recombinant CCN2 protein as an immunogen. The subclass of the antibody was determined to be human IgG2 and to specifically recognize the VWC module of human CCN2 [11].
2. Other antibodies against CCN2 as well as other CCN proteins can be tested. In the case of CCN protein predicted to inhibit tumor metastasis, CCN protein itself may be used.

3. The medium change is important for maintaining cell viability.
4. It is important to disperse the cells completely. Aggregated cells cause embolism after heart injection.
5. The depth of anesthesia needs to be such that there is no body motion. However, one should avoid deeper anesthesia that lowers blood pressure extremely. Under such condition, it is difficult to detect the backward flow of the arterial blood into the syringe, which is important to confirm proper injection into the heart.
6. It is important that there is symmetry of the mouse position.
7. The point of making is the slightly side of injection to keep the point sterilized.
8. Rest the hand on the table to prevent shaking of the tip of the needle, and puncture only by moving the finger tip.
9. Confirmation of the successful insertion into left cardiac ventricle is careful observation of the backward flow of arterial blood or the pulsatility movement of the cell suspension in the syringe.
10. To prevent injury to the heart ventricle or leaking of cells into the pericardium, do not move the needle.
11. After injection of the cells, pull the needle directly out of chest quickly and then apply gentle pressure on the injection site by using sterilized gauze for hemostasis.
12. These three groups comprise mice injected with neutralizing CCN2 mAb (JMAb 31), those given control IgG, and those left uninjected.
13. Start the administration on day 0 of tumor inoculation and continue throughout the experiment.
14. Recently, luciferase, green fluorescent protein (GFP), and live animal imaging based on 3D micro-computed tomography (μCT) and micro-positron emission tomography (μPET) have been used and can provide valuable information [12].

Acknowledgements

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Chapter 43

Analysis of Pathological Activities of CCN2/CTGF in Muscle Dystrophy

María José Acuña and Enrique Brandan

Abstract

CCN2 or connective tissue growth factor (CTGF) is a matricellular protein that regulates several cellular processes. In skeletal muscle, CTGF is a key modulator of fibrogenesis, is increased in pathological conditions such as muscular dystrophies, and plays a major role in the pathology outcome. Overexpression of CTGF in skeletal muscle of wild-type mice results in muscle damage, fibrosis, and reduction of strength. In contrast, a decrease in CTGF in dystrophic mice increases strength and reduces damage and fibrosis. Thus, CTGF is a relevant target to study in skeletal muscle pathology and its possible modulation by different treatments or potential new drugs to develop new strategies for the treatment of muscular dystrophies. We summarize the techniques used to detect CTGF in the skeletal muscle of dystrophic *mdx* mice.

Key words CCN2, CTGF, Skeletal muscle, Muscular dystrophy, *mdx* mice

1 Introduction

The matricellular protein CCN2, also known as connective tissue growth factor (CTGF), regulates several cellular processes, such as proliferation, migration, adhesion, and differentiation, and achieves these effects by modulating the activity of various growth factors, such as transforming growth factor type beta (TGF-β), bone morphogenetic proteins (BMP), and vascular endothelial growth factor (VEGF), among many others [1, 2]. CTGF participates in the physiopathology of different tissues; in adult skeletal muscle, under normal conditions CTGF is almost absent but is increased in pathological conditions, such as muscular dystrophies [3, 4], and is a key modulator of muscle fibrogenesis [4, 5]. In this context, overexpression of CTGF in the tibialis anterior of wild-type mice results in muscle damage, fibrosis, and reduction of strength. However, the reduction of CTGF in dystrophic muscle increases strength and reduces damage and fibrosis. Thus, studying CTGF in muscular dystrophies and its possible modulation by different treatments or potential new drugs is a critical issue.

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In this chapter, we summarize how to study CTGF messenger expression by real-time PCR analysis, protein levels in muscle extract by western blotting, and localization in skeletal muscle cryosections by immunohistochemistry techniques. We provide detailed step-by-step protocols.

2 Materials

2.1 Animals

We use the mice model for Duchenne muscular dystrophy, *mdx* mice (C57BL/10ScSn-Dmd^{mdx}mice), and wild-type C57BL/10J.

2.2 Muscle mRNA Extraction cDNA Synthesis and Real-Time qPCR

1. Ultra-Turrax homogenizer.
2. RNase Zap (*see* **Note 1**).
3. TRIzol.
4. Nuclease-free water or DEPC-treated water.
5. Chloroform, analytical grade.
6. Isopropanol, analytical grade.
7. 70% Ethanol solution prepared in nuclease-free water.
8. DNase I amplification-grade kit (Invitrogen).
9. RNase Out, ribonuclease inhibitor.
10. Moloney murine leukemia virus reverse transcriptase (M-MLV) kit (Invitrogen).
11. dNTPs mix, 10 mM.
12. Random primers.
13. SYBR Green Master Mix 2×.
14. CTGF primers: Fwd: 5′-CAG-GCT-GGA-GAA-GCA-GAG-TCGT-3′; Rev: 5′-CTG-GTG-CAG-CCA-GAA-AGC-TCAA-3′.
15. 18s primers: Fwd: 5′-TGA-CGG-AAG-GGC-ACC-ACC-AG-3′; Rev: 5′-CAC-CAC-CAC-CCA-CGG-AAT-CG-3′.

2.3 Muscle Protein Extraction

1. Tris-EDTA extraction buffer: 50 mM Tris, 10 mM EDTA, pH 8.3.
2. 2× Treatment buffer: 0.125 M Tris, 4% SDS, 20% glycerol, pH 6.8.
3. Ultra-Turrax homogenizer.
4. Distilled water.
5. Ethanol 70%, technical grade.

2.4 Muscle Dissection and Cryosection

1. Common surgical material, such as tweezers and scissors.
2. Isopentane (2-methylbutane).
3. Optimum cutting temperature (OCT) compound.

4. SuperFrost glass slides.
5. Cryostat.

2.5 Immunostaining of CCN2 in Tissue Cryosections

1. Anti-CTGF antibody (Goat-anti-CTGF, Santa Cruz Biotechnology, Cat#SC-14939).
2. Rabbit anti-goat HRP.
3. 5 % Bovine Serum Albumin (BSA) diluted in phosphate buffer saline.
4. 0.05 % Tween 20 diluted in phosphate buffer saline (Tween-PBS).
5. Cold acetone, –20 °C, analytical grade.
6. Methanol-H_2O_2 3 % solution.
7. Powervision Poly-HRP anti-rabbit IgG (Novocastra).
8. 3′,3′-Diaminobenzidine tetrahydrochloride (DAB) liquid system.
9. Hematoxylin.
10. Ethanol 70 %, analytical grade.
11. Ethanol 100 %, analytical grade.
12. Xylol, analytical grade.
13. Entellan mounting medium.

3 Methods

3.1 Muscle RNA Extraction cDNA Synthesis and Real-Time qPCR

3.1.1 RNA Extraction

1. Clean the Ultra-Turrax with ethanol and then with nuclease-free water (*see* **Note 2**).
2. Weigh the muscle and calculate the TRIzol volume to be used; for the tibialis anterior muscle, use 2 mL of TRIzol (*see* **Note 3**).
3. Set the Turrax at maximum speed and make the homogenate on an ice-cooled rack. Leave the homogenate on ice until the next step.
4. Centrifuge the homogenates at 4 °C, at 12,000 × *g* for 15 min.
5. Take the supernatant and place it in a new tube. Discard the pellet (*see* **Note 4**).
6. Add 0.2 mL of chloroform per every 1 mL of TRIzol. Mix with inversion and repeat the centrifugation in **step 4**.
7. Recover the soluble phase (transparent phase); avoid aspirating the non-soluble phase (*see* **Note 5**). Add an equal volume of cold isopropanol (keep isopropanol at –20 °C) to precipitate the RNA. Leave overnight at –20 °C (*see* **Note 6**).
8. Centrifuge at 4 °C at 12,000 × *g* for 15 min (*see* **Note 7**).
9. Discard the supernatant and wash the pellet with 0.5 mL of ethanol 70 %.

10. Centrifuge at 4 °C at 12,000 × *g* for 15 min.
11. Discard the supernatant and dry at room temperature for about 15 min or until the ethanol dries out.
12. Suspend the pellet in 20 μL of DNase-free water. Heat the samples on a dry bath at 65 °C for 10 min and then place them on ice.

3.1.2 cDNA Synthesis

1. Before beginning the cDNA synthesis, test the RNA integrity by running the samples (1 μL) on 1 % agarose gel. Visualize the integrity by assessing ribosomal RNA (*see* **Note 8**).
2. Measure the RNA concentration in the samples. Make sure to start with the same amount of RNA for all samples (*see* **Note 9**).
3. Treat samples using the DNase I kit following the manufacturer's instructions.
4. Prepare complementary DNA using the M-MLV kit manufacturer's instructions.

3.1.3 Real-Time Quantitative PCR

1. Make a cDNA dilution 1:10 in nuclease-free water. Use these dilutions to perform qPCRs (*see* **Note 10**).
2. Use 2 μL of cDNA per every 10 μL of reaction, and 1 μL for the housekeeping gene (*see* **Note 10**).
3. Use the primers at a final concentration of 500 nM (*see* **Note 11**).
4. Add the 2× Master Mix for SYBR Green to a final 1× concentration.
5. Add nuclease-free water to reach a final 10 μL volume (*see* **Note 12**).
6. The program used is the following (*see* **Note 13**):
   - UDG incubation: 50 °C 2 min 1 cycle.
   - Polymerase activation: 95 °C 10 min 1 cycle.
   - PCR cycling step 1: 95 °C 15 s 40 cycles.
   - PCR cycling step 2: 60 °C 1 min 40 cycles (data recollection).
   - Melting curve step 1: 95 °C 15 s 1 cycle.
   - Melting curve step 2: 55 °C 15 s 1 cycle (data recollection).
   - Melting curve step 3: 95 °C 15 s 1 cycle.

3.2 Muscle Protein Extraction

1. Clean the homogenizer with ethanol and then with distilled water. Repeat this step after every sample.
2. Weigh the muscle and add 10 mL of extraction buffer per gram of muscle (*see* **Note 14**).
3. Add a cocktail of protease inhibitors according to the volume used to extract.

4. Make a homogenate using Ultra-Turrax maximum velocity. Make sure that the muscle gets milled (*see* **Note 15**). This step is performed with the tubes on ice.
5. Add one volume of 2× treatment buffer (*see* **Note 16**).
6. Incubate the samples at 50 °C for 20 min. Then mix by pipetting and reheat for 5 more minutes.
7. Centrifuge at room temperature at 16,000 × *g* for 20 min.
8. Keep the supernatant and discard the pellet.
9. Calculate the sample protein concentration using the standard protocol (*see* **Note 17**).
10. Prepare a 10% gel SDS-PAGE and load 50–100 μg of total protein.
11. Transfer the proteins in a PVDF membrane, and use the standard protocol.
12. Block the membrane with 5% fat-free milk in Blotto 1×, and incubate overnight with CTGF antibody at 1:500 dilution at 4 °C.
13. Wash the membrane three times with Blotto 1× for 10 min at room temperature.
14. Incubate the secondary antibody (anti-goat HRP) 1:5000 for 1 h at room temperature.
15. Wash the membrane three times with Blotto 1× for 10 min at room temperature.
16. Wash the membrane with PBS 1×.
17. Use the standard detection method (*see* **Note 18**).

3.3 Muscle Cryosectioning

1. Freeze isopentane on liquid nitrogen in 5 mL tubes (*see* **Note 19**).
2. Dissect the skeletal muscle of interest, from the hind limb or the diaphragm (*see* **Note 20**); Fig. 1.
3. To freeze large hind limb muscles on isopentane, cut them into half with a cryostat blade or any sharp blade, and directly freeze them on the isopentane (Fig. 1). For cryomolds, fill the cryomold with OCT to accommodate the muscle in the correct position and submerge the cryomold in the frozen isopentane (*see* **Note 21**).
4. Set the cryostat temperature between –23 °C and –20 °C.
5. Put a layer of OCT on the specimen disc, and let it freeze until a thin layer is still melted. Put the muscle tendon on the melted OCT and freeze it quickly by using the fast freezing function (*see* **Note 22**).
6. Set the size of the slides on 7 μm. Start to cut the specimen (*see* **Note 23**).

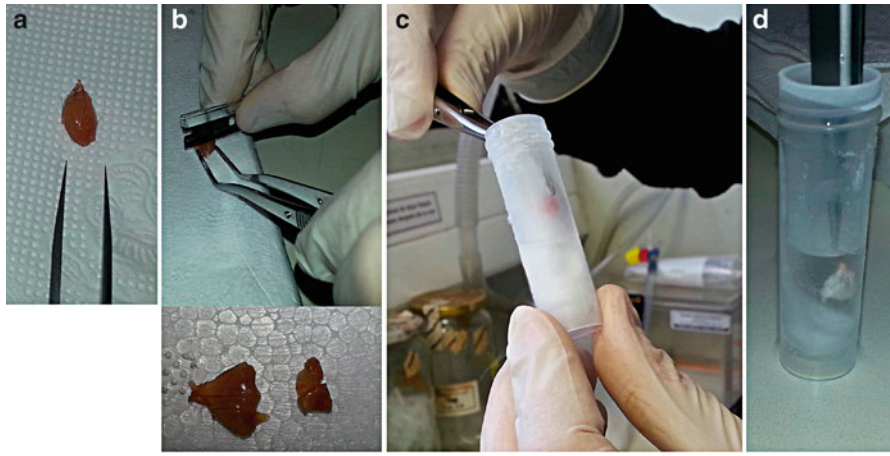

Fig. 1 Steps for freezing muscle for cryosectioning. (**a**) Dissect the muscle of interest (in this case, the gastrocnemius). (**b**) Cut the muscle into half (for large muscles). (**c**) Directly submerge the muscle in frozen isopentane. (**d**) The isopentane melts, and the muscle is frozen. Immediately after, store the muscle at –80 °C

7. After accommodating the muscle cryosections on the glass slide, store the specimens at –20 °C. However, if they are going to be stored for more than a month, they should be kept at –80 °C.

3.4 Immunostaining in Tissue Cryosections

1. Fix the tissue cryosections for 20 min with cold acetone at –20 °C (*see* **Note 24**).
2. Wash three times with Tween 20 0.05% in PBS (Tween-PBS).
3. Block with PBS-5% BSA at room temperature for 30 min.
4. Incubate with primary antibody 1:100 diluted in 5% BSA in PBS, overnight at 4 °C.
5. Wash three times with Tween-PBS.
6. Incubate for 30 min at room temperature with rabbit anti-goat HRP 1:100 diluted in PBS-5% BSA.
7. Wash three times with Tween-PBS.
8. Block endogenous peroxide activity by incubating for 15 min with methanol-3% H_2O_2 at room temperature (*see* **Note 25**).
9. Incubate for 30 min at room temperature with Powervision poly-HRP anti-rabbit IgG.
10. Wash three times with Tween-PBS.
11. Incubate with DAB for 10 min at room temperature. Stop the reaction with distilled water (*see* **Note 26**).
12. Stain the nuclei with a hematoxylin solution for 5 min.
13. Wash with running tap water for 10 min.

14. Dehydrate with an alcohol battery from 70 to 100% and a final step with xylol (*see* **Note 27**).
15. Mount with Entellan mounting medium.

4 Notes

1. Before beginning with RNA extraction, wash the bench and all working materials, such as pipettes, homogenizer, and tube rack, with RNase Zap solution in order to eliminate the ribonuclease activity and prevent RNA from degrading. In addition, working with nuclease-free plastic material is required, which should be autoclaved before use.
2. Make sure to clean the Ultra-Turrax homogenizer thoroughly and use nuclease-free water. After every use, wash the homogenizer with ethanol and water.
3. For RNA extraction, keep the muscle in liquid nitrogen or dry ice until use (once the muscle is dissected, freeze it immediately in liquid nitrogen and store in nuclease-free tubes at –80 °C). Use autoclaved tubes to make the homogenate. TRIzol is used in the following ratio: 1 mL for 500 mg of tissue. Normally, half of the tibialis anterior or half of the gastrocnemius muscle is used. If the diaphragm is used, it should weigh at least 20 mg. Use 0.4 mL of TRIzol to make the homogenate.
4. Discard the TRIzol waste with the appropriate lab's safety protocol for chemicals since TRIzol contains phenol and toxic compounds. NEVER use common trash cans or discard liquid wastes in the sink. In addition, discard pipette points properly.
5. It is better to lose some material instead of mixing it with the non-soluble phase, because it contaminates the RNA.
6. It can be kept for several days in isopropanol at –80 °C, but do not wait for more than 3–4 weeks to extract the RNA.
7. Normally, the pellet is visible at the bottom of the tube.
8. Ribosomal RNA is abundant, and three bands can be visualized using SYBR Green or similar staining. If smearing is observed, the RNA is degraded. Therefore, do not use that sample because it can lead to bad conclusions.
9. The RNA concentration can be measured before or after the integrity gel is made. It is up to the user's criterion. Use NanoDrop or similar techniques, such as Take3 BioTek plates, because it saves time and requires a small amount of sample. Often, cDNA is prepared starting with 2–4 μg RNA. Always start with the same quantity of RNA for every sample you will compare.
10. The optimal dilution should be determined for every set of primers, but usually the dilutions for this protocol are between

1:10 and 1:25. If the cDNA is too concentrated, the qPCR reaction is not efficient because the reaction becomes inhibited. Often 1 μL is used for a 10 μL reaction for the housekeeping gene and 2–3 μL for the target gene; however, the current recommendation is the use of at least two housekeeping genes. If TaqMan chemistry is used, both genes can be made in the same reaction as long as the target gene and housekeeping probes have different fluorophores; however, here we describe the conditions for SYBR Green qPCR.

11. Verify the primer concentrations with the melting curve at the end of the PCR reaction. If no primer dimers have formed (it looks like an elbow before the peak in the curve), then 500 nM is optimum. A range between 500 and 200 nM can be used to minimize the formation of dimers.
12. Some manufacturers indicate that the minimum volume of reaction is 20 μL. Verify first whether your equipment permits the use of less volume. Some can work with a minimum volume of 13–15 μL.
13. Usually for the wild-type condition, CTGF ct is around cycles 27–28, and for the *mdx* it appears around cycles 22–25. The predicted length of the PCR product is 141 base pairs, and the melting temperature is 82 °C.
14. If you weigh 50 mg, you should use 500 μL of extraction buffer. Usually half of the tibialis anterior or the gastrocnemius is used, which weighs between 50 and 70 mg if obtained from adult mice.
15. Around 30 s should be enough. The same length of time should be used for homogenization every time.
16. Do this immediately after finishing the homogenization step, and mix by pipetting.
17. Do not use the Bradford method because SDS interferes with these reagents. The recommended method is bicinchoninic acid.
18. We use the West Dura reagent (Thermo Fisher) according to the manufacturer's instructions, and use a ChemiDoc detection system. Usually, the exposure times vary from 30 s to 3 min.
19. The isopentane is frozen in tubes submerged in liquid nitrogen; keep frozen until use. For the soleus, EDL, and small muscles, use cryomolds, and freeze the muscle in OCT. In this case we use a precipitate glass containing the isopentane.
20. Avoid leaving the dissected muscles on any aqueous solution such as saline or PBS. This keeps water crystals from forming and damaging the tissue. Just dissect the muscles and freeze them immediately.
21. This step is key to obtain a good histology. The muscles should be submerged on isopentane quickly but avoid bending or twisting the muscle. So that it is easier to cut the specimen in

the right direction, store the muscle on a cooled tube. If a cryomold is used, place directly on the frozen isopentane with the muscle embedded on OCT. Once the muscle is frozen, leave the tubes on liquid nitrogen or immediately freeze at –80 °C.

22. Only the tendon or a very little surface of the muscle should touch the melted OCT, because this part of the tissue gets unfrozen, and little holes are formed on the tissue.
23. Accommodate the specimen disc with the muscle to cut it on the transversal.
24. Use the anti-roll plate or a paintbrush to extend the cut muscle on the cooled glass slides. To adhere the specimen, just put the glass slides on your wrist, and the muscle will rapidly attach to the glass slide.
25. This step is critical to avoid unspecific staining. In addition, a negative control without a primary antibody should be made in parallel with the samples of interest.
26. The length of time for exposure to DAB should be established empirically, usually between 5 and 10 min. When the brown color appears, stop the reaction (all samples should be done at the same time if possible in the same slide). Too much time will result in a dark background and difficulty visualizing the staining.
27. The battery we use is 70% ethanol 1 and 70% ethanol 2, 100% ethanol 1, 100% ethanol 2, xylol 1, and xylol 2.

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Chapter 44

Method for Analysis of Matrix Degradation by CCN2 Through the MMP/TIMP System

Susan V. McLennan, Danqing Min, Xiaoyu Wang, and Stephen M. Twigg

Abstract

Many studies have shown effects of members of the CCN family on matrix synthesis and accumulation but few have examined degradative pathways. This scarcity of information is in part due to the lack of suitable model systems. Here we describe methods for making rhCCN2 and also for the preparation of a biosynthetically labeled matrix substrate that can be used to measure the effect of CCN on cellular or secreted degradative pathways.

Key words CCN2, Connective tissue growth factor, Matrix metalloproteinase, Tissue inhibitor of matrix metalloproteinase, Matrix degradation pathways

1 Introduction

Expansion of extracellular matrix (ECM) is a hallmark of many of the complications of diabetes and it is particularly relevant in the kidney where it can cause structural changes that impair renal function. In the presence of high glucose concentrations, matrix accumulation occurs because of an imbalance between synthetic and degradative pathways [1–3]. This imbalance occurs, at least in part, because of altered expression of growth factors. CCN2, a member of the CCN family and the IGFBP superfamily [4], has been shown to play a role in ECM synthesis [5] as well as ECM degradation [6, 7].

The methodologies described in this chapter are used to demonstrate the novel data (shown schematically in Fig. 1) that CCN2 inhibits degradation of ECM through induction of the tissue inhibitor of MMPs, known as TIMP-1, and that it contributes to the inhibition of ECM degradation caused by elevated extracellular glucose. They describe the production and purification of rhCCN2 as well as the production of a biosynthetically labeled matrix for study of extracellular matrix degradation. A description of endpoint studies to investigate how CCN2 inhibits such degradation through upregulation of TIMP-1 is also included. In our hands the methodology is robust, highly reproducible, and user friendly.

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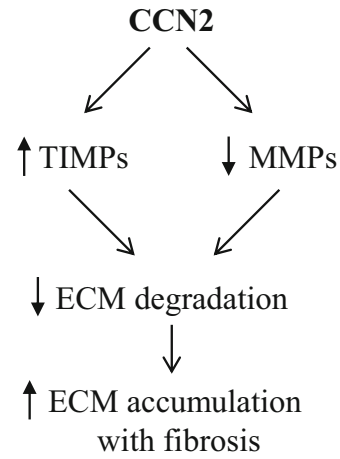

Fig. 1 Schematic diagram summarizing how CCN2 may regulate cell and matrix TIMPs and MMPs to decrease matrix degradation and increase fibrosis. Note that TIMP induction to inhibit ECM degradation will mainly function by the specific TIMP binding to particular MMPs, thus inhibiting MMP bioactivity

2 Materials

2.1 Synthesis and Quantitation of rhCCN2

1. AdEasy Adenoviral Vector System [8, 9].
2. Full-length CCN2 suitable for cloning into the shuttle vector pAdTrack-CMV [8, 9].
3. *E. coli*. BJ5183 cells.
4. E1-transformed human embryonic retinoblasts: 911 cells.
5. Tissue culture flasks T150.
6. Dulbecco's Modified Eagle's medium (DMEM) complete medium containing 4.5 g/l D glucose, 4 mM L-glutamine, 10% (v/v) fetal calf serum (FCS).
7. Heparin-sepharose affinity chromatography column, 1 ml (*see* **Note 1**).
8. Phosphate-buffered saline (PBS): The PBS solution can be made as a 1× solution or as a 10× stock. To prepare 1 l of either 1× or 10× PBS, dissolve the reagents listed below in 800 ml of H_2O. Adjust the pH to 7.4 with HCl, and then add H_2O to 1 l. The solution can then be sterilized by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature (Table 1).
9. Bovine serum albumin (BSA), 0.05%: 0.25 g BSA in 50 ml PBS.
10. Protease inhibitors: One CompleteTM tablets (*see* **Note 2**).
11. Binding buffer, 10 mM sodium phosphate pH 7.4: 1.28 g Monopotassium phosphate, 1.58 g dipotassium phosphate in 1 l of distilled H_2O, adjust pH to 7.4.

Table 1
Preparation of phosphate-buffered saline (PBS) solution

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM

12. Low-salt elution buffer, 10 mM sodium phosphate, 0.1 M NaCl, pH 7.0: 1.28 g Monopotassium phosphate, 1.58 g dipotassium phosphate, 5.844 g NaCl in 1 l of distilled H_2O, adjust pH to 7.0.
13. High-salt elution buffer (10 mM sodium phosphate, 2 M NaCl, pH 7.0): 1.28 g Monopotassium phosphate, 1.58 g dipotassium phosphate, 116.88 g NaCl in 1 l of distilled H_2O, adjust pH to 7.0 as required.
14. SDS-PAGE gels: 12.5%.
15. Polyclonal or monoclonal CCN2 antibody.
16. Coomassie Blue Stain: 0.25 g Coomassie Brilliant Blue R-250 in 90 ml methanol:H_2O (1:1 v/v) and 10 ml glacial acetic acid. Filter solution through a Whatman No. 1 filter paper to remove particulate matter.
17. Low-molecular-weight protein standards.

2.2 Biosynthetically Labeled Extracellular Matrix Substrate

1. Mesenchymal cells (*see* **Note 3**).
2. RPMI complete media containing 10% FCS.
3. Trypsin.
4. [S]methionine 35 (Tran)S-label.
5. Ammonium hydroxide (NH_4OH), 0.25 N: Dilute 16.67 ml of concentrated NH_4OH (14.8 M, 28%NH_3) to 1 l in distilled H_2O.
6. Ethanol, 70%: Dilute 73.68 ml of 95%ETOH to 100 ml in distilled H_2O.

2.3 Endpoint Studies

1. Beta counter.
2. MMP inhibitors: *o*-Phenanthroline monohydrate (1–10 mM), EDTA (10 mM), EGTA (10 mM).
3. RPMI complete media containing 5 mM or 25 mM glucose.

Table 2
Primer sequences for measurement of human fibronectin, MMPs/TIMPs, and 36B4 by RT-PCR

Name	Forward primer	Reverse primer
Fibronectin	5′-tccttgctggtatcatggca-3′	5′-agacccaggcttctcatactt-3′
TIMP-1	5′-caaccagaccacctttacc-3′	5′-gagtgccactctgcagtttg-3′
TIMP-2	5′-ggcgttttgcaatgcagatg-3′	5′-catctggtacctgtggttcag-3′
TIMP-3	5′-tcaagtcctgctactacctg-3′	5′-gggaagagttagtgtccaag-3′
MMP-2	5′tggattcgagaaaaccgcagtgg3′	5′-cgccgtcgcccatcatcaagt-3′
36B4	5′-tcgtggaagtgacatcgtctt-3′	5′-tctgctcccacaatgaaaca-3′

4. Anti-CCN2 blocking antibody.
5. Anti-TIMP-1 antibody (Clone 147-6D11).
6. Anti-MMP-2 antibody.
7. Control IgG.
8. RNA extraction reagent: TriReagent or TriZol.
9. Reverse transcription reagents: Reverse transcriptase, buffers, dNTPs, oligo dT.
10. Real-time RT-PCR reagents: Taq polymerase, buffers, forward and reverse primers as listed in Table 2.

3 Methods

3.1 Synthesis and Purification of rhCCN2 Protein

1. The 1047 bp cDNA open reading frame of CCN2 was originally cloned from an Hs578T human breast cancer cDNA library and sequenced [8, 9].
2. The fragment coding for full-length human CCN2 was then subcloned into the Kpn1 and Xho1 restriction sites of the shuttle vector pAdTrack-CMV.
3. The resultant plasmid can then be linearized by digestion with restriction endonuclease Pme I, and subsequently co-transformed with an adenoviral backbone plasmid, pAdEasy-1, into *E. coli* BJ5183 cells.
4. Recombinants are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses and DNA sequencing [8].

The linearized recombinant plasmid is transfected into adenovirus packaging cell line, 911 cells, using the techniques described below.

3.1.1 Production of rhCCN2 by 911 Cells

1. Seed 911 cells into T150 flasks with DMEM complete medium in 5% CO_2/95% air. The cells are grown to 70–80% confluence.
2. The 911 cells are then infected with the rhCCN2 by addition of sense adenovirus (at a concentration of approximately 1×10^9 viral particles per flask) in DMEM supplemented with 0.05% (v/v) BSA.
3. After 48 h collect the conditioned media and add protease inhibitors (*see* **Note 2**).
4. The media is then spun at 450 g for 20 min to pellet the cell debris and the supernatant containing the rhCCN2 is collected for purification of rhCCN2.

3.1.2 Purification of rhCCN2 Using Heparin Sepharose Affinity Chromatography

Perform each of the following steps in a cold room at 4 °C.

1. Prepare the HiTrap column by filling the pump tubing with binding buffer (10 mM sodium phosphate, pH 7.0) and connecting it to the column and attaching to the pump. The binding buffer is then pumped through the column at a rate of 0.5 ml/min. Approximately 10 ml will be sufficient to wash and equilibrate the column.
2. The media containing the rhCCN2 is then pumped onto the column at a rate of 0.2–0.3 ml/min at 4 °C.
3. Binding buffer (approximately 10 ml), followed by 6 ml of the low-salt elution buffer, is then pumped through the column to remove any non-bound protein (*see* **Note 4**).
4. The rhCCN2 is then eluted from the column with 6 ml of the high-salt elution buffer (10 mM sodium phosphate, 2 M NaCl, pH ~7.0). As before 1 ml fractions are collected for analysis. The purified rhCCN2 is generally eluted in the second 1 ml fraction of the 2.0 M NaCl elution buffer.

3.1.3 Quantification of Purified rhCCN2

1. To determine which fraction contains the rhCCN2 15 μl of each of the eluted fractions are run on a 12.5% SDS-PAGE gel. The gel is stained with Coomassie Brilliant Blue and the fraction containing a single band at approximately 40 kDa is then tested for the presence of the purified rhCCN2 by Western blot.
2. To quantitate the amount of rhCCN2 serially diluted aliquots of the purified protein are run on a 12.5% SDS-PAGE gel and the band intensities are determined from the digitized image and the concentration after staining with Coomassie Brilliant Blue is calculated by comparison with the band intensity of a protein ladder standard with known quantities of protein (LMW Electrophoresis Calibration Kit, Pharmacia Biotech).
3. Prior to use the fractions containing rhCCN2 are desalted by dialysis in 1 l of PBS (*see* **Note 5**).
4. To establish the bioactivity of rhCCN2, human fibroblasts are plated onto a 6-well plate and grown to confluence. rhCCN2

(100–1000 ng/ml) is added and after 24 and 48 h the expression of fibronectin (see primer table materials section) is measured by qRT-PCR. Cells not cultured in the presence of rhCCN2 are used as control (*see* **Note 6**).

3.2 Synthesis of the Biosynthetically Labeled Matrix Substrate

Mesangial cells (*see* **Note 3**) are cultured in T25cm flasks at 37 °C in 95 % air and 5 % CO_2 in RPMI complete media. After the third passage the cells are washed three times with 10 ml of sterile PBS prior to trypsinization (1 ml/flask). The cells are washed three times in RPMI complete media and resuspended in 5 ml of RPMI complete media.

3.2.1 Production of the Radiolabeled Matrix Substrate

1. Plate 10,000 cells into each well of a 6-well tissue culture plate in 2 ml of RPMI complete media. After the cells have attached (4–6 h) remove the media and replace it with fresh RPMI complete media containing [S]methionine 35 (Tran S-label) at final concentration of 10 μCi/ml. Five days later decant the media and the cells and matrix are washed three times with 1 ml of sterile PBS.
2. To remove the cells and leave the labeled matrix substrate attached to the well add 1 ml of 0.25 N NH_4OH to each well and incubate the tissue culture plates at 37 °C for 30 min.
3. The cells will detach from the matrix and can easily be aspirated away (*see* **Note 7**).
4. The labeled matrix remaining on the plate is then washed three times each with 1 ml of sterile PBS.

The biosynthetically labeled matrix can then be used for degradation studies as described in the following section.

3.3 Endpoint Studies

3.3.1 To Examine the Effect of CCN2 on Matrix Degradative Capacity

Shown schematically in Fig. 2 the labeled matrix substrate can be used to examine a) the effect of CCN2 on cell degradative activity or b) the degradative activity of conditioned media collected from cells treated with CCN2 as follows:

1. To examine the effect of CCN2 on cell-degradative capacity, cells (1×10^6 cells/well) are plated onto the prepared biosynthetically labeled matrix. After 4 h when cells are attached the media is removed and replaced with 1 ml of serum-free RPMI in the presence of rhCCN2 (0–1000 ng/ml). After 72 h, the media is collected for study measurement of degradative activity by counting release of ^{35}S methionine to the culture media. The cells are washed with cold PBS before extraction of RNA for measurement of MMP and TIMP gene expression, described in detail below.
2. To examine the effect of CCN2 on secreted degradative capacity the conditioned medium is collected from the cells grown in serum-free RPMI in the presence of rhCCN2 (0–1000 ng/ml).

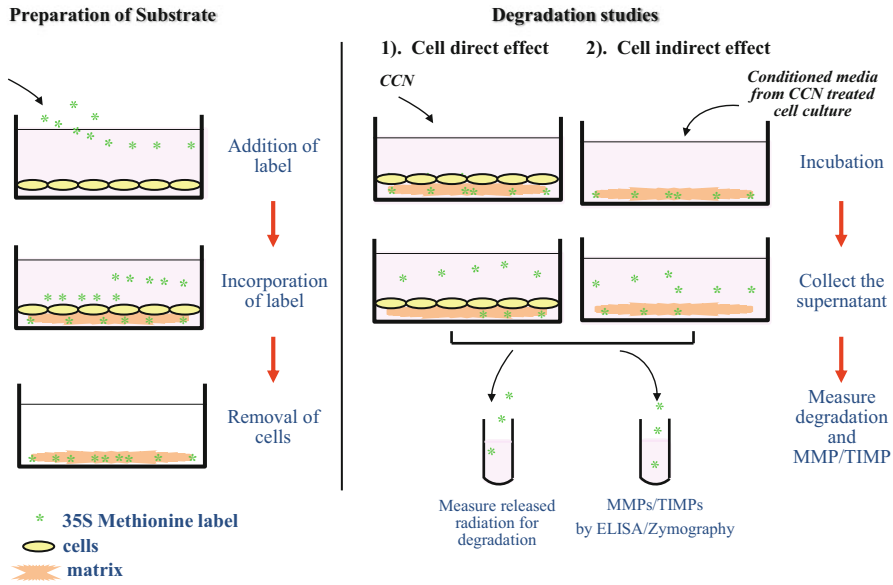

Fig. 2 Schematic diagram summarizing the preparation of a biosynthetically labeled matrix substrate and its use in analysis of the effects of CCN2 on the activity of the MMP/TIMP system

After 72 h, the media is collected and the degradative activity is determined by placing 1 ml of the collected media on the biosynthetically prepared radiolabeled matrix substrate. Medium not exposed to cells is used as control. Use of this technique enables the analysis of medium MMP activities in the presence of the TIMPs. The degradative capacity is determined by counting the radioactivity released to the culture media, and results are expressed as a percentage of the total count incorporated into the matrix. Using specific inhibitors, we have previously shown that the majority of degradative capacity measured by this assay is due to the action of MMPs [10].

3. To address a potential role for CCN2 in the inhibition of matrix degradation by high glucose, mesangial cells are cultured in the presence of either 5 or 25 mmol/l D-glucose, each with 30 μg/ml of an anti-CCN2 antibody (known to neutralize rhCCN2) (500 ng/ml) [11], or with 30 μg/ml of control IgG. For each experiment, the anti-CCN2 antibody or control IgG is added 30 min before addition of either 25 mM glucose or the rhCCN2. After another 72 h of cell culture, the conditioned medium is collected for degradation studies and the RNA is extracted from the cells for measurement of gene expression of the MMPs and TIMPs.

3.3.2 To Examine the Effect of Anti-TIMP-1 Antibody on Matrix-Degradative Capacity

1. Cells are cultured in serum-free RPMI media in the presence of either 25 mM D-glucose or rhCCN2 (0–1000 ng/ml) and an anti-TIMP-1 antibody (20 μg/ml total IgG, clone 147-6D11) or control IgG (20 μg/ml).
2. The conditioned media (1 ml) is then added to the radiolabeled matrix substrate for 24 h.
3. The degradative capacity is determined by counting the radioactivity released to the culture media, and results are expressed as a percentage of the total count incorporated into the matrix.

Effect of CCN2 on MMP and TIMP Gene Expression

1. Culture cells in the presence of rhCCN2 (0–1000 ng/ml).
2. After 72 h, the cells are washed with cold PBS and scraped from the plate. Extract the RNA using TRI Reagent. RNA is transcribed to cDNA using oligo dT (10 pmol) and a reverse transcriptase enzyme.
3. For each study at least three independent experiments are carried out to generate data. For each experiment, the treatment conditions are tested in triplicate wells of cells. For the studies where mRNA is examined all samples are analyzed in duplicate by quantitative, real-time RT-PCR.

The expression of MMPs and TIMPs is determined by quantitative real-time RT-PCR using SYBR green fluorophore. Specifically, all amplicons are amplified using Platinum Quantitative PCR SuperMix-UDG and 20 pmol of each forward and reverse primer and the following PCR conditions:

1. For MMP-2, TIMP-1, TIMP-2, and TIMP-3 (1 μl first-strand cDNA), 50 °C for 2 min and 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s.
2. For TIMP-1, fibronectin, and 36B4 (0.5 μl first-strand cDNA), 50 °C for 2 min and 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.
3. The expression of the housekeeping gene β-actin is also measured as control for loading and RT efficiency. The primers used have been shown to generate a single appropriately sized cDNA band on DNA agarose gels.
4. Negative control RT-PCRs are performed by omitting either the reverse transcriptase enzyme or the RNA from the reaction mixture. All negative controls failed to produce PCR amplicons.
5. All samples for analysis generated cycle thresholds that are on the linear part of the serial dilution curves, and an equivalent gradient of dilution curves with the housekeeper is confirmed in each case. The inter-assay coefficient of variation for real-time runs is maximally 8.2 ± 3.1 %.

Results were expressed using the delta-delta method as a ratio corrected for abundance of the housekeeping gene β-actin (31).

3.3.3 Effect of CCN2 on MMP and TIMP Protein

The concentration of MMPs and TIMPs in the culture medium and cell lysates is determined by ELISA and Western blot as described. MMP-2 activity and TIMP concentration in the media are also examined by zymography and reverse zymography as previously described in detail [12] (*see* **Note 8**).

For Western blot analysis, mouse monoclonal anti-MMP-2 antiserum is used as primary antibodies and the bands are visualized with horseradish peroxidase-conjugated goat anti-mouse IgG and chemiluminescence.

TIMP-1 and TIMP-2 protein levels in the cell-conditioned media are determined by ELISA (according to the manufacturer's instructions). These assays measure the MMP/TIMP complexes as well as free TIMP-1 or TIMP-2, respectively. TIMPs in the conditioned media are also determined by reverse zymography, using standard methods [12].

4 Notes

1. In our studies we have used HiTrap Heparin HP, Amersham Biosciences, and found them to be easy to use and reliable.
2. The addition of a proteolytic inhibitor is essential to prevent degradation of protein. We find that one CompleteTM tablet is sufficient for the inhibition of the proteolytic activity in 50 ml of conditioned medium. These tablets are useful as they cover all proteolytic enzymes.
3. In this work we have usually used primary mesangial cells but any cell of mesenchymal origin which produces large amounts of extracellular matrix could also be used.
4. We routinely collect the fractions from the low-salt elution step in 1 ml volumes to check that we are not eluting the protein of interest.
5. To prevent the rhCCN2 sticking to the wall of the dialysis tubing we add BSA to give a final solution in the tube of 0.05 %. In addition to avoid loss of protein with multiple thaws the desalted rhCCN2 is aliquoted into smaller volumes and stored at −80 °C.
6. The dose, found to increase the fibronectin mRNA [11] level at least two times greater than control, is considered to be the amount required to induce a biological effect. In our studies this is generally seen at 500 ng/ml.
7. For this step tip the cellular layer is visible as an opaque solution. To remove tip the plate at a 45° angle and pipette from the side to avoid aspirating the attached matrix.
8. The technique of zymography for measurement of MMP-2 and MMP-9 has the advantage over an ELISA in that you are able to measure the biologically active form of the MMP as the smaller (approximately 10 kDa) form.

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Chapter 45

An Analysis of Pathological Activities of CCN Proteins in Joint Disorders: Mechanical Stretch-Mediated CCN2 Expression in Cultured Meniscus Cells

Takayuki Furumatsu and Toshifumi Ozaki

Abstract

The multifunctional growth factor CYR61/CTGF/NOV (CCN) 2, also known as connective tissue growth factor, regulates cellular proliferation, differentiation, and tissue regeneration. Recent literatures have described important roles of CCN2 in the meniscus metabolism. However, the mechanical stress-mediated transcriptional regulation of CCN2 in the meniscus remains unclear. The meniscus is a fibrocartilaginous tissue that controls complex biomechanics of the knee joint. Therefore, the injured unstable meniscus has a poor healing potential especially in the avascular inner region. In addition, dysfunction of the meniscus correlates with the progression of degenerative knee joint disorders and joint space narrowing. Here, we describe an experimental approach that investigates the distinct cellular behavior of inner and outer meniscus cells in response to mechanical stretch. Our experimental model can analyze the relationships between stretch-induced CCN2 expression and its functional role in the meniscus homeostasis.

Key words CCN2, Meniscus, Meniscus cells, Mechanical stretch, Transcriptional regulation

1 Introduction

An articulating joint has many essential components such as the cartilage, meniscus, ligament, synovium, and subchondral bone. The joint homeostasis is regulated by physiological mechanical stimuli involved in weight-bearing, gait, and joint motion. Excessive mechanical stresses induced by malalignment, obesity, and muscle weakness can progress the degenerative joint disorders. Recent literatures have described that the dysfunction of the meniscus increases the contact pressure of the knee joint and induces cartilage degradation [1, 2]. In addition, extrusion (subluxation or displacement) and complex tear of the medial meniscus correlate with the progression of the knee osteoarthritis and joint space narrowing [3, 4]. Based on these studies, minimizing the meniscal damage may protect the onset of degenerative knee joint disorders including spontaneous osteonecrosis and osteoarthritis of the knee.

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We consider that an experimental approach for understanding the behavior of meniscus cells in response to mechanical stresses can lead to the prevention of degenerative knee joint disorders.

The meniscus is a fibrocartilaginous tissue that plays an important role in controlling complex biomechanics of the knee such as tensile strain, compression, and shear stress [5]. In the adult human meniscus, a perimeniscal capillary plexus supplies the outer one-third of the meniscus, whereas the inner two-thirds of the meniscus are composed of avascular tissue [6]. Avascular inner region of the meniscus has a more chondrocytic phenotype as compared with the outer meniscus [7–9]. Inner meniscus cells derived from the inner meniscus have chondrocytic morphology and an ability to produce type II collagen, a cartilage-specific extracellular matrix (ECM) component. On the other hand, outer meniscus cells show fibroblastic morphology and mainly synthesize type I collagen that can resist a circumferential tensile stress [8]. Previous studies demonstrate that mechanical stimuli regulate the expression of growth factors and ECM proteins in the menisci [7, 10–12]. Larger tensile strains (>7%) are computed in the inner region of the lateral meniscus compared with the outer regions [13]. However, the distinct regulation of growth factor and ECM production in response to mechanical stretch remains unclear in the meniscus.

CCN2, also known as connective tissue growth factor, is a classical member of the CYR61/CTGF/NOV (CCN) family that has a multifunctional role in cellular proliferation, differentiation, ECM synthesis, and tissue regeneration [14]. We have previously reported that CCN2 promotes the proliferation of meniscus cells [15] and anterior cruciate ligament-derived cells [16]. CCN2-incorporated fibrin glue enhances the healing of rabbit meniscal tears in the avascular inner zone [17]. In a sheep model, CCN2/transforming growth factor (TGF)-β3-releasing scaffolds restore inhomogeneous mechanical properties of the regenerated meniscus by inducing fibrochondrocyte differentiation of bone marrow-derived mesenchymal stem cells [18]. On the other hand, mechanical stress regulates the gene expression of CCN2 and TGF-β that is involved in CCN2 transactivation [19]. The TGF-β-regulated Smad2/3 signaling is considered a central inducer of CCN2 expression via association with the Smad-binding element (SBE) on the CCN2 promoter [14, 15, 20]. Effect of mechanical stretch on CCN2 expression is dependent on cell types and stretching force [10, 16, 21]. Secreted CCN2 stimulates the expression of several types of collagen genes in a cellular phenotype-dependent manner [16, 22]. Based on these literatures, mechanical stretch-induced CCN2 expression seems to have an important role in promoting the zone-specific meniscal healing. However, the mechanical stretch-dependent epigenetic CCN2 transactivation is unclear in the meniscus [23].

In this chapter, we describe an experimental approach that investigates the distinct cellular behavior of human inner and outer meniscus cells in response to mechanical stretch. Our experimental model can analyze the relationships between stretch-induced CCN2 expression and its functional role in the meniscus.

2 Materials

2.1 Meniscus Cell Preparation

1. Phosphate-buffered saline containing 2% penicillin/streptomycin.
2. 0.2% Collagenase solution.
3. Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
4. Cell strainer (pore size, 70 μm) or an equivalent.
5. ELP sterile disposable scalpel (No. 11 blade, Akiyama, Tokyo, Japan) or an equivalent.
6. Sterilized fine forceps and surgical scissors.

2.2 Stretching Experiment

1. Cell stretching system (STB-140, Strex, Osaka, Japan). *See* **Note 1**.
2. Polydimethylsiloxane (PDMS) stretch chambers (STB-CH-04/10, Strex). *See* **Note 1**.
3. 100 μg/mL of rat tail type I collagen solution in 0.2 N acetic acid. Store at 4 °C.

2.3 Chromatin Immunoprecipitation Assay

1. 37% Formaldehyde solution.
2. 2.5 M Glycine.
3. PBS. Keep on ice.
4. Cytoplasmic extraction buffer: 2 M KCl, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH = 7.5), 1 M $MgCl_2$, 100× protease inhibitor cocktail (PIC). Prepare the cytoplasmic extraction buffer at a final concentration of 10 mM KCl, HEPES, 1.5 mM $MgCl_2$, and 1× PIC. Keep on ice.
5. Nuclear extraction buffer: 5 M NaCl, 1 M HEPES, 1 M $MgCl_2$, 100× PIC. Prepare the nuclear extraction buffer at a final concentration of 420 mM NaCl, 10 mM HEPES, 1.5 mM $MgCl_2$, and 1× PIC. Keep on ice.
6. Sonication system (Handy Sonic UR-20P) or an equivalent.
7. Antibodies for chromatin immunoprecipitation (ChIP) use.
8. Protein G agarose (e.g., protein G plus-agarose immunoprecipitation reagent, Santa Cruz).
9. TE buffer: 10 mM Tris–HCl and 1 mM EDTA buffer (pH = 7.5).

10. IP elution buffer 1: TE buffer (pH = 8.0), 1% SDS.
11. IP elution buffer 2: TE buffer (pH = 8.0), 0.67% SDS.
12. Proteinase K (20 mg/mL).
13. 4 M LiCl.
14. Phenol, chloroform, isoamyl alcohol, ethanol.
15. Specific primer sets spanning 100–150 bp length.

3 Methods

3.1 Isolation of the Zone-Specific Meniscus Cells

Cells derived from the meniscus have several distinct populations such as rounded chondrocyte-like cells (inner meniscus cells, fibrochondrocytes), elongated fibroblast-like cells (outer meniscus cells), superficial zone cells, spindle-shaped progenitor cells, and vascular endothelial cells [8, 24, 25].

1. Wash the meniscal tissue by 2% penicillin/streptomycin-contained PBS (*see* **Note 2**).
2. Remove the anterior and posterior horns of the meniscus to prevent the contamination of ligamentous tissues using a sterile scalpel (Fig. 1a).

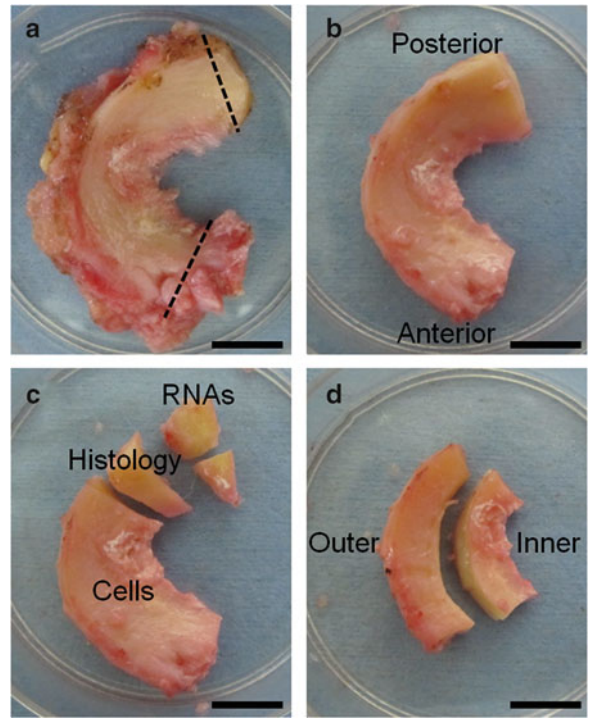

Fig. 1 (**a**) The lateral meniscus obtained from the right osteoarthritic knee. *Dashed lines* denote the margins of ligamentous insertions. (**b**) Prepared meniscal sample. (**c**) Divided samples for several experiments. IHC, immunohistochemistry. (**d**) Inner and outer meniscal tissues. Bars, 1 cm

3. Remove the synovial-meniscus transition zone and degenerated surface carefully using scissors and scalpel (Fig. 1b, *see* **Note 3**).
4. Keep small segments of the meniscal tissue for histological and other analyses (Fig. 1c).
5. Divide the remaining meniscal tissue into the inner and outer regions (Fig. 1d). Inner and outer halves are prepared by careful cut at the middle point of meniscal width (5–7 mm in length from the inner edge) using a scalpel.
6. Collagenase digestion: Divided inner and outer meniscal tissues are individually minced at the size of 2–3 mm fragments using scissors and scalpel. Minced meniscal tissues are digested with 0.2% collagenase solution using a rotator for 2 h at 37 °C. Centrifuge the digested samples and cells. Remove the supernatants. Add new collagenase solution to the samples. Rotate the digesting mix for 2–4 h at 37 °C (*see* **Note 4**).
7. Centrifuge the digesting mix and remove the supernatant. Add the FBS-contained DMEM.
8. Filter the samples mixed in the DMEM using cell strainers for removing the debris.
9. The filtrates contain the meniscus cells. Seed the cells at a density of 2×10^3 cells/cm^2 on a tissue culture dish. Harvest the cells for 1–2 weeks at 37 °C/5% CO_2 in a humidified atmosphere (*see* **Note 5**). Change the medium every 2–3 days.
10. Passage the cells when the primary culture reaches a confluent condition. Inner meniscus cells have a chondrocytic, small triangular morphology. Outer meniscus cells show a fibroblastic, slender morphology (*see* **Note 6** and ref. 4).
11. Cells passaged 1–4 are useful for the following experiments.

3.2 Cell Stretching Experiments

Coating efficiency, cellular density, and stretching force and duration affect the experiments. Other coating materials such as fibronectin and gelatin may be useful. A new chamber may be required to perform preliminary coating several times for obtaining successful cellular attachments on the well.

1. Stretch chamber coating: Sterilize the chambers using an autoclave machine. Plasma processing on the chambers will increase cellular adhesion on the surfaces. Fill the chamber wells with 100 μg/mL of type I collagen solution. Coat the PVDF membrane at room temperature for 12–24 h in a clean cabinet. Wash the wells twice with PBS. Dry up the wells in a clean cabinet. Seed the meniscus cells at 60–80% confluent conditions (*see* **Note 7**).
2. Attach the cells to the coated chambers for 12–24 h in a CO_2 incubator.

3. Set the chambers to STB-140 stretching system.
4. Choose a stretching pattern involved in the frequency (Hz) and elongation percentage (*see* **Note 8**).
5. Start cell stretching experiments in a CO_2 incubator. Stretching force may affect the expression pattern of interested genes immediately. Preliminary experiments must be required for the optimum combination of stretching frequency, degree, and time. CCN2 gene expression in inner meniscus cells is gradually up-regulated by 2–4-h treatment under 5 % stretching condition (*see* ref. 15).
6. Stop the stretching system.

3.3 ChIP Assay for the CCN2 Promoter

Cells derived from four STB-CH-10 chambers may be suitable for a single ChIP assay. Sufficient number of the cells may increase the success rate of ChIP assay. The effect of mechanical stretch on the CCN2 promoter activity seems to be higher in inner meniscus cells than in outer meniscus cells (*see* ref. 15). This phenomenon may be caused by the constant and relatively higher expression of CCN2 in outer meniscus cells. The location of specific primer sets for ChIP assay should be selected according to several putative DNA-binding sequences on the human CCN2 promoter (*see* ref. 20, 26).

1. Cross-link formation: After the stretching treatment, add formaldehyde at 1 % final volume in the medium. Prepare unstretched controls.
2. Shake the chambers gently for 20 min at room temperature.
3. Add glycine solution at a final concentration of 125 mM. Color of the medium will change into yellow.
4. Shake gently for 5 min at room temperature.
5. Wash the wells with cold PBS. Aspirate the PBS.
6. Scrape the cells with cold PBS and put them into a microtube.
7. Centrifuge for 3 min (800 × *g*) at 4 °C. Remove the supernatant.
8. Add cytoplasmic extraction buffer to the “cellular pellet” and pipet gently. Keep on ice for 10 min.
9. Centrifuge for 5 min (2000 × *g*) at 4 °C. Remove the supernatant.
10. Add 200 μL of nuclear extraction buffer to the “nuclear pellet” and pipet gently. Keep on ice for 10 min.
11. Sonicate (10-s sonication, 2–3 times) the nuclear mixture on ice (*see* **Note 9**).
12. Centrifuge for 10 min (15,000 × *g*) at 4 °C. Transfer the supernatant to a new tube (approx. 200 μL).

13. Keep 10% volume of the sample as an input fraction (20 μL). Divide the remaining sample into half as ChIP sample and negative control (90 μL each).
14. Add an antibody that recognizes transcriptional complexes on the CCN2 promoter region into the ChIP sample according to the manufacturer's protocol (endogenous molecules, *see* ref. 17; overexpressed proteins, *see* ref. 22). For negative control sample, add a control IgG derived from same species of the antibody.
15. Add 10 μL of protein G plus-agarose reagent into the ChIP and control samples.
16. Rotate the samples for 1–12 h at 4 °C.
17. Wash the agarose pellets with nuclear extraction buffer twice. Short spin. Remove the supernatants.
18. Add 50 μL of IP elution buffer 1 to the agarose pellet. Keep the samples at 65 °C for 15 min. Centrifuge for 3 min (15,000 × *g*). Transfer the supernatant to a new tube.
19. Add 75 μL of IP elution buffer 2 to the agarose pellet. Keep the samples at 65 °C for 15 min. Centrifuge for 3 min (15,000 × *g*). Transfer the supernatant to the tube of **step 18**. Mix gently.
20. Add TE buffer (pH = 7.5, 100 μL) and 10% SDS (5 μL) to the input fractions (*see* **Note 10**).
21. Keep the tubes at 65 °C for 4–8 h to reverse the cross-link. Centrifuge for 3 min (15,000 × *g*).
22. Add 2 μL of Proteinase K solution to digest protein complexes on the DNA fragments. Keep the samples at 37 °C for 2 h.
23. Add 12.5 μL of 4 M LiCl to the samples. Add 140 μL of phenol/chloroform/isoamyl alcohol (25:24:1) solution. Invert the mixture several times. Centrifuge for 2 min (15,000 × *g*). Transfer the upper aqueous phase to a new tube.
24. Add 140 μL of chloroform/isoamyl alcohol (24:1) solution. Invert the mixture several times. Centrifuge for 2 min (15,000 × *g*). Transfer the upper aqueous phase to a new tube.
25. Add 300 μL of 100% ethanol. Mix well. Keep the samples at −20 °C for 1–12 h.
26. Centrifuge at 4 °C for 1 h (15,000 × *g*). Aspirate the supernatant carefully.
27. Dry the DNA fragments. Add 10 μL of sterilized distilled water and dissolve the DNA samples. Store at −20 °C.
28. PCR reactions using the specific primer sets for detecting interested regions of the CCN2 promoter.

4 Notes

1. The STB-140 cell stretching system can induce mechanical stimuli to cultured cells using PDMS stretch chambers. Stretch ratio (degree of elongation) and stretching frequency (repetition frequency/interval of stretch) can be selected from several programmed patterns.
2. Meniscal samples are usually obtained at total knee arthroplasty in elderly patients suffering from medial compartmental osteoarthritis of the knee. The lateral meniscus may be useful for experiments. However, excessive degeneration and calcification are sometimes observed even in the lateral meniscus. These highly degenerated menisci are not suitable for cellular experiments.
3. The superficial zone (approximately 100 μm thickness) of the meniscus is often lost in the end stage of osteoarthritic knees.
4. Additional exchange of collagenase solution may be required for sufficient digestion of meniscal samples. However, an excessive digestion time will decrease the survival rate of meniscus cells.
5. If cell isolation using the collagenase digestion does not work, explant cultures may be suitable. Minced meniscal tissues are seeded on dishes and maintained in the culture media for 2 weeks. Meniscal tissues show the outgrowth of each cell.
6. CCN2 has a distinct effect on type I and II collagen gene expressions in each meniscus cell (*see* ref. 15). Recombinant human CCN2 (BioVender, Candler, NC) increased the expression of type I and II collagen genes in inner meniscus cells. However, the expressions of these genes were not affected by CCN2 treatment in outer meniscus cells (*see* ref. 15).
7. Cellular density is critical for each experiment. Subconfluent condition is suitable for mRNA, protein, immunoprecipitation, and enzyme-linked immunosorbent assays under several stretching conditions. Sparse (30–50% confluent) condition is recommended to immunohistological analysis.
8. Note the relationship between the chamber size and stretching length. One millimeter elongation is 5% stretch for STB-CH-04. In the use of STB-CH10, 1.6 mm elongation is 5% tensile strain. Meniscus cells may be detached by severe stretching condition (more than 6-h treatment under 10–15% strain).
9. The degree of sonication (power, duration, and the number of times) may affect the fragmentation status of the chromatin. Preliminary experiments are required to reduce nonspecific PCR fragments in negative control samples. Heat may induce

the damage of transcriptional complexes on interested DNA fragments.

10. Three tubes (input, ChIP sample, and negative control sample; 125 μL each) are prepared from one material.

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Chapter 46

Analysis of CCN Protein Expression and Activities in Vasoproliferative Retinopathies

Sangmi Lee, Menna Elaskandrany, Afruja Ahad, and Brahim Chaqour

Abstract

The retina is a complex neurovascular structure that conveys light/visual image through the optic nerve to the visual cortex of the brain. Neuronal and vascular activities in the retina are physically and functionally intertwined, and vascular alterations are consequential to the proper function of the entire visual system. In particular, alteration of the structure and barrier function of the retinal vasculature is commonly associated with the development of vasoproliferative ischemic retinopathy, a set of clinically well-defined chronic ocular microvascular complications causing blindness in all age groups. Experimentally, the retinal tissue provides researchers with a convenient, easily accessible, and directly observable model suitable to investigate whether and how newly identified genes regulate vascular development and regeneration. The six mammalian CCN gene-encoded proteins are part of an extracellular network of bioactive molecules that regulate various aspects of organ system development and diseases. Whether and how these molecules regulate the fundamental aspects of blood vessel development and pathology and subsequently the neurovascular link in the retina are open-ended questions. Sophisticated methods have been developed to gain insight into the pathogenesis of retinal vasculopathy. This chapter describes several useful methodologies and animal models to investigate the regulation and potential relevance of the CCN proteins in vasoproliferative diseases of the retina.

Key words CCN, Angiogenesis, Retinopathy, Transgenic reporter mice, Diabetes, Antibodies

1 Introduction

The retina is a neurovascular tissue that lines the back of the eye. It contains numerous cell types including light-sensitive cells, rods, and cones, which collect and allow for the processing of visual information (Fig. 1). The macula is a small, central portion of the retina, which contains a specialized network of rod cells that allow for focused vision. The rest of the retina is responsible for peripheral vision. Behind the macula is the optic nerve that transmits visual information from the retina to the brain. The retina is in fact an extension of the brain tissue. It also is metabolically active and highly sensitive to changes in oxygen and nutrient levels. The vascularization of this tissue is assumed by two vessel systems [1]: the

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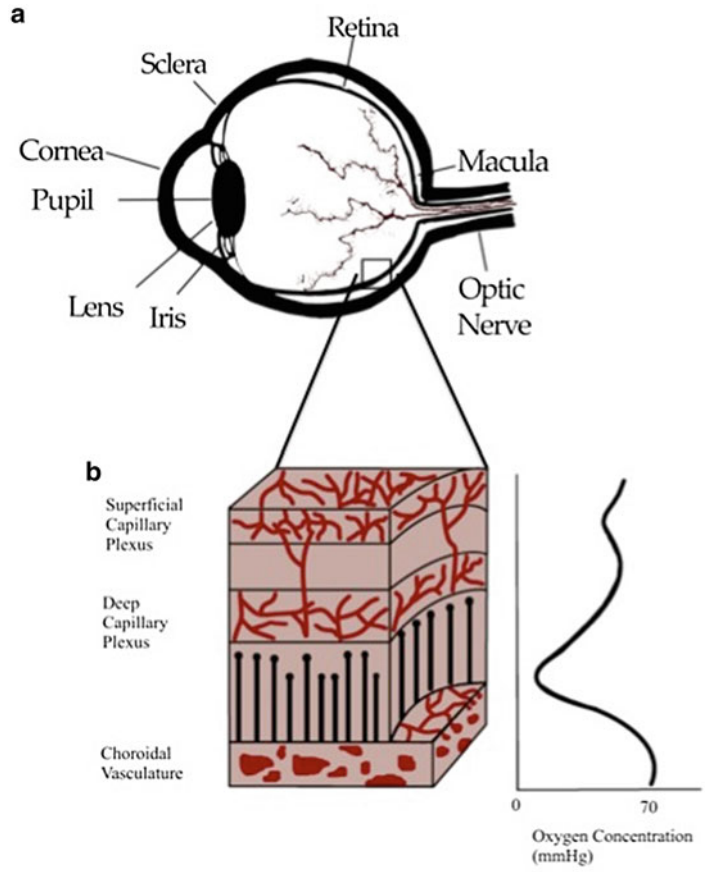

Fig. 1 Schematic representation of the eye and retina. (**a**) Cross section of the eye. (**b**) Cross section of the retina with a graphical representation of retinal and choroidal vessels as well as oxygen levels

choroidal and the retinal circulation, which are vital for the continuous supply of oxygen and nutrients to the neural retina. The choroidal arteries arise from long and short posterior ciliary arteries that pierce the sclera around the optic nerve to form three vascular layers of the choroid [2]. On the other hand, a central retinal artery branches to form superficial arteries that invest the retina in three vascular plexuses (superficial, intermediary, and deep plexuses). The choroid receives the largest blood flow (65–85%) and nourishes the outer part of the retina (photoreceptors), while the smaller part of the flow (20–30%) received by retinal vessels maintains the inner layers of the retina [3].

Retinopathies are ocular diseases that damage the structure of the retina, resulting in its deterioration and gradual loss of vision. They are characterized by vaso-obliteration and subsequent overgrowth of new but leaky blood vessels which further cause retinal detachment and blindness. Vascular disorders of the retina include

retinopathy of prematurity (ROP), proliferative diabetic retinopathy, wet age-related macular degeneration, and retinal vein or artery occlusion.

The CCN (short for CYR61/CTGF/NOV) family of genes comprises six members whose encoded proteins share several structural features, but they all are functionally distinct [4–7]. By far, the first two members, CCN1 and CCN2, have been the most studied with respect to their role in vasoproliferative disorders of the eye because their expression fluctuates during vascular tissue development and in vascular diseases [8, 9]. Approaches using mouse genetics showed that CCN1, 2, and 5 were required for embryonic development and exhibit vascular specific functions while the other three CCN proteins (CCN 3, 4, and 6) were found to exhibit relevant functions in adult tissues [8, 10]. However, more studies are needed to better understand the role of all these molecules in vasoproliferative retinopathy and their potential use as disease markers or therapeutic targets.

This review describes some of the sophisticated methodologies and animal models useful to study the regulation and function of the CCN proteins in ischemic retinopathy. These methods include target modification of bacterial artificial chromosomes (BAC), the Cre/lox P recombinase system, oxygen-induced retinopathy (OIR) model, choroidal neovascularization (CNV), streptozotocin (STZ)-induced diabetic retinopathy, and immunostaining immunochemistry.

1. Targeted modification of BAC and generation of CCN promoter reporter transgenic mouse strains that express green fluorescent protein (GFP) under a CCN gene promoter allow for the analysis of expression of various CCN genes. They also allow for the identification of cellular sources of their encoded proteins in the retina.
2. The Cre/lox P system allows for the design and implementation of precise genetic manipulations in the mouse and helps determine the function of a given CCN gene in a specific cell type or tissue [11, 12]. In conjunction with inducible systems for controlling Cre recombinase expression and function, this recombination-based strategy can provide useful animal models of gain- or loss-of-function situations.
3. The mouse model of OIR is used to study the regulation and function of the CCN proteins during vaso-obliteration and ischemia-induced neovascularization [13]. Mice are exposed to hyperoxic conditions, which results in vaso-obliteration of the retina, which can then be dissected and studied.
4. CNV aims to induce vascular alterations, which are associated with wet macular degeneration in humans, by inflicting cuts to the Bruch's membrane in the retina.

5. STZ injection allows for the induction of diabetes, and analysis of the associated effects of hyperglycemia on the retinal vasculature.
6. Immunohistochemistry evaluates the cell and tissue localization of the CCN proteins alone or with other proteins in retinal tissues.

2 Materials

2.1 Wild-Type and Transgenic CCN Promoter Reporter Mouse Strains

1. Wild-type and knockout mice in the C57BL/6 background (*see* **Note 1**).
2. Modified BAC RP23-2P12, RP24-96J1, and RP23-235B13 for CCN1, CCN2, and CCN3, respectively.
3. Expression vectors encoding CCN1, 2, 3.
4. Green fluorescent protein (GFP) reporter gene.

2.2 Tamoxifen and Streptozotocin (*See* Note 2)

1. Tamoxifen solution: Tamoxifen, 100% ethanol, corn oil (5–10 mg/kg).
2. Streptozotocin (50 mg/kg).

2.3 Hyperoxia Chamber and Laser-Induced Choroidal Neovascularization

1. Oxygen chamber (PRO-OX, Biospherix Ltd) or an equivalent.
2. Argon green ophthalmic laser.
3. A slit-lamp biomicroscope delivering laser spots (100 μm each, 532 nm, 150 mW, 0.1 s).

2.4 Immunostaining Immunohistochemistry

1. Blocking buffer: 2% Fetal bovine serum, 1% bovine serum albumin, and 0.01% Triton X-100 in phosphate buffer solution (PBS).
2. 4′,6-Diamidino-2-phenylindole (DAPI).
3. Fluorescence microscope.
4. ImagePro Plus (version 5.1) software.
5. Cryopreservative solution (OCT compound).
6. Cryostat.

3 Methods

3.1 Targeted Modification of BAC and Generation of CCN Promoter Reporter Transgenic Mice

Transgenic reporter mice expressing GFP or a reporter gene under the control of CCN gene promoter represents a powerful resource for analyzing the expression of the various CCN genes and identifying the cellular sources of their encoded proteins in complex organ systems such as the retina. Mouse genotypes are modified to contain multiple copies of a modified BAC vector in which the

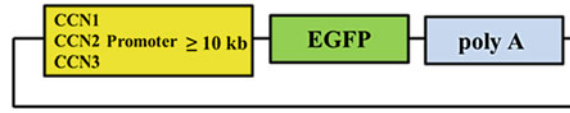

Fig. 2 Schematic representation of the CCN-promoter GFP-reporter construct used to generate transgenic CCN-GFP mice

GFP reporter gene is inserted immediately upstream of either CCN1, CCN2, or CCN3 promoter [14] (Fig. 2). Visualization of GFP expression in specific neuronal or vascular cell populations in the retina provides compelling evidence that this strategy can result in the identification of the cellular sources of CCN proteins, as the detailed morphology of GFP-expressing cells is apparent. There are three available transgenic CCN-promoter GFP-reporter mouse strains that have been developed under the NINDS-funded GENSAT BAC transgenic project and include CCN1-, CCN2-, and CCN3-promoter reporter transgenic mice. The use of these transgenic mice helps map CCN gene expression at cellular resolution in the neural and vascular beds and allows for the isolation of, if needed, specific CCN-positive cell populations by fluorescence-activated cell sorting. Using these transgenic lines, we and others were able to pinpoint the cellular sources of CCN1 and CCN2 in the retina [10, 15]. CCN1 promoter-driven GFP reporter localized largely in the endothelium in the retina while CCN2 promoter-driven GFP reporter expression was found in multiple ocular tissues with relatively high levels in the corneal endothelium, lens subcapsular epithelium, and vasculature of the iris and retina. Development of transgenic CCN4, 5, and 6 reporter mouse lines is needed to map out the expression and cellular sources of these remaining molecules.

The basic strategy for the generation of transgenic CCN promoter-reporter mice consists of:

1. Selecting the gene to be analyzed (e.g., CCN1, 2, 3, 4, 5, or 6).
2. Preparing modified BACs and substituting the CCN gene sequence by the GFP reporter gene.
3. Generating transgenic lines carrying each reporter construct.
4. Analyzing reporter gene expression in several lines during development and in the adult animal under physiological conditions or in response to a specific stimulus/challenge, e.g., ischemia, hypoxia, diabetes, and hyperlipidemia.

This approach presents the advantage of increased sensitivity of the reporter gene, particularly in BAC transgenic lines carrying multiple copies of the BAC transgene. Therefore, detection of sites of expression that are not evident using for instance in situ hybridization experiments is paramount (*see* **Note 3**).

3.2 Cre/lox P Recombination System

Cre-lox P recombination is the targeting of a specific gene sequence and splicing it out with the help of the cyclization recombination (Cre) protein. The Cre recombinase is a site-specific enzyme that causes DNA recombination at specific loxP sites, which contain specific binding sequences for the Cre gene, and flank the gene of interest. Specific exon (s) of the gene of interest is (are) flanked by loxP sites (floxed sites) that are inserted by homologous recombination. The gene can then be altered in the forms of insertions, deletions, or translocations. Strains expressing the Cre recombinase under the control of specific promoters are created by either classic microinjection of an exogenous transgenic construct or by placing the Cre gene under the control of an endogenous promoter. In the presence of a floxed allele, the expressed Cre protein binds to the two loxP sites and precisely removes the DNA between them, leaving a nonfunctional or truncated gene behind that carries a single loxP site (Fig. 3).

Mice with floxed CCN1, CCN2, and CCN3 genes have been generated and used in various ways to determine the gene function in a specific cell lineage [16–18]. Mice with CCN floxed genes are healthy with normal CCN function. A particularly powerful feature of conditional CCN gene inactivation strategy using Cre is that the same loxP-tagged mouse can be used for gene ablation independently in a large number of different tissues, or at different developmental stages, by simply breeding it with a corresponding Cre transgenic mouse that displays the desired tissue or temporal specificity of Cre expression. By the same token, bypassing embryonic lethality of CCN gene deletion requires an inducible conditional

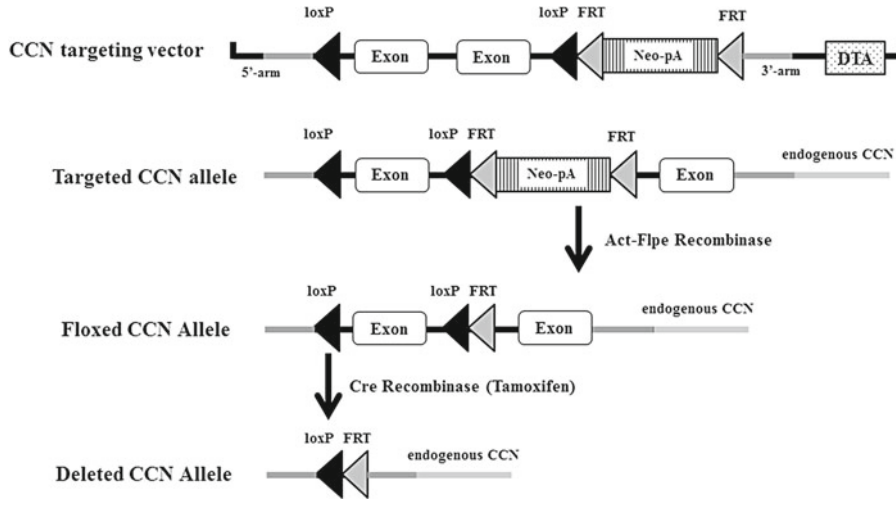

Fig. 3 Schematic representation of conditional gene deletion by Cre recombinase. The target endogenous gene (e.g., CCN gene) is modified by homologous targeting in embryonic stem cells so that the first exon is flanked by two directly repeated loxP sites. Mice containing such a modified gene are then crossed with mice expressing Cre in the desired target tissue, and Cre-mediated excision results in tissue-specific gene ablation

Table 1
Transgenic Cre mouse line commonly used to target Cre recombinase expression to specific tissues

Cre strain	Target cells	Reference
Lyz2tm1(cre)Ifo	Macrophages	[43]
Tg(Cdh5-cre/ERT2)1Rha	Endothelium	[44]
Tg(Myh11-cre/ERT2)1Soff	Smooth muscle	[45]
Tg(GFAP-cre/ERT2)505Fmv	Astrocytes	[46]
Chattm1(cre)Lowl	Amacrine cells	[47]
Tg(Pcp2-cre)3555Jdhu	Rod bipolar cells	[48]
Tg(Pdgfra-cre)1Clc	Müller glial cells	[49]
Tg(Grik4-cre)G32-4Stl	Ganglion cells	[50]
Tg(Tyrp1-cre/ERT2)1Mtz	Retinal pigment epithelium (RPE)	[51]

knockout approach combining the CCN-floxed mouse model with an inducible tissue-specific promoter mouse line (e.g., $CreER^{T2}$). As $CreER^{T2}$ is inactive until exposed to tamoxifen, this combination of alleles allows tissue-specific deletion of a CCN gene at different stages of development and adult life, permitting a high degree of manipulability (Table 1). Using this strategy, Chintala et al. have shown that endothelium-specific deletion of CCN1 results in severe retinal vascular defects including loss of hierarchical organization of blood vessels in arteries, capillaries, and veins and formation of a syncytium-like vasculature with wide lumen vessels [10]. Using a conditional deletion mouse model of CCN2, Fontes et al. demonstrated a minor role of CCN2 in heart fibrosis [19]. This same strategy can be applied successfully to other CCN genes as well.

The following protocol is used for the induction of tissue-specific gene deletion through administration of tamoxifen:

1. Dissolve 10 mg of tamoxifen in 250 μl of 100% ethanol and add 750 μl of corn oil. For injection, dilute stock solution at ratio of 1:10 in corn oil.
2. Using a 30-gauge needle insulin syringe, inject 50 μl of tamoxifen solution into the intraperitoneal cavity.
3. Repeat the injection once every day for 3 days.
4. Animals are then left untreated or challenged with a stimulus, e.g., hyperglycemia, hypoxia, and hyperlipidemia. The retinal vascular phenotype is then analyzed (*see* **Note 4**).

3.3 Model of OIR

The mouse model of OIR is well suited to study the regulation and function of the CCN proteins during hyperoxia-induced vaso-obliteration and subsequent ischemia-induced

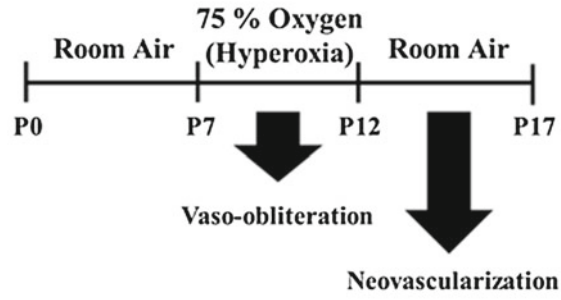

Fig. 4 OIR model. Neonatal mice are placed in normoxic conditions from P0 to P7. From P7 to P12, they are then placed in a hyperoxia chamber (75 % oxygen), followed once more by normoxic conditions from P12 to P17. Retinal vasculature is analyzed at P17 when maximal neovascularization occurs

neovascularization [20]. This model has been commonly used to replicate ROP in humans. In this model, mouse pups are placed in a 75 % oxygen environment for up to 5 days (Fig. 4). The hyperoxic environment results in vaso-obliteration of the central retinal area with capillary loss as well as peripheral vascularization. Subsequent return to normoxic conditions leads to regeneration of blood vessels that grow abnormally outside the retina, a characteristic feature of ROP [21, 22]. Mouse retinas are dissected, and the effects of OIR can be observed by immunohistochemical staining of blood vessels [23]. Using this model, our group has previously demonstrated that following OIR, CCN1 and CCN2 gene expression was repressed during the hyperoxic phase [24, 25]. The hyperoxic environment primed the CCN1 gene to remain repressed during the subsequent ischemic phase of OIR. Conversely, CCN2 gene expression was upregulated concomitantly with the development of abnormal blood vessels during the ischemic phase. This model is well suited to study differential CCN1 and CCN2 gene expression and regulation and the transcriptional or/posttranscriptional events involved in this process.

The following protocol is used to effectively induce OIR in mice:

1. Keep neonatal mouse pups under normal oxygen conditions from P0 to P7.
2. At P7, transfer mice to oxygen chamber.
3. Set the oxygen level to 75 %.
4. Keep mice in the oxygen chamber until P12.
5. At P12, bring back mice to normoxic conditions until P17.
6. Sacrifice the mice and enucleate the eyes.
7. Dissect retinas following immunostaining protocol (*see* Subheading 3.6).

3.4 Model of CNV

Choroidal neovascularization (CNV) is the formation of new blood vessels in the choroids of the eye. CNV recapitulates the vascular alterations associated with wet macular degeneration, one of the leading causes of visual loss in adult humans [26]. CNV occurs rapidly upon injury to Bruch's membrane, a unique laminar structure strategically located between the retinal pigment epithelium (RPE) and the fenestrated choroidal capillaries of the eye [27]. Subsequently, subretinal blood vessel growth from the choroid occurs. This results in the formation of new leaky and disorganized blood vessels that hemorrhage which deteriorates central vision [13]. CNV can be replicated in animal models through laser injury that perforates the Bruch's membrane.

The following protocol is used to induce CNV in mice:

1. Anesthetize adult mice (10–12 weeks) through intraperitoneal injection of ketamine (14 mg/kg) and xylazine (30 mg/kg).
2. Dilate pupils with ophthalmic tropicamide (0.5%) and phenylephrine (2.5%).
3. Use an argon green ophthalmic laser coupled with a slit lamp (100 ms pulse at 200 mW with a 50 μm spot size) to create ruptures in Bruch's membrane. The ruptures should be created at noon, 3, 6, and 9 O'clock positions around the optic nerve. Efficacy of laser-induced injury is confirmed upon appearance of a bubble [26].
4. Sacrifice the mice at different time points. The eyes are dissected to isolate the posterior segment consisting of the retinal pigment epithelium, the choriocapillaris, and the sclera and analyzed for histological and structural alterations.
5. To detect the vessels within the CNV lesion, the posterior cups should be cut with 4–7 radial slices, and mounted flat on microscope slides and stained for digital image capture [28, 29] (*see* **Note 5**).

3.5 Experimental Models of Diabetic Retinopathy

Injection of STZ to rodents destroys β-cells in the pancreas and inhibits the production of insulin which generates type 1 diabetes model animals. STZ is a glucosamine-nitrosourea compound that was originally identified as an antibiotic, but is also known to have an anticancer effect [30]. The STZ model of autoimmune diabetes allows studies of the early effects of hyperglycemia on the retinal vasculature. The following protocol is used to induce diabetic retinopathy in mice:

1. Weigh the appropriate amount of STZ, so the final concentration in sodium citrate buffer will be 7.5 mg/ml.
2. Inject STZ solution 50 mg/kg intraperitoneally for 5 days to fasting mice.
3. Measure glucose level for 4 weeks post-injection for sufficient levels of hyperglycemia.

STZ-induced experimental diabetes can be induced in both rats and mice. In the mouse model, the blood glucose level reaches over 500 mg/dl after 1 month of diabetes, compared with about 100–120 mg/dl in control mice. Retinas can be studied over the course of 14 weeks after the onset of diabetes [31, 32] (*see* **Note 6**). STZ-induced hyperglycemia commonly results in reactive endothelium, dilated thickened capillaries with edema, acute inflammation, and apoptosis of the inner retinal neurons [33]. However, pathological neovascularization does not develop in these animals, which is typically found in severe diabetic patients. Several studies have shown that CCN1 and CCN2 were upregulated in the retina upon STZ-induced diabetes and that combining anti-CCN2 with anti-VEGF therapy was beneficial in reducing basement membrane thickness and vessel permeability in the STZ model of diabetes [34, 35]. Chemically induced diabetes is appropriate to use when testing drugs or therapies targeting CCN proteins to lower blood glucose in a non-beta-cell-dependent manner (*see* **Note 7**).

3.6 Immunostaining and Immunohistochemistry

Immunohistochemistry (IHC) is commonly and extensively used to evaluate the colocalization of the CCN proteins with other proteins in retinal tissues. The method allows for easy immunodetection of proteins, although the use of highly specific antibodies is required. Tissue processing and staining are performed on flat-mounted or cross-sectioned retinas as follows:

1. Place the enucleated eyes in a dish filled with PBS.
2. Fix the tissue for 30 min in 4% paraformaldehyde (PFA) and PBS.
3. Isolate the intact retinas under a dissecting microscope.
4. Flatten the retina through four equally distant cuts using scissors.
5. Wash the retinas three times in PBS for 10 min and incubate overnight in appropriate blocking buffer.
6. Incubate the retinas with primary antibody in blocking buffer overnight at 4 °C.
7. Wash the retinas three times in PBS for 10 min and incubate with secondary antibody in blocking buffer overnight at 4 °C.
8. Wash three times in PBS for 10 min and then mount retinas with DAPI using a cover slip.
9. Capture the images using a fluorescence microscope.

Cross-sectioning and staining of retina are performed as follows:

1. After dissection, incubate the retinas overnight in 10% sucrose.
2. Incubate the retinas overnight in 20% sucrose/PBS.
3. Incubate the retinas again overnight in /30% sucrose/PBS.

4. Embed a retina block with Neg 50 tissue freezing solution.
5. Cut 7 μm frozen retinal sections using a cryostat.
6. Wash the retina sections three times with PBS for 10 min.
7. Block the retinas using blocking buffer for 1 h at room temperature.
8. Incubate primary and secondary antibodies as described above for flat-mounted retinas and proceed to visualization under a fluorescence microscope.

4 Notes

1. The pattern and time course of vascularization of the retina can vary among different mouse strains. Differences in genetic background in mice result in variation in branch patterning of the retinal arterial circulation, and these differences predict strain-dependent differences in the retinal vascular response to injurious stimuli [36]. It is highly recommended to use inbred mice in the C57BL/6J as their genetic homogeneity allows researchers in different laboratories to independently replicate results without the genetic background of the model being a confounding factor.
2. Tamoxifen and streptozotocin are presumed human reproductive toxicant (teratogen) and are classified as hazardous drugs. Appropriate controls, personal protective equipment, and disposal techniques are required when handling these drugs as described in their Material Safety Data Sheets.
3. A reproducible pattern of gene expression must be verified using multiple transgenic lines as position effects of integration of a transgene into the genome may give rise to different gene expression patterns among mouse lines.
4. Neovascularization and angiogenesis occur in the first few days after birth of mice, so in order to study the formation of vessels and early plexuses, tamoxifen must be injected on 3 consecutive days (P1–P3). However, if the study is more focused on vessel remodeling and the formation of deeper plexuses, tamoxifen must be injected on 4 consecutive days (P5–P8) as vascular remodeling occurs between P8 and P15. Vascular homeostasis occurs in adult (>8 weeks old) mice, so if that is to be investigated, mice are injected with tamoxifen on 5 consecutive days.
5. The CNV model recapitulates many of the clinical manifestations of CNV in humans. This laser-induced model develops neovascularization in a relatively short period of time (days to weeks). Thus, it is ideal for testing drugs targeting CCN proteins [28].

There are several other models of CNV (e.g., surgically induced or transgenic mouse models) [37]. However, these models present several caveats related to the time to develop CNV (measured in months to years), course of progression, and variability of the size and appearance of the CNV lesion.

6. Alternatively, alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil) can be used instead of STZ to induce diabetes [38]. The diabetic effect of alloxan is mainly attributed to rapid uptake by the β-cells and the formation of free radicals, which β-cells have poor defense mechanisms against [39]. In either STZ- or alloxan-induced diabetes model, diabetes affects virtually neuronal, glial, and vascular cells in the retina.
7. There are numerous other diabetes models of animals that can be used to study the regulation and function of the CCN proteins. These include the non-obese diabetic (NOD) mouse and the biobreeding (BB) rat, two models of spontaneous autoimmune type 1 diabetes [40]; the AKITA mouse that was derived in Akita, Japan, from a C57BL/6NSlc mouse with a spontaneous mutation in the insulin 2 gene preventing correct processing of pro-insulin [41]; $Lep^{ob/ob}$ mouse, which is deficient in leptin; and the $Lepr^{db/db}$ mouse and Zucker diabetic fatty rat, which are deficient in the leptin receptor [42]. These and other animal models of diabetes may be appropriate and valuable models for studies of the functional relevance of the CCN proteins in the associated retinal vascular alterations.

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