CCN1 Regulates Chondrocyte Maturation and Cartilage Development

Yongchun Zhang1,2, Tzong-jen Sheu1,3, Donna Hoak1,3, Jie Shen4, Matthew J Hilton5, Michael J Zuscik1,3, Jennifer H Jonason1,3,*, and Regis J O’Keefe4

1Center for Musculoskeletal Research, University of Rochester Medical Center, Rochester, NY, USA
2Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, NY, USA
3Department of Orthopaedics and Rehabilitation, University of Rochester Medical Center, Rochester, NY, USA
4Department of Orthopaedic Surgery, Washington University School of Medicine, St. Louis, MO, USA
5Department of Orthopaedic Surgery, Duke University, Durham, NC, USA

Abstract

WNT/β-CATENIN signaling is involved in multiple aspects of skeletal development, including chondrocyte differentiation and maturation. Although the functions of β-CATENIN in chondrocytes have been extensively investigated through gain-of-function and loss-of-function mouse models, the precise downstream effectors through which β-CATENIN regulates these processes are not well defined. Here, we report that the matricellular protein, CCN1, is induced by WNT/β-CATENIN signaling in chondrocytes. Specifically, we found that β-CATENIN signaling promotes CCN1 expression in isolated primary sternal chondrocytes and both embryonic and postnatal cartilage. Additionally, we show that, in vitro, CCN1 overexpression promotes chondrocyte maturation, whereas inhibition of endogenous CCN1 function inhibits maturation. To explore the role of CCN1 on cartilage development and homeostasis in vivo, we generated a novel transgenic mouse model for conditional Ccn1 overexpression and show that cartilage-specific CCN1 overexpression leads to chondrodysplasia during development and cartilage degeneration in adult mice. Finally, we demonstrate that CCN1 expression increases in mouse knee joint tissues after meniscal/ligamentous injury (MLI) and in human cartilage after meniscal tear. Collectively,
our data suggest that CCN1 is an important regulator of chondrocyte maturation during cartilage development and homeostasis.

Keywords
CCN1; β-CATENIN; CHONDROCYTE; CARTilage

Introduction
During endochondral ossification, chondrocytes differentiate from condensing mesenchymal stem cells (MSCs) to form a cartilage template for bone formation. A key step in chondrogenesis involves expression of the transcription factor SOX9. SOX9 directly induces the expression of Col2a1 and Acan, markers of committed, nonhypertrophic chondrocytes.\(^1,2\) Committed chondrocytes then proliferate rapidly until those most central to the template exit the cell cycle and undergo hypertrophy. Hypertrophic chondrocytes express Col10a1 and Vegf, as well as other factors that lead to mineralization and vascular invasion of their matrix. As hypertrophic chondrocytes terminally differentiate, they begin to express Mmp13 and osteopontin and eventually undergo apoptosis. Vascular channels grow into the lacunae, replacing apoptotic chondrocytes. MSCs associated with these channels differentiate into osteoblasts and form bone on the ossified cartilage template.\(^3\) At the molecular level, WNT/β-CATENIN, TGF-β/BMP, IHH, FGF, and NOTCH signaling pathways, among others, are involved in regulating the complex processes of chondrocyte maturation and bone formation during embryonic skeletogenesis.\(^4\)

Postnatally, a secondary ossification center (SOC) develops in the epiphyses of the limbs separating the growth plate cartilage from the articular cartilage. The articular cartilage is avascular and divided into distinct zones varying in matrix composition. Unlike growth plate chondrocytes, articular chondrocytes in the unmineralized articular cartilage matrix do not proliferate rapidly or readily undergo hypertrophy. Although there are hypertrophic chondrocytes in the mineralized articular cartilage layer, they do not promote vascular invasion or bone formation like the hypertrophic chondrocytes of the growth plate. Many of the same signaling pathways that guide limb development during embryogenesis are also important in maintenance of the postnatal articular cartilage. Disruption or hyperactivation of these signaling mechanisms, however, can lead to aberrant articular chondrocyte hypertrophy and pathogenesis of the cartilage. In osteoarthritis (OA), for example, the articular chondrocytes exhibit reduced expression of important genes that maintain the cartilage matrix, including Sox9, Col2a1, and Acan. Instead, the markers of hypertrophic chondrocytes, Col10a1 and Runx2, are expressed, along with catabolic genes Mmp13 and Adams4/5. Thus, OA is associated with a shift in chondrocyte gene expression toward a state of hypertrophy and cartilage catabolism. Further understanding of the growth factors and signals involved in the maturation of chondrocytes during the onset and progression of OA will define key targets to control this disease process.\(^5\)

WNT/β-CATENIN signaling controls myriad events in the development and homeostasis of multiple tissues, including stem cell self-renewal and cell differentiation.\(^6\) Murine models
of β-CATENIN loss-of-function (LOF) or gain-of-function (GOF) reveal that β-CATENIN either inhibits or promotes endochondral bone formation in a cell-type and stage-specific manner. For example, β-CATENIN inhibits differentiation of mesenchymal stem cells to all lineages but promotes osteogenesis of osteochondroprogenitor cells while suppressing chondrogenesis.\(^7,8\) Once a committed chondrocyte, however, β-CATENIN promotes chondrocyte survival, proliferation, and maturation.\(^9\) Postnatally, chondrocyte-specific β-catenin gene deletion in mice results in apoptosis of articular chondrocytes and cartilage degeneration.\(^10\) In contrast, constitutive activation of β-CATENIN signaling in articular chondrocytes promotes the expression of Col10a1 and Mmp13, markers of chondrocyte hypertrophy.\(^11\) The knee joints of these mice display phenotypes consistent with the development of OA, such as degeneration and osteophyte formation. Thus, an appropriate balance of β-CATENIN signaling is necessary for normal articular cartilage metabolism and for maintenance of the joint surface. Although β-CATENIN function in the chondrocyte has been well characterized, the precise molecular mechanisms and downstream mediators involved are still unclear.

CCN1 (also known as CYR61; cysteine-rich protein 61) is a member of the family of CCN (CYR61, CTGF, and NOV) proteins, which includes six members, CCN1-6.\(^12\) All are matricellular proteins that share common structural domains. They are localized to the extracellular and pericellular matrix, where they bind to both growth factors as well as to distinct membrane receptors, including integrins. The binding of matricellular proteins to membrane receptors leads to the activation of signaling pathways that modulate cell functions, such as cell proliferation, adhesion, migration, survival, and differentiation. CCN1 is an essential factor for placental development and angiogenesis.\(^13\) It has also been reported to promote chondrogenesis from mesenchymal stem cells,\(^14\) and recent studies showed that CCN1 expression is promoted by WNT/β-CATENIN signaling to accelerate osteoblast differentiation.\(^15\) Here, we have found that CCN1 is also induced by β-CATENIN signaling in chondrocytes. By using biochemical methods and a novel transgenic mouse model, we show that CCN1 plays a significant role in chondrocyte maturation and cartilage development.

**Materials and Methods**

**Mouse models**

All animal studies were performed according to the protocol approved by the University of Rochester Committee on Animal Resources. All mice were housed in a room using Microisolator Technology kept at 70°F to 73°F. They had free access to food (LabDiet 5010) and water (Hydropac) at all times. β-cateninfx-(exon3)/fx(exon3), β-cateninfx/fx, Aggrecan-CreERT2, Col2a1-CreERT2, and Col2a1-Cre mouse strains were described previously.\(^16-20\) To induce β-catenin GOF and LOF in embryos, β-cateninfx(exon3)/fx(exon3) and β-cateninfx/fx mice were bred with Col2a1-CreERT2 mice, respectively, and tamoxifen was injected into pregnant female mice at E13.5 (0.1 mg/g body weight in corn oil).\(^21\) Embryos were harvested at E18.5. Col2a1-CreERT2; β-cateninfx(exon3)/+ and Col2a1-CreERT2; β-cateninfx/fx were designated as β-catenin GOF and LOF, respectively, whereas their Cre-negative littermates were designated wild-type control. To induce β-
CATENIN GOF in adult mice, 1-month-old Aggrecan-CreERT2; β-cateninfx(exon3)/+ mice were administered tamoxifen for 5 consecutive days (0.1 mg/g body weight). To generate transgenic mice with inducible CCN1 overexpression, mouse CCN1 cDNA was inserted into the multiple cloning site of the iZEG vector. This vector was sent to Cyagen Biosciences (Santa Clara, CA, USA) for pronuclear injection and generation of transgenic founder mice. Meniscal/ligamentous injury (MLI) was performed as previously described. Briefly, a 5-mm incision was made on the medial aspect of the joint. The medial collateral ligament was then transected and the medial meniscus detached at the anterior tibial attachment site. Sham joints received only the incision.

Numbers of animals per experimental group are indicated: E18.5 Col2a1-CreERT2; β-cateninfx(exon3)/+ (Cre+, n = 4; Cre−, n = 6), E18.5 Col2a1-CreERT2; β-cateninfx/fx (Cre+, n = 4; Cre−, n = 5), P60 Acan-CreERT2; β-cateninfx(exon3)/+ (Cre+, n = 4; Cre−, n = 4), E18.5 Col2a1-Cre; Ccn1Tg-1 (Cre+, n = 5; WT, n = 4), P1 Col2a1-Cre; Ccn1Tg-2 (Cre+, n = 3; n = 3; n = 3), 2-month-old Col2a1-Cre; Ccn1Tg-2 (Cre+, n = 4 WT, n = 4), 6-month-old Col2a1-Cre; Ccn1Tg-2 (Cre+, n = 3; WT, n = 4).

Cell isolation and culture
Primary chondrocytes from the sterna and ribs of P3 mice were isolated as described and cultured in DMEM (Life Technologies, Grand Island, NY, USA) containing 10% FBS and 1% penicillin/streptomycin (Life Technologies). Ad5-CMV-Cre and Ad5-CMV-GFP adenoviruses were purchased from Baylor Vector Development Laboratory (Houston, TX, USA) and used at an MOI of 1000. Forty-eight hours after infection, adenovirus was replaced with standard culture media supplemented with 50 mg/mL ascorbic acid. To screen for angiogenic factors induced by β-CATENIN, culture medium from β-cateninfx(exon3)/fx primary chondrocytes infected with Ad5-CMV-Cre or Ad5-CMV-GFP was incubated with the Proteome Profiler Mouse Angiogenesis Array Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

ATDC5 cells were cultured in DMEM/F-12 (Life Technologies) containing 10% FBS and 1% penicillin/streptomycin. For chondrogenic differentiation, ATDC5 cells were grown in medium containing 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and insulin-transferrin-selenium (ITS-G, Life Technologies). To perform Alcian blue and Alizarin red staining, cells were first fixed in 10% NBF for 30 minutes and then incubated with a 1% Alcian blue/3% acetic acid solution or a 2% Alizarin red in ammonium water, respectively, for 30 minutes. Cultures were then rinsed with 70% ethanol followed by ddH2O (Alcian blue) or with PBS (Alizarin red) and air-dried before imaging. Control siRNA (SignalSilence, #6568) and β-catenin siRNA (SignalSilence, #6387) were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at a concentration of 100 nM. Ad-Null and Ad-Ccn1-V5 were purchased from SignaGen Laboratories (Rockville, MD, USA) and used at an MOI of 300.

Histology and immunohistochemistry
Embryonic tissues were harvested and fixed in 10% neutral-buffered formalin (NBF) for 24 hours followed by decalcification in 14% EDTA for 24 hours. Postnatal knee joints were
harvested and fixed in 10% NBF for 3 days followed by decalcification in 14% EDTA for 14 days. Tissues were then processed, embedded into paraffin, and sectioned at 5 mm. Alcian blue/hematoxylin/Orange G eosin staining was performed on sections for visualization of cartilage and bone. For immunohistochemistry, sections were deparaffinized and rehydrated. Antigen retrieval was performed in Tris/EDTA buffer, pH 9.0, in a pressure chamber (anti-CCN1) or with 0.1 mg/mL hyaluronidase (anti-MMP13) followed by quenching of endogenous peroxidase activity in 3% H2O2. Sections were incubated overnight with a 1:500 dilution of anti-CCN1 antibody (ab24448, Abcam, Cambridge, MA, USA), a 1:200 dilution of anti-MMP13 antibody (MS-825, Thermo Scientific, Waltham, MA, USA), a 1:200 dilution of anti-COL2A1 (MS235-P, Thermo Scientific), or a 1:200 dilution of anti-COL10A1 (Quartett, Berlin, Germany). Sections were rinsed in phosphate-buffered saline (PBS) and incubated at room temperature with a 1:1000 dilution of biotinylated goat anti-rabbit secondary antibody (anti-CCN1) or of biotinylated horse anti-mouse secondary antibody (anti-MMP13) for 30 minutes. After a final rinse with PBS, antigen was detected with DAB after application of horseradish peroxidase–conjugated streptavidin. Slides were counterstained with hematoxylin. TUNEL staining was performed using the In Situ Cell Death Detection Kit, Fluorescein per the manufacturer’s specifications (Roche, Mannheim, Germany). Quantification of cell numbers and sizes were determined using OsteoMeasure software (OsteoMetrics, Decatur, GA, USA).

Western blotting

Western blotting was performed as described previously. Primary antibodies were used as follows: mouse α-CCN1 (1:200; sc-13100, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse α-β-ACTIN (1:5000; Sigma-Aldrich, St. Louis, MO, USA).

Quantitative real-time RT-PCR

Total RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed on a Rotor-Gene 6000 real-time DNA amplification system (Qiagen) using the PerfeCTa SYBR Green SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Supplemental Table S1 includes a list of primers used.

Human cartilage samples

An IRB-approved protocol was executed to collect discarded cartilage tissue from orthopedic surgery patients. Normal cartilage was collected from hip fracture patients. Knee articular cartilage was collected from patients undergoing arthroscopic surgery 4 weeks after meniscal injury. No identifiers are associated with the tissues. Tissue was fixed, decalcified, and processed for embedding into paraffin. A tissue microarray was then created using 2 to 3 cores from 11 normal cartilage samples and 14 injured cartilage samples.
Statistics

Data are presented as the mean ± SEM or SD. Statistical significance was determined by Student’s t tests or one-way ANOVA followed by Fisher’s LSD multiple comparisons test as indicated in the figure legends; p values of less than 0.05 were considered significant.

Results

β-catenin signaling promotes CCN1 expression in chondrocytes

Our previous studies showed that β-CATENIN signaling promotes vessel invasion into the developing secondary ossification center. To follow up on these observations, we performed a screen aimed at identifying possible angiogenic factors involved in β-CATENIN-mediated vascularization. Specifically, we incubated conditioned culture media from β-cateninfx(exon3)/fx(exon3) primary sternal chondrocytes infected with adenovirus encoding GFP (control) or adenovirus encoding Cre recombinase (β-CATENIN GOF) with a commercial angiogenic antibody array. We found that CCN1 secretion was highly increased in β-CATENIN GOF chondrocytes compared with control chondrocytes (Fig. 1A and Supplemental Fig. S1). CCN1 was shown to be a direct transcriptional target of β-CATENIN in osteoblast progenitor cells. To confirm transcriptional regulation of Ccn1 by β-CATENIN signaling in chondrocytes, we measured Ccn1 mRNA levels in β-CATENIN GOF chondrocytes and in control chondrocytes. We found that Ccn1 gene expression was significantly increased in β-CATENIN GOF chondrocytes (Fig. 1B). We then treated wild-type primary sternal chondrocytes with WNT3A to activate β-CATENIN signaling and also observed a significant increase in Ccn1 gene expression (Fig. 1C).

To determine whether CCN1 expression is regulated by β-CATENIN in vivo, we performed immunohistochemistry (IHC) for CCN1 on E18.5 proximal humerus limb sections from Cre-negative control (WT) and chondrocyte-specific β-CATENIN GOF mice (Fig. 1Da–f and Supplemental Fig. S2). In WT limbs, CCN1 is expressed throughout the growth plate with the highest levels in hypertrophic chondrocytes, especially those at the chondro-osseous junction, and their surrounding matrix. CCN1 immunostaining was markedly upregulated in the cartilage tissues of β-CATENIN GOF mice. The increase was observed in the prehypertrophic and hypertrophic zones of the growth plate with ectopic expression throughout the epiphysis of the proximal humerus.

To determine if loss of β-CATENIN affects Ccn1 expression, we transfected chondrogenic ATDC5 cells with siRNA designed to target β-catenin. This resulted in roughly a 50% reduction of β-CATENIN protein levels as well as a significant decrease in Ccn1 mRNA expression (Fig. 1E). In vivo, β-CATENIN LOF limbs exhibit reduced expression of CCN1 in prehypertrophic and hypertrophic chondrocytes as well as in chondrocytes at the chondro-osseous junction (Fig. 1E).

β-CATENIN signaling not only regulates chondrocyte maturation during embryonic development but also maintains the postnatal articular cartilage. Aberrant activation of β-CATENIN has been linked to the maturation of articular chondrocytes and to the degradation of articular cartilage. To determine if CCN1 expression is regulated by β-CATENIN signaling in chondrocytes postnatally, we performed IHC on knee joints of 2-
CCN1 promotes chondrocyte maturation

To test whether CCN1 is involved in chondrocyte maturation, we cultured ATDC5 chondrogenic progenitor cells in differentiation medium for 21 days. The cultures exhibit progressive expression of chondrogenic markers consistent with maturation of the cells (Fig. 2A). Col2a1 expression increases over time and peaks at day 14, whereas Col10a1 expression is first detected at day 7 and peaks at day 17. Mmp13, a marker of terminally differentiated hypertrophic chondrocytes, gradually rises throughout the culture period with maximal levels at day 21 (Fig. 2A). Ccn1 transcription is reduced from day 0 to day 3 but increases from day 3 to day 21, suggesting that CCN1 might play a role during chondrocyte maturation. CCN1 protein levels are increased at day 8 compared with day 0, suggesting CCN1 is expressed by hypertrophic chondrocytes, consistent with the CCN1 expression pattern in vivo (Fig. 2B).

To further explore whether CCN1 plays a role in chondrocyte maturation, ATDC5 cells were treated with a neutralizing antibody to block the function of CCN1 during culture. Proteoglycan and mineralized matrix production were reduced by blocking CCN1 function, as indicated by a reduction in Alcian blue and Alizarin staining, respectively. Gene expression of chondrocyte differentiation markers Col2a1, Col10a1, and Mmp13 were also reduced, suggesting CCN1 is required for chondrocyte maturation (Fig. 2C). We then infected ATDC5 cells with adenovirus encoding CCN1 or a null control virus. In contrast to treatment with the neutralizing antibody, over-expression of CCN1 resulted in an increase in mineralized matrix as well as in Col10a1 and Mmp13 gene expressions (Fig. 2D). Col2a1 expression decreased upon CCN1 overexpression. Collectively, these data suggest that CCN1 can promote chondrocyte hypertrophy.

CCN1 overexpression leads to chondrodysplasia and cartilage degradation

To determine if CCN1 regulates chondrocyte maturation and cartilage development in vivo, we generated a conditional CCN1 overexpression transgenic mouse. Two founder colonies were generated and crossed to the Col2a1-Cre transgene to over-express CCN1 specifically in chondrocytes (Fig. 3A). Both transgenic lines exhibited a generalized chondrodysplasia phenotype and increased CCN1 expression compared with their littermate controls, although the phenotype of the first transgenic line was slightly more severe than that of the second (Fig. 3B and Supplemental Fig. S3). The resting, proliferating, and hypertrophic zones are hypocellular in both transgenic lines (Fig. 3C, D). The proliferating zones of the transgenic mice are especially disorganized compared with the columns of proliferating chondrocytes.
in their WT littermates (Fig. 3Bi–l). Interestingly, the hypertrophic chondrocytes, and to a lesser degree the resting and proliferative chondrocytes, of the transgenic mice appear enlarged with an expanded cytoplasm (Fig. 3Bm–p, C, D).

To determine if the loss of cellularity within the growth plates of the transgenic mice is the result of increased cell death, we performed a TUNEL assay. An increase in the number of TUNEL-positive cells was indeed observed throughout the epiphyses of the mutant mice (Fig. 4). We next performed immunohistochemistry for the cartilage extracellular matrix proteins COL2A1 and COL10A1 to determine if a defect in matrix protein synthesis or secretion could account for the enhanced cell size observed in the transgenic mice. No change in COL2A1 expression was apparent, and only a slight reduction in COL10A1 was observed (Supplemental Fig. S4).

To determine whether CCN1 is also involved in postnatal articular cartilage development and homeostasis, mice were maintained into adulthood. Because Ccn1Tg-1 died shortly after birth because of the severity of the chondrodysplasia, we focused on Ccn1Tg-2 in these studies. Knee joints of Ccn1Tg-2 and littermate control mice were harvested at 2 and 6 months of age. At 2 months of age, the articular cartilage of the Ccn1Tg-2 mice is hypocellular with many of the cells in unmineralized matrix appearing hypertrophic (Fig. 5Aa–d). We also observed cartilage loss and possible fibrosis in the distal femur. By 6 months of age, the articular cartilage destruction is more severe and joint architecture compromised with possible osteophyte formation (Fig. 5Ba–f). The number of chondrocytes within the articular cartilage of the transgenic mice is significantly reduced (Fig. 5D). The growth plate at this time point is highly disorganized with reduced cellularity. Additionally, expression of MMP13, a COL2A1 collagenase, is increased in the articular cartilage and meniscus of the Ccn1Tg-2 mice at this time point (Fig. 5C).

To further examine a relationship between CCN1 expression and cartilage degradation, CCN1 IHC was performed on joint sections from mice subjected to MLI or sham surgery. CCN1 expression was increased in the cartilage matrix and inflamed synovium just 1 week after injury (Fig. 6A). Additionally, we examined human cartilage tissues obtained from patients undergoing surgery for recent meniscus injury. Compared with normal cartilage tissue, CCN1 was highly increased post-injury, further suggesting that CCN1 is upregulated in the inflammatory period after joint injury before onset of osteoarthritis (Fig. 6B).

Discussion

Our findings establish that WNT/β-CATENIN signaling induces Ccn1 expression in chondrocytes. By blocking CCN1 function or overexpressing CCN1 in chondrogenic ATDC5 cells, we demonstrate that CCN1 promotes chondrocyte maturation. Our chondrocyte-specific CCN1 transgenic mouse model further provides evidence that CCN1 plays a significant role in cartilage development. Finally, we show enhanced expression of CCN1 in both human and mouse joint tissues after meniscal injury.

Murine β-CATENIN GOF and LOF genetic models have been used to study the role of WNT/β-CATENIN signaling in chondrocyte differentiation and maturation.9 Our previous
work demonstrated that β-CATENIN signaling promotes SOC formation, which accompanies chondrocyte maturation and vascular invasion. The appearance of vascular cartilage canals within the humeral epiphyseal area of β-CATENIN GOF mutants suggests that β-CATENIN signaling promotes angiogenic factor secretion. Here, we used an antibody array to discover that CCN1 secretion is highly upregulated by β-CATENIN signaling in chondrocytes. The fold increase of CCN1 secretion in β-CATENIN GOF cells was second only to that of VEGFA, a factor known to be regulated by β-CATENIN in chondrocytes and other cell types. Interestingly, CCN1 expression is highest in hypertrophic chondrocytes and chondrocytes at the chondro-osseous junction in wild-type limbs where other angiogenic factors are expressed and vascularization of the cartilage matrix occurs. In β-CATENIN GOF limbs, we observed enhanced expression of CCN1 throughout the epiphysis of the proximal humerus, suggesting to us that CCN1 could be a mediator of β-CATENIN signaling to promote cartilage canal and SOC formation. Surprisingly, we did not observe early SOC formation in the CCN1 transgenic mice, however, suggesting that CCN1 alone is insufficient to drive SOC formation and that the early SOC formation observed in the chondrocyte-specific β-CATENIN GOF mice is likely attributable to the coordination of several downstream targets of β-CATENIN signaling. Whether CCN1 plays an essential role in this process will be a focus of future studies.

β-CATENIN likely induces transcription of Ccn1 in chondrocytes. Ccn1 mRNA was elevated in β-CATENIN GOF chondrocytes and in chondrocytes treated with Wnt3a. Alternatively, loss of β-CATENIN via RNA interference reduced Ccn1 mRNA expression. Others have identified two TCF/Lef binding elements in the Ccn1 promoter and demonstrated that in the hepatocellular carcinoma cell line, HepG2, β-CATENIN binds to these elements to directly regulate promoter activity. In mesenchymal C3H10T1/2 cells, Si and colleagues showed that Wnt3a induces Ccn1 expression, as well as the binding of β-CATENIN to the Ccn1 promoter.

In vitro, we used ATDC5 cells, a committed chondrocyte progenitor cell line, to examine the role of CCN1 in later stages of chondrocyte differentiation. Blocking the function of CCN1 with a neutralizing antibody led to a reduction in chondrocyte differentiation and maturation. In contrast, overexpression of CCN1 in ATDC5 cells during chondrogenic differentiation resulted in an increase in the expression of the chondrocyte hypertrophy markers Col10a1 and Mmp13, demonstrating that CCN1 facilitates the process of chondrocyte maturation. In vivo, overexpression of CCN1 in chondrocytes results in a generalized chondrodysplasia phenotype similar to that of β-CATENIN GOF embryos, although far less severe. The cartilage of CCN1 transgenic mice is highly disorganized with a reduction in cell number and an increase in cell size throughout the growth plate, which could be consistent with a role for CCN1 in promoting hypertrophy. It is also possible, however, that CCN1 overexpression enhances cell death or promotes senescence, roles that have been described for CCN1 in other cell types. Indeed, we observed enhanced TUNEL staining of the epiphyseal chondrocytes in the Ccn1 transgenic mice. Because the growth plates of the β-CATENIN GOF embryos are also disorganized with enlarged cells (Supplemental Fig. S5), it will be an interesting future direction to determine the contribution of CCN1 to these phenotypes.
Postnatally, we observed progressive cartilage degeneration and fibrosis in the knee joints of mice with CCN1 overexpression. By 6 months of age, the joint architecture is also affected with synovial expansion and osteophyte formation. Additionally, immunohistochemistry revealed increased expression of MMP13 in both cartilage and meniscus. This osteoarthritis-like phenotype is reminiscent of the postnatal chondrocyte-specific β-CATENIN GOF phenotype, where constitutive β-CATENIN activation in adult mice led to inappropriate articular chondrocyte hypertrophy and cartilage degeneration.(11) Our findings also reveal increased expression of CCN1 in synovium and cartilage matrix after MLI, as well as in human articular cartilage after traumatic joint injury, indicating that CCN1 may play a role in the genesis of posttraumatic OA. In fact, others have recently reported that Ccn1 mRNA is increased in human OA cartilage and that CCN1 immunoreactivity directly correlates with Mankin score and chondrocyte cloning.(33) Additionally, CCN1 levels were shown to increase in synovial tissue from patients with both OA and rheumatoid arthritis.(34,35)

CCN1 is one of six members of a family of matricellular proteins that bind to extracellular matrix (ECM) proteins to regulate a diverse array of cellular processes. All are expressed in chondrocytes at some point in development,(36) but the specific roles they play are largely unknown. Like CCN1, CCN2 is positively regulated by β-CATENIN signaling.(37) It is expressed in hypertrophic chondrocytes and is required for proper cartilage ECM organization.(37,38) CCN3 is thought to antagonize the actions of CCN2 and direct the differentiation of articular chondrocytes.(39,40) Even less is known about other CCN family members and their roles in cartilage. Future studies, including the generation of novel conditional mouse models, are, therefore, required to further elucidate the likely important roles of CCN proteins in cartilage development and homeostasis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Fig. 1. WNT/β-CATENIN signaling promotes CCN1 expression in chondrocytes. (A) Primary sternal chondrocytes from β-cateninflox(exon3)/floxflox(exon3) mice were infected with Ad5-CMV-GFP (Control) or Ad5-CMV-Cre (β-CAT GOF) and cultured for 6 days. Culture medium was then collected to detect CCN1 secretion using a membrane array spotted with antibodies to angiogenesis factors. Anti-CCN1 antibody spots from membranes incubated with Control or β-CAT GOF culture media are shown. (B) β-cateninflox(exon3)/floxflox(exon3) primary sternal chondrocytes were infected with Ad5-CMV-GFP (Control) or Ad5-CMV-Cre (β-CAT GOF) and harvested for mRNA isolation and quantitative real-time RT-PCR analyses of Ccn1 gene expression. p < 0.05, Student’s t test. (C) Wild-type primary sternal chondrocytes were
treated with vehicle (Control) or WNT3A (100 ng/mL) for 48 hours and harvested for mRNA isolation and quantitative real-time RT-PCR analyses of Ccn1 gene expression. **p < 0.01, Student’s t test. (D) CCN1 immunohistochemistry of proximal humerus sections from E18.5 β-cateninfx(exon3)/+ (WT) and Col2a1-Cre+/−ERT2; β-cateninfx(exon3)/+ (β-CAT GOF) littermates administered tamoxifen at E13.5. Da and Db, 5× images; Dc–f, high-magnification images (20×) from the corresponding boxed regions in Da and Db. Arrows in Df point to ectopic epiphyseal CCN1 expression. (E) ATDC5 cells were transfected with 100 nM Control or β-catenin siRNA and then harvested 72 hours later for isolation of mRNA or protein. Quantitative real-time RT-PCR analyses was performed for analyses of Ccn1 gene expression. Western blotting was performed for the indicated proteins. **p < 0.01, Student’s t test. (F) CCN1 immunohistochemistry of proximal humerus sections from E18.5 β-cateninfx/fx (WT) and Col2a1-Cre+/−ERT2; β-cateninfx/fx (β-CAT LOF) littermates administered tamoxifen at E13.5. (G) CCN1 immunohistochemistry of knee joint sections from 2-month-old (P60) β-cateninfx(exon3)/+ (WT) and Acan-Cre+/−ERT2; β-cateninfx(exon3)/+ (β-CAT GOF) littermates administered tamoxifen at age 1 month. Fa and Fb, 5× images; Fc–f, high-magnification images (20×) from the corresponding boxed regions in Fa and Fb.
Fig. 2. CCN1 promotes chondrocyte maturation. (A) ATDC5 cells were cultured in chondrogenic differentiation media for the indicated number of days and then harvested for mRNA isolation and quantitative real-time RT-PCR analyses of Col2a1, Col10a1, Mmp13, and Ccn1 gene expressions. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Fisher’s LSD multiple comparisons test. (B) ATDC5 cells were cultured in chondrogenic differentiation media for 0 or 8 days and then harvested for Western blotting of the indicated proteins. (C) ATDC5 cells were cultured in chondrogenic differentiation media and treated with control IgG or an anti-CCN1 neutralizing antibody. After 6 days, the cultures were stained with Alcian blue and Alizarin red or harvested for mRNA isolation and quantitative real-time RT-PCR analyses of Col2a1, Col10a1, and
Mmp13 gene expressions. **p < 0.01, ***p < 0.001, Student’s t test. (D) ATDC5 cells were infected with Ad-Null or Ad-CCN1-V5 adenoviruses and cultured in chondrogenic differentiation media. After 6 days, the cultures were harvested for Western blotting, Alizarin red staining, or mRNA isolation and quantitative real-time RT-PCR analyses of Col2a1, Col10a1, and Mmp13 gene expressions. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.
Fig. 3.
CCN1 overexpression leads to chondrodysplasia and aberrant cartilage development. (A) Diagram of the conditional Ccn1 transgene. The CMV early enhancer/chicken b-actin promoter (CAG) drives Ccn1 expression from this transgene after removal of a loxP-flanked STOP cassette by Cre recombinase. Two independent transgenic mouse strains were generated and bred with Col2a1-Cre mice (designated Ccn1Tg-1 and Ccn1Tg-2). (B) Alcian blue/hematoxylin/Orange G staining of tibial growth plate sections from E18.5 Ccn1Tg-1 (Col2a1-Cre+/−; Tg+/−) and wild-type (WT) littermates (left panels) or P1 Ccn1Tg-2 (Col2a1-Cre+/−; Tg+/−) and wild-type (WT) littermates (right panels). (C, D) Quantification of cell number and size in PZ and HZ regions.
(Col2a1-Cre+/−, Tg+/−) and wild-type (WT) littermates (right panels). Be-Bp are high-
magnification images (40×) of the boxed regions in Ba–d. (C) Quantification of proliferating
zone and hypertrophic zone cell number and size in E18.5 Col2a1-Cre; Ccn1Tg-1 embryos
(Cre+, n = 5; WT, n = 4). (D) Quantification of proliferating zone and hypertrophic zone cell
number and size in P1 Col2a1-Cre; Ccn1Tg-2 neonates (Cre+, n = 3; WT, n = 3). Values for
the Tg mice were normalized to those of the WT mice and are presented as a fold change. *p
< 0.05, **p < 0.01, ***p < 0.001, Student’s t test. RZ = resting zone; PZ = proliferating
zone; HZ = hypertrophic zone.
Fig. 4.
CCN1 overexpression leads to increased epiphyseal chondrocyte apoptosis. TUNEL staining of tibial growth plate sections from E18.5 Ccn1Tg-1 mice and WT littermates (a and b) or P1 Ccn1Tg-2 mice and WT littermates (c and d).
Fig. 5.
CCN1 overexpression leads to postnatal cartilage degradation. (A) Alcian blue/hematoxylin/Orange G staining of knee joint sections from 2-month-old WT (Aa, Ac) and Ccn1Tg-2 (Ab, Ad) mice. Aa and Ab, 5× images; Ac and Ad, high-magnification images (20×) from the corresponding boxed regions in Aa and Ab. Articular cartilage degeneration/fibrosis on the distal femur (black arrows) and enlarged chondrocyte size (orange arrows) are indicated. (B) Alcian blue/hematoxylin/Orange G staining of knee joint sections from 6-month-old WT (Ba, Bc, Be) and Tg-2nd (Bb, Bd, Bf) mice. Ba and Bb, 5× images; Bc–f, high-magnification images (20×) of the articular and growth plate cartilages from the corresponding boxed regions in Ba and Bb. Articular cartilage degeneration/fibrosis on the distal femur (black arrow) is indicated. (C) MMP13 immunohistochemistry of knee joint sections from 6-month-old WT and Ccn1Tg-2 mice. Ca and Cb, nonspecific IgG primary antibody; Cc and Cd, anti-MMP13 primary antibody. (D) Quantification of articular chondrocyte numbers in 6-month-old Col2a1-Cre; Ccn1Tg-2 mice (Cre+, n = 3; WT, n = 4). Numbers for the Tg mice were normalized to those of the WT mice and are presented as a fold change. **p < 0.01, Student’s t test.
Fig. 6.
Meniscal injury leads to elevated CCN1 expression in articular cartilage and synovium. (A) CCN1 immunohistochemistry of knee joint sections from mice subjected to sham or MLI at age 10 weeks. Joints were harvested 1 week post-injury. (B) Representative results of CCN1 immunohistochemistry on a tissue microarray of normal human cartilage or cartilage from patients undergoing arthroscopic surgery 4 weeks after meniscal injury.