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The matricellular protein connective tissue growth factor (CTGF/CCN2) is essential for angiogenesis and cartilage matrix secretion during development

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A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in Molecular, Cell and Developmental Biology

by

Faith Lauren Hall-Glenn

2012

ABSTRACT OF THE DISSERTATION

Connective tissue growth factor (CCN2/CTGF) is essential for angiogenesis and cartilage matrix secretion during development

by

Faith Lauren Hall-Glenn

Doctor of Philosophy in Molecular, Cell and Developmental Biology

University of California Los Angeles, Los Angeles, 2012

Professor Karen M. Lyons, Chair

Members of the C(Cyr61) C(CTGF) N(Nov) family of matricellular proteins are involved in multiple aspects of embryonic and postnatal development and disease pathologies. CCN2, or connective tissue growth factor, is the second member of the family and has gained importance due to its prominent role in fibrotic disease. CCN2 has been extensively studied both *in vitro* and *in vivo*, but its specific roles remain unclear due to discrepancies in cell specific signaling mechanisms and experimental design. The current use of CCN2 blocking antibodies in clinical trials to treat symptoms associated with fibrosis and kidney function during diabetes warrants a clearer understanding of the function of CCN2 during normal physiology in order anticipate side effects. In order to investigate the functions of CCN2 during development, we have further analyzed the *Ccn2* global knockout mouse and found that CCN2 plays essential roles in angiogenesis during vascular remodeling and cellular stress during endochondral ossification. CCN2 plays a dual role in vascular remodeling, first through mediating platelet derived growth factor (PDGF) signaling between endothelial cells and pericytes, and second through inducing

the secretion of provisional and permanent vascular basement membrane components. Further analysis of the ECM defect observed in the growth plates of *Ccn2* mutant mice revealed that the loss of CCN2 results in increased endoplasmic reticulum (ER) stress. *Ccn2* mutants also exhibited decreased Nuclear Factor κB (NFκB) and autophagy-mediated cellular survival. Conversely, the overexpression of CCN2 results in attenuated ER stress and increased cellular survival during chemically induced ER stress. These results highlight a novel protective role for CCN2 during chondrocyte differentiation. Taken together, these results demonstrate that CCN2 plays important physiological roles *in vivo* and these roles should be considered during therapeutic interventions.

The dissertation of Faith Lauren Hall-Glenn is approved.

John S. Adams

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2012

Dedicated to my family

Thank you all for your love and support

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Chapter 1

The CCN family of matricellular proteins: roles in development and disease

Matricellular proteins are matrix proteins that do not contribute structurally to the extracellular matrix (ECM), but serve as key modulators of cell function and cell-matrix interactions during development [1]. Matricellular proteins are expressed at high levels during embryonic development and in response to postnatal injury. Matricellular proteins act in cell specific contexts by binding to cell surface receptors, ECM molecules, growth factors proteases and cytokines. Classical matricellular proteins include thrombospondins (TSP) 1 and 2, secreted protein acidic and rich cysteine (SPARC) and osteopontin (OPN). Global depletions of these classical matricellular proteins result in subtle phenotypes that are worsened during injury and repair [1,2].

The C(Cyr61) C(Connective tissue growth factor) N(Nephroblastoma overexpressed) family of matricellular proteins were initially classified as immediate early gene products and/or growth factors [3]. This introduction discusses the roles of CCN matricellular proteins during embryonic and postnatal development, mechanisms of action and their roles during pathological diseases. And finally, the current and emerging therapeutic innovations and clinical trials towards treating CCN related pathologies will be discussed.

CCN Family Structure: Modular Domains in action

The CCN family is comprised of six members, CCN1-6. The name CCN is derived from a unified nomenclature based on the first three members: Cysteine-rich 61 (Cyr61/CCN1), Connective tissue growth factor (CTGF/CCN2) and Nephroblastoma overexpressed (Nov/CCN3) [4]. Members of the CCN family are modular proteins that were formed through exon shuffling, and share structural properties at both the gene (30-50 percent) and protein levels (40-60 percent) [5]. CCN proteins are about 38-42 kDaltons in mass and consist of an amino-terminal secretory peptide followed by four modular domains (Figure 1) [6]. These modular

domains directly influence cellular proliferation, adhesion, migration and survival through interactions with growth factors, integrins, ECM molecules and receptors (Figure 2). The insulin-like growth factor binding protein (IGFBP) domain shares a 32 percent sequence homology with classical IGF binding proteins 1-6, but the binding observed is relatively weak and may be dependent upon experimental conditions [3,7]. The second domain is the von Willebrand type C/chordin-like cysteine rich domain (VWC), which is present in mucins, collagens and thrombospondins [3]. This domain binds to integrins and growth factors, such as transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMPs) [8]. The third domain is the thrombospondin type 1 repeat (TSR), which contains a motif that mediates cell adhesion through integrins, sulfated proteoglycans [9], lipoprotein related protein receptors (LRPs) [10,11] and growth factors such as vascular endothelial growth factor (VEGF). The final domain is the C-terminal (CT) domain, which consists of six cysteine residues that form a cysteine knot motif that is also present in nerve growth factor (NGF), TGFβ and platelet derived growth factor (PDGF) [12]. The C-terminal domain also contains both dimerization and receptor binding regions that interact with heparin sulfate proteoglycans (HSPGs), integrins, Notch 1 receptor [13] and LRPs [3]. CCN5 lacks this module, but the loss does not seem to affect its function [14]. The N-terminal (IGFBP/VWC) and C-terminal (TSR/CT) domains are separated by a hinge region that is cleaved by most proteases, including matrix metalloproteases (MMPs) 1,2,3,7,9 and 13, elastase and plasmin [15]. These cleaved domains show distinct biological properties, which may explain why CCN proteins exhibit cell specific functions during multiple physiological and pathological conditions [6]. These truncated domains are also preferentially expressed during fibrosis and certain cancers, making them important diagnostic markers for disease progression and treatment [6].

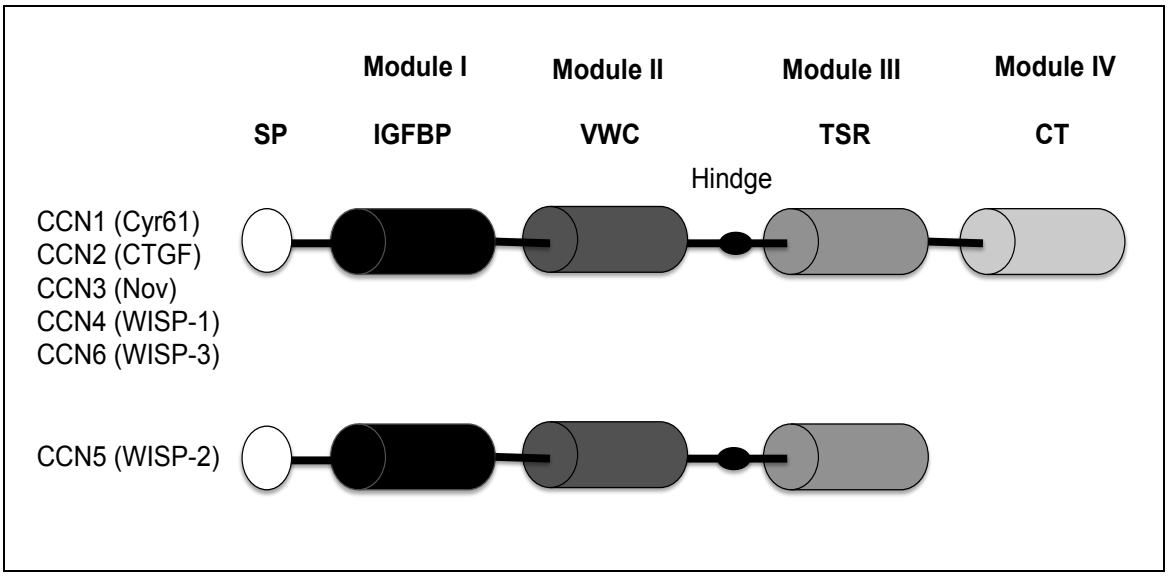

Figure 1: The modular domains of the CCN family of matricellular proteins.

Schematic representation of the CCN protein structure. The CCN family structure consists of a secretory peptide followed by four modular domains: (I) the insulin-like growth factor binding protein (IGFBP), (II) The von Willebrand factor C domain (VWC), (III) the thrombospondin type 1 repeat (TSR), and (IV) the carboxy-terminal domain (CT) containing a cysteine knot, which is not present in CCN5. A hinge region is susceptible to cleavage by matrix metalloproteases (MMPs), plasmin and elastase. (Modified from Kular et al., 2011)

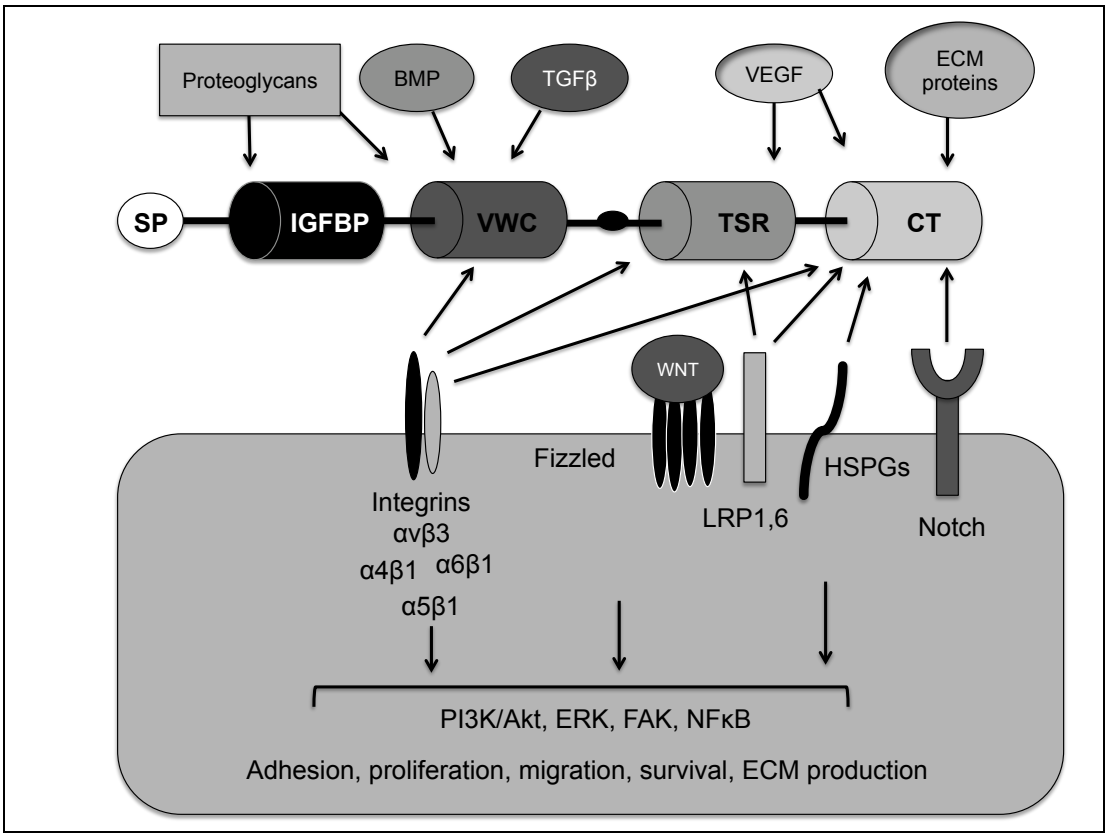

Figure 2: Molecular interactions mediated through the modular domains of CCN proteins. CCNs are able to interact with multiple binding partners including integrins, WNT co-receptors lipoprotein receptor-related proteins (LRPs), extracellular matrix (ECM) proteins such as heparin sulfate proteoglycans (HSPGs), fibronectin and perlecan; growth factors such as bone morphogenetic proteins (BMPs), transforming growth factor β (TGFβ) and vascular endothelial growth factor (VEGF), and the Notch1 extracellular domain. The modular domains of CCN proteins allow them to interact with these various factors, receptors and ECM molecules to activate multiple intracellular signaling pathways. These pathways ultimately induce cellular adhesion, proliferation, migration, survival, ECM production and apoptosis in multiple physiological and pathological processes. (Modified from Jun and Lau, 2011)

CCN expression and functions during development

CCN1 (Cyr61)

Ccn1 was described in 1985 as a gene that was expressed in mouse Balb/c3T3 cells induced by serum and PDGF [16]. CCN1 is a potent angiogenic factor with expression in the developing cardiovascular system [17]. CCN1 is also expressed in cartilaginous elements, the developing central nervous system, and the placenta [17]. A global depletion of *Ccn1* results in embryonic lethality due to major defects in angiogenesis, including failure of proper chorioallantoic fusion, placental vascular defects, and cardiac atrioventricular septal defects (AVSD) [18,19]. CCN1 can stimulate chondrogenesis and osteogenesis in limb bud mesenchyme cultures [20,21]. However, due to the early embryonic lethality of CCN1 mice, its role in chondrogenesis has not been investigated. Unpublished work from our laboratory analyzing a cartilage specific depletion of CCN1 revealed no major defects in chondrogenesis. Cartilage specific CCN1 mutant mice perish soon after birth, possibly due to cardiac defects resulting from the early expression of the collagen type II promoter in the atria (data not shown) [22]. These results indicate that CCN1 does not play a major role in chondrogenesis, but further investigation of this phenotype is warranted. In the adult, CCN1 is expressed in the kidneys, skeletal muscle, lung and uterus [23].

CCN2 (CTGF)

CCN2 was discovered through the screening of a cDNA library from serum stimulated NIH-3T3 cells in 1988, and was later reported in 1991 as a factor that cross-reacted with PDGF when isolated from human umbilical vein endothelial cells (HUVECs) [24,25]. CCN2 is expressed throughout embryonic development in multiple tissues including the developing vasculature, bone and cartilage, mesangial cells (MCs) of the kidneys, brain, mammary glands and lungs [26]. CCN2 promotes endochondral and intramembranous bone formation *in vivo* [27,28]. A global

depletion of *Ccn2* results in perinatal lethality as a result of severe skeletal abnormalities caused by impaired chondrocyte proliferation, ECM production and growth plate angiogenesis [27]. *In vitro*, CCN2 mediates chondrocyte extracellular matrix production in part through controlling the expression and activation of integrin α5β1 [29]. Consistent with its high expression in vascular elements, CCN2 plays critical roles in endothelial cell adhesion to pericytes, as well as the formation and integrity of the vascular basement membrane [30]. In the adult mouse, CCN2 continues to be expressed in major and minor vascular elements, lungs, articular and growth plate cartilage, intervertebral disks and cranial sutures [31].

CCN3 (Nov)

Ccn3 was first discovered in 1992 as an overexpressed gene in virally induced nephroblastomas in avian embryos [32]. CCN3 expression has been observed in the developing nervous, cardiovascular and urogenital systems, retina, skeleton, muscle and tendon [33-35]. A full knock down of *Ccn3* has not been generated but mice carrying a targeted mutation of *Ccn3* within the VWC domain were investigated [36]. These CCN3 mutants were viable at birth, but exhibited enlarged skeletal elements including rib cage malformations and abnormal joint articulation. These skeletal defects persisted into adulthood. In addition to these abnormalities, adult CCN3 mutant mice exhibit age-dependent cardiomyopathy, muscle degeneration, and cataracts [36]. Isolated *Ccn3* mutant differentiated fibroblasts and limb bud micromasses exhibited accelerated chondrogenesis and osteogenesis, suggesting that CCN3 plays an inhibitory role during mesenchymal differentiation [36].

CCN4 [Wnt Inducible Secreted Protein-1 (WISP-1)]

CCN4/WISP1 was first identified in 1996 as a Wnt-1 regulated oncogene in metastatic murine melanoma cells [37]. CCN4 expression during embryonic and postnatal development has not

been characterized nor has a targeted deletion in mice been developed to determine its developmental function. CCN4 is aberrantly expressed in multiple human cancers, idiopathic pulmonary fibrosis and cancer derived cell lines [3].

CCN5 (WISP-2)

CCN5 was identified in heparin treated rat vascular smooth muscle cells (VSMCs) during a subtractive hybridization screen in 1997 [38]. *Ccn5* is expressed all embryonic tissues including cardiac muscle, intestinal smooth muscle and epithelium, lung bronchioles and during late endochondral ossification [39]. In adult tissues, CCN5 expression is observed in vascular elements including epithelial and smooth muscle cells, pancreas, spleen, skeletal muscle, reproductive organs, nervous system, kidney and digestive tract [40]. A targeted depletion of *Ccn5* in mice has not been reported [14].

CCN6 (WISP-3)

Ccn6 was identified in humans during a screen of an expressed sequence tag database using *Ccn4* [38]. CCN6 is the only CCN family member directly associated genetically with human disease. Humans with point mutations and deletions in *Ccn6* develop a rare autosomal recessive disorder called progressive pseudorheumatoid dysplasia (PPD), also known as spondyloepiphyseal dysplasia tarda with progressive arthropathy (SEDT-PA) [41,42]. Humans with PPD show no overt clinical phenotypes at birth, but the disorder begins to manifest in early childhood with malformations, swelling and stiffness of joints, platyspondyly and early onset osteoarthritis (OA) [43]. Articular chondrocytes isolated from PPD patients show increased proliferation and decreased apoptosis [42]. These observations indicate that the loss of *Ccn6* results in accelerated chondrocyte differentiation, which may explain the joint degeneration and early onset of OA observed in PPD patients. CCN6 expression during development has not been

characterized, but based on the defects observed in humans, appears to be expressed in developing endochondral elements and joints [44]. Surprisingly, mice with a targeted deletion or overexpression of *Ccn6* show no overt phenotype and do not recapitulate any features of PPD patients [44]. The presence of a phenotype in humans but not in mice is likely related to the observation that CCN6 is expressed in human articular cartilage, but not in mouse articular cartilage [44]. These studies demonstrate that there are fundamental differences between mice and humans with respect to with respect to CCN family member function and/or models of disease, but they also recognize CCN6 as an important regulator of mesenchymal differentiation and postnatal maintenance of bone and cartilage.

CCN Mechanisms of Action

Cell Adhesion, migration and proliferation

CCNs 1, 2 and 3 can promote adhesion in multiple cell types including smooth muscle cells, endothelial cells, platelets, monocytes, pancreatic stellate cells and hepatic stellate cells (Figure 2) [45]. CCN1 and CCN2 promote adhesive signaling in fibroblasts through α6β1 and HSPGs to induce focal adhesion (FAK) signaling, small GTPase Rac, actin cytoskeletal rearrangements, filapodia and lamellipodia formation [46]. CCN2 promotes fibroblast and chondrocyte adhesion to fibronectin through α6β1 and α5β1 integrins, respectively [29,47].

In addition to supporting adhesive properties in cells, CCNs can also induce cell migration and proliferation. CCNs 1, 2 and 3 can stimulate cell migration and chemotaxis in most mesenchymal cell types [48]. Conversely, CCN3 and CCN5 inhibit cell migration of VSMCs and various cancer cell lines [49-51].

The affects of CCN proteins on proliferation are cell type specific. CCN1, 2 and 3 can enhance DNA synthesis in fibroblasts through integrins, but do not posses the intrinsic ability to

induce mitogenesis without the presence of other growth factors [48]. CCN5 inhibits cellular proliferation and seems to have antagonistic functions against other CCNs [14]. Although the functions of CCNs during cell adhesion, migration and proliferation are highly contextual in nature, they do present interesting avenues to circumvent tumorigenesis and metastasis.

Cell survival and Apoptosis

The ability of CCNs to regulate cell adhesion invariably promotes cell survival. Paradoxically, CCN1, 2 and 3 can induce apoptosis as cell adhesion substrates in fibroblasts by interacting with α6β1 and syndecan-4 [52]. This interesting phenomenon is due to CCN1, 2 and 3 synergizing with the inflammatory cytokine tumor necrosis factor α (TNFα) to induce apoptosis [52]. Specifically, CCN1 is able to convert the anti-apoptotic adhesive functions of TNFα, to a pro-apoptotic response through Rac-1 and p53 induction of reactive oxygen species (ROS) [52]. CCN4 may work antagonistically in this process, as CCN4 has been found to inhibit TNFα-induced apoptosis in cardiomyocytes [53]. Therefore, CCNs can activate cell survival or death in cell-specific and integrin dependent contexts. These studies also demonstrate the CCN specific effects on biological outcomes during inflammatory processes.

Angiogenesis

CCN1, 2 and 3 are all potent inducers of angiogenesis, in part through binding to integrin αvβ3 on endothelial cells to induce cell migration and tube formation *in vitro* [9,46,54]. CCN1 and 2 are secreted by endothelial cells and stimulated by angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF [46]. Both *Ccn1* and *2* knockout mice exhibit defects in vessel formation caused by defective ECM deposition, degradation and remodeling [19,27,30]. The anti-proliferative effect of CCN5 in VSMCs raises the intriguing possibility that CCN5 may regulate angiogenesis *in vivo* [50]. The angiogenic functions of CCNs make them interesting

therapeutic targets for treatment of vascular diseases. Conditional depletions of CCN1, 2 and 5 in specific populations of vascular cells (i.e. endothelial cells, pericytes or VSMCs) should be generated in order to further elucidate the cell-specific and autocrine functions of CCNs during angiogenesis.

Chondrogenesis and Osteogenesis

CCN proteins display both positive and negative roles in cartilage and bone formation [45,55]. The most severe chondrodysplasia resulting from the loss of a matricellular protein is observed in *Ccn2* knockout mice [27]. As discussed above, homozygosity for a modified allele of CCN3 results in enhanced chondrogenesis and osteogenesis [36]. However, it is not clear whether this allele encodes a protein with reduced function, no function, or novel functions. CCN3 is upregulated in the absence of CCN2 in the growth plate, and this is accompanied by delayed differentiation and defective proliferation [56]. However, overexpression of CCN2 (conditional overexpression in bone) and CCN3 (global transgenic) antagonize both BMP and Wnt signaling pathways, resulting in osteopenia [36,57]. The mechanisms responsible for the ability of CCN2 and CCN3 to antagonize these pathways *in vivo* are unknown. However, the VWC domain allows CCNs to interact with BMPs and TGFβ, which are indispensible for embryonic and postnatal endochondral bone formation and maintenance [8,58], and this interaction inhibits BMP signaling. In turn, CCN2 expression is induced by TGFβ-mediated signaling owing to the presence of a SMAD binding element in the *Ccn2* promoter region [59]. CCNs can also regulate Wnt signaling during endochondral bone formation through binding to the Wnt co-receptor LRP6 [11,21], but whether this occurs *in vivo* is unknown. Wnt proteins regulate aspects of bone formation during embryonic development and postnatal bone remodeling by controlling the activity of osteoblasts and osteoclasts [60]. In turn, CCN2 expression is induced by Wnt

signaling in chondrocytes [61]. Thus, CCN2 appears to interact physically with BMPs, TGFβ, and Wnts. These interactions are thought to antagonize Wnt and BMP signaling but promote TGFβ signaling. The physiological relevance of these interactions remains to be established. Furthermore, CCN2 expression is induced by Wnts and TGFβ in skeletal cells, suggesting that CCNs are components of feedback loops involving BMPs and Wnts. As the functions of CCNs in embryonic skeletogenesis and adult bone maintenance continue to be elucidated, it would be of interest to determine the compensatory roles they play in these processes.

Stem Cell maintenance and Differentiation

CCNs regulate the differentiation of bone, cartilage and adipose from mesenchymally derived tissues [62]. CCN2 can also drive the differentiation of bone marrow mesenchymal stem cells into fibroblasts [63]. CCN3 plays an important role in the self-renewal of hematopoietic stem cells from umbilical chord blood [64]. The mechanisms by which CCN2 and CCN3 mediate these conversions are unknown. The role of CCN3 and integrin-mediated signaling may present interesting future studies on stem cell engraftment [62,64]. Future studies are also needed to further illuminate the roles of CCNs in stem cell renewal and maintenance in bone marrow and hematopoietic microenvironments.

Roles in Pathological Diseases

CCN proteins are highly expressed during inflammation and tissue repair. Aberrant expression of CCNs is a hallmark of multiple pathological conditions including fibrosis, cancer, arthritic inflammation and cardiovascular disease. The mechanisms of CCN action during these processes are not completely understood, but their roles remain specific and complex.

Wound Healing and Fibrosis

Wound healing is characterized by three distinct and overlapping phases to facilitate healing and repair. During the first phase, inflammation at the site of injury occurs. Resident fibroblasts are induced by inflammatory factors and differentiate into contractile myofibroblasts, which deposit ECM to promote tissue integrity during the healing process. During fibrosis, ECM deposition by myofibroblasts is exacerbated, resulting in scarring and eventual loss of tissue function [65]. CCN2 is major player in fibrosis and exacerbates fibrosis in multiple organs. CCN2 is secreted by platelet α-granules, resident fibroblasts and myofibroblasts during wound healing. CCN2 is also activated by other inflammatory cytokines and growth factors such as TGFβ [66]. Mouse models studying the tissue specific effects of CCN2 overexpression during fibrosis have yielded ambiguous results. Transgenic mice exhibiting tissue specific overexpression of CCN2 in parenchymal cells do not exhibit spontaneous fibrosis in most organs, but upon injury, fibrotic responses are initiated and exacerbated [66]. On the other hand, a fibroblast specific overexpression of CCN2 can drive spontaneous fibrosis in the skin, lung, kidneys and arteries [67]. The most likely explanation for these differing outcomes is the different levels of CCN2 expression observed in the transgenic models.

CCN4 is also involved in fibrotic processes and is upregulated during human and mouse models of pulmonary fibrosis [68]. In addition, the administration of recombinant CCN4 increases ECM deposition in fibroblasts [68]. Future studies should focus on the relationship between CCN2 and CCN4 to discover if they act in concert to regulate cell specific responses during fibrosis.

CCN1 acts as a senescence switch during wound healing and fibrosis, where it induces activated myofibroblasts to adopt a senescent secretory phenotype, which involves the secretion

of MMPs and decreased ECM deposition [69]. CCN1 may thus play an anti-fibrotic role by inducing the resolution of fibrotic tissue to promote healing.

CCN3 and CCN5 appear to also exert anti-fibrotic effects. CCN3 blocks the pro-fibrotic effects of CCN2 and collagen deposition in mesangial cells in an *in vitro* model of renal disease [70]. CCN5 reduces cardiac fibrosis, possibly through inhibition of TGFβ signaling. CCN2 and CCN5 play pro- and anti-fibrotic roles, respectively, during cardiac hypertrophy [71]. These investigations should be expanded to studying fibrosis in other tissues, as similar antagonistic mechanisms may be at work.

Cancer

Aberrant expression of CCNs is observed in multiple tissues and organs of the following cancers: colorectal, gallbladder, gastric, ovarian, pancreatic, prostate, breast, hepatocellular carcinoma, melanoma, cartilaginous tumors, lymphoblastic leukemia, non-small cell lung and squamous cell carcinoma and gliomas [45]. CCNs play both pro- and anti- tumorigenic roles that are organ specific and depend on the availability of angiogenic factors and other agents in the tumor microenvironment. Generally, CCN1, CCN2 and CCN4 have been associated with tumor cell proliferation and growth, with CCN3, CCN5 and CCN6 playing inhibitory roles during these processes [48].

CCNs can promote tumor growth by augmenting tumor angiogenesis. CCN1 and CCN2 can induce or suppress angiogenesis in a tumor dependent fashion. CCN1 overexpression in gastric adenocarcinoma [72] and MCF-7 breast cancer cells enhances their tumorigenicity through increased vascularization [73]. A newly developed anti-CCN1 monoclonal antibody inhibited the proliferation, migration, and invasion of a highly malignant human breast cancer cell line [74]. CCN2 is overexpressed in human pancreatic cancer and a CCN2 monoclonal

blocking antibody was shown to inhibit pancreatic tumor growth and metastasis in mice [48,75]. CCN1 and CCN2 are prognostic markers for secondary osteolytic bone metastasis from breast cancer [76], with neutralizing antibodies suppressing bone metastasis and micro-vascularization [77].

CCNs can also enhance tumor cell survival by activating anti-apoptotic mechanisms. In addition to enhancing tumor vascularization, ectopic CCN1 expression can induce apoptotic resistance in MCF-7 breast cancer cell lines by up-regulating XIAP, an anti-apoptotic protein [78].

CCNs can also inhibit tumor growth by suppressing proliferation. CCN3 is ectopically expressed in gliomas, resulting in reduced tumor size [79], but paradoxically, can also promote melanoma metastasis in mouse xenograft models [80]. Increased CCN3 expression in patients is linked to poorer disease prognosis in Ewing's sarcoma, breast cancer and melanoma [62]. CCN3 can also sensitize cancer cells to anti-cancer drugs. For example, in myeloid leukaemia cells CCN3 halts proliferation, restores cell growth control and sensitizes the cells to cancer drug induced apoptosis [81]. Consistent with its role in inhibiting the growth of VSMCs [50], CCN5 can also inhibit the growth of breast, leiomyoma and myometrial cancer cell lines [62]. CCN6 can also inhibit tumor growth in breast carcinomas in part through the inhibition of IGF-1 signaling, which can augment breast cancer survival and metastasis [82].

In summary, the mechanisms underlying these pro- and anti-tumorigenic roles are unknown. It is likely that some of these effects reflect the pro-angiogenic functions of CCN proteins. In other cases, CCN proteins have direct effects on tumor cells, promoting proliferation, migration, survival, or apoptosis. These different cellular activities may be mediated through

engagement of different integrins. Given their potent effects on several cell types, CCNs are emerging as important therapeutic targets for cancer treatments.

Inflammatory Diseases

Inflammation is a transient physiological reaction of tissues that enables the immune system to remove toxic or pathological stimuli and initiate the healing process [83]. Uncontrolled or sustained inflammation can extensively damage tissues and lead to the progression of multiple chronic pathologies. CCNs can either activate or be activated by inflammatory cytokines in multiple cell dependent contexts. CCNs play significant roles in osteoarthritis, kidney disease and neuroinflammation [83].

Osteoarthritis (OA) is a common degenerative disease characterized by cartilage loss in joints. CCN2 is highly expressed in OA cartilage and chondro-osteophytes [84]. An injection of CCN2 into the synovial lining of mouse knee joints resulted in transient fibrosis and cartilage damage [84]. However, in a model of induced OA in rats, the injection of CCN2 stimulated cartilage repair [85]. Due to these conflicting results, further investigations are needed in order to determine if CCN2 plays protective or destructive roles during OA, as the affects of CCN2 during OA are tightly regulated and highly stage and dosage dependent.

CCN3 was identified as a marker of late stage OA, suggesting that CCN3 may play a critical role in the progression of OA [86]. CCN4 expression is also increased in human and experimental models of OA and contributes to cartilage damage by increasing the production of MMPs in chondrocytes and macrophages [87].

Progressive renal fibrosis is characterized by increased matrix deposition and inflammatory cell infiltration within the kidney interstitium and glomerulus, ultimately causing the loss of kidney function. CCN2 is produced by mesangial cells (MCs) and can drive renal

disease in response to TGFβ [70]. CCN2 is also involved in the recruitment of inflammatory cells from the renal interstitium through NFκB-mediated secretion of cytokines and chemokines [88]. CCN3 was shown to also be secreted by MCs and can negatively regulate CCN2 stimulation by TGFβ, and therefore block the fibrotic response [70]. More studies are needed to address these antagonist roles *in vivo*.

Chronic inflammation of the nervous system is characterized by the activation of resident microglia and astrocytes. Neuroinflammation has been implicated in the progression of various pathological conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, and amyotrophic lateral sclerosis. CCNs are highly expressed in the central nervous system, and although no neurodevelopmental defects were observed in CCN mutants, they may play significant roles in neuroinflammation [48]. Elevated expression of CCN2 was observed in AD brain neurons and astrocytes, which correlates with the progression of AD dementia and the deposition of amyloid plaques [89]. These studies suggest that CCN2 contributes to the progression of β-amyloidosis and AD prognostic outcome. The roles of CCNs during CNS development and neuroinflammation remain to be elucidated.

CCNs represent a class of novel and potent inflammatory modulators in multiple disease pathologies. Future studies should focus on more *in vivo* analyses of the separate and overlapping functions of CCNs during transient and chronic inflammatory diseases.

Cardiovascular Disease

CCNs are involved in many vascular disease pathologies including atherosclerosis, restinosis and various cardiomyopathies. CCN1 and CCN2 are overexpressed in VSMCs of atherosclerotic plaques and promote neointimal hyperplasia of the vascular walls after injury [83]. CCN1 siRNA knock down results in decreased neointimal hyperplasia after vascular injury, making it a

potential therapeutic target in treating restenosis [45]. *Ccn3* null mice exhibit increased neointimal thickening after thrombosis induction, indicating an inhibitory role in neointimal hyperplasia [51].

CCN1 and CCN2 are up-regulated in the cardiomyocytes of patients during cardiac remodeling after myocardial infarction and ischemic injury [90,91]. CCN2 and CCN5 play opposing roles during cardiac hypertrophy and induced pressure overload, with CCN2 playing a pro-fibrotic role and CCN5 playing an anti-fibrotic role. Interestingly, a deletion of the C-terminal domain of CCN2 conferred anti-fibrotic and hypertrophic responses similar to CCN5; and conversely, CCN5 develops a CCN2 like pro-fibrotic phenotype by adding the C-terminus. These results show that the C-terminal domain of CCN2 is the mediator of CCN2-mediated cardiac hypertrophy and possibly the fibrotic responses in other tissues [71].

Therapeutic Innovations and Clinical Trials

The secretory properties of CCNs make them well suited for the production of functional blocking antibodies. Blocking antibodies that directly target integrins or their binding sites in CCN domains (i.e. VWC, TSR and C-terminal domains), may be another approach to block the activation of CCNs [48]. Small interfering RNA (siRNA) and anti-sense oligonucleotides against CCNs also present interesting therapeutic targets, as they are more specific and decrease the side effects that may occur with the use of monoclonal antibodies [48].

Most drug targeting approaches are directed at CCN2, due to the prominent role it plays in multi-organ fibrosis. A phase I clinical study is currently in progress to determine the efficacy of a CCN2 monoclonal blocking antibody (FG-3019) in the treatment of mild to moderate idiopathic pulmonary fibrosis. Initial studies are promising and phase II clinical trials are underway [48]. Additional trials investigating the affect of FG-3019 for the treatment of liver

fibrosis caused by chronic hepatitis B infection are entering their early stages of development. The CCN2 FG-3019 antibody has also been used in another clinical trial for the treatment of diabetes patients with albuminuria, which can compromise kidney function. Initial studies are promising and show that blocking CCN2 results in a significant decrease in albuminuria [48].

Anti-sense oligonucleotides against CCN2 are also being utilized in several phase II clinical trials studying the effects of blocking CCN2 to reduce scarring after breast and abdominoplasty surgeries with promising results [48].

The targeting approaches applied to the treatment of CCN2 during fibrosis can also be applied to other CCNs. In addition, the blocking activities of CCN5 may also be utilized to inhibit the functions of other CCNs and presents an interesting therapeutic approach to inhibiting the harmful effects of other CCNs.

Summary

CCNs present a unique class of matricellular proteins that are involved in numerous developmental and disease states. CCNs function in concert and antagonistically with one another to regulate various aspects of cell proliferation, migration, adhesion and apoptosis. These properties make the functions of CCNs in different physiological and pathological conditions difficult to ascertain, but also highlight the general complexities associated with integrated pathways and ECM signaling molecules in the whole organism. Future studies should focus on the cell specific functions of CCNs during development, as these processes are recapitulated during stem cell renewal and differentiation, wound healing, cancer and metastasis.

CCNs also play roles in various fibrotic disorders, cancers and inflammatory diseases, making them important diagnostic markers for monitoring disease progression and therapeutic efficacy. The clinical trials in progress with CCN2 in the treatment of fibrotic disorders are

promising. However, other CCNs are also involved in similar disease states and have opposing functions against CCN2 in these contexts. Research and therapies targeting the function of other members of the CCN family should be continued, as their therapeutic potential is far from being unlocked.

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Chapter 2

Roles for CCN2 in normal physiological processes

Preface

Roles for CCN2 in normal physiological processes is a review article that was written in order to discuss the current literature pertaining specifically to the functions of CCN2/CTGF during embryonic and postnatal development. The roles of CCN2 during pathological fibrosis are also discussed, which are variable depending on the stage, tissue type and mouse model. This review also contains new data on the expression of CCN2 during postnatal development through the analysis of CCN2-enhanced green florescent protein (CCN2-eGFP) mice. The overview is divided into the following sections: (1) CCN2 structural domains and functions; (2) CCN2 expression; (3) CCN2 and fibrosis; (4) CCN2 function in development; (5) Roles in angiogenesis; and (6) Insights and future directions.

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Cellular and Molecular Life Sciences

MULTI-AUTHOR REVIEW

Roles for CCN2 in normal physiological processes

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Abstract CCN2, also known as connective tissue growth factor, is a member of the CCN (CCN1–6) family of modular matricellular proteins. Analysis of CCN2 function in vivo has focused primarily on its key role as a mediator of excess ECM synthesis in multiple fibrotic diseases. However, CCN2 and related family members are widely expressed during development. Recent studies using new genetic models are revealing that CCN2 has essential roles in the development of many tissues. This review focuses on current and emerging data on CCN2 and its functions in chondrogenesis and angiogenesis, and on new studies showing that CCN2 has essential functions during embryonic and postnatal development in a number of epithelial tissues.

Keywords CCN2 · CTGF · Connective tissue growth factor

Introduction

CCN2, also known as connective tissue growth factor (CTGF) is a member of the CCN (CCN1–6) family of modular matricellular proteins [1]. Analysis of CCN2 function in vivo has focused primarily on its key role as a mediator of excess extracellular matrix (ECM) synthesis in multiple fibrotic diseases. However, CCN2 and related family members are widely expressed during development, and modulate cell adhesion, proliferation, survival, migration, and extracellular matrix production in diverse cell types. A role for CCN2 as an essential regulator of chondrogenesis during development has been established, but recent studies have provided new insights into the mechanisms of CCN2 action in cartilage. Other studies are revealing that CCN2 has essential roles in the development of many other tissues. This review focuses on current and emerging data on CCN2 and its functions throughout embryonic and postnatal development.

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CCN2 structural domains and functions

CCN2 and the five other members of the CCN family (CCN1/Cyr61, CCN3/Nov, CCN4/WISP-1/ELM1, CCN5/WISP-2/CTGF-L and CCN6/WISP-3) are cysteine-rich matricellular proteins that contain an N-terminal secretory peptide, followed by four multi-functional domains with a diverse array of binding partners that potentially impact multiple signaling mechanisms [2]. Proteins that interact with CCN2 through recognition of these domains include integrins, low-density lipoprotein receptor-related proteins (LRPs), growth factors, and ECM components (Fig. 1). The first domain shares homology to insulin-like growth factor binding proteins (IGFBPs), but has very low affinity for IGF [3]. The second domain encodes a von Willebrand type C (VWC) repeat. This motif mediates CCN2 interactions with integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and with growth factors such as bone morphogenetic proteins (BMPs) and transforming growth factor β (TGFβ) [1, 4–6]. Recently, the IGFBP and VWC domains, which comprise the N-terminal half of CCN2, have been shown to bind to aggrecan, the major proteoglycan produced by chondrocytes [7]. The

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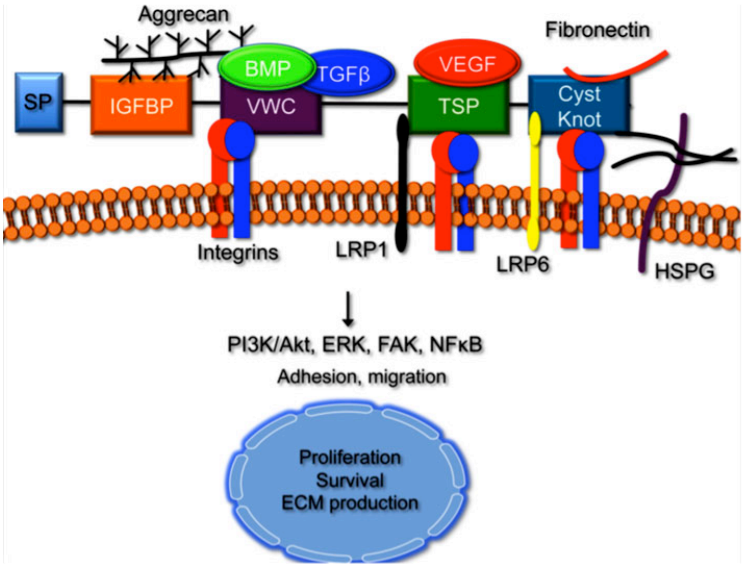

Fig. 1 Functional Domains of CCN Family Members. The CCN family is comprised of six members that contain four conserved functional domains. Each domain is likely to contribute both independently and cooperatively to the overall function of the CCN family members. The insulin-like growth factor binding protein (IGFBP) domain and the von Willebrand factor C (VWC) domain both bind to aggrecan. The VWC domain also binds to BMPs and TGFβ, integrins and matrix metalloproteinases (MMPs). The third motif is the thrombospondin (TSP) domain, which interacts with integrins, growth factors, vascular endothelial growth factor (VEGF) and low-density lipoprotein receptor-related proteins (LRPs). The last structural module is the C-terminal domain, which contains the cysteine knot motif. This motif binds to integrins, heparin sulfate proteoglycans (HSPGs), fibronectin and LRPs. CCN5 lacks this structural motif, but can still function similarly to other CCN family members. Downstream signaling via integrins activates multiple cell signaling pathways that mediate cell survival, migration, and ECM production

third domain is a type-1 thrombospondin (TSP) repeat, known to mediate the ability of TSP to bind to ECM proteins, matrix metalloproteinases (MMPs), and integrins. This domain modulates CCN2 interactions with VEGF and LRP1 [6, 8]. The final C-terminal (CT) motif contains a cysteine knot similar to those present in many growth factors, including members of the TGFβ super family, platelet derived growth factor (PDGF), and nerve growth factor (NGF). This domain is also found in other secreted proteins, including WISE, slit, and mucins [9–11]. It mediates CCN2 interactions with LRP6, fibronectin, perlecan, and fibulin-1 [12–14].

Interactions between CCN2 and these binding partners mediate the effects of CCN2 on cell proliferation, survival, differentiation, adhesion, migration, and ECM production [15]. However, to date, none of the in vivo activities of CCN2 have been unequivocally attributed to any of these specific interactions. This is due in part to the fact that the in vivo roles of CCN2 in development and homeostasis have not been completely defined, and because CCN2 most likely mediates its effects through multiple mechanisms that are tissue specific.

CCN2 expression during embryonic and postnatal development

CCN2 is conserved in chordates but is not found in invertebrates. Where studied, its pattern of expression appears to be largely conserved. For example, in zebrafish, *Xenopus*, and mouse, CCN2 expression is first detected in somites, floorplate, and notochord [16–18]. The role of CCN2 in these tissues is essentially unknown. In zebrafish, the injection of morpholinos against CCN2 led to developmental delays and distortion of the notochord. However, the nature of these defects has not been clarified, and they are not seen in *Ccn2* null mice or in *Xenopus* embryos treated with morpholinos [1, 12, 18, 19]. CCN2 is co-expressed with the related family member CCN1 in the notochord (unpublished data), raising the possibility of overlapping functions for these CCN proteins in early stages of notochord formation.

CCN2 is expressed in a wide variety of structures at later stages of development. In the mouse, CCN2 is expressed at high levels in the developing cartilage and vasculature [18, 20, 21]. Other prominent sites of expression include various

epithelial structures, such as bronchial and alveolar epithelium of the lung, pancreatic duct and endocrine cells, and salivary glands [20, 22]. As discussed below, recent studies indicate that CCN2 plays essential roles in the development of many of these structures. CCN2 is also expressed in maturing neurons, and in sensory organs such as the otic placode [6, 16, 17, 20]. To date, the functions of CCN2 in neural and sensory structures have not been investigated.

CCN2 expression is maintained in adult tissues, and is increased in pathological conditions such as fibrosis, atherosclerosis, osteoarthritis, and certain cancers [23]. In the adult mouse, CCN2 is expressed in the reproductive system, where it has been implicated in control of uterine cellular growth, adhesion, migration, and ECM production during the estrous cycle and pregnancy [24].

CCN2 and fibrosis

In vivo studies of CCN2 function have focused almost entirely on its role in fibrosis. Fibrosis is caused by excessive extracellular matrix (ECM) deposition mediated by activated contractile fibroblasts termed myofibroblasts [25]. CCN2 over-expression is a hallmark of fibrosis in multiple tissues, including skin, liver, heart, lung, and kidney, and is widely thought to be required to mediate the profibrotic effects of TGFβ. However, the extent to which CCN2 causes fibrosis, (or is merely a marker of fibrosis in different organs), is unknown. A number of excellent reviews on this topic have been published recently [15, 26, 27]. Hence, we focus here on the development of several new transgenic models of CCN2 over-expression, which have revealed unexpected actions of CCN2 in the onset and progression of fibrosis in vivo.

The expression of CCN2 during normal wound healing and in various fibrotic diseases [25, 28] has prompted many studies of CCN2 function in wound healing and in chemically induced fibrosis [25]. However, in vivo studies have been limited, in part because suitable transgenic models have not been available. Mice over-expressing CCN2 in podocytes [28] or liver [29] are normal. Increased fibrosis was observed in these transgenic models only upon tissue injury. These findings may indicate that CCN2 alone is not sufficient to induce fibrosis in these organs. An alternative explanation is that levels of CCN2 expression were not high enough to induce fibrosis. A new transgenic model, described below, suggests the latter possibility may be true with respect to the kidney.

Mice over-expressing CCN2 under the control of the fibroblast-specific enhancer of type I collagen (Col1-CCN2) develop systemic multi-organ fibrosis. Excess ECM production was noted in dermis, kidney, and lung. Immunostaining revealed a large increase in the number of myofibroblasts, and consistent with previous in vitro studies, fibroblasts from transgenic mice exhibited increased migratory capability and excessive ECM production [30]. Unlike the podocyte-specific transgenic mice discussed above [28], basement membrane thickening and increased ECM deposition around blood vessels were noted in the kidneys of Col1-CCN2 transgenics. Consistent with the notion that the difference in susceptibility to kidney fibrosis between these two transgenic models relates to CCN2 levels, these effects were dose-dependent, as mice carrying two copies of the Col1-CCN2 transgene insertion had a shorter lifespan and earlier onset of fibrosis than did mice heterozygous for the transgene insertion [30].

Recently, Liu et al. generated a conditional mouse model in which CCN2 was deleted specifically in fibroblasts and smooth muscle cells [31]. Consistent with the findings that CCN2 over-expression in fibroblasts induced systemic fibrosis [30], loss of CCN2 resulted in a marked decrease in bleomycin induced-skin fibrosis [31]. Remarkably, these effects were not TGFβ-dependent, nor did the loss of CCN2 impact the ability of TGFβ to induce alpha smooth muscle actin (α-SMA) or collagen type I expression [31]. The attenuated fibrosis in *Ccn2* conditional mutants was attributed to defective myofibroblast migration and recruitment to the site of fibrosis [31]. Thus, these gain- and loss-of-function studies suggest that CCN2 is sufficient to induce fibrosis, and challenge the dogma that CCN2 is required to mediate the pro-fibrotic effects of TGFβ.

The Col1-CCN2 transgenic phenotype described above contrasts with that seen in transgenic mice in which CCN2 expression from the endogenous locus can be increased or decreased [30, 32]. This latter mouse model was developed by introduction of a 3′UTR cassette into the *Ccn2* locus that alters *Ccn2* mRNA stability relative to the native *Ccn2* 3′UTR. Cre-mediated recombination excises this 3′UTR and replaces it with one that yields increased *Ccn2* mRNA stability compared to the native *Ccn2* 3′UTR. This novel strategy, when used in conjunction with *Ccn2* null mice, permits analysis of the effects of altering *Ccn2* expression from 30% of normal to up to ninefold higher than normal [32]. Mice with 30% of normal *Ccn2* expression were indistinguishable from wild-type littermates, whereas ninefold over-expression led to developmental delay, craniofacial defects, and early embryonic lethality [32]. The cause of lethality was not investigated in this study, but both the time of lethality and the presence of hemorrhage and altered microvasculature in transgenic over-expressers suggests a cardiovascular defect. The few over-expressers that survived to adulthood exhibited moderate cranial and axial skeletal defects, but no fibrosis. However, these surviving mice were found to exhibit incomplete Cre-mediated recombination [32]. While the present study demonstrates that CCN2 has potent effects during development, it was not

informative with respect to adult tissues. However, when used in conjunction with tissue-specific inducible Cre alleles, this transgenic model is likely to be highly informative with respect to CCN2 function in adult tissues that normally express this protein.

Transgenic models have led to unexpected insights into CCN2 function in the heart. Although CCN2 is highly expressed in the developing heart, it is unknown whether CCN2 is required for cardiac development. CCN2 is also markedly elevated during cardiac fibrosis, and can stimulate cardiac smooth muscle cell proliferation [33]. Two recent papers reported the generation of transgenic mice over-expressing CCN2 in the heart [34, 35]. Surprisingly, neither study found any evidence of fibrosis, either basally, or in response to cardiac ischemia/reperfusion injury or acute pressure-overload. In the first model, there was no fibrosis, but induction of an adaptive hypertrophic response that may have contributed to the finding that transgenics were no more susceptible than wild-type littermates to ischemia/reperfusion injury [34]. In the second transgenic model, although CCN2 transgenic hearts exhibited increased myocardial pro-collagen and fibronectin expression, this was counteracted by increased MMP3 levels [35]. When challenged with cardiac ischemia/reperfusion injury, CCN2 over-expression was actually cardio-protective [35].

The discrepancies between these models may be a consequence of the different promoters used. Both alpha-myosin heavy chain (α-MHC) and myosin light chain-2 (MLC-2) are expressed early in developing cardiomyocytes, but as development proceeds, α-MHC expression is confined mainly to the atria, whereas MLC-2 is expressed predominantly in the ventricle [36]. These promoters may also be differentially regulated during cardiac hypertrophy. Nonetheless, these results clearly indicate that CCN2 is not always profibrotic, and can have protective functions in adult tissues. Studies to elucidate the mechanisms underlying these cardioprotective functions are clearly warranted.

Overall, the effects of CCN2 over-expression appear to be both dose-dependent and context-specific. While the above models have confirmed the expected contribution of CCN2 to the onset and/or progression of fibrosis in several tissues in adults, they have led to the discovery of protective functions in others. Given the vast number of growth factors, ECM components, integrins, and other receptors that can interact with CCN2 in vitro, it is likely that CCN2 can exert very different functions at different doses within a given tissue.

CCN2 function in development

The most prominent sites of CCN2 expression in developing embryos are the skeletal and cardiovascular systems [20, 22, 37], and expression in these tissues persists in adults [38]. Essential functions for CCN2 have been demonstrated in multiple aspects of skeletogenesis and angiogenesis. Other recent studies are revealing roles in epithelial tissues. We review some of the major findings from in vivo studies below.

Lung: Both gain- and loss-of-function studies support an essential role for CCN2 in lung formation. Similar to the effects of CCN2 over-expression in hepatocytes, CCN2 adenoviral over-expression in the adult mouse lung is unable on its own to induce chronic fibrosis, but does increase susceptibility to fibrosis following chemical treatment [39, 40]. In contrast, CCN2 over-expression in airway epithelium [41] or alveolar type II epithelial cells [42] during the first 2 weeks of postnatal life in transgenic mice led to thicker alveolar septa, characterized by increased cell proliferation, collagen and fibronectin deposition, myofibroblast differentiation, and fibrosis. Increased levels of integrin-linked kinase (ILK) activation were noted, raising the possibility that the phenotype reflects the ability of CCN2 to engage integrins directly, and/or to increase the expression of other ECM components that bind to these integrins. Moreover, increased stabilization and nuclear translocation of β-catenin, a well-known target of ILK [43] that mediates fibrosis in multiple organs (e.g., [44–46]) was seen in CCN2 transgenic lungs. These findings, along with the TGFβ-independent of ability of CCN2 to induce fibrosis found in the Col1-CCN2 transgenics discussed previously [30], suggest that CCN2 may mediate its pro-fibrotic effects to a significant degree through Wnt pathways. A number of studies have shown that CCN2 expression is induced by canonical Wnt signaling [47, 48]. The demonstration that CCN2 over-expression in turn induces stabilization of β-catenin raises the possibility that CCN2 is an essential component of a positive feedback loop regulating levels of canonical Wnt signaling.

A loss-of-function study also provides strong support for an essential role for CCN2 in alveolarization during lung maturation [49]. Examination of *Ccn2* null mice revealed hypoplastic lungs, with reduced cell proliferation, a phenotype opposite to that seen in the above transgenic over-expression models. This study did not examine the consequences of loss of CCN2 on canonical Wnt pathways. However, the authors reported decreased expression of PDGF receptor beta (PDGFRβ), which is primarily expressed by pericytes [49, 50]. Whether the decreased expression of PDGFRβ in *Ccn2*−/− lungs translates to altered levels of PDGF-mediated signal transduction is unknown. However, an investigation of the role of CCN2 in PDGF signaling is warranted, especially in light of the potent pro-fibrotic properties of PDGF [51].

Skeletal tissues: CCN2 appears to have essential functions in multiple skeletal tissues. CCN2 function has been studied most extensively in cartilage, but recent studies have demonstrated roles in osteoblasts, osteoclasts, intervertebral discs, and cranial sutures.

Cartilage: Endochondral bone formation is a dynamic process in which mesenchymal cells condense and differentiate, resulting in the formation of a growth plate. Chondrocytes in the growth plate are arranged in distinct zones, which include a slowly proliferating reserve population, the rapidly proliferating columnar population, followed by a post-mitotic prehypertrophic zone and finally a terminally differentiated hypertrophic zone. Hypertrophic chondrocytes produce a matrix that subsequently promotes vascular invasion and osteoblast migration, leading to replacement of the cartilage template with bone [52, 53].

CCN2 is not expressed in mesenchymal condensations, but can be detected at the earliest stages of growth plate formation [18]. CCN2 is expressed in proliferating chondrocytes at low levels, and at higher levels in hypertrophic cells [37]. The expression of *Ccn2* in growth plate chondrocytes was shown recently to be positively regulated by canonical Wnt pathways via a TCF/LEF motif in the *Ccn2* promoter [48]. This finding accounts for the high levels of expression of CCN2 in hypertrophic chondrocytes, which contain the highest levels of stabilized β-catenin in the growth plate [48].

Ccn2 null mice exhibit lethality at birth, accompanied by severe chondrodysplasia [18]. *Ccn2*−/− chondrocytes exhibited decreased rates of proliferation and survival. These defects were attributed to defective production of multiple ECM components, including type II collagen and aggrecan, the major collagen and proteoglycan, respectively, in cartilage [18, 54]. While the precise mechanisms by which CCN2 mediates its essential role in maintenance of ECM production in the growth plate are unknown, integrins are likely to play key roles. CCN2 is a ligand for integrin $\alpha 5\beta 1$ in chondrocytes, and this integrin is important for chondrocyte adhesion and survival [54]. Moreover, the loss of CCN2 results in a significant decrease in $\alpha 5$ integrin levels and in levels of activation of the downstream effectors of integrins, including focal adhesion kinase (FAK) and extracellular regulated protein kinase in chondrocytes [54].

One of the most unexpected findings in *Ccn2* null mice is the expansion of the hypertrophic zone. This was accompanied by decreased expression of VEGF in these cells [18]. VEGF is essential for growth plate angiogenesis [55], and *Ccn2*−/− growth plates exhibit defective vascularization of the growth plate. Thus, a key function for CCN2 in the hypertrophic zone is to maintain VEGF levels. We investigated the mechanisms by which CCN2 induces VEGF expression in chondrocytes. Hypoxia-inducible factor-1α (HIF-1α) is expressed by chondrocytes and required for chondrocyte survival [52]. It is also a potent inducer of VEGF in many tissues. We found that HIF-1α levels are decreased in the growth plates of *Ccn2* mutants; similar results are seen in isolated chondrocytes where recombinant CCN2 induces HIF-1α, demonstrating that the effects of CCN2 on HIF-1α are direct in chondrocytes [56]. The mechanisms underlying CCN2-mediated regulation of HIF-1α are unknown.

While the above results demonstrate that CCN2 is essential for chondrogenesis during development, whether it is required for maintenance of cartilage in adults is an important unanswered question. We have analyzed CCN2 expression in postnatal cartilage using a BAC transgenic line in which green florescent protein (GFP) is expressed under the control of the *Ccn2* locus [57] (Fig. 2). In 1 to 2-month-old mice, CCN2 is expressed in the intervertebral disc (Fig 2a), a derivative of the notochord (which also expresses CCN2) in the mouse. It is also expressed in all appendicular and axial cartilaginous elements, with the highest levels of expression in hypertrophic zone in both axial (rib) and appendicular elements (Fig. 2b,c). CCN2 expression persists in articular cartilage, suggesting an essential role in cartilage maintenance in adults.

On the one hand, CCN2 is expressed in articular chondrocytes and can stimulate articular cartilage repair and regeneration in vivo [58]. On the other hand, CCN2 levels are elevated in osteoarthritic (OA) cartilage [58, 59], and adenoviral over-expression of CCN2 in knee joints leads to transient fibrosis and cartilage damage [59]. These disparate outcomes most likely reflect different levels of CCN2 expression, which in turn, may lead to different levels of HIF activity. For example, low levels of HIF-1α are anabolic for cartilage [60]. However, HIF-2α promotes aspects of hypertrophy in the growth plate, and OA in articular cartilage [61]. It is thus conceivable that low levels of CCN2 are required to maintain levels of HIF activity that promote anabolic functions in growth plate and articular cartilage, but that the higher levels of CCN2 found in the hypertrophic zone and in OA cartilage promote HIF-2α expression, leading to cartilage damage.

Bone: CCN2 also appears to have effects on bone formation in vivo. This was initially demonstrated by Kawaki et al. using *Ccn2* null mice [62]. Owing to neonatal lethality, this analysis did not address the role of CCN2 in adult bone remodeling. However, *Ccn2* null mice were shown to exhibit a reduction in bone matrix synthesis, osteoblast proliferation, maturation, and mineralization in vivo. At least some of these effects are likely to be direct, as opposed to a secondary consequence of defective endochondral ossification, as the osteogenic response in calvarial osteoblasts isolated from *Ccn2* mutants was significantly reduced in vitro [62]. These studies were

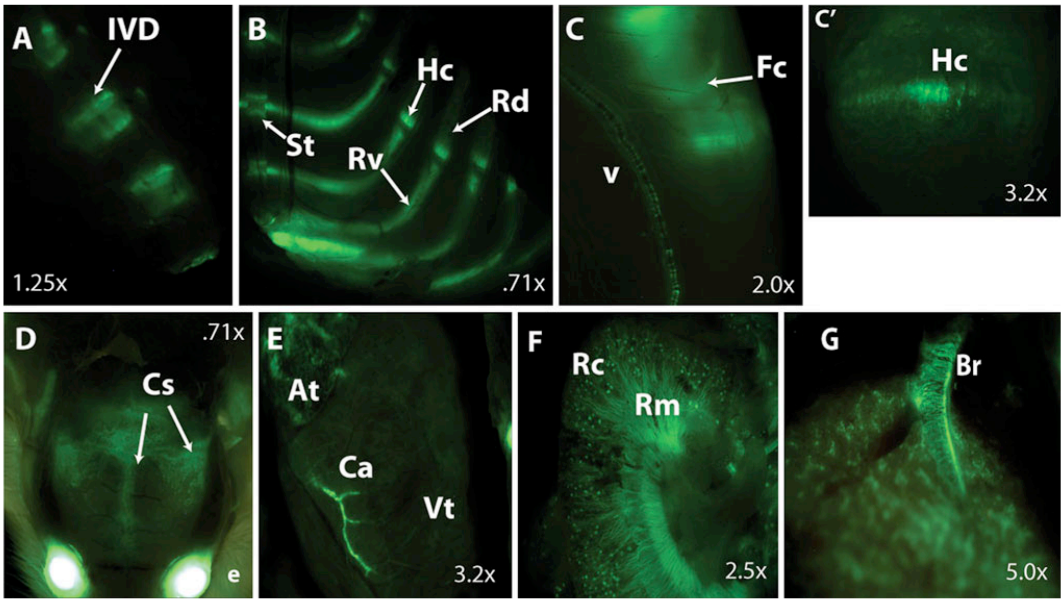

Fig. 2 CCN2 eGFP Expression in the adult mouse. CCN2 expression was examined in 1-month-old male mice. **a** Strong CCN2 expression was observed in vertebral bodies and intervertebral discs. **b** CCN2 is expressed in ribs within the cartilaginous ventral segments, as compared to undetectable expression in the ossified dorsal rib segments. **c**, **c′** Expression in hypertrophic chondrocytes in the femoral growth plate and in articular cartilage. **c′** is a magnification of the growth plate in **c**. **d** CCN2 is highly expressed in cranial sutures. Expression was also observed in both major and minor vasculature including coronary arteries (**e**), kidneys (**f**) and lungs (**g**). Intervertebral disc (IVD), sternebra (St), rib ventral segment (Rv), rib dorsal segments (Rd), femoral condyle (Fc), vessel (v), hypertrophic chondrocytes (Hc), cranial sutures (Cs), atria (At), coronary artery (Ca), ventricle (Vt), renal cortex (Rc), renal medulla (Rm), bronchus (Br). Images were taken on a Leica MZ 16F bright field/GFP dissecting scope

extended recently by Canalis et al. to examine consequences of postnatal loss of CCN2 in osteoblasts [63]. In this study, the authors observed osteopenia, but only in male adult mice [63]. As in the study by Kawaki et al., isolated calvarial osteoblasts were found to exhibit diminished osteoblast function. Of note, the conditional allele of CCN2 used in the study by Canalis et al. led to reductions in *Ccn2* mRNA levels ranging from 20 to 80%, and inactivation throughout the limb bud reportedly led to a phenotype similar to that seen in *Ccn2*+/− mice [63]. This is in contrast to the bent bones seen in *Ccn2* null mice [18]. Thus, while the full extent of CCN2 action in bone is as yet uncertain, it is clear that CCN2 is an important regulator of osteoblast function. Its mechanism of action is completely unknown in this tissue.

CCN2 is abundantly expressed in cranial sutures (Fig. 2d). Premature mineralization of cranial sutures leads to craniosynostosis, which restricts skull growth and causes disfigurement. A recent study showed that CCN2 expression can prevent craniosynostosis in a rat model [64]. While sutures appear to be unaffected in *Ccn2*−/− neonates (unpublished data), it is conceivable that CCN2 plays a role postnatally in regulating suture patency.

Roles in angiogenesis

CCN2 was first isolated from human umbilical vascular endothelial cells (HUVECs) [21], and is highly expressed in all major and micro-vasculature including coronary vessels of the heart, vasculature of the kidneys, lung, and liver (Fig. 2e–g) [20, 22, 65]. The physiological roles of CCN2 in angiogenesis are unclear, however, as CCN2 has both pro- and anti-angiogenic activities in vitro. For example, CCN2 induces angiogenesis in the cornea [66], and induces neovascularization in vitro through engagement of integrins. CCN2 is a ligand for $\alpha 6\beta 1$ and $\alpha v\beta 3$ in endothelial cells [21, 65, 66]. However, anti-angiogenic activities have also been reported. CCN2 binds to and sequesters VEGF in an inactive form [8] and combined administration of CCN2 and VEGF in a mouse model of hindlimb ischemia inhibits VEGF induced angiogenesis

[67]. Taken together, these studies implicate CCN2 as an essential regulator of angiogenesis, but do not address its role in vivo.

CCN2 global knock out mice exhibit defective growth plate angiogenesis as a result of decreased levels of vascular endothelial growth factor (VEGF) in hypertrophic cartilage [18]. However, whether CCN2 also plays a direct role in the vasculature has not been demonstrated. One study compared basal and VEGF-induced outgrowth of vessels from metatarsals of wild-type and *Ccn2* mutant neonatal mice to conclude that CCN2 is not required for angiogenesis [68]. However, the large amount of variation in outgrowth that was observed may obscure real differences, and the images shown in the paper reveal considerably less outgrowth from *Ccn2−/−* metatarsals treated with VEGF [68].

On the other hand, a second study by the same group showed that the thickening of the basal lamina of retinal capillaries that occurs during streptozotocin (STZ)-induced diabetes in mice was completely prevented in *Ccn2+/−* mice [69]. Similarly, glomerular basement membrane thickening was prevented in diabetic *Ccn2+/−* mice compared to WT littermates [70]. *Ccn2−/−* mice could not be analyzed in these studies owing to their neonatal lethality. Nonetheless, these results raise the possibility of a role for CCN2 in the formation of endothelial basement membranes. Other evidence for a role for CCN2 in endothelial basement membrane formation comes from the findings that one of the most prominent features of Col1-CCN2 [30] transgenic mice was a thickening of endothelial basement membranes.

Each of these studies addresses a role for CCN2 in vascular fibrosis; over-expression promotes basement membrane thickening, whereas decreased CCN2 expression prevents it. However, whether CCN2 plays an essential role in normal angiogenesis has not been established. The survival of *Ccn2−/−* mice to birth indicates that vasculogenesis, the initial formation of blood vessels during development, is not impaired. However, we have analyzed vasculature from *Ccn2−/−* mice, and have uncovered evidence that CCN2 is required for vascular remodeling, a process that takes place at midgestation stages and postnatally in vivo (manuscript in preparation).

Insights and future directions

Genetic models are revealing that CCN2 has essential functions for tissue formation and maintenance that extend beyond its ability to induce tissue fibrosis. It is likely that developmental functions for CCN2 beyond those discussed here will be discovered. For example, a role has recently been discovered for CCN2 in the formation of the endocrine pancreas [71]. CCN2 is widely expressed in other secretory epithelial tissues, raising the possibility that CCN2 has a much broader role during development than currently appreciated [24]. A major unknown is the extent to which CCN2 and other members of the family have overlapping functions. Members of the CCN family can engage similar sets of integrins and bind to similar ECM components to mediate similar effects in vitro [23], and their patterns of expression overlap in many tissues [17, 72]. Given the many integrins, growth factors and their receptors, and ECM components that have been shown to interact with CCNs, whether individual CCN proteins have similar versus distinct functions will likely prove to be cell-type specific. Finally, the mechanisms by which CCNs mediate their effects in vivo are essentially unknown. While we uncovered a role for integrins as mediators of the effects of CCN2 in chondrogenesis, it is highly likely that CCN2 uses additional mechanisms in this and other tissues.

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Chapter 3

CCN2/Connective Tissue Growth Factor is Essential for Pericyte Adhesion and Endothelial Basement Membrane Formation during Angiogenesis

Preface

A global knockout of *Ccn2* in mice was generated in our laboratory in 2003. These mice die at birth due to severe defects in cartilage differentiation, matrix production, growth plate angiogenesis and subsequent bone formation [1]. The defective growth plate angiogenesis observed was attributed to two separate mechanisms: the first was defective chondrocyte proliferation and differentiation, which results in a disorganized growth plate with defective structural properties; the second, resulted from a decrease in the secretion of the angiogenic factors vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9) from terminally differentiating chondrocytes, leading to defective growth plate angiogenesis [1].

This interesting vascular phenotype led us to explore further the function of CCN2 in other vascular beds throughout embryonic development. Previous *in vitro* and *ex vivo* studies investigating the role of CCN2 during angiogenesis were ambiguous, as both pro- and anti-angiogenic activities were observed [2,3]. This chapter provides evidence that CCN2 is a critical mediator of angiogenesis. The first mechanism is revealed in the analysis of CCN2 mutant vasculature showing defective endothelial cell and pericyte adhesion. These effects were mediated in part, by the ability of CCN2 to induce platelet derived growth factor (PDGF) secretion by endothelial cells and subsequent PDGF/Akt-signaling in vascular smooth muscle cells (VSMCs), both of which are required for vessel stability [4]. The second mechanism we observed was decreased expression of the vascular basement membrane components fibronectin and collagen type IV in CCN2 mutants. The proper formation of the vascular basement membrane is essential for cell adhesion and vessel integrity [5]. We conclude that CCN2 plays duel roles in angiogenesis, first by mediating PDGF signaling between endothelial cell and perictyes, and second, by controlling the formation of vascular basement membrane components.

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CCN2/Connective Tissue Growth Factor Is Essential for Pericyte Adhesion and Endothelial Basement Membrane Formation during Angiogenesis

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Abstract

CCN2/Connective Tissue Growth Factor (CTGF) is a matricellular protein that regulates cell adhesion, migration, and survival. CCN2 is best known for its ability to promote fibrosis by mediating the ability of transforming growth factor β (TGFβ) to induce excess extracellular matrix production. In addition to its role in pathological processes, CCN2 is required for chondrogenesis. CCN2 is also highly expressed during development in endothelial cells, suggesting a role in angiogenesis. The potential role of CCN2 in angiogenesis is unclear, however, as both pro- and anti-angiogenic effects have been reported. Here, through analysis of *Ccn2*-deficient mice, we show that CCN2 is required for stable association and retention of pericytes by endothelial cells. PDGF signaling and the establishment of the endothelial basement membrane are required for pericytes recruitment and retention. CCN2 induced PDGF-B expression in endothelial cells, and potentiated PDGF-B-mediated Akt signaling in mural (vascular smooth muscle/pericyte) cells. In addition, CCN2 induced the production of endothelial basement membrane components *in vitro*, and was required for their expression *in vivo*. Overall, these results highlight CCN2 as an essential mediator of vascular remodeling by regulating endothelial-pericyte interactions. Although most studies of CCN2 function have focused on effects of CCN2 overexpression on the interstitial extracellular matrix, the results presented here show that CCN2 is required for the normal production of vascular basement membranes.

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Introduction

CCN2, also known as connective tissue growth factor, is a member of the CCN (CCN1-6) family of matricellular proteins. CCN family members are cysteine-rich and contain an N-terminal secretory peptide, followed by four multi-functional domains that interact with a diverse array of binding partners [1,2]. Proteins that interact with CCN2 through recognition of these domains include integrins, low-density lipoprotein receptor-related proteins (LRPs), growth factors, and extracellular matrix (ECM) components. The first domain shares homology to insulin-like growth factor binding proteins (IGFBPs), but has very low affinity for IGF [3]. The second domain encodes a von Willebrand type C (VWC) repeat. This motif mediates CCN2 interactions with growth factors such as bone morphogenetic proteins (BMPs) and transforming growth factor β (TGFβ) [4]. The third domain is a type-1 thrombospondin (TSP) repeat, known to mediate the ability of CCN2 to bind to ECM proteins, matrix metalloproteinases (MMPs) and integrin α6β1 [5,6] The final C-terminal (CT) motif contains a cysteine knot similar to those present in many growth factors, including members of the TGFβ superfamily, platelet derived growth factor (PDGF), and nerve growth factor (NGF). This motif mediates interactions with integrins αvβ3, α5β1, and α6β1 [7–13].

CCN2 was originally isolated from human umbilical vein endothelial cells (HUVECs) [14]. *In situ* hybridization and immunohistochemical studies demonstrated that CCN2 is expressed predominantly in endothelial cells in embryonic and adult

vasculature [15–18]. The physiological role of CCN2 in angiogenesis is unclear, however, as it appears to have both pro- and anti-angiogenic activities *in vitro*. For example, CCN2 induces corneal angiogenesis, and anti-CCN2 antibodies block angiogenesis in the chick chorioallantoic membrane assay [19,20]. On the other hand, anti-angiogenic activities have been reported; although *Ccn2* expression is induced by VEGF [21], CCN2 binds to and sequesters VEGF in an inactive form [5], and combined administration of CCN2 and VEGF inhibits VEGF-induced angiogenesis [22]. The role of CCN2 in angiogenesis *in vivo* is unknown.

The majority of studies have focused on the role of CCN2 as a stimulator of excess ECM production in the context of pathological fibrosis [23]. CCN2 is overexpressed in all fibrotic conditions described to date, and depending on the tissue involved, induces collagen type I deposition and increased susceptibility to injury [24]. Conversely, the loss of CCN2 in fibroblasts results in decreased collagen deposition and resistance to chemically induced skin fibrosis [25,26]. In addition to its role as a mediator of fibrosis, CCN2 is required for ECM production in cartilage [27]. *Ccn2* knockout mice survive in Mendelian ratios throughout gestation, but die within minutes of birth. They exhibit severe chondrodysplasia as a result of decreased collagen type II and aggrecan expression by chondrocytes *in vivo* and *in vitro* [27,28]. CCN2 regulates cell survival, adhesion, migration, and ECM production in multiple cell types by regulating integrin expression and activation [13]. In *Ccn2* mutant chondrocytes, integrin $\alpha5\beta1$ expression and downstream focal adhesion kinase (FAK) and extracellular signal-related kinase (ERK1/2) signaling are decreased, indicating that CCN2 regulates ECM production through integrins [28].

In endothelial cells, CCN2 mediates adhesion, migration and survival through binding to integrin $\alpha v\beta3$ [7]. CCN2 is also a ligand for $\alpha5\beta1$ and $\alpha6\beta1$ [13], and these integrins are required for endothelial basement membrane formation and vessel stabilization *in vitro* [29]. Taken together, these studies implicate CCN2 as an important regulator of cellular adhesion and ECM production during angiogenesis, but do not address its role *in vivo*. As CCN2 is the major mediator of excess ECM production during fibrosis, and has also been implicated in tumor angiogenesis [30], it is important to understand its function in normal tissues. Therefore, the function of CCN2 in angiogenesis was investigated through analysis of *Ccn2* mutant mice.

Results

CCN2 is expressed in the developing vasculature

Using transgenic mice in which lacZ expression is driven by the 4 kb proximal *Ccn2* promoter [31], CCN2 expression was seen throughout the vasculature and microvasculature at E16.5 (Figure 1A). Expression was observed in large vessels, arterioles and capillaries at all stages examined (E13.5-P0). CCN2 was detected as early as E13.5 in developing dermal microvasculature (Figure 1B), where lacZ is present in large and small caliber vessels (Figure 1A,B). Similar results were seen using bacterial artificial chromosome (BAC) transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the *Ccn2* locus (CCN2-EGFP) [32]. This analysis revealed *Ccn2* expression in endothelium of arterial and venous elements, and in capillaries. In large arteries, CCN2-EGFP was expressed in both endothelial and vascular smooth muscle cells (vSMCs) (Figure 1C,E). CCN2 was also expressed in developing capillary networks (Figure 1D). Endothelial-specific expression in microvasculature was also shown by immunostaining for CCN2 (Figure 1F–H). Specificity of the antibody was confirmed by the absence of staining sections from *Ccn2* mutants (Figure 1H). Punctate intracellular staining was observed, most likely within the Golgi and in secretory vesicles, as reported previously [33]. Cell-associated expression was also seen on the abluminal surface of the endothelium (Figure 1G). Co-immunostaining with the endothelial-specific marker PECAM (CD31) revealed CCN2 expression in endothelial cells and in mural cells (Figure S1A). Thus, *Ccn2* is expressed in both endothelial and mural cells in blood vessels and capillaries during development.

Ccn2 mutant mice exhibit vascular defects

Ccn2 mutant mice exhibit perinatal lethality due to a severe chondrodysplasia [27]. CCN2 expression in developing blood vessels raised the possibility of an additional role in vascular development. *Ccn2*−/− embryos were examined to investigate this possibility. No overt differences between *Ccn2* mutants and WT littermates were apparent during the initial formation of the vasculature from E9.5–E13.5 (data not shown). Moreover, placentas were normal in appearance, weight, and vascularity throughout development (Figure S1B,C, and data not shown). However, beginning at E14.5, minor enlargement of vessels was observed in mutants (Figure S1D,E), which became more pronounced at later stages (Figure 2A,B). Local edema was seen in E18.5 mutant dermis (Figure 2C,D). Immunofluorescence analysis of the vSMC marker smooth muscle actin (SMA) and PECAM (CD-31) did not reveal obvious evidence that SMC coverage of large vessels was affected in mutants (Figure S1F–I). However, comparison of hematoxylin and eosin-stained sections of the aorta at thoracic and lumbar levels from E16.5 embryos showed defects in the organization of the tunica media (Fig. 2E–H). In WT embryos, SMCs had a spindle-like morphology and were circumferentially oriented around the vessel lumen in distinct layers (Figure 2E,G). In mutants, SMCs failed to adopt this spindle-like morphology, were more heterogeneous in size, and were not organized into distinct layers (Figure 2F,H). The large vessel phenotype will be reported in more detail elsewhere. Here we focus on the microvascular phenotype.

Morphological examination (Figure S1J,K) revealed that arterial-venous identity appeared to be maintained in mutants (see also Figure S1H,I). Ephrin B2 (expressed on arterial elements) and EphB4 (preferentially expressed on veins) staining demonstrated no defects in arterial-venous identity (Figure S1L,M, and data not shown). However, inspection of E18.5 dermal microvasculature revealed evidence of defective remodeling in *Ccn2* mutants. Consistent with a defect in remodeling, vessel density was increased in *Ccn2* mutants (Figure 2I–L and Figure S2A–C). Moreover, mutant capillaries had multiple protrusions along their surfaces (Figure 2M,N). Electron microscopy revealed numerous luminal and abluminal protrusions in mutant capillaries, consistent with the confocal analysis (Figure 2O,P).

CCN2 mutants exhibit defects in vascular remodeling

PCNA labeling and TUNEL analyses were performed to assess whether defects in proliferation and/or survival might contribute to the microvascular abnormalities in *Ccn2* mutants. No differences were detected in mutants in comparison to WT littermates (Figure S2D–G). During vascular remodeling, immature vascular beds become less dense, arterioles become smaller in diameter than venules, and pericytes form stable associations with endothelial tubes [34]. Angiopoetin 1 (Ang1) is required for stabilizing endothelial-pericyte interactions and is expressed primarily by mural cells [35]. *Ang1* mRNA levels were diminished in *Ccn2*−/− skin (Figure S2H). No differences were detected in levels of

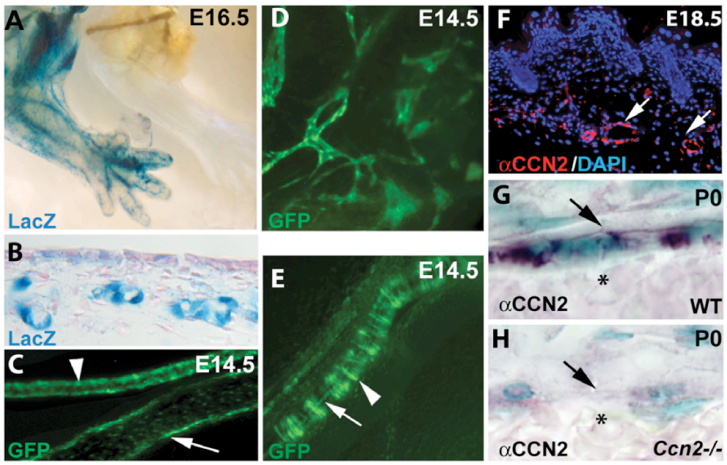

Figure 1. Expression of *Ccn2* in developing vasculature. (A) β-galactosidase activity in *Ccn2-lacZ* transgenic mice reveals *Ccn2* promoter activity throughout the vasculature in E16.5 embryos. (B) *Ccn2-lacZ* expression in dermal microvessels at E13.5. (C–E) EGFP fluorescence in CCN2-EGFP BAC transgenic mice demonstrates CCN2 expression in the endothelium of arterial elements (C and E), venous elements (C), and developing capillary networks (D). Arrowheads in (C) and (E) demarcate arterial element. Arrow in (C) identifies endothelial cells of a venous element. Arrowhead in (E) highlights EGFP expression in mural cells in the arterial element. Arrow in (E) highlights expression in endothelial cells in the arterial element. (F) Immunofluorescence and (G,H) immunohistochemical staining with an αCCN2 antibody on paraffin sections through dermis, demonstrating CCN2 expression in endothelial cells. Arrows in (F) highlight endothelial cells in E18.5 microvasculature. Specificity of the αCCN2 antibody is demonstrated by the absence of reactivity in the *Ccn2*−/− section (H). Arrows in (G) and (H) demarcate abluminal surface of the endothelium. Asterisks in (G) and (H) identify blood cells within the vessels. αCCN2 staining in (G) shows punctate intracellular expression, presumably with the Golgi, in addition to the surface expression marked by the arrow.
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expression of *Tie2*, the endothelial-specific receptor for Ang1 (data not shown). However, levels of the mRNA encoding the bio-active VEGF isoform 164 were elevated in mutants (Figure S2I). Versican is the principal chondroitin sulfate proteoglycan in blood vessels and exists in at least four isoforms, V0, V1, V2, and V3 [36]. Embryonic endothelial cells express more V0 than other isoforms, and V0 expression declines during vascular maturation [37]. No differences were seen in levels of versican *V1* in *Ccn2* mutants and WT littermates (Figure S2J); however, *Ccn2* mutants exhibited increased levels of *V0* (Figure S2K). Therefore, the loss of *Ccn2* leads to diminished expression of vessel maturation marker *Ang1* and elevated expression of markers of immature vasculature, indicative of a potential defect in vascular remodeling.

The vascular phenotype in *Ccn2* mutants bears some resemblance to mice lacking platelet-derived growth factor-B (PDGF-B) or its receptor, PDGFRβ [38,39]. In particular, defective pericyte recruitment is seen in these mice. Therefore, we examined pericyte recruitment in *Ccn2* mutants. Pericytes, which express NG2 and desmin, become associated with small diameter vessels during vessel maturation [40]. Consistent with the gene expression analysis described above, confocal analysis of desmin expression revealed incomplete coverage of microvessels by pericytes in the dermis of *Ccn2* mutants at E16.5 and E18.5 (Figure 3A–C; data not shown). Similar results were seen for NG2 expression in the lung liver, and brain microvasculature (Figure 3D–F, and data not shown). Thus, the loss of CCN2 affects the microvasculature in multiple tissues. Flow cytometric analysis of lung, liver, and brain samples from E16.5 embryos for cells negative for the endothelial cell marker PECAM, but expressing the pericyte markers NG2 and PDGFRβ [41] revealed normal numbers of endothelial cells and pericytes in *Ccn2* mutants (Figure S3, and data not shown). This suggests that the reduced pericyte coverage in *Ccn2* mutants is not caused by a decrease in pericyte number or migration, but possibly by defects in the ability of pericytes to make stable associations and elongate along endothelial cells in *Ccn2* mutant mice.

Confocal analysis of E16.5 dermal and lung microvasculature co-stained with NG2, desmin, and PECAM supports this possibility. NG2 staining demonstrated that pericytes associated with WT vessels were in close contact with the capillary endothelium and appeared elongated along the endothelial surface (Figure 3G). In contrast, pericytes associated with capillaries in mutants were more rounded and exhibited less elongation (Figure 3H). Immunostaining with desmin also suggested a defect in pericyte association with endothelial cells in mutants. In WT capillaries, pericytes were elongated and covered the surface of endothelial tubes (Figure 3I,J and Figure S4A,B). In contrast, pericytes on mutant capillaries were rarely elongated, and vessel coverage was incomplete (Figure 3K,L and Figure S4C,D). Taken together, these findings indicate that the ability of pericytes to form stable associations with microvascular endothelium is defective in *Ccn2* mutants.

CCN2 potentiates PDGF signaling in vascular cells

PDGF-B, produced by endothelial cells, and its receptor, PDGFRβ expressed in pericytes, are required for pericyte recruitment to nascent vessels [38]. CCN2 was originally identified as a protein that competes with PDGF-B for binding to NIH 3T3 cells, leading to the suggestion that CCN2 binds to PDGF receptors [42]. However, subsequent studies using a C-terminal isoform of CCN2 showed no interaction between CCN2 and PDGF receptors [43]. We tested whether full-length CCN2 interacts with PDGF-B or its receptor through co-immunoprecipitation and found no evidence for a direct physical interaction

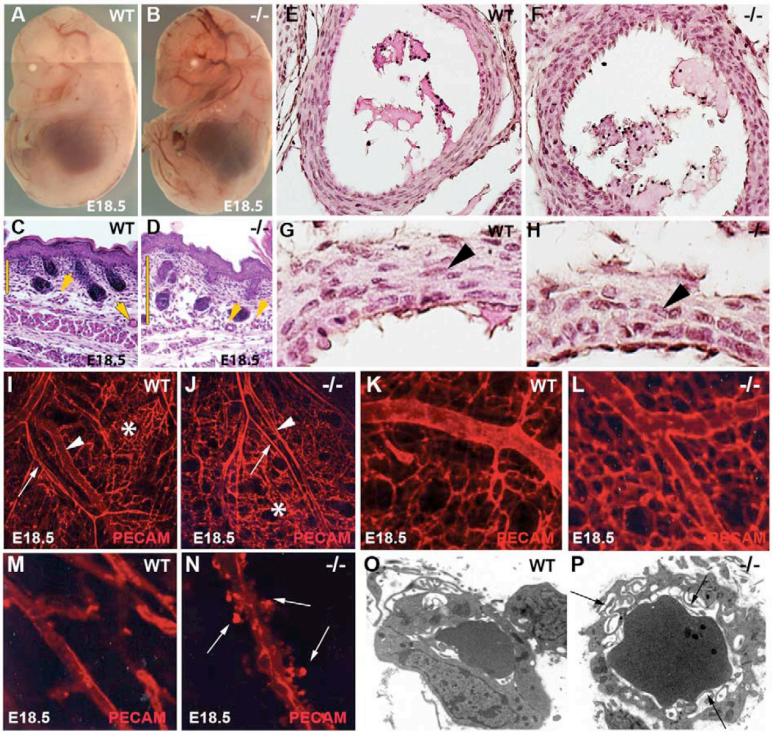

Figure 2. Vascular abnormalities in *Ccn2* mutant embryos. (A) E18.5 WT and (B) *Ccn2*−/− littermate, showing vessel dilation throughout the mutant embryo. (C, D) H&E-stained paraffin sections through the lumbar dorsal dermis of (C) E18.5 WT and (D) *Ccn2*−/− littermate. Arrowheads point to vessels. Bars highlight the enlarged distance between the hypodermal and epidermal layers in the mutant, indicative of local edema. (E,F) Hematoxylin and eosin-stained sections through E16.5 WT (E) and *Ccn2*−/− (F) descending aorta at thoracic level. Smooth muscle cells in the tunica media are spindle-shaped and arranged in layers in the WT embryo, but are more cuboidal and disorganized in the *Ccn2*−/− littermate. (G,H) Higher magnification images through aorta at lumbar level in E16.5 (G) WT and (H) *Ccn2*−/− littermate showing spindle-shaped smooth muscle cells (arrowheads) in WT that have a cuboidal shape in the mutant. (I,J) Confocal images of PECAM-stained dorsal dermal vasculature in (I) WT and (J) *Ccn2*−/− littermates. Arrows demarcate arterial elements; arrowheads demarcate venous elements; asterisks identify capillary beds. (K,L) Higher magnification confocal images of (K) WT and (L) *Ccn2*−/− dorsal dermal capillary beds, showing increased capillary density in the mutant. (M,N) High magnification confocal image of (M) WT and (N) *Ccn2*−/− dorsal dermal capillaries, showing numerous abluminal protrusions (arrows in (N)) on the mutant capillary. (O,P) Electron micrographs of newborn (P0) (O) WT and (P) *Ccn2*−/− dermal capillaries, showing abluminal and luminal (arrows in (P)) protrusions.
doi:10.1371/journal.pone.0030562.g002

(Figure S5, Methods S1). These findings suggest that CCN2 does not influence PDGF signaling by interacting directly with PDGF-B or PDGFRβ.

Next, we investigated whether CCN2 could induce PDGF-B expression in endothelial cells. Recombinant CCN2 (rCCN2) induced PDGF-B protein expression in human umbilical vein endothelial cells (HUVECs) at 1 and 4 hours of stimulation (Figure 4A). This was confirmed using HUVECs transfected with a CCN2-GFP adenovirus (adCCN2GFP). AdCCN2GFP-transfected cells induced PDGF-B protein expression at all time points tested, and the level of PDGF-B induction correlated with levels of CCN2 expression (Figure 4B). Given that CCN2 induces PDGF-B expression in endothelial cells, the potential effects of CCN2 on PDGF signaling pathways in mural cells, which express PDGFRβ, were investigated. CCN2 on its own did not activate Stat3, ERK1/2, or AKT, whereas PDGF activated all of these pathways. Furthermore, CCN2 had no effect on PDGF-B-induced ERK1/2 or Stat3 activation, but Akt activation was elevated and prolonged upon treatment with PDGF and CCN2 (Figure 4C). Thus CCN2 can potentiate PDGF signaling between endothelial cells and mural cells.

Components of the endothelial basement membrane are compromised in *Ccn2* mutants

Decreased expression of PDGF-B and reduced PDGF signaling are unlikely to be the entire basis for the *Ccn2* mutant phenotype because endothelial-specific loss of PDGF-B is compatible with survival, and mice having as much as a 90% decrease in pericyte number survive as adults [44]. The basement membrane is essential for coordinating key signaling events that stabilize the vasculature during angiogenesis [45]. The expression of fibronectin (FN) by endothelial cells is an early event in vascular basement membrane formation [46]. The provisional fibronectin matrix provides organizational signals to endothelial cells, and establishes a framework for the incorporation of permanent basement membrane components such as collagen type IV [29,46,47]. Defects in basement membrane formation lead to severe defects in angiogenesis [48–51]. Because overexpression of CCN2 leads to

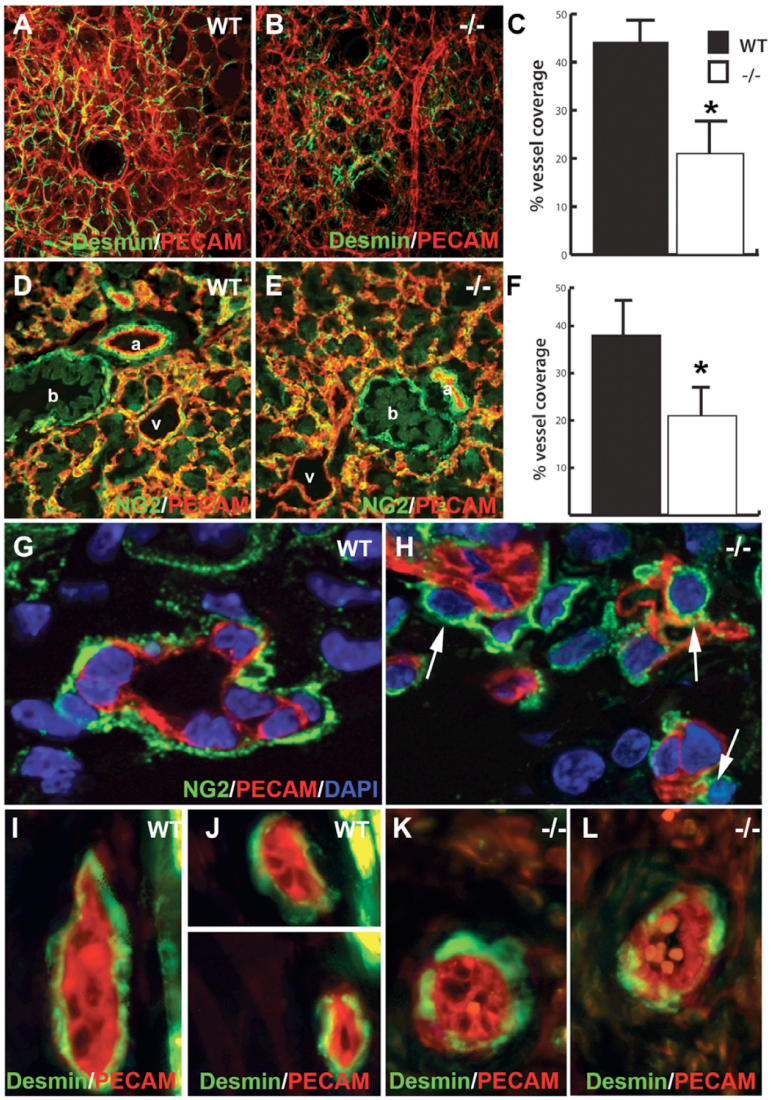

Figure 3. Defective endothelial-pericyte interactions in *Ccn2* mutants. (A, B) Co-immunofluorescence staining for desmin and PECAM in E18.5 dermis from (A) WT and (B) *Ccn2*−/− mice analyzed by confocal microscopy. (C) Quantification of vessel coverage by pericytes in E18.5 dermis; asterisk, $p<0.05$. (D, E) Co-immunofluorescence staining for NG2 and PECAM in E16.5 lung from (D) WT and (E) *Ccn2*−/− mice analyzed by confocal microscopy. (F) Quantification of vessel coverage by pericytes in E16.5 lung; asterisk, $p<0.05$. (G,H) Confocal analysis of NG2 and PECAM immunostaining in (G) WT and (H) *Ccn2*−/− E16.5 dermis. Pericytes are elongated around the microvessel in (G), whereas in mutants (H), pericytes (arrows) are associated with the endothelium, but are rounder, and fewer of them have elongated along the endothelial surface. (I–L) Confocal sections through E16.5 dermis analyzed for desmin (green) and PECAM (red) immunofluorescence. (I,J) WT desmin positive pericytes appear elongated and cover most of the surface of the microvessels. (K.L) *Ccn2*−/− desmin-positive pericytes have a rounder appearance and show less extensive coverage of the surface of the endothelium.
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thickening of glomerular and retinal capillary basement membranes in diabetic mice [52,53], we investigated whether CCN2 is required for the formation of endothelial basement membranes during development.

Electron microscopy provided evidence for defects in microvascular endothelial basement membrane assembly in *Ccn2* mutants. In WT microvessels, the interstitial matrix was compact and localized near the surface of the plasma membrane (Figure 5A). It was more diffuse in mutants (Figure 5B). Therefore, expression of FN and Col4α2 was investigated through confocal analysis. FN expression and association with vessels is significantly decreased in E16.5 *Ccn2* mutant skin and lung vasculature (Figure 5C–F, and data not shown). Collagen type IV expression was also diminished and discontinuous in vascular basement

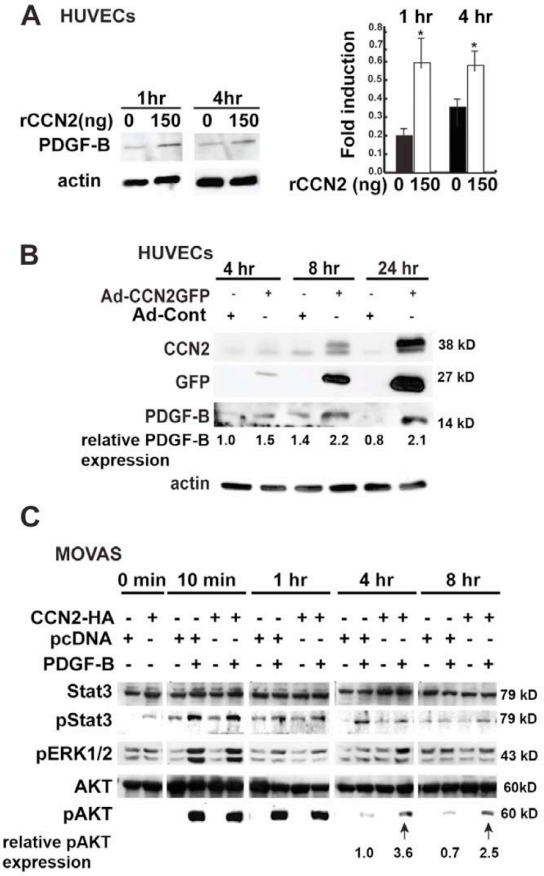

Figure 4. CCN2 potentiates PDGF-B signaling. (A) rCCN2 induces PDGF-B expression in HUVEC cells. Right panel, representative Western blot. Left panel, Quantification of relative expression levels of PDGF-B in cells treated with or without rCCN2 from three separate experiments. *, $p<0.02$. (B) Adenovirally expressed CCN2 induces PDGF-B expression in HUVECs compared to transfection with an empty adenoviral control. The extent of PDGF-B induction correlated with levels of CCN2 expression. As reported previously, a higher molecular weight isoform of CCN2, presumably a result of post-translational modification [1], is detected 4 and 8 hours post-infection. Relative level of PDGF-B expression was assessed using ImageJ software. The experiment was repeated three times, with similar results each time. The induction of PDGF-B in the presence of CCN2 was statistically significant for each time point; $p<0.05$. A representative Western blot is shown. (C) Effects of rPDGF-B, and/or pcDNA3-CCN2-HA expression on activation of PDGF pathways in MOVAS cells. PDGF-B stimulated activation of Stat3, ERK, and Akt, whereas CCN2-HA on its own had no effect. However, combined treatment with PDGF-B and CCN2-HA led to prolonged Akt activation (arrows). Relative levels of pAKT expression were assessed using ImageJ software. All experiments were performed in triplicate and repeated three times, with similar results each time. The increase in pAKT levels in the presence of CCN2 was statistically significant at each time point; $p<0.05$. A representative Western blot is shown.
doi:10.1371/journal.pone.0030562.g004

membranes in mutants (Figure 5G–J). Western blot analysis of Ad-CCN2GFP-transfected cells demonstrated that CCN2 induced expression of FN in HUVECs compared to empty vector-transfected controls (Figures 5K and S6). CCN2 had no apparent effect on Col4α2 expression (Figures 5K and S6).

Discussion

Endothelial cells proliferate and migrate toward the sources of angiogenic signals during development. Upon removal of the angiogenic trigger, a switch to a maturation phase occurs, involving cessation of cell proliferation and migration, followed by the recruitment of mural cells to the vessels, and deposition of the basement membrane. Although the importance of the basement membrane in vascular maturation is widely accepted, the roles of specific ECM components have been difficult to ascertain, especially *in vivo* [45]. Here we show that the matricellular protein CCN2 is a crucial regulator of vascular remodeling.

The results reported here suggest that CCN2 is required for pericyte recruitment in part by potentiating PDGF signaling. We have shown that CCN2 induces expression of PDGF-B in endothelial cells. In turn, CCN2 is induced in pericytes in response to serum or TGFβ [54]. Thus, PDGF and CCN2 appear to be components of a positive feedback loop that operates between endothelial cells and pericytes.

In addition to regulating levels of PDGF-B expression, CCN2 potentiates Akt activation by PDGF-B in vSMCs. Our findings extend previous studies [42] that indicate CCN2 does not interact directly with PDGF-B or PDGFRβ in vascular cells. Thus, CCN2 most likely potentiates the ability of PDGF-B to activate PDGFRβ in mural cells through indirect mechanisms. One of the most plausible of these involves interactions between CCN2 and integrin αvβ3. This integrin is expressed in endothelial cells and pericytes [55,56]. CCN2 binds to integrin αvβ3 to promote endothelial cell migration and proliferation [9]. Moreover, αvβ3 associates with and potentiates signaling through PDGFRβ [55]. Although our *in vivo* studies cannot address the physiological consequences of altered Akt signaling to the *Ccn2*−/− vascular phenotype, the *Ccn2*−/− phenotype is consistent with the possibility that reduced activation of Akt makes a contribution; *Akt1*−/− vasculature is characterized by an incomplete basement membrane [57].

As discussed above, reduced PDGF signaling alone cannot explain the severity of the *Ccn2*−/− endothelial phenotype. Rather, the data indicate an essential role for CCN2 in formation of the vascular provisional ECM and basement membrane. The relationship between CCN2 and FN expression and function is likely to be complex. CCN2 binds to FN and FN receptors (integrins α4, α5 and β1) [12,58,59]. Moreover, loss of CCN2 leads to defective adhesion and spreading of cells on FN, suggesting that these physical interactions are essential for certain cell types, at least *in vitro* [28,59]. Other studies have shown that CCN2 is required for FN protein and mRNA expression in pathological processes *in vivo* [60,61]. Studies employing siRNA knockdown approaches demonstrate that CCN2 induces FN expression in various cell types [25,62]. The studies reported here show that CCN2 induces FN expression in endothelial cells, and that CCN2 is required for normal levels of FN expression during development *in vivo*. While we have focused here on the role of CCN2 as a mediator of FN production by vascular cells, decreased FN synthesis was also seen in fibroblasts in *Ccn2*−/− dermis (Figure 5E,F). These data are consistent with previous studies showing that CCN2 is required for FN synthesis in fibroblasts *in vitro* [61]. Additional studies employing tissue-specific CCN2 knockouts will be required to determine whether the defect in FN synthesis in dermal fibroblasts has physiological consequences.

The reduced deposition of collagen IV in *Ccn2* mutants reveals that CCN2 is an essential regulator of vascular basement membrane formation. The underlying mechanisms by which

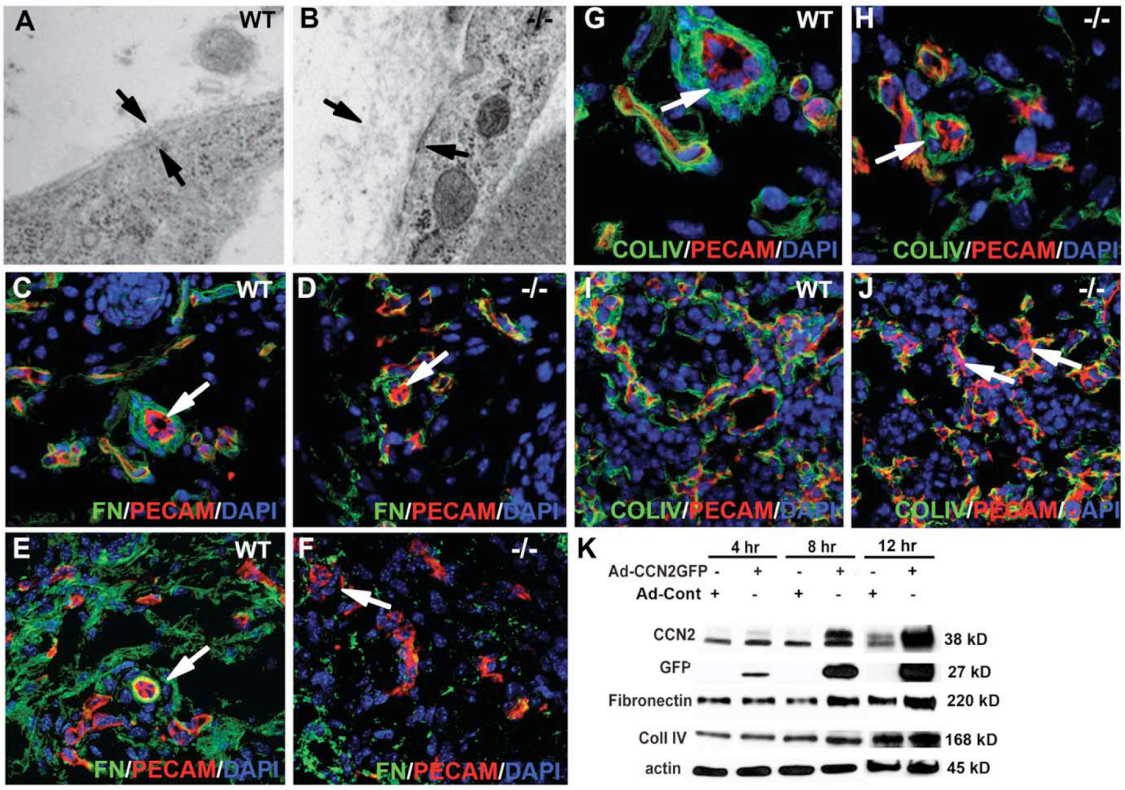

Figure 5. Endothelial basement membrane defects in *Ccn2* mutants. Electron microcopic images of endothelial basement membranes in dermal capillaries of E16.5 (A) WT and (B) *Ccn2*−/− littermates. Arrows demarcate the plasma membrane (bottom arrow) and top of the interstitial matrix (top arrow). (C,D) Confocal images of dermis of E16.5 WT (C) and *Ccn2*−/− (D) mice analyzed by immunofluorescence for fibronectin (FN) and PECAM. Arrows identify an arteriole. The arteriole in (C) is surrounded by several layers of FN. The arteriole in (D) is incompletely invested with FN. (E,F), Lower magnification confocal images through (E) WT and (F) *Ccn2*−/− E16.5 dermis, illustrating less fibronectin throughout the dermis in mutants. (G,H) Confocal images of dermis of E16.5 (G) WT and (H) *Ccn2*−/− mice analyzed by immunofluorescence for ColIV (Col4α2) and PECAM. Arrows identify an arteriole. ColIV coverage of the mutant vasculature is incomplete. (I,J) Confocal images of lungs of E16.5 (I) WT and (J) *Ccn2*−/− mice analyzed by immunofluorescence for ColIV and PECAM. Most of the vascular elements in the WT lung are surrounded by ColIV. Coverage is incomplete in the *Ccn2* mutant lung. Arrows in (J) identify vessels lacking coverage by ColIV. (K) CCN2 induces expression of FN and ColIV in HUVECS. HUVECs were infected with Ad-CCN2-GFP or Ad-control. Lysates were collected at the indicated time points post-infection. Levels of FN are elevated 8 hours after infection, concomitant with accumulation of CCN2. There appeared to be an increase in FN levels at 12 hours in the presence of CCN2 in the blot shown, but this was not seen in every experiment and the result did not reach statistical significance at this time point. Similarly, there was a trend towards increased expression of Col IV at 12 hr, but this increase did not reach statistical significance ($p = 0.065$). The experiment was repeated three times. A representative blot is shown. Quantification of levels of FN and Col IV are shown in Figure S6.
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CCN2 mediates basement membrane formation are unknown. Our studies indicate that CCN2 does not directly regulate levels of expression of *Col4α2*. Therefore, the loss of collagen IV expression in vascular basement membranes may be a secondary consequence of altered FN synthesis and folding. As discussed above, CCN2 directly interacts with FN and its receptors. Increased expression of matrix metalloproteinases (MMPs) that target type IV collagen might also contribute to reduced type IV collagen deposition in endothelial basement membranes. Additional *in vivo* studies will be required to evaluate these possibilities. A growing body of literature implicates CCN2 in abnormal basement membrane thickening in pathological processes. Glomerular basement membrane thickening is prevented in diabetic *Ccn2*+/− mice compared to WT littermates [52]. Moreover, one of the most prominent features in transgenic mice overexpressing CCN2 from the type I collagen promoter is a thickening of endothelial basement membranes [63]. Taken together with the data reported here, CCN2 appears to be a critical mediator of basement membrane formation. CCN2 is required for normal elaboration of the basement membrane during developmental angiogenesis, but CCN2 overexpression leads to basement membrane thickening in multiple fibrotic processes.

The formation of mature endothelial basement membranes involves both pericytes and endothelial cells. While we have focused here on effects of CCN2 in endothelial cells *in vivo*, it is very conceivable that primary defects in both endothelial cells and pericytes in *Ccn2*−/− mice contribute to the basement membrane defects seen in these mutants. It is likely that CCN2 has direct effects on ECM production in pericytes, as CCN2 promotes ECM production and fibroblast activation *in vitro* [64]. Moreover, our preliminary analysis reveals that in addition to the microvasculature, large vessels are impacted by loss of CCN2. This finding

raises the possibility that CCN2 plays a direct role in SMCs in addition to pericytes. It is noteworthy that the related matricellular protein CCN1 (Cyr61) is expressed in major vessels, and *Ccn1*−/− mice die early in embryogenesis as a result of defects in large vessel integrity [65]. Although vascular basement membranes have not been investigated in *Ccn1*−/− mice, the defects in vessel integrity raise the possibility that CCN1 and CCN2 will exhibit functional redundancy in vascular elements. It will thus be of interest in future studies to investigate vascular cell recruitment and basement membrane assembly in *Ccn1* and *Ccn1/Ccn2* mutants.

Finally, the use of tissue-specific *Ccn2* knockouts and co-culture experiments will be required to understand the physiological relevance of CCN2 produced by endothelial and mural cells in large vessels.

Methods

Ethics Statement

All the experiments related to mice were performed in accordance with National Institutes of Health guidelines for care and use of animals, and also approved by the UCLA Institutional Animal Care and Use Committee (IACUC), protocol #95-018.

Transgenic Mice

***Ccn2*−/− mice.** The generation of *Ccn2*−/− mice was described previously [27]. As previously described, *Ccn2*+/− mice appear indistinguishable from WT littermates, and are viable and fertile [27]. *Ccn2*−/− embryos and neonates were obtained by intercrossing *Ccn2*+/− mice. The 4 kb proximal promoter LacZ mice were generated and genotyped as previously described [31]. CCN2-eGFP mice were ordered from the Mutant Mouse Resource Center (MMRC, UC Davis) [32]. All mice were treated and euthanized in accordance with the UCLA Institutional Animal Care and Use Committee (ARC # 1995-018-52A), and the Association of Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines.

Histochemical and Immunofluorescent Staining. Freshly isolated embryos were fixed and embedded in paraffin wax as described previously [27]. 5 μm sections were stained with hematoxylin and eosin using standard protocols. LacZ staining was performed as described [66]. Immunofluorescence was performed as described previously [27]. Briefly, paraffin, sections were boiled for 15 min in citrate buffer. Sections were blocked with 5% goat or donkey serum for 1 hour and incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 hour at room temperature, then with fluorophore for 30 minutes at room temperature. The following antibodies were used: PECAM (1:500; MEC 13.3, BD Biosciences), CCN2 (1:500; L-20 Santa Cruz Biotechnology), NG2 (1:100; Abcam), Collagen IV (1:500; Abcam and Santa Cruz Biotech), Desmin (1:1000; Abcam), anti-Smooth Muscle Actin-FITC (1:500; Sigma), Col4α2 (1:1,000; Abcam) and Fibronectin (1:1,000; Santa Cruz Biotech). Secondary antibodies were conjugated with Alexa-Fluor-555 and Alexa-Fluor-488 (Invitrogen). Sections were counterstained with DAPI (Vectashield). Immunofluoresence was visualized on a Leica TCS-SP Confocal Microscope. For TUNEL staining, the fluorescein In Situ Cell Death Detection Kit (Roche) was used according to manufacturer's protocol. PCNA staining was performed on paraffin sections as described previously [27] using an anti-PCNA antibody (Zymed) and, vessels were identified by PECAM immunofluorescence. The percentage of TUNEL- or PCNA-positive endothelial cells (PECAM-positive) was quantified on digital photomicrographs processed with Photoshop software (Adobe), using Image-Pro software. Pericyte coverage of microvasculature was quantified as described [67]. Capillary density was quantified as the area of PECAM1-positive cells on anti-PECAM1 immunostained images as described [68]. Ten images each for WT and *Ccn2*−/− mice, obtained from 5 independent pairs of littermates, were analyzed. Statistical analysis was performed using Student's *t* test. A p value of less than 0.05 was considered statistically significant.

Confocal Microscopy. Confocal laser scanning microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy Shared Resource Facility at UCLA, supported with funding from NIH-NCRR grant (CJX1-443835-WS-29646) and NSF grant (CHE-0722519). Representative images are shown.

Real-time quantitative polymerase chain reaction. RNA was isolated using TRIZOL (Invitrogen) according to the manufacturer's protocol. Synthesis of cDNA was performed with Superscript III (Invitrogen). Semi-quantitative PCR was performed with 20 ng reverse-transcribed RNA. Amplifications were performed for 30 cycles, followed by a 5 min extension at 72°C. Reaction products were gel electrophoreses and quantified using Image Quant software (Molecular Dynamics). Primers for the genes investigated by semi-quantitative RT-PCR were: VegfA and C: VEGFACF 5′-GAA GTC CCA TGA AGT GAT CAA G-3′, VEGF164 5′-CAA GGC TCA CAG TGA TTT TCT GGC-3′; ANG1: ANG1F 5′-CAT TCT TCG CTG CCA TTC TG, ANGR 5′-GCA CAT TGC CCA TGT TGA ATC-3′; PECAM: PECAMF 5′- GAG CCC AAT CAC GTT TCA GTT T-3′, PECAMR 5′-TCC TTC CTG CTT CTT GCT AGC T-3′; Versican0: V_0F 5′-TTC ACA GAA CGC CAC CCT TGA GTC C-3′, V_0R 5′-CTA GCT TCT GCA GCT GGC CGG GTC C-3′; Versican1-3: V1F 5′- GCA GCT TGG AGA AAT GGC TTT GAC C-3′, V_1R 5′- CGA GTA GTT GTG GGT GAT TCC GTG G-3′; PDGFBF 5′-GATCCGCTCCTTTGATGATC-3′, PDGF-BR 5′-GTCTCACACTTGCATGCCAG-3′; PDGFRbetaF 5′-AATGTCTCCAGCACCTTCGT-3′, PDGFRbetaR 5′-AGC-GGATGTGGTAAGGCATA-3′ [69]; GAPDH, GapdhF 5′-GCA GTG GCA AAG TGG AGA TT-3′; GapdhR 5′-AGT GGA TGC AGG GAT GAT GT. cDNA was amplified using Sybr Green I PCR Master Mix (Applied Biosystems). Amplicons were generated and analyzed with the ABI 7000 Real-time PCR system (Applied Biosystems). Data were normalized to the levels of *Gapdh*. Triplicate assays were run and analyses were repeated three times. Specificity was tested by measurement of Tm-values and by gel electrophoresis of the amplicons. Data are represented as the means of relative levels of expression+the S.E. of the mean, and statistical analysis was performed with Student's *t* test. A p value of less than 0.05 was considered statistically significant.

Flow Cytometry. FACS analysis was performed as previously described [70]. Brain, liver and lung samples were harvested from E16.5 CCN2 wild type and mutant embryos. Single cell suspensions were created by serial syringe digestion in 0.2% Collagenase (Sigma *Clostridium histolyticum* C2674-6), 0.05% Dispase (Invitrogen 17105-041), 0.0075% DnaseI (Sigma D4513), 0.02% Penicillin Streptomycin (GIBCO-Invitrogen 15140148) in 1× PBS/10%Fetal Bovine Serum (GIBCO-Invitrogen 10437-028). Cell suspensions were incubated with the following primary antibodies: CD45-APC Cy7 (1:200;Abcam); NG2 (1:200; Abcam); CD31-PE (1:200; Abcam); PDGFRβ-APC (1:50; Invitrogen). A secondary goat anti-rabbit conjugated antibody 488 (Invitrogen) was used for the unconjugated NG2 antibody. FITC, APC, APC-Cy7, PE control beads (Invitrogen) and 488 secondary alone were used as controls to correct for background florescence and gate parameters. FACS sorting was performed using the LSRII FACS

analyzer and cell counts were plotting by FlowJo analysis (TreeStar).

Transmission Electron Microscopy. Ultrastructural analysis was performed on dermal microvasculature by the University of California, Los Angeles, Electron Microscopy Core Facility. 10 images were taken from each E18.5 embryo. Four *Ccn2*−/− and four WT littermates were examined. Representative images are shown.

Cell lines and treatments. Human umbilical vein endothelial cells (HUVECs a gift from Dr. Jau-Nian Chen) were maintained in HUVEC culture media (Sigma) as described previously [71]. HUVECs were maintained in 0.5% serum for 12 hr prior to treatment with recombinant protein. Cells were treated with 150 ng/ml recombinant (r) CCN2 (Peprotech) and/or 150 ng/ml rPDGF-B (Peprotech), using serum free treated cells as control. Mouse vascular smooth muscle (MOVAS) (ATTC) cells were cultured in DMEM, 10% FBS. MOVAS cells were washed with Hepes buffered saline (HBS) containing 5 mM $MgCl_2$ (HBS+Mg), and treated with or without 150 ng/ml rPDGF-B in DMEM, 0.5% FBS for the indicated times. In other experiments, MOVAS cells were transiently transfected with pcDNA3-CCN2-HA [72] using Lipofectamine (Invitrogen), and treated with 150 ng/ml rPDGF-B 24 hrs later for the indicated time periods. Each experiment was performed in triplicate and repeated at least twice. HUVECs were also transfected with CCN2-GFP adenovirus and adenoviral control vectors at a multiplicity of infection (MOI) of 200 (a kind gift of Dr. Fayez Safadi).

Western blot analysis. Cells were lysed with RIPA buffer with 1× protease (Complete Mini Roche) and 1× phosphatase inhibitors (Cocktail 2, Sigma). Lysates were separated by 6–12% SDS-PAGE and transferred to nitrocellulose membrane (0.45 um; BioRad). Membranes were incubated with antibodies against CCN2 (L-20; 1:2,000, Santa Cruz Biotechnology), PDGF-B (1:2000, Cell Signaling), PDGFR β (1:2,000 Cell Signaling), STAT3 (1:1,000, Cell Signaling), pSTAT3 (1:2,000, Cell Signaling), total AKT (1:2,000, Cell Signaling), phospho-AKT (1:2000, Cell Signaling), phospho-ERK1/2 (1:2,000, Cell Signaling), Collagen type IV (1:2,000; Abcam), Fibronectin (1:2,000; Santa Cruz Biotech) and actin (1:5,000, Sigma). Antibody-antigen complexes were detected with HRP-conjugated secondary goat and rabbit antibodies (Bio-Rad). Western blots were performed in triplicate and normalized to actin. Quantification was performed using ImageJ. Statistical analysis was performed using the Student's t-Test, and a p-value less than 0.05 was considered significant. Representative western blots are shown.

Supporting Information

Methods S1 Methods for co-immunoprecipitation and western blot analysis (Figure S5).
(DOCX)

Figure S1 Expression of CCN2 in vasculature and vascular defects in *Ccn2* mutants. (A) Confocal image of dermal microvasculature immunostained for CCN2 (green) and PECAM (red). Yellow indicates co-expression in endothelial cells. The staining is punctate, as reported previously [30]. Associated mural cells expressing CCN2 (green) are indicated by arrows. Endothelium demonstrating CCN2 expression is indicated by arrowheads. (B,C) Confocal images of fetal placenta from E16.5 WT (B) and *Ccn2*−/− (C) littermates immunostained for NG2 (green) and PECAM (red) and counterstained with DAPI showing no obvious changes in vascular organization. (D) E14.5 WT and (E) *Ccn2*−/− littermate. Arrows highlight dilation of cerebral vessels in the mutant. Dilated vessels are apparent in the mutant. (F–I) Confocal images of immunofluorescence staining for αSMA (green) and PECAM (red) in dorsal dermis of newborn (P0) WT (F,H,) and *Ccn2*−/− (G,I,) littermates. Arrows in (F–I) indicate arteries; arrowheads demarcate veins. (J,K) Confocal images of immunofluorescence staining for αSMA (green) and PECAM (red) in dorsal dermis of newborn (P0) WT (J) and *Ccn2*−/− (K) littermates showing paired arterioles (arrows) and venules (arrowheads). (L,M) Confocal images of immunofluorescence staining for EphB4 (green) and PECAM (red) of E16.5 WT (L) and Ccn2−/− littermate (M) dorsal dermal microvasculature.
(TIF)

Figure S2 Altered gene expression in Ccn2 mutants. (A) Quantification of microvessel density. (B,C) Additional representative confocal images of PECAM-immunostained dorsal dermal microvasculature from WT (B) and *Ccn2*−/− (C) E18.5 littermates showing increased vessel density in mutants. (D) Representative image of paraffin section through E16.5 dorsal dermis analyzed by αPECAM and αPCNA co-immunofluorescence and counterstained with DAPI, used to assess endothelial cell proliferation. Image from WT dermis is shown. Arrows point to PCNA-positive endothelial cells. (E) Quantification of PCNA-positive cells revealed no differences in proliferation in WT versus mutant vessels. (F) Representative images of paraffin section through E16.5 dorsal dermis analyzed by immunostaining for PECAM and TUNEL-positive endothelial cells and counterstained with DAPI. Image from WT dermis is shown. (G) Quantification of TUNEL-positive endothelial cells revealed no evidence for altered levels of cell death in *Ccn2* mutant vasculature. (H–K) Quantitative RT-PCR analysis of relative levels of expression of (H) *Angl*, (I) *Vegf164*, (J) *Versican1*, and (K) *Versican0* mRNA in WT and *Ccn2*−/− E16.5 vasculature. *, $p<0.05$.
(TIF)

Figure S3 FACS analysis of pericyte or endothelial cell number in *Ccn2* mutants. (A, C) FACS analysis of (A) WT and (C) *Ccn2*−/− skin samples analyzed for expression of PDGFRβ. (B, D) FACS analysis of (B) WT and (D) *Ccn2*−/− skin samples analyzed for expression of NG2. (E) Quantification of percentages of PDGFRb, NG2, and PECAM-expressing cells revealed no differences.
(TIF)

Figure S4 Defective pericyte association with endothelium in *Ccn2* mutants. Paraffin sections through E16.5 dermis immunostained with desmin (red) and counterstained with DAPI. (A,B) WT desmin positive pericytes appear elongated and cover most of the surface of the microvessels. (C,D) *Ccn2*−/− desmin-positive pericytes have a rounder appearance and desmin staining has a less uniform appearance.
(TIF)

Figure S5 No physical interaction between CCN2 and PDGF-B or PDGFRβ. (A) No physical interactions between CCN2 and PDGF-B. MOVAS cells were infected with a lentiviral vector encoding CCN-HA (M-CCN2 cells). Non-crosslinked or DSP-crosslinked lystaes (see Supplementary Materials and Methods) were immunoprecipitated with αHA antibody. Western blots of the immunoprecipitates were probed with αCCN2 and αPDGFB antibodies. First lane in each panel shows rCCN2 and rPDGFB standards. TXsol and TX insol, triton X-soluble and –insoluble pellets, respectively. (B) No direct interactions between CCN2 and PDGFRβ. M-CCN2 cells were treated with or without PDGF-B, followed by immunoprecipitation with αHA antibody. Western blots of the immunoprecipitates were probed with αPDGFRβ (PDGFR) or αphospho (Y751) PDGFRβ antibody.
(TIF)

Figure S6 CCN2 induces fibronectin expression in endothelial cells. Quantification of relative levels of expression of fibronectin (FN) and Col IV in endothelial cells in the presence or absence of CCN2. See legend to Figure 5 for experimental details. Induction of FN was seen by 8 hrs. There was a trend towards increased FN at 12 hrs ($p<0.06$), but this did not reach statistical significance. *, $p<0.05$. There was no significant increase in Col IV levels at any time point.
(TIF)

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Author Contributions

Conceived and designed the experiments: KML MLI-A HM. Performed the experiments: FH-G RAD B-LH BvH JJH TTC JRO AC PDB. Analyzed the data: FH-G MLI-A HM PDB KML. Contributed reagents/materials/analysis tools: PDB HM MLI-A. Wrote the paper: FH-G KML.

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Supporting Information Methods

Co-Immunoprecipitation Assays and Western blot analysis. For analysis of physical interactions between CCN2 and PDGF, the M-CCN2 cell line was derived from MOVAS cells infected with a lentiviral construct encoding HA-tagged CCN2 regulated by a CMV promoter (OPEN Biosystems). Cells were selected and cultured in DMEM, 10% FBS with 4 ng/mL puromycin. Immunoprecipitations were performed by incubation of M-CCN2 cells with or without 45ng rPDGF for 5 minutes. Crosslinking was performed by rocking cells for 15 minutes in HBS+Mg containing DSP (Dithiobis, Pierce). DSP solution was replaced with ice-cold HBS+Mg containing 50 mM ammonium chloride, pH 7.5 for 10 minutes. Cells were lysed with lysis buffer containing 50 mM NH_4Cl. The TritonX 100-insoluble pellet was resuspended in non-reducing sample buffer. Insoluble material was removed by centrifugation. 4 volumes of acetone were added to the supernatant and held overnight at -80°C. Precipitated crosslinked proteins were dissolved in 0.25% SDS in HBS. The protein solution was denatured by boiling and sonication. Lysates were immunoprecipitated with Protein-G magnetic beads (Millipore) displaying HA antibody (Bethyl). Antibody beads without exposure to lysates were used as a negative control. rPDGF and whole cell lysates of M-CCN2 cells were used as standards for rPDGF and CCN2, respectively. Cells were lysed in HBS, 5mM $MgCl_2$, 1% Triton X-100, 1% Protease (Roche), phosphatase inhibitors (Sigma) for 20 min with rotation at 4°C. In some experiments, the pellet was re-extracted with Triton X to generate TX and TX-insoluble pellets. Lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (BioRad). Membranes were incubated with antibodies against CCN2 (L-20 Santa Cruz Biotechnology), PDGF-B (1:2000, Cell Signaling), PDGFR β (1:2,000 Cell Signaling). Experiments were performed in triplicate. Representative blots are shown.

Supporting Information Figures

Figure S1: Expression of CCN2 in vasculature and vascular defects in *Ccn2* mutants

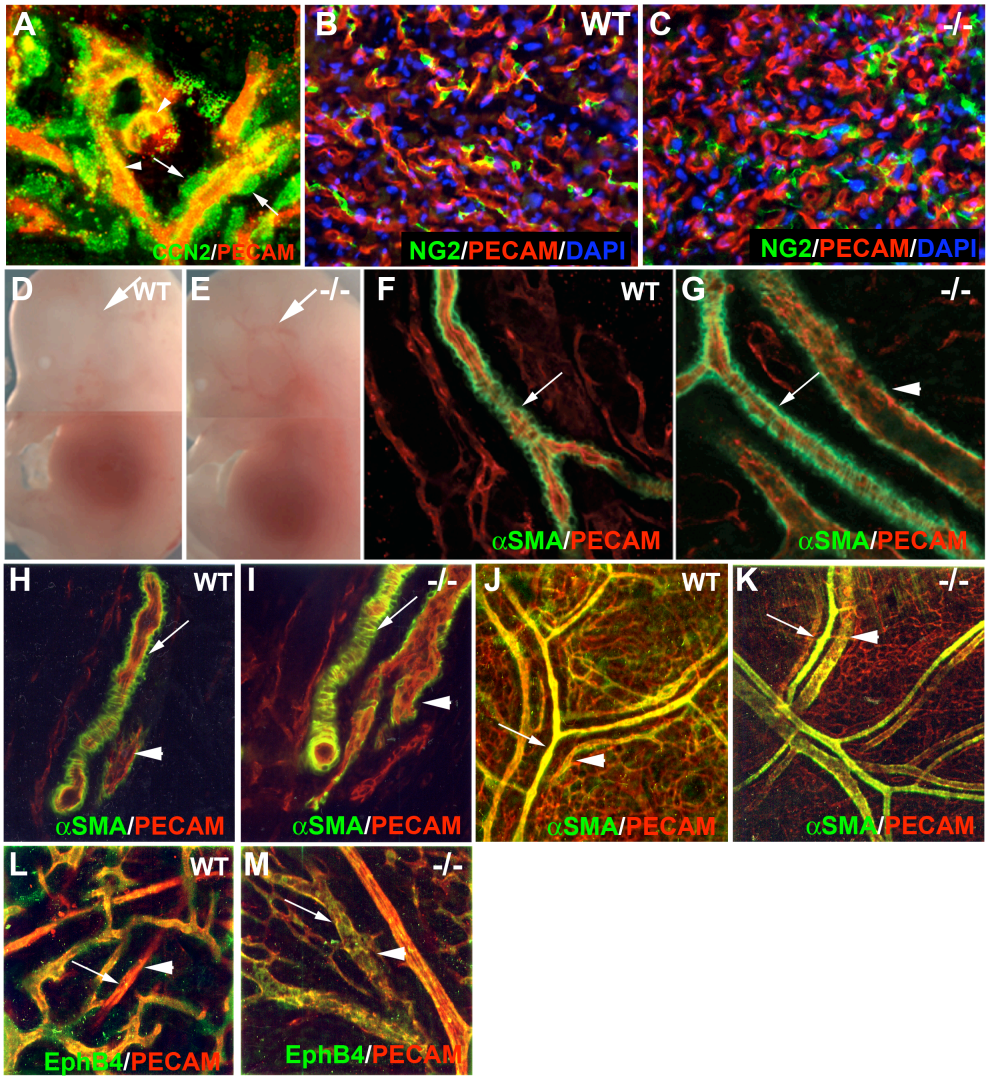


Figure S2: Altered gene expression in *Ccn2* mutants.

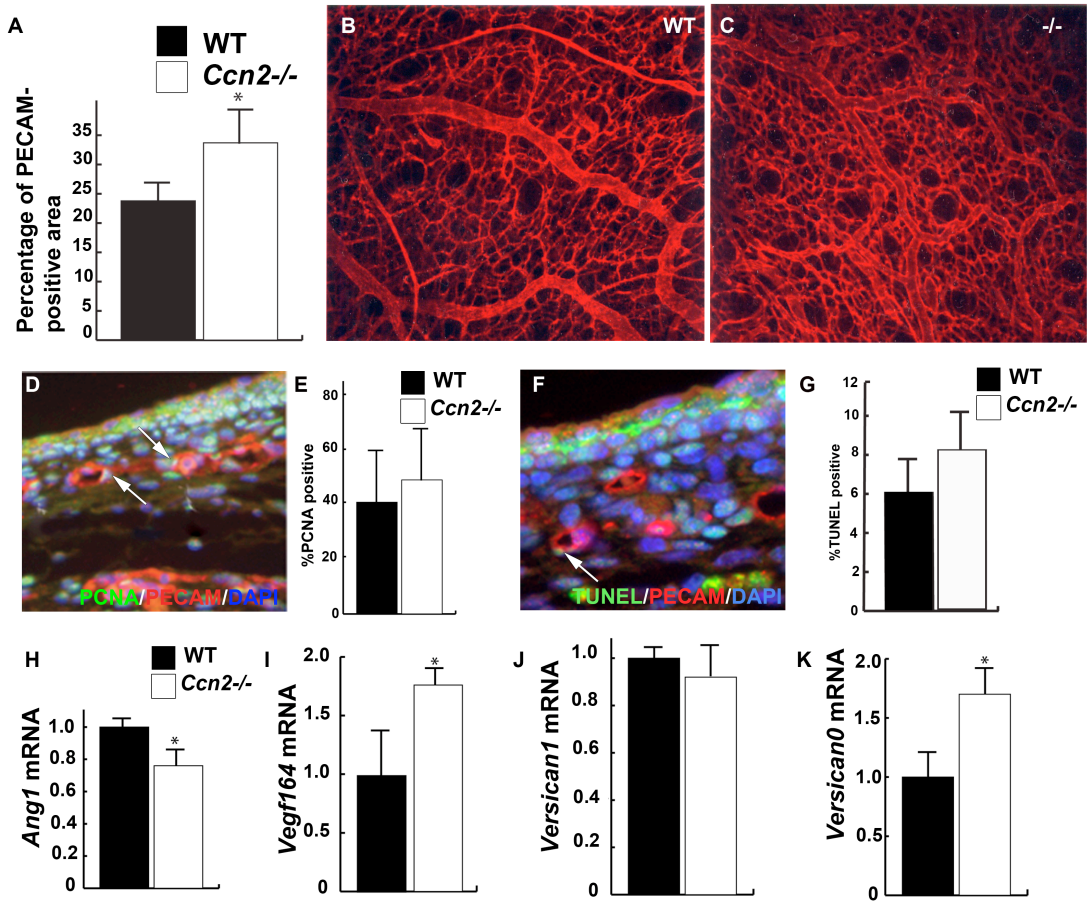


Figure S3: FACS analysis of pericyte or endothelial cell number in *Ccn2* mutants.

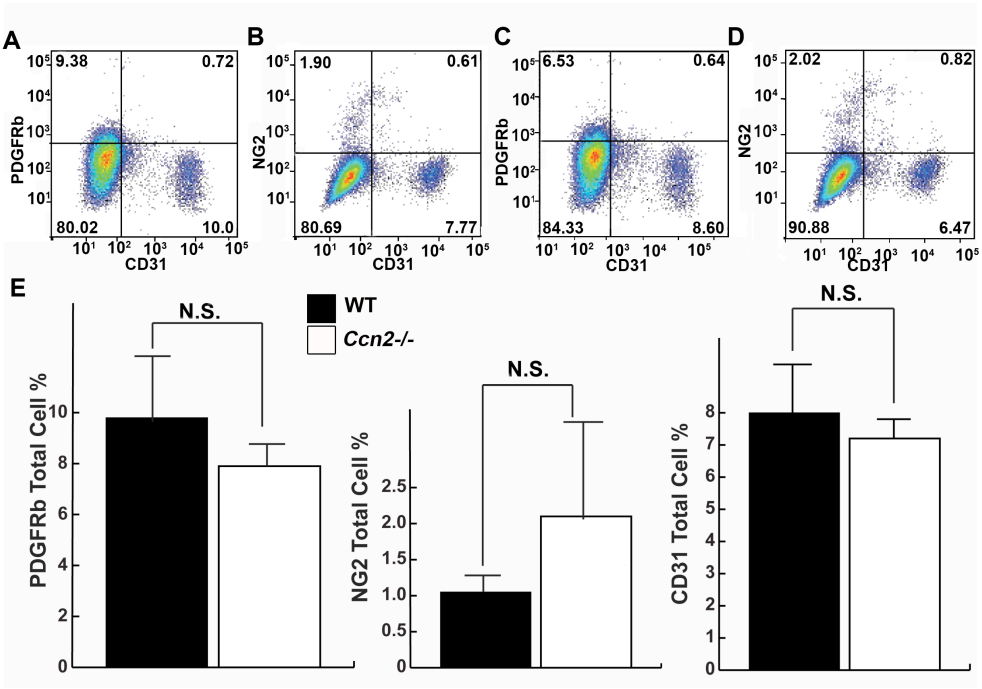


Figure S4: Defective pericyte association with endothelium in *Ccn2* mutants

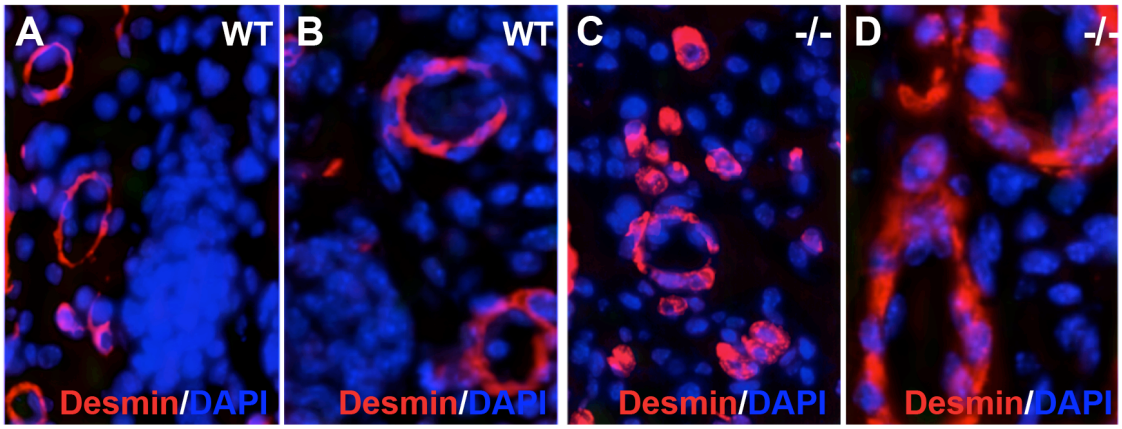


Figure S5: No physical interaction between CCN2 and PDGF-B or PDGFRβ

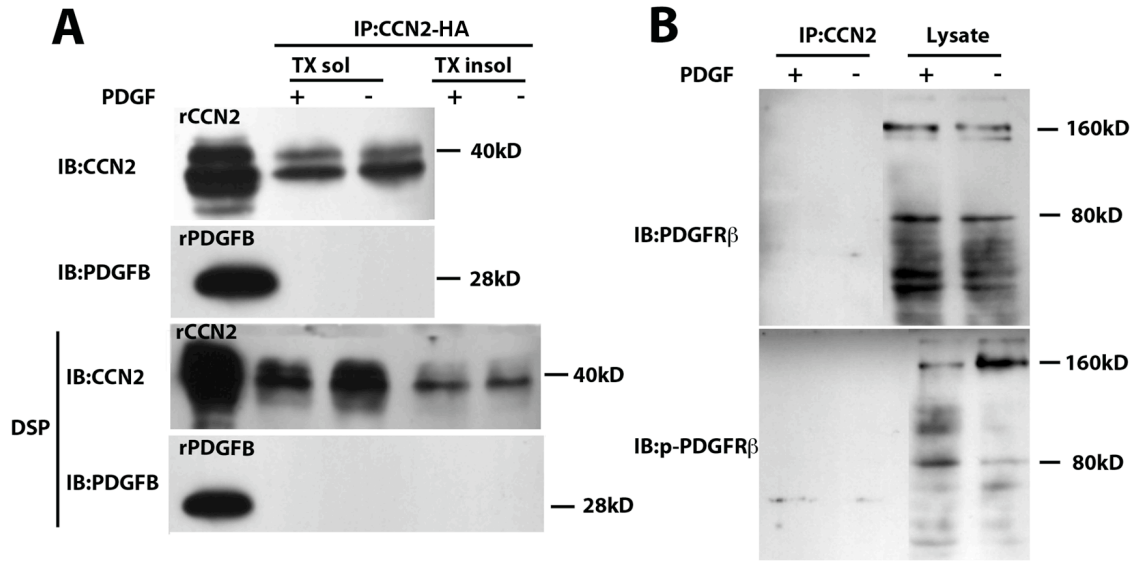
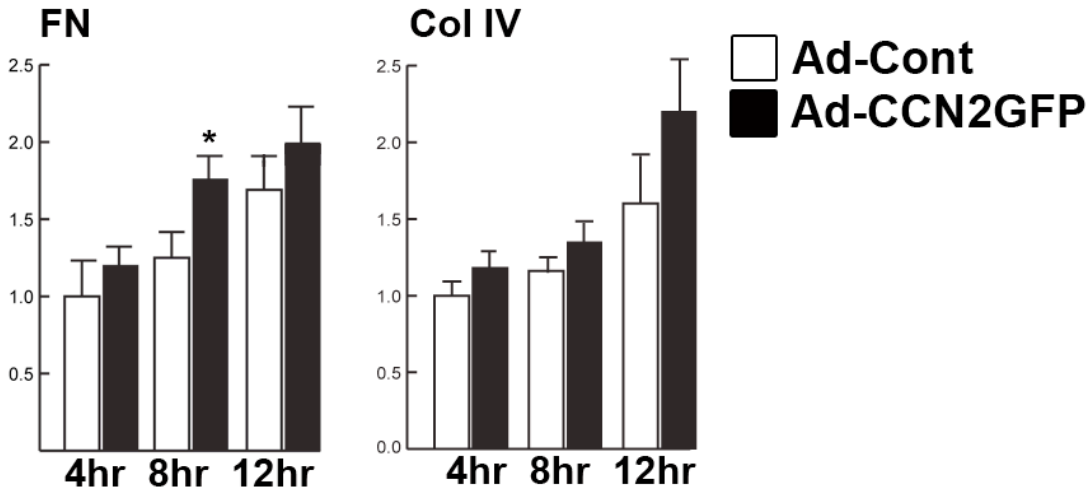


Figure S6: CCN2 induces fibronectin expression in endothelial cells



Chapter 4

CCN2/CTGF is required for matrix secretion and cellular survival during chondrogenesis

Preface

Analysis of the *Ccn2* global knockout mouse revealed defects in cartilage extracellular matrix (ECM) production, with overall decreases in total ECM as evidenced by decreased Safranin O staining, and specifically aggrecan and link protein (stabilizes aggrecan protein formation) [1]. We wanted to further investigate this phenotype by analyzing *Ccn2* mutant growths plates on the ultrastructural level using electron microscopy. Surprisingly, we discovered enlarged endoplasmic reticula (ER), a prime indicator of ER stress. In addition, we observed upregulated expression of the upstream ER stress regulator, GRP78/BiP and downstream pro-apoptotic protein, CHOP. We determined that *Ccn2* is an ER stress responsive gene that is upregulated during chemically induced ER stress with the same kinetics as *BiP* and *CHOP*. Since the loss of CCN2 resulted in ER stress, we wanted to determine if the overexpression of CCN2 attenuated ER stress. We generated mice overexpressing CCN2 specifically in cartilage and found that ER stress was attenuated. These results demonstrate that CCN2 plays a protective role during ER stress. We were also able to determine that these protective effects were mediated by CCN2 partially through integrin-mediated chondrocyte adhesion, and also through controlling the expression of nuclear factor κB (NF κB) and autophagy-mediated cellular survival factors.

This manuscript is currently under review at *Osteoarthritis and Cartilage*.

1. Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, et al. (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130: 2779-2791.

Introduction

Chondrocytes secrete large amounts of extracellular matrix (ECM) as they differentiate. Disruptions in this process often result in chondrodysplasias, which account for an estimated 0.15% of congenital birth defects per year[1]. The most common causes of chondrodysplasias are mutations that lead to structural alterations in genes that encode ECM proteins. These alterations prevent proper protein processing, leading to their retention in the endoplasmic reticulum (ER), or the incorporation of the mutated product into the ECM, interfering with assembly[2].

ER enlargement is a hallmark of defective protein folding and ER stress. ER stress activates the Unfolded Protein Response (UPR), an adaptive mechanism to restore cell homeostasis and viability[3]. The UPR ameliorates ER stress by attenuating protein synthesis and by increasing chaperone protein levels to facilitate correct protein folding and clearing of misfolded protein aggregates. The glucose-regulated protein 78 (GRP78) also known as BiP, is a resident ER chaperone and the main initiator of the UPR[4]. BiP activates UPR effector molecules inositol requiring enzyme-1 (IRE1), pancreatic ER eukaryotic translation initiation factor 2a kinase (PERK) and activating transcription factor 6 (ATF6). ER stress activates the expression of chaperones and anti-oxidative stress genes, which promote survival; however, prolonged ER stress leads to activation of CCAAT/enhancer-binding protein-homologous protein (CHOP) and caspase-12 to induce cell death[3]. Although ER sensing of unfolded proteins is the most common inducer of ER stress, other mechanisms can activate the UPR, including hypoxia and detachment from ECM[5, 6]. ER stress is a major player in multiple pathologies including osteoarthritis[3] and identifying proteins involved in ER stress pathways may provide new therapeutic targets.

Nuclear factor κB (NFκB/p65/relA) is a transcription factor that plays pivotal roles in cell proliferation, differentiation and survival[7]. ER stress activates NFκB via tumor necrosis factor-α

(TNF-α) receptor associated factor 2 (TRAF2) and IRE1 *in vitro*[8]. The role of NFκB activation under conditions of ER stress is unclear, but several studies show that NFκB exerts pro-survival functions[9, 10]. In the growth plate, NFκB is essential for chondrocyte viability[11, 12]. However, the mechanisms that control NFκB activity in the growth plate are unknown.

Autophagy is a lysosomal degradation pathway used to recycle cellular components and activate cell survival under conditions of starvation and stress[13]. Autophagy is essential for maintaining ER homeostasis, and evidence indicates that during prolonged ER stress, autophagy is activated to assist in the removal of misfolded protein aggregates[14]. Crosstalk between ER stress, autophagy and NFκB-mediated cellular survival has been confirmed previously[10, 15], but whether such crosstalk occurs in chondrocytes is unknown.

The term "matricellular" was coined to encompass a structurally diverse group of proteins that reside in the ECM, but serve no structural functions[16]. Members of the CCN (Cyr61/CTGF/Nov) family of matricellular proteins are critical regulators of cell-ECM interactions in many cell types. The six members of the CCN family contain four conserved domains that mediate interactions with integrins, growth factors, and ECM components including heparin sulfate proteoglycans, fibronectin and aggrecan[17]. Connective tissue growth factor (CTGF/CCN2) is best known for its role in fibrosis, where its overexpression exacerbates excess collagen deposition in multiple organs[18]. CCN2 overexpression is involved in many other pathological processes, such as atherosclerosis, osteoarthritis and certain cancers[19].

CCN2 is indispensible for endochondral bone formation. Global loss of CCN2 in mice results in severe chondrodysplasia and lethality at birth[20]. This lethality is due to a significant decrease in chondrocyte proliferation, delayed chondrocyte differentiation, impaired ECM production and insufficient vascular invasion[20]. Subsequent studies showed that loss of CCN2

leads to decreased expression of hypoxia inducible factor 1α (HIF1α)[21], a pro-survival factor in chondrocytes[22].

CCN2 induces NFκB activity in ATDC5 cells through integrin αvβ3-mediated mechanisms to enhance migration[23]. Reciprocally, the CCN2 promoter contains an NFκB responsive element that activates CCN2 expression during the mechanical stretch response in smooth muscle cells[24]. These studies raise the possibility that CCN2 may regulate NFκB in the growth plate. Given the essential role of HIF1α and NFκB in chondrocyte survival, and evidence that CCN2 induces expression and activity of HIF1α and NFκB in multiple cell types, we investigated whether CCN2 plays a role in survival of growth plate chondrocytes. We show that CCN2 is essential for chondrocyte survival, protecting growth plate chondrocytes from pro-apoptotic pathways activated by ER stress, and promoting chondrocyte survival through activation of NFκB. Additionally, we provide evidence that these effects are mediated through the ability of CCN2 to engage integrins.

Method

Mouse strains and cell lines. *Ccn2* knockout mice were generated and maintained as described[20]. *Ccn2*-/- embryos were obtained from timed heterozygote matings, with embryonic day (E) 0.5 representing the detection of a post-copulatory plug. Genotyping of embryos was performed as described[20].

CCN2 floxed inducible transgenic mice (*Ccn2fxTg*) were generated as described[25, 26]. These mice were crossed to *Col2a1Cre* mice[27] to induce overexpression of CCN2 in chondrocytes. Genotyping was performed on DNA isolated from tail biopsies with the following primers: Forward 5'-TCT TCT GCG ATT TCG GCT CC-3'; Reverse 5'- AAT GTG TCT TCC AGT CGG TAG-3.' Mouse embryonic fibroblasts (MEFs) from *Ccn2fxTg* embryos were

isolated and cultured as described[28]. MEFs were infected for 24 hours with adenoviral Cre-recombinase (Ad-CRE) and empty vector controls (Ad-CNT) (University of Iowa Gene Transfer Vector Core) at a multiplicity of infection of 300. RNA was isolated using Qiagen RNeasy Kit, and RT-PCR was used to quantify relative CCN2 expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described[29].

Experiments on mice were performed with four sets of WT and *Ccn2*-/- littermates at E16.5, E18.5 and postnatal day (P) 0 (N=8 per genotype). Animals were treated in accordance with the National Institutes of Health guidelines for care and use of animals, and approved by the UCLA Institutional Animal Care and Use Committee (IACUC- #95-018).

Transmission electron microscopy. WT and *Ccn2*-/- E18.5 hindlimbs were prepared for electron microscopy as described[30]. Ultrastructural analysis was performed at the Electron Microscopy Core Facility at the International Space Science Institute (ISSI) in Bern, Switzerland.

Immunofluorescent staining. Freshly isolated embryos were fixed and paraffin embedded as described[20]. 7μ sections were generated using a Leica GM40 Microtome. For fluorescent staining, Tyramide Signal Amplification was performed according to the manufacturer's protocol (Invitrogen). The following antibodies were used: CCN2 L-20 (1:1,000; Santa Cruz Biotechnology), BiP (1:500; Cell Signaling), CHOP (1:400; Cell Signaling), Calnexin (1:300; Chemicon), NFκB/RelA/p65 (1:400; Cell Signaling), Apg/Atg12 (1:400; Chemicon) and Apg8b/LC3 (1:200; Abgent). Secondary antibodies were conjugated with AlexaFluor-594 and 488 (Invitrogen). Sections were counterstained with DAPI (Sigma) and mounted with Prolong Gold antifade reagent (Sigma). Immunofluorescence was visualized on an Olympus Bx60 Microscope.

Sternal chondrocyte isolation. E16.5 sterna were isolated and pooled by genotype into conical tubes. Garnet bead mix (Invitrogen) was added to 15ml of HEPES (Sigma) buffered DMEM (HDMEM) (GIBCO). The connective tissue from the sterna was removed by mechanical shaking for 30 minutes. The media was aspirated, and 0.03% bacterial collagenase (Chlostridium; Sigma) in HDMEM was added. Additional mechanical shaking was performed for 10 minutes. Cleared sterna were further digested in 0.01% collagenase overnight. The following day, chondrocytes were filtered (70μ Fisher) and plated.

Alginate chondron cultures. Sternal chondrocytes were isolated as described above. Chondrocytes were then suspended in 1.1% sodium alginate in 1X Phosphate Buffered Saline (PBS) (Sigma)[31]. The cell/alginate suspension was extruded drop-wise into 0.1M Calcium Chloride (Sigma) and beads were polymerized at room temperature for 10-15min. The alginate beads were then washed with 1X PBS, transferred to T25 flasks and incubated in differentiation media containing 50ng/ml ascorbic acid (Sigma). After 7-10 days in culture, the chondrons were released using 10mM sodium citrate (Sigma). Chondron clusters were washed with PBS, plated on an adhesive surface (Cell-tak BD Biosciences) and cultured for 1-3 days for immunofluorescence and thapsigargin (THG, Sigma)-induced ER stress[32].

Thapsigargin-induced ER stress in ATDC5 cells. ATDC5 chondrosacrcoma cells (RCB0565- Riken Cell Bank) were cultured described[33]. ER stress was induced by thapsigargin (THG, Sigma) diluted in DMSO (Sigma) at the following concentrations: 0, 40, 80 and 160nM over a 24-hour time period[32].

Integrin blocking assays. Sternal chondrocytes were isolated as described above. Integrin blocking assays were performed as previously described[34]. Briefly, sternal chondrocytes were plated and serum starved for 8 hours. Chondrocytes were then treated for 24 hours with an anti-

rat integrin α5 blocking antibody (1:100 dilution; CD49e BD Pharmingen) or 5% rat control serum (Sigma). Cells were lysed and RNA extracted using Qiagen RNA purification kit following the manufacturer's instructions. cDNA was generated using Superscript III (Invitrogen). The following primers sequences were amplified: Integrin alpha 5 Forward primer: 5'-AGCGCATCTCTCACCATCTT-3' and Reverse primer: 3'-TCAGGTTCAGTGCGTTCTTGT-5' and normalized to GAPDH as described[34].

CCN2 overexpression in ATDC5 cells. ATDC5 cells were infected with a bicistronic adenovirus expressing CCN2 and green florescent protein (AdCCN2-GFP) adenovirus or an adenoviral control (Ad-CNT) vector for 24 hours at a multiplicity of infection of 200. After 24 hours, cells were either analyzed by immunofluorescence or treated with THG for ER stress induction[32].

Western blot analysis. Proteins were isolated from *Ccn2fxTg* sterna through lysis in RIPA buffer with 1X protease (Complete Mini Roche) and 1X phosphatase inhibitors (Cocktail 2, Sigma). 30μg of protein lysates were separated by gel electrophoresis and transferred to 0.45μ nitrocellulose membranes (Biorad). Membranes were blocked in milk and incubated at a 1:2,000 dilution of the following primary antibodies overnight at 4°C: CCN2 (L-20; Santa Cruz Biotech) and β-actin (Sigma). The blots were incubated with the following secondary antibodies: Donkey anti-goat horseradish peroxidase (HRP) and Goat anti-rabbit HRP (1:5,000; Biorad). Membranes were developed using Pierce ECL Plus HRP chemoluminescent reagent (ThermoScientific).

Reverse transcriptase PCR. RNA from ATDC5 cells and dissociated sterna were isolated using Qiagen RNA purification kit (according to the manufacturer's protocol). Synthesis of cDNA was performed with Superscript III (Invitrogen). Quantitative reverse transcriptase PCR

(qRT-PCR) reactions were performed with a SYBR Green Real-time PCR Master Mix (Fermentas) with a Mx3005P QPCR System (Stratagene). Relative expression of CCN2, BIP and CHOP were quantitated and normalized to GAPDH as previously described[29, 32].

Statistical Analysis

Immunofluorescent quantitation of the levels of CCN2, BiP and CHOP expression was performed through ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. All experiments were performed in triplicate at least twice. Five images were taken per sample and representative images are shown. RT-PCR experiments were performed in triplicate and normalized to GAPDH. All graphs are represented as fold inductions over normalized untreated controls and presented as the mean ± SD. Statistical analysis was performed using the Student's *t*-test. P-values less than 0.05 were considered statistically significant.

Results

Loss of CCN2 results in defects in ECM assembly and ER stress in the growth plate

Ccn2 mutant growth plates and cultured chondrocytes exhibit decreases in ECM production[20, 34]. However, the consequences of decreased ECM on the overall organization of the cartilage ECM were not investigated. Therefore, transmission electron microscopy was performed on E18.5 WT and *Ccn2* mutant growth plates. This analysis revealed major defects in ECM assembly in mutants. Fewer collagen fibrils were observed throughout the growth plate in *Ccn2* mutants (Fig. 1A,B and data not shown). Unexpectedly, ultrastructural examination also revealed enlarged and distended ERs in *Ccn2* mutant growth plates (Fig. 1C-F and data not shown). WT proliferative chondrocytes contained an organized rough ER (rER) with a limited amount of protein evenly distributed throughout the cisternae (Fig. 1 C,E). However, in *Ccn2*

mutants, rER cisternae were dilated with large amounts of amorphous substances (Fig. 2B,D). Large vacuoles filled with an electron-lucid granular substance were also observed in mutants, indicative of accumulated intracellular proteins (Fig. 1F). Moreover, the nuclear chromatin in mutant chondrocytes was uniformly condensed (Fig. 1F), indicating the chondrocytes were undergoing cell death.

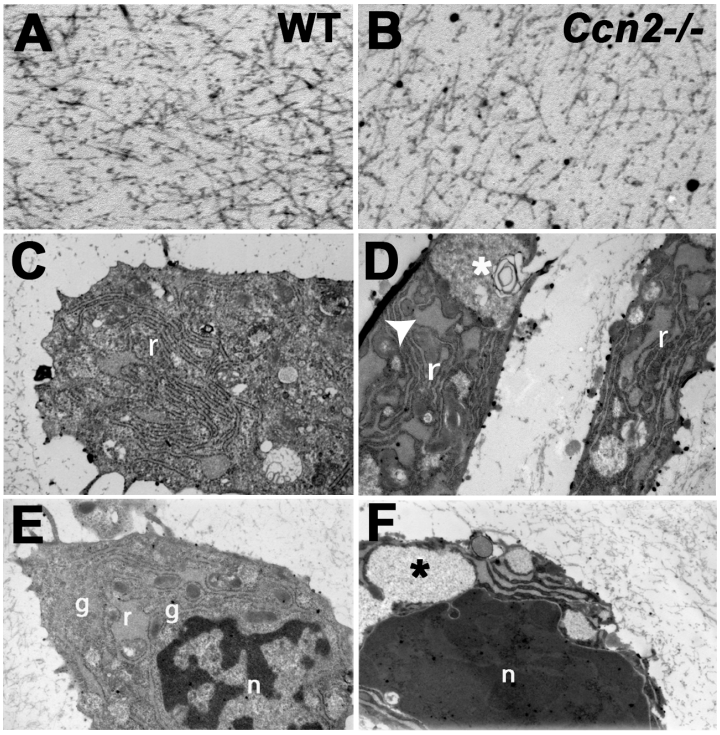


Figure 1. The loss of CCN2 results in an enlarged endoplasmic reticulum. Electron microscopy was performed on CCN2 WT and mutant proliferative chondrocytes in E18.5 growth plates. (A) Collagen fibrils in interstitial ECM in the proliferative zone of a WT embryo. (B) CCN2 mutant chondrocytes exhibit fewer collagen fibrils compared to WT control matrix. (C) WT and (D) *Ccn2-/-* proliferative chondrocytes. The *Ccn2* mutant ER appears devoid of ribosomes and is engorged with an electron lucid substance, which is presumably composed of misfolded protein aggregates (red arrow). Large vacuoles are also present and filled with granulated material (asterisk). (E,F) WT (E) and *Ccn2* mutant chondrocyte showing electron dense nuclear heterochromatin characteristic of chondrocytes undergoing apoptosis. g, golgi, n; nucleus; r, endoplasmic reticulum. Representative images are shown.

The distended ER suggested that *Ccn2*-/- chondrocytes experience ER stress as a direct consequence of loss of CCN2. We observed ER stress in all zones of the growth plate. We confirmed previously that CCN2 protein is expressed the proliferative zone, in addition to the strong expression observed in the hypertrophic zone[20] (Fig. 2A). As ER stress in chondrocytes has been associated with mutations in ECM components[2], but not with a complete loss of these proteins, we confirmed that CCN2 protein is absent in mutant growth plates (Fig. 2B)[20]. Next, we tested whether the distended ER in *Ccn2* mutant chondrocytes is associated with activation of ER stress pathways by examining expression of UPR activator, BiP, and the apoptosis inducing protein, CHOP. Consistent with previous studies[35], low levels of BiP were observed in the hypertrophic zone (Fig. 2C), and CHOP (Fig. 2E) was undetectable in E16.5 WT growth plates. Increased expression of BiP (Fig. 2D) and CHOP (Fig. 2F) were observed in the hypertrophic zones of *Ccn2* mutant growth plates. Expression of calnexin, a calcium dependent ER chaperone, has not been examined in the growth plate, but is known to be upreguated by ER stress in chondrocytes[36]. Calnexin was elevated throughout the growth plates of *Ccn2* mutants (Fig. 2H) compared to WT (Fig. 2G). These results show that the absence of CCN2 in the growth plate results in defective ECM assembly and ER stress, suggesting that the UPR is activated in the growth plates of *Ccn2* mutants.

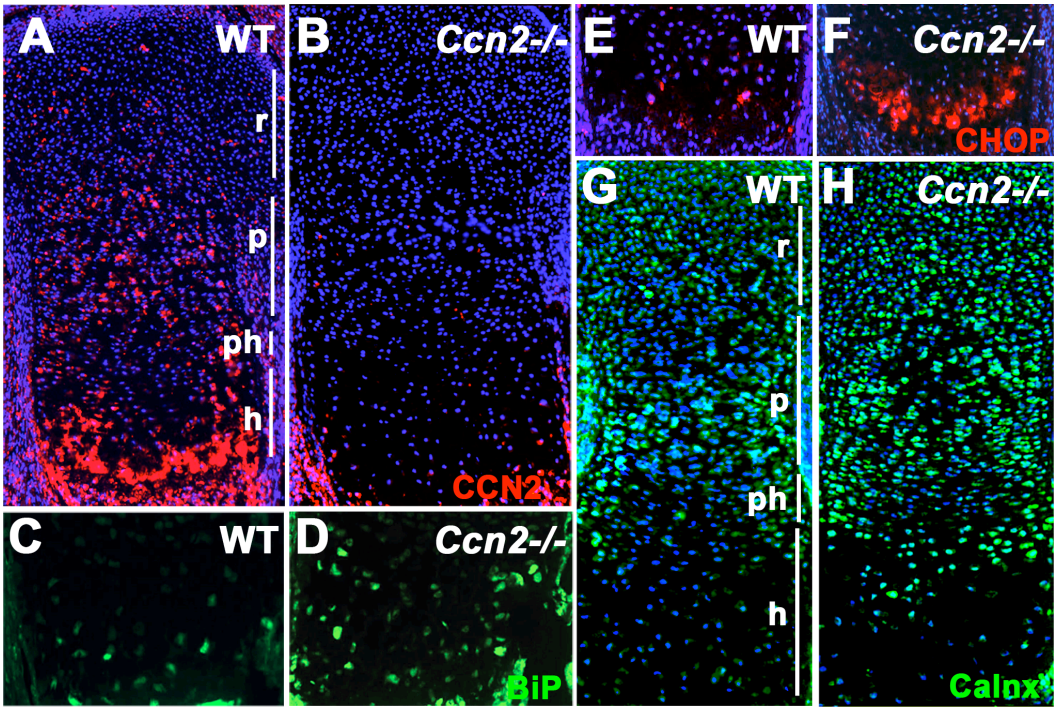


Figure 2. Depletion of CCN2 results in increased ER stress and defective chaperone expression *in vivo*. All images are E16.5 tibial growth plates. Immunfluorescence staining for CCN2 in (A) WT and (B) *Ccn2-/-* growth plates, showing expression of CCN2 in proliferative chondrocytes in addition to hypertrophic chondrocytes. (B) The *Ccn2* mutant allele is transcriptionally null, and in accordance, no CCN2 expression is observed in *Ccn2-/-* growth plates. (C,D) Immunofluorescence staining for BiP reveals very low levels of expression in WT growth plates (C), and elevated expression in the hypertrophic zones of *Ccn2* mutant littermates (D). (E,F) The pro-apoptotic marker CHOP is seen at very low levels, restricted to the hypertrophic zone, in WT growth plates (E), but is strongly upregulated in the hypertrophic zone in *Ccn2* mutant littermates (F). (G,H) Expression of the resident ER chaperone calnexin is restricted to lower proliferative zone chondrocytes in WT growth plates (G), but is upregulated throughout the growth plates of *Ccn2* mutants (H). Representative images are shown.

CCN2 is an ER stress responsive gene

The finding that loss of CCN2 was associated with ER stress raised the possibility that CCN2 may be a primary component of ER stress in chondrocytes. We therefore tested whether CCN2 is upregulated in chondrocytes during chemically induced ER stress. Thapsigargin (THG) induces ER stress by inhibiting sarco/endoplasmic reticulum calcium ATPases, thus blocking calcium release from ER stores, eventually leading to cell death[37]. Prior to cell death, the transcription of ECM components is downregulated and protective ER stress response genes such as BiP are activated by THG[37]. ATDC5 chondrosarcoma cells were treated with increasing concentrations of THG. BiP and CHOP levels were examined using quantitative reverse transcriptase-PCR (qRT-PCR). Consistent with previous studies, THG induced dose-dependent increases in *BiP* (Fig. 3A) and *Chop* mRNA (Fig. 3B)[32]. In contrast to the expression of ECM components, which are downregulated by THG[38], *Ccn2* mRNA levels were increased by THG with the same kinetics as *BiP* and *Chop* (Fig. 3C), suggesting that CCN2 is an ER stress response gene. These results confirm that CCN2 is induced by ER stress, as opposed to structural ECM proteins, whose expression is down regulated by ER stress[37].

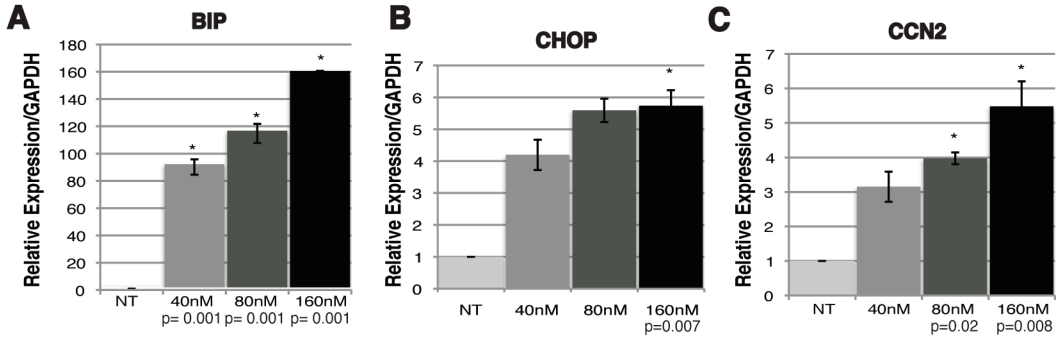


Figure 3. CCN2 is an ER stress responsive gene in chemically induced ER stress. ATDC5 chondrocytes were cultured for 24 hours in the presence of Thapsigargin (THG) at the indicated concentrations. mRNA expression of *Ccn2* (A), *BiP* (B) and *Chop* (C) were assayed by Quantitative

Reverse Transcriptase PCR (qRT-PCR). Assays were performed in triplicate and a p value of >0.05 was considered significant compared to no THG (*). p values shown are for 160nM THG.

CCN2 depletion induces ER stress in chondron cultures

The above experiments demonstrate that CCN2 expression is induced by ER stress. However, they do not demonstrate that the ER stress observed in *Ccn2-/-* growth plates is due to a direct role for CCN2 in chondrocytes, rather than an indirect one as a result of defective growth plate angiogenesis. Recent studies have shown that the presence of ECM protects chondrocytes from ER stress[39]. Therefore, a 3-dimensional culture system was developed in which chondrocytes maintained in alginate beads, yielding chondrocyte clusters (chondrons) (Fig. 4)[31]. The accumulation of ECM components can be observed (Fig. 4A,B). THG treatment induced ER stress in chondrons, with BiP localization transitioning from the ER lumen in control cells (Fig. 4'C), to the nucleus in THG treated chondrons (Fig. 4D). The re-localization of BiP to the nucleus has not been reported previously in chondrocytes, but is consistent with previous reports in other cell types[40]. CHOP levels were also increased in THG treated chondrons (Fig. 4F), compared to untreated controls (Fig. 4E). Therefore, chondrons in alginate are sensitive to THG-induced ER stress.

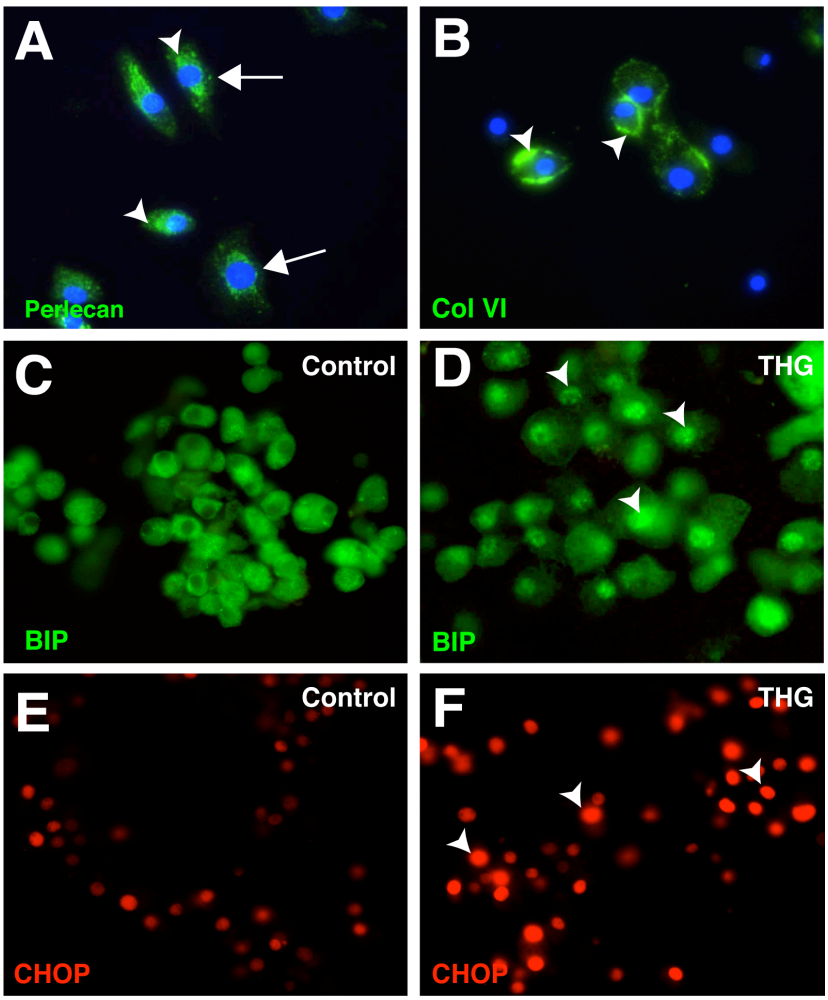


Figure 4. Alginate chondron system to analyze ER stress markers *in vitro*. Primary chondrocytes cultured for 10 days in alginate. (A) Perlecan, a major ECM component (green) is seen intracellularly (arrowheads) and in a punctate pattern at the cell surface. (B) Collagen 6a1 staining (green) is seen extracellularly. (C-F). Control and Thapsigargin (THG) treated chondrons stained for BiP and CHOP. BiP expression relocalizes from the cytoplasm to the nucleus during ER stress induction (C,D). Control and THG treated chondrons showing induction of CHOP (E,F) (blue = nuclear stain). Representative images are shown.

Next, the impact of loss of *Ccn2* on THG-induced ER stress was tested. First, we confirmed that CCN2 is expressed in WT chondrons (Fig. 5A) and is absent in *Ccn2-/-* chondrons (Fig. 5B,C). WT chondrons expressed low levels of BiP (Fig. 5D). *Ccn2-/-* chondron clusters displayed both upregulated BiP expression and a higher proportion of nuclear BiP-positive cells (Fig. 5E,F). CCN2 mutant chondrons also exhibit increased expression of CHOP (Fig. 5H-I). These results confirm that CCN2 plays a direct role in the protection of chondrocytes against ER stress.

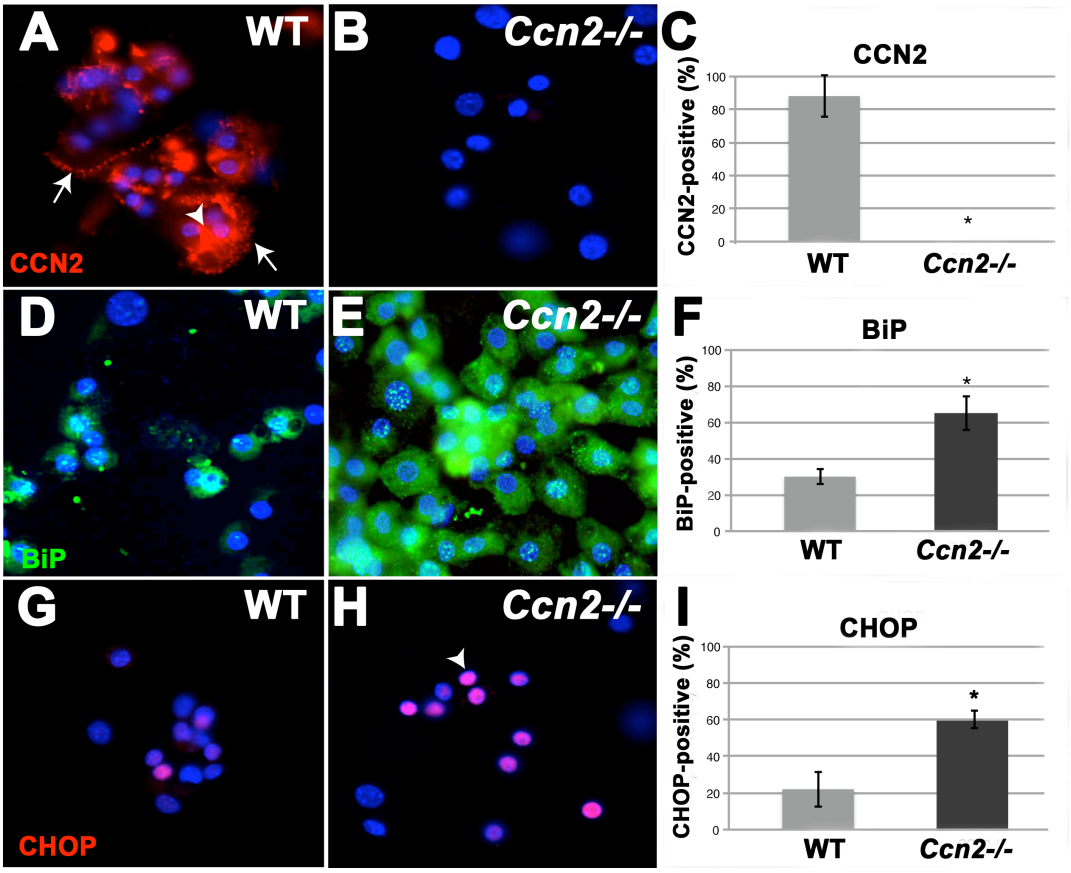


Figure 5. CCN2 depleted chondrons exhibit elevated ER stress. Primary sternal chondrocytes were isolated from E16.5 WT and *Ccn2-/-* sterna, and cultured in alginate for 10 days to yield chondrons. CCN2, BiP and CHOP protein expression were analyzed through immunofluorescence. (A-C) CCN2 expression. CCN2 protein expression was abundant in WT chondrons (A), where punctate CCN2 protein expression was observed intracellularly (arrowhead) and pericellularly (arrows). CCN2 protein was not

detected in *Ccn2* mutant chondrons (B) (C) Quantitation of number of CCN2-expressing cells. (D-F) BiP expression in WT and *Ccn2-/-* chondrons. (D) BiP is detected in the ER in some cells in WT chondron clusters. (E) Nearly all *Ccn2-/-* chondrocytes express BiP, and many nuclei are positive. (F) Quantitation of the percentage of nuclear BiP-expressing cells. (G-I) CHOP expression. (G) Few WT chondrocytes express CHOP. In contrast, most *Ccn2-/-* chondrocytes express CHOP (H). (I) Quantitation of the percentage of CHOP-expressing chondrocytes. (C,F,I) CCN2, BiP, and CHOP expression was analyzed in triplicate using ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. Asterisks indicate statistical significance with a p-value of 0.003. Representative images are shown.

The protective effect of CCN2 on ER stress may be mediated through integrin α5

CCN2 is thought to mediate its functions by acting as a ligand for integrins[17]. CCN2 induces expression of integrin α5, and binds to integrin α5β1 to promote chondrocyte proliferation and ECM secretion[34]. Integrin α5β1 is also required for chondrocyte survival[41]. Recent data demonstrate that ER stress pathways are activated as a survival mechanism during cell detachment from the ECM[6]. Taken together, these findings suggest that one of the pro-survival functions of integrin α5β1 in chondrocytes is suppression of ER stress. To test this possibility, sternal chondrocytes were treated with or without an integrin α5-blocking antibody for 24 hours (Fig. 6A). *BiP* and *Chop* mRNA expression levels were upregulated in response to treatment with the integrin α5-blocking antibody (Fig. 6A). Fibronectin (FN) is the primary ligand for α5β1, and we showed previously that exogenous CCN2 induces FN expression in chondrocytes[34]. However, FN distribution was not altered in *Ccn2*-/- growth plates (Fig. 6B,C). However, *Ccn2*-/- growth plates exhibit decreased levels of integrin α5[34]. These results suggest that CCN2 may control ER stress in part through integrin α5 but not through regulation of FN

levels.

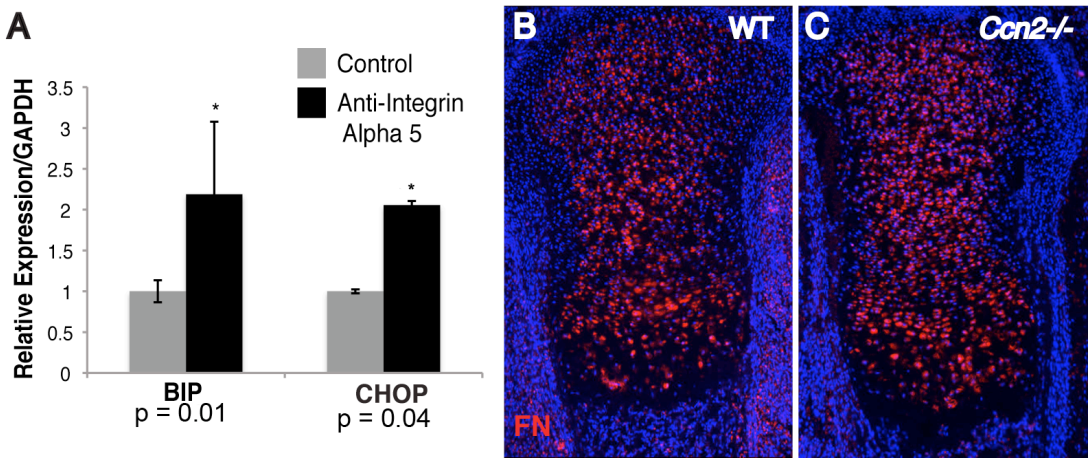


Figure 6. Blocking integrin α5 results in ER stress. Primary sternal chondrocytes were serum starved and treated with anti-integrin α5 blocking antibody or goat serum (control) for 24 hours, after which BiP and CHOP mRNA levels were quantitated by qRT-PCR and normalized to GAPDH. BiP and CHOP expression were increased by treatment with the blocking antibody (p=0.01 and p=0.04 respectively). Immunofluorescence staining for fibronectin in WT (B) and *Ccn2-/-* growth plates (C) counterstained with DAPI.

Overexpression of CCN2 attenuates ER stress

To provide additional evidence that CCN2 protects chondrocytes from ER stress, we generated transgenic mice that overexpress CCN2 in chondrocytes. An inducible transgenic construct was generated, consisting of a floxed transcriptional STOP signal placed upstream of the CCN2 coding sequence (Fig 7A). Transgenic lines were established and mated to *Col2Cre* mice[27] to induce chondrocyte-specific overexpression of CCN2 (Fig 7A). A 3-5 fold induction of CCN2 protein was observed in *Ccnfx2Tg;Col2Cre* mice (Fig. 7B). The specificity of induction

was confirmed in isolated mouse embryonic fibroblasts (MEFs) from *Ccn2fxTg* embryos infected with adenoviral Cre (Ad-CRE) (Fig. 7C). *Ccn2fxTg;Col2Cre* mice are viable and exhibit no obvious morphological differences at birth (Fig. 7D), but exhibit progressive overgrowth of cartilage elements leading to vertebral fusion in adults (data not shown). A complete characterization of these transgenics will be presented elsewhere. The growth plates of P0 *Ccn2fxTg* tibiae have normal chondrocyte organization (Fig. 7F), although both the hypertrophic zone and chondrocytes within it are slightly smaller than in WT littermates (Fig. 7E). Consistent with a protective function for CCN2, levels of BiP were reduced in *Ccn2fxTg;Col2Cre* mice (Fig. 7H) compared to WT controls (Fig. 7G).

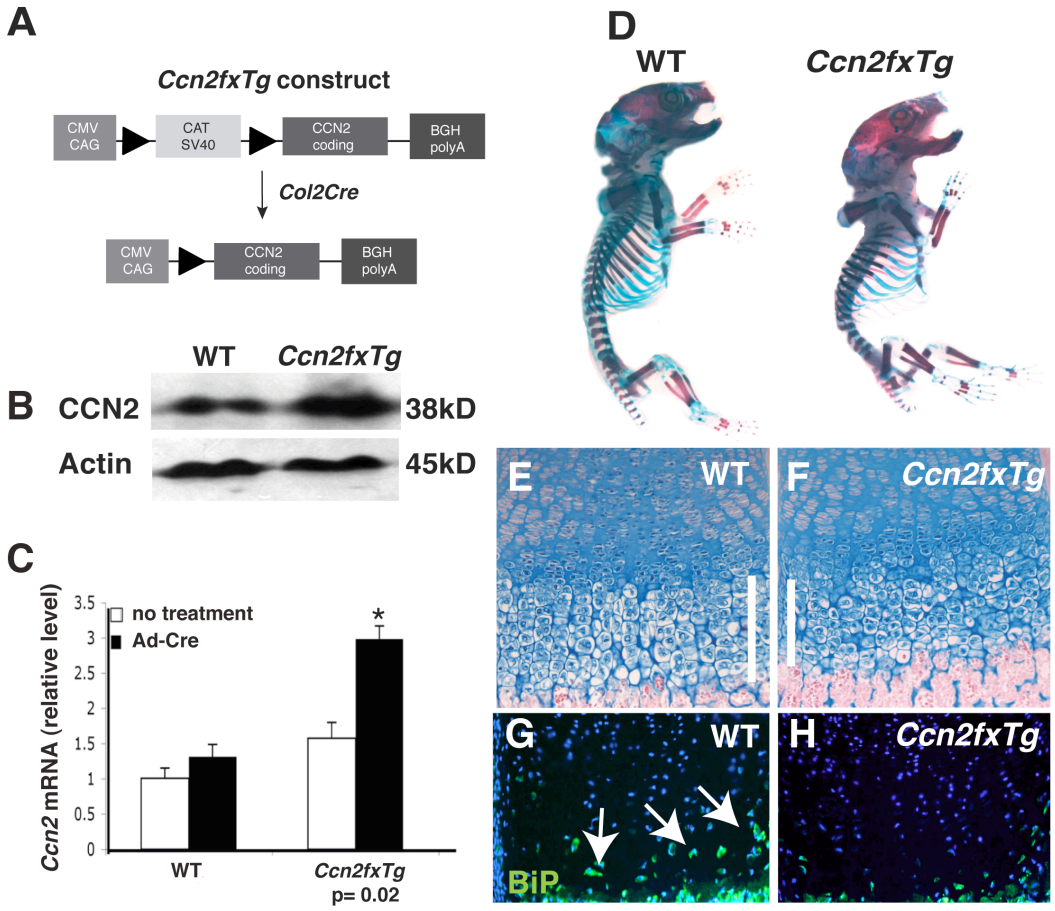


Figure 7. CCN2 overexpression attenuates ER stress *in vivo*. (A) Schematic of the CCN2 floxed transgenic construct. A floxed cassette consisting of a STOP cassette and a chloroacetyltransferase

(CAT) gene is flanked by loxP sites and inserted between,the CMV/chicken β actin (CAG) promoter and the murine CCN2 coding sequence, followed by a bovine growth hormone polyA signal sequence. Upon crossing to a *Col2Cre* mouse, the silencer is excised, leading to transcription of CCN2 from the transgene in cartilage. (B) Western blot of protein isolated from sterna of WT and *Ccn2fxTg;Col2Cre* P0 pups with actin controls. (C) *Ccn2* mRNA levels in mouse embryonic fibroblasts (MEFs) from E11.5 *CCN2fxtg* E11.5 embryos infected with AdCre or Adcontrol. *Ccn2* mRNA levels are not significantly different in WT vs. *Ccn2fxTg* MEFs in the absence of Cre, but are increased approximately 2.5-fold in *Ccn2fxTg* mice in the presence of Cre. (D, E) Skeletal preparations of postnatal day 0 (P0) *Ccn2fxTg;Col2Cre* and WT littermates, showing no apparent morphological differences. (E,F) Growth plate histology of P0 WT (E) and *Ccn2fxTg;Col2Cre* (F) littermate. The hypertrophic zone is slightly shorter and hypertrophic chondrocytes are smaller in transgenics. (G, H) Overexpression of *Ccn2* results in decreased BiP expression (H) compared to WT littermate (G) in P0 growth plates.

CCN2 regulates chondrocyte survival through NFκB and autophagy

NFκB is required for chondrocyte survival[11, 12]. CCN2 induces nuclear localization of NFκB and activates pro-survival mechanisms, while inhibiting stress-mediated apoptosis in a number of cell types[42]. NFκB is activated by integrin α5β1 in breast cancer epithelial cells[43], and α5β1 is a major receptor for CCN2 in chondrocytes[43]. We therefore investigated whether CCN2 might exert its pro-survival function in chondrocytes at least in part through regulation of NFκB levels. NFκB localization was examined in E16.5 WT and mutant growth plates. In WT proliferative zones, NFκB was predominantly localized to the nucleus (Fig. 8A), similar to previous reports[11, 12]. In *Ccn2-/-* growth plates, NFκB is present in the cytoplasm, but the number of cells exhibiting nuclear localization is decreased (Fig. 8B,C). These data are thus consistent with previous *in vitro* studies showing that CCN2 induces NFκB activation[42], and raise the

possibility that CCN2 exerts its pro-survival, ER stress-protective effects in chondrocytes at least in part through NFκB.

Autophagy is an important survival mechanism in normal chondrocytes and in response to stress[44]. We examined the expression of autophagosome markers Atg12 and LC3 (Atg8), which conjugate with one another to stabilize autophagosome formation[13]. In E16.5 WT growth plates, both Atg12 and LC3 proteins were expressed throughout the growth plate, with the highest levels in the hypertrophic zone (Fig. 8D,F). This expression is consistent with previous literature showing that autophagy is required for terminal chondrocyte differentiation[45]. However, in *Ccn2-/-* growth plates Atg12 and LC3 expression levels were greatly diminished (Fig. 8E,G).

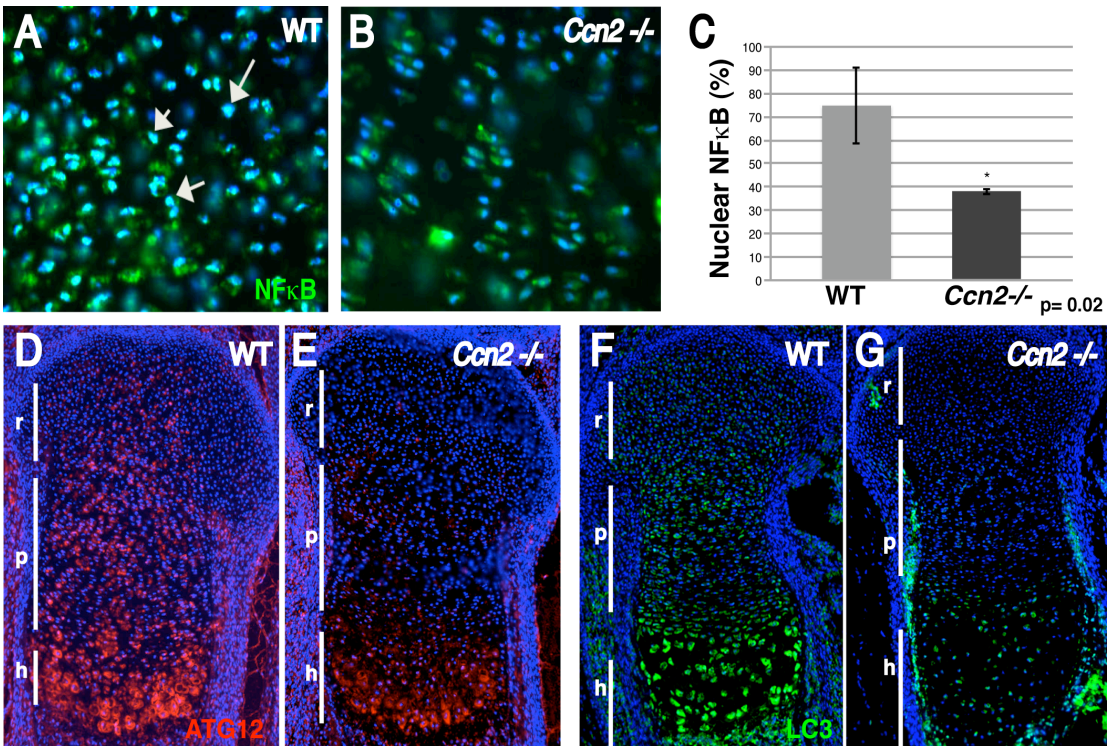


Figure 8. Decreased NFκB and autophagy-mediated cell survival in *Ccn2* mutants. NFκB expression was visualized by immunofluorescence in E16.5 WT and *Ccn2* mutant growth plates. (A) In

WT growth plates, NFκB was primarily localized to the nucleus throughout the proliferative zone. (B) In *Ccn2-/-* growth plates, NFκB expression in the proliferative zone was decreased and localized mainly to the cytoplasm. (C) NFκB nuclear expression throughout the growth plate was quantitated using Image J analysis as the percentage of DAPI positive nuclei. Growth plate immunostaining was performed in triplicate; asterisk denotes a p-value of 0.003, indicating statistical significance. (D,E) E16.5 WT (D) and *Ccn2-/-* (E) growth plates immunostained for Atg12. (F,G) E16.5 WT (F) and *Ccn2-/-* (G) growth plates immunostained for Atg8 (LC3). All sections were counterstained with DAPI.

The above results suggest that CCN2 regulates autophagy by controlling the expression of autophagy-related genes. Because both NFκB and autophagy have pro-survival functions in chondrocytes, we investigated whether CCN2 induces NFκB and the expression of autophagy genes *in vitro*. ATDC5 cells infected with a CCN2 overexpressing adenovirus (Ad-CCN2-GFP) exhibited increased CCN2 expression within 24 hours after infection (Fig. 9A,B). Consistent with *in vivo* studies (Fig. 8), CCN2 expression induced increased nuclear NFκB (Fig. 9C,D). Furthermore, CCN2 stimulated the expression of LC3 (Fig. 9E,F). In summary, these data suggest that CCN2 protects against pro-apoptotic ER stress pathways by upregulating pro-survival NFκB and autophagy pathways in chondrocytes.

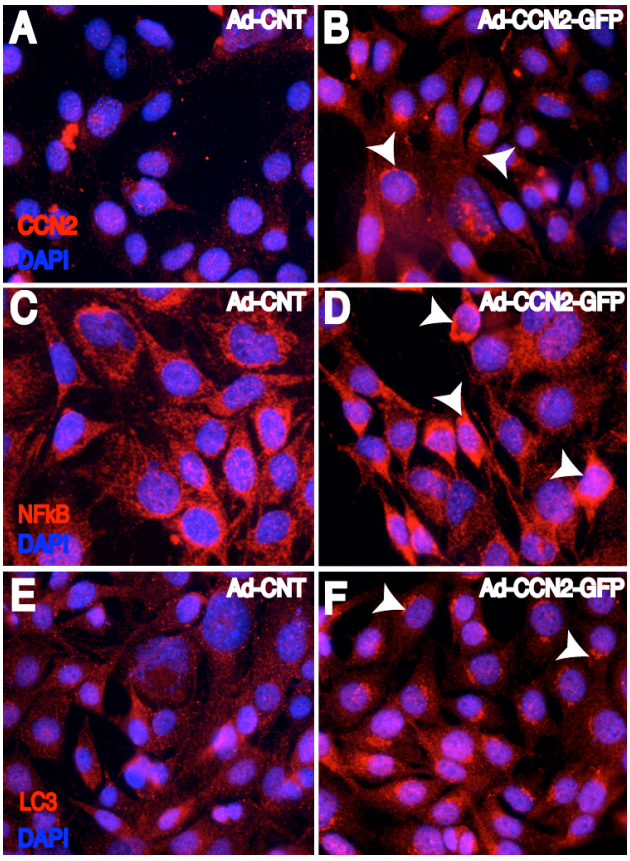


Figure 9. Ectopic CCN2 expression results in increased NFκB and expression of autophagy genes in ATDC5 cells. NFκB and autophagy marker LC3 were examined in ATDC5 cells infected with Ad-CCN2-green florescent protein (Ad-CCN2-GFP) and Ad-control (Ad-CNT) constructs. (A,B) CCN2 expression is upregulated in Ad-CCN2-GFP ATDC5 cells (B) compared to Ad-CNT treated cells (A). (C,D) Nuclear NFκB is increased in Ad-CCN2-GFP cells (D) compared to control cells (C). (E,F) Expression of LC3/Atg8 is increased in Ad-CCN2-GFP cells (F) compared to Ad-CNT treated cells (E). (Red- CCN2, NFκB and LC3); Blue= DAPI counterstain). Representative images are shown.

Discussion

The data reported here provide evidence that CCN2 is required for ECM assembly and chondrocyte survival in the growth plate. Ultrastructural analysis revealed that loss of CCN2 leads to defective ECM assembly and chondrocyte death, accompanied by ER stress. The activation of pro-apoptotic ER stress pathways *in vivo* was confirmed by elevated levels of BiP and CHOP in *Ccn2-/-* growth plates. These results were recapitulated *in vitro*, where loss of *Ccn2* in chondron cultures also resulted in ER stress. This latter finding demonstrates that the role of CCN2 in protection from ER stress in chondrocytes is direct, rather than an indirect consequence of defective growth plate angiogenesis. This is the first demonstration of a role for a matricellular protein in protection against ER stress. The finding that *Ccn2* expression is induced by ER stress, unlike structural ECM proteins, whose expression is downregulated, provides further evidence that the primary function of CCN2 is to mediate chondrocyte survival. This is also supported by the finding that transgenic mice overexpressing CCN2 exhibit lower levels of BiP in the growth plate.

The precise mechanisms by which CCN2 protects against ER stress are unclear. ER stress leads to decreased ECM deposition and could account for the ECM defects seen in *Ccn2-/-* growth plates. On the other hand, decreased ability of cells to attach to the ECM has been shown to lead to ER stress[5, 6]. Evidence that CCN2 exerts its protective effects through modulation of cell-ECM attachment comes from the finding that treatment of chondrocytes with an integrin α5-blocking antibody leads to ER stress. Integrin α5β1 is essential for chondrocyte survival *in vitro*[41]. Currently, no *in vivo* studies have been conducted to assess the role of α5 containing integrins in cartilage. The loss of β1 integrin leads to reduced ECM production, growth plate disorganization and impaired survival[46], consistent with a potential role for α5β1 in ECM

assembly and chondrocyte survival. We have shown that CCN2 exerts effects on chondrocyte proliferation and adhesion through α5β1[34]. However, mice lacking FN in chondrocytes exhibit no abnormalities[46]. Furthermore, FN levels were normal in *Ccn2* mutant growth plates. Therefore, we propose that CCN2, rather than FN, is the major ligand for integrin α5β1 in chondrocytes.

In addition to induction of pro-apoptotic ER stress pathways, the loss of *Ccn2* also results in decreased expression of nuclear NFκB and autophagy genes. In accordance, overexpression of *Ccn2* in chondrocytes results in induction of nuclear NFκB and autophagy gene expression. Both NFκB and autophagy exert pro-survival functions in growth plate chondrocytes[11, 45]. The precise mechanisms by which CCN2 induces the expression of NFκB in chondrocytes are unclear but are probably mediated through integrin signaling, as previously observed in other cell types[42, 47].

CCN2 expression is correlated with the onset of osteoarthritis (OA) and synovial damage[48, 49]. Paradoxically, recombinant CCN2 expedites repair of articular cartilage during chemically induced knee OA[38]. These opposing effects of CCN2 may be due to its ability to modulate NFκB. While NFκB has pro-survival functions in chondrocytes[11, 12], it also promotes the expression of pro-inflammatory genes in OA chondrocytes[7]. Whether or not NFκB has a pro-survival function in OA chondrocytes is unknown. Further investigations of the mechanism by which CCN2 promotes survival in growth plate chondrocytes, and its role in the maintenance of articular cartilage may provide new avenues to treat and prevent OA.

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Chapter 5:

Discussion and Future Directions

CCN2 is a matricellular protein that plays multiple roles during embryonic and postnatal development, as well as crucial roles in multiple pathological disease states. This dissertation explored the physiological roles of CCN2 during angiogenesis and chondrocyte matrix secretion and survival through the investigation of *Ccn2* global knockout mice.

Angiogenesis is essential for embryonic development as well as postnatal maintenance and injury repair. Factors that can promote or disrupt angiogenesis may provide new therapeutic targets, as angiogenesis plays major roles in tumorigenesis. Previous data on the role of CCN2 during angiogenesis were ambiguous, with both pro- and anti-angiogenic functions reported; the functions were variable depending at least in part on cellular specificity and experimental design [1,2]. The data presented in this dissertation demonstrate that CCN2 has direct functions during vascular remodeling. CCN2 controls cell signaling between endothelial cells, pericytes and vascular smooth muscle cells, in part through the modulation of platelet derived growth factor (PDGF) levels and downstream PDGF/Akt signaling. CCN2 played no role in *de novo* blood vessel formation, as evidenced by the by normal vasculogenesis in *Ccn2* mutants at early stages of development. However, CCN2 was found to induce the secretion of provisional and permanent vascular basement membrane components, which are required for blood vessel remodeling and stabilization. In conclusion, CCN2 plays two roles during angiogenesis: first CCN2 mediates signaling between vascular cells and second, CCN2 induces the formation of vascular basement membrane components to aid in vessel stability and integrity. These properties make CCN2 an interesting target for angiogenic therapies that may employ the use of CCN2 blocking antibodies that disrupt angiogenesis during cancer treatments. In order to fully investigate the cell autonomous functions of CCN2 during angiogenesis specifically in vascular smooth muscle, endothelial cells and perictyes, future experiments should include using *Ccn2*

conditional knockout mouse models. Most angiogenesis-associated pathologies occur in the adult, and *Ccn2* mutant mice exhibited late gestational defects in vascular remodeling, but not in early embryonic vasculogenesis. Future research should focus on inducible depletion of CCN2 postnatally in order to explore its contribution during adult vascular maintenance and injury. CCN1 also plays a prominent role in vascular development, with *Ccn1* global knockout embryos perishing early in development due to major defects in vasculogenesis [3]. As CCN1 and CCN2 are co-expressed in at least some vessels at early and later stages of vasculogenesis and angiogenesis, the generation of double mutants is an important future goal. CCN3 has been described as another potent inducer of angiogenesis, and can promote endothelial cell adhesion, migration and tube formation *in vitro* through integrin-mediated signaling [4]. Other CCNs appear to play inhibitory roles in vascular elements, such as CCN5, which has been shown to inhibit vascular smooth muscle cell migration [5]. Future research should focus on the contribution of all CCN family members to vascular development and remodeling, which will provide therapeutic insights into cancer and inflammatory diseases.

CCN2 is essential for endochondral bone formation and the loss of CCN2 results in a severe chondrodysplasia that eventually leads to perinatal lethality [6]. This lethality is due to major defects in chondrocyte proliferation, differentiation and terminal maturation [6]. This dissertation provides evidence that the defects observed in *Ccn2* mutant chondrocytes are due in part to defects in ECM secretion and decreased cellular survival. Again, this study highlights the multifunctional roles of CCN2 in cell and tissue specific contexts, as CCN2 also works through two different mechanistic pathways to control chondrocyte stress and survival. First, CCN2 controls cellular survival by mediating and responding to endoplasmic reticulum (ER) stress. We show that CCN2 is an ER stress responsive gene that is activated upon chemically induced ER

stress. CCN2 may also activate ER stress pathways, as we found that the loss of CCN2 results in ER stress and activation of the downstream unfolded protein response (UPR). Conversely, the overexpression of CCN2 results in the attenuation of ER stress. Taken together, these results show that CCN2 regulates ER stress and this control is mediated in part through integrin α5 signaling due to the increased ER stress observed upon blocking integrin α5 signaling *in vitro*. The increased ER stress observed in mutant growth plate chondrocytes is also due to a disruption in Nuclear Factor κB (NFκB) signaling. NFκB is required for chondrocyte survival [7]. Decreased nuclear NFκB was observed in *Ccn2* mutant growth plates, indicating that CCN2 regulates NFκB signaling *in vivo*. CCN2 was also able to induce nuclear localization of NFκB. Autophagy is a lysosomal degradation and recycling system of cellular compartments that is required for cellular homeostasis. Autophagy can be activated by prolonged ER stress [8], and is required for chondrocyte differentiation in the growth plate [9]. We found that components of the autophagy pathway are decreased in *Ccn2* mutant growth plates, demonstrating that CCN2 may control cellular survival through mediating autophagy.

In summary, these data show that CCN2 plays a novel protective role during ER stress through direct control of the ER stress response, integrin-mediated signaling and through NFκB and autophagy-mediated cellular survival. ER stress is emerging as an important therapeutic target in the treatment of multiple disease including neurodegenerative diseases, cancer, diabetes and chondrodysplasias [10]. CCN2 is overexpressed in multiple diseases like osteoarthritis and certain cancers and is associated with poor prognosis. Our data suggest that the increases in CCN2 observed in these tissues may represent an attempt by the injured cells to activate cellular survival mechanisms. Based on the mild chondrodysplasia we observed in *Ccn2* overexpressing mice, it is also possible that proper levels of CCN2 need to be maintained in order to activate

protective mechanisms, with very high levels of CCN2 promoting pathological disease progression. Trials utilizing CCN2 blocking antibodies should take into account the possible pro-survival effects that CCN2 may provide during pathogenesis [11]. Due to the overlapping functions and compensatory mechanisms of other CCNs in the absence of CCN2 [12], and the expression of CCNs during inflammation, injury and fibrotic conditions [13], it is likely that other CCNs play roles in regulating ER stress and controlling cellular survival in these processes. These mechanisms should be explored in future experiments using conditional knockout mouse models and *in vitro* studies examining other CCNs that are upregulated in response to different forms of chemically induced ER stress.

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