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Taking Aim at the Extracellular Matrix: CCN Proteins as Emerging Therapeutic Targets

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Abstract

The CCN family of matricellular proteins is critical for embryonic development and plays important roles in inflammation, wound healing, and injury repair in the adult. Deregulation of their expression or activities contributes to the pathobiology of myriad diseases, many of which may arise when inflammation or tissue injury becomes chronic, including fibrosis, arthrosclerosis, arthritis, diabetic nephropathy and retinopathy, and cancer. Emerging studies indicate that targeting CCN expression or signaling pathways holds promise in the development of diagnostics and therapeutics for such diseases. This review summarizes the biology of CCN proteins, their roles in various pathologies, and potential as therapeutic targets.

INTRODUCTION

Beyond serving as a scaffold for the organization of cells into tissues, the extracellular matrix (ECM) is also a multifunctional regulator of cellular behavior. ECM proteins can modulate the activity or bioavailability of extracellular signaling molecules such as growth factors, cytokines, chemokines, and extracellular enzymes, or directly bind to and signal through cell surface receptors to regulate cell functions. A subset of ECM proteins, known as matricellular proteins, is dynamically expressed and serves primarily regulatory rather than structural roles[1, 2]. Among known matricellular proteins are members of the CCN family, a group of highly conserved secreted proteins identified by differential expression screening whose synthesis is regulated by mitogenic growth factors or oncogenic transformation[2–4]. The acronym CCN is derived from the first three members of the family described, namely CYR61 (cysteine-rich 61/CCN1), CTGF (connective tissue growth factor/CCN2), and NOV (nephroblastoma overexpressed/CCN3). Together with a set of three Wnt-inducible signaling pathway proteins (WISP1/CCN4, WISP2/CCN5, and WISP3/CCN6), they comprise a family of six homologous cysteine-rich proteins in mammals that have been renamed CCN1–6 by international consensus[5]. CCN proteins share a modular structure, with an N-terminal secretory peptide followed by four conserved domains with sequence homologies to insulin-like growth factor binding proteins (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type I repeat (TSR), and a carboxyl-terminal domain (CT) that contains a cysteine-knot motif (Box 1). A non-conserved, protease-sensitive central hinge region bisects the proteins into two halves that bind distinct cell surface receptors. The expression of CCN proteins is exquisitely regulated on transcriptional, post-transcriptional, and translational levels in response to changes in environmental stimuli, including those encountered in tissue injury repair (Box 1, Fig. 1).

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At the cellular level, CCN proteins regulate cell adhesion, migration, proliferation, differentiation, apoptosis, survival, senescence, and gene expression. By modulating one or more aspects of these cellular functions in a cell type-specific manner, CCNs coordinate complex biological processes, including cardiovascular and skeletal development during embryogenesis, as well as inflammation, wound healing, and tissue injury repair in the adult. Aberrant CCN expression is associated with a remarkable diversity of seemingly unrelated diseases. A useful albeit simplistic framework with which to rationalize the roles of CCNs in this broad array of diseases is that many pathological conditions arise when inflammation or tissue injury becomes chronic, and in this process CCNs are concomitantly deregulated. Studies in animal models and human patients have confirmed that CCNs are involved in many diseases related to inflammation and injury repair, including arthritis, atherosclerosis, restenosis after vascular injury, fibrosis, cancer, and diabetic nephropathy and retinopathy. Modulating CCN expression or function has yielded significant benefits in animal models of disease, suggesting that CCNs are promising therapeutic targets in these pathologies. Clinical trials targeting CCN2 using a humanized monoclonal antibody (FG-3019) have begun to show encouraging results in several human diseases, such as diabetic nephropathy[6]. However, the therapeutic potential of other CCN proteins have yet to be evaluated in a clinical setting. In this review, we discuss recent insights into the regulation and function of CCN proteins in various biological processes, summarize the evidence for their participation in disease pathologies, explore their potential as diagnostic markers and therapeutic targets, and discuss various targeting strategies as applied to these proteins.

FUNCTIONS OF CCN PROTEINS AND THEIR MECHANISMS OF ACTION

As members of the CCN family serve both distinct and overlapping biological roles, these highly conserved proteins bind many of the same receptors and function through similar mechanisms to regulate a common set of biological processes. However, the specific biological responses to some of the CCN proteins may be similar in some cell types but rather different in others, and at times diametrically opposed. Nevertheless, some generalizations can be made about how CCNs act. First, many CCN activities are mediated through their direct binding to integrin receptors on the cell surface, with the participation of one or more coreceptors in some contexts. The convergence of signals initiated from multiple receptors in some cases lead to unique biological responses not achieved with a single receptor, and the requirement of multiple receptors for certain CCN functions contributes to target cell specificity. Second, CCNs can regulate the expression of bioactive molecules such as growth factors, cytokines, and MMPs, and physically interact with some of them to modulate their bioavailability and activity (Fig. 2). In addition, CCNs can interact with and tether to other ECM proteins, including decorin, fibronectin, vitronectin, and perlecan, potentially functioning as a local scaffold that coordinates interaction of specific bioactive molecules or ECM proteins with the target cell (Fig. 2). Third, CCNs can profoundly alter the biological activities of cytokines, and possibly growth factors, through signaling crosstalk (Fig. 3). Through these and other mechanisms (see Box 1), CCN proteins integrate and modulate multiple signaling pathways to elicit cell type-specific responses.

Cell surface receptors

Many activities of CCN proteins are mediated through their direct binding to integrin receptors, with the involvement of coreceptors in some contexts (Table 1). At least eight integrins have been shown to bind CCN proteins[2]. Since distinct integrins are differentially expressed in various cell types and may mediate disparate activities, CCNs can achieve remarkable functional versatility through their interaction with different integrins in a cell type- and context-specific manner. Although CCN proteins do not contain the canonical RGD sequence that binds several integrins, they interact with integrins through non-canonical binding sites, many of which have been identified (Box 1). Site-directed mutations

in these binding sites abolish the specific activities for which the cognate integrins are responsible both *in vitro* and *in vivo*[7, 8], providing compelling evidence for the role of these integrins in mediating CCN functions.

At least two types of coreceptors have been identified for CCNs. The first of these are cell surface heparan sulfate proteoglycans (HSPGs), of which syndecan-4 has been identified as crucial for CCN functions[7, 9]. LRPs, which are endocytic receptors that cooperate with many growth factors receptors and integrins to modulate cellular responses[10], are the second class of coreceptor[11]. CCN2 can interact with TrkA in human mesangial cells to enhance TGF-β signaling[12] and in glioma cells to activate NFκB[13]. CCN2 binds to TrkA in a complex with β_1 integrins, indicating that TrkA also functions as a coreceptor with integrins[13]. Additionally, CCN3 can bind Notch and suppress myoblast and osteoblast differentiation[14, 15]; whether integrins are involved in these signaling events is unknown.

Cell adhesion, migration, and proliferation—As cell adhesive proteins of the ECM, CCNs support cell adhesion and promote cell spreading in many cell types (Table 1). The process of cell adhesion may also lead to other cellular responses, including cell migration, proliferation, and altered gene expression. The adhesion of human skin fibroblasts to CCN1 and CCN2 is mediated through $\alpha_6\beta_1$-HSPGs, and results in cell adhesive signaling events including the rapid formation of $\alpha_6\beta_1$-containing focal adhesion complexes, actin cytoskeleton reorganization, formation of filopodia and lamellipodia, and activation of focal adhesion kinase, paxillin, and RAC[16]. The effects of CCN proteins on cell proliferation and migration are cell type-specific. In fibroblasts, CCN1, CCN2, and CCN3 have no intrinsic ability to induce mitogenesis on their own, but can enhance DNA synthesis induced by other mitogenic growth factors by acting through integrin $\alpha_v\beta_3$[2]. Whereas CCN2 alone is capable of promoting DNA synthesis in chondrocytes and osteoblasts[17, 18], it also induces a G1-cell cycle arrest in mesangial cells[19]. CCN1, CCN2, and CCN3 stimulate cell migration or chemotaxis in fibroblasts and endothelial cells, and promote the invasiveness of certain cancer cells[2]. Although CCN3 inhibits the proliferation of Ewing's sarcoma cells, it promotes their migration and invasion[20]. Whereas CCN1 and CCN2 enhance vascular smooth muscle cell (VSMC) proliferation and migration[21, 22], CCN3 and CCN5 inhibit them[23, 24].

Cell survival, apoptosis, and cellular senescence—Cell adhesion to ECM molecules promotes cell survival, whereas detachment from the ECM induces cell death by anoikis in many cell types. Although adhesion of endothelial cells to CCN1, CCN2, or CCN3 through integrin $\alpha_v\beta_3$ supports cell survival[2], these CCN proteins can also promote apoptosis as cell adhesion substrates in fibroblasts by acting through $\alpha_6\beta_1$[9]. The apoptosis-promoting activity may be most relevant in the context of inflammation, as CCN1, CCN2, and CCN3 all have the unusual ability to enable the inflammatory cytokine TNFα to induce apoptosis without inhibiting NFκB signaling or *de novo* protein synthesis, conditions required for TNFα cytotoxicity in normal cells *in vitro*[25] (Fig. 3). These CCN proteins also enable the apoptotic activity of lymphotoxin and enhance that of other TNF family cytokines such as FasL and TRAIL[26, 27]. Knockin mice expressing an apoptosis-defective *Ccn1* allele are substantially resistant to TNFα- or Fas-mediated hepatic apoptosis *in vivo*, indicating that CCN1 is a physiologic regulator of TNFα and FasL cytotoxicity[7, 26]. By contrast, CCN4 inhibits TNF-induced cell death in cardiomyocytes[28], suggesting that the interplay between various CCNs and the TNF family of cytokines may profoundly affect the biological outcome during inflammatory responses.

A recent unexpected finding is that CCN1 can induce cellular senescence in fibroblasts by acting as a cell adhesion molecule[8]. Through binding to $\alpha_6\beta_1$ and cell surface HSPGs, CCN1 activates the RAC1-dependent NADPH oxidase 1 (NOX1) to induce a robust and

sustained level of reactive oxygen species (ROS), which leads to the activation of p53 and pRb, resulting in senescence (Fig. 3). An important feature of senescent cells is the expression of the senescence-associated secretory phenotype (SASP), characterized by the increased expression of ECM-degrading enzymes such as matrix metalloproteinases (MMPs), inflammatory cytokines and chemokines, and down-regulation of ECM components such as collagen, thus imposing a matrix-degrading phenotype[29]. Consistently, CCN1-induced myofibroblast senescence functions as a mechanism for limiting fibrosis during wound healing[30] (see below).

Angiogenesis, chondrogenesis and osteogenesis—CCN1, CCN2, and CCN3 are potent angiogenic inducers, functioning through direct binding to integrin $\alpha_v\beta_3$ in endothelial cells to promote proliferation and induce chemotaxis and formation of tubules[31, 32]. They can also regulate the expression[33] and activities of other angiogenic factors such as VEGF-A and VEGF-C[34, 35]. Through their angiogenic activities, these CCNs may be involved in embryonic development, inflammatory diseases, and tumorigenesis[32, 36].

Both CCN1 and CCN2 promote chondrogenic and osteoblastic differentiation [37, 38]. CCNs can interact with members of the BMP and TGF-β family, most likely through the chordin-like homology found in the VWC domain, and modulate their binding affinity for their respective receptors[39]. CCN2 and CCN3 can bind BMP2 and inhibit its functions in promoting chondrogenic and osteogenic differentiation[15, 40], respectively, whereas CCN4 binds BMP2 and enhances its function in osteogenesis[41]. CCN2 regulates Wnt signaling by interaction with the Wnt receptor complex through direct binding to the Wnt coreceptor LRP6[11]. CCN1 inhibits osteoclastogenesis[42], whereas CCN3 stimulates it through a process that may involve calcium flux[43]. In transgenic mice, overexpression of CCN2 or CCN3 in osteoblasts antagonizes both BMP and Wnt-signaling and result in osteopenia[44, 45].

Inflammation—Accumulating evidence indicate that CCN proteins modulate the inflammatory response[36]. CCN1 and CCN2 are induced by inflammatory cytokines or upon viral or bacterial infection, and CCNs in turn regulate the activity and expression of cytokines and chemokines. Clear examples include the ability of CCNs to greatly alter the cytotoxicity of TNF family of cytokines both *in vitro* and *in vivo*[7, 26], and CCN1 to reprogram macrophages toward M1 polarization and activate the expression of proinflammatory cytokines[46]. CCN1 also regulates immune cell infiltration *in vivo* in an experimental model of autoimmune myocarditis[47]. CCN3 inhibits NFκB activation in endothelial cells, suggesting that it may regulate endothelial inflammation[48]. Furthermore, CCNs are involved in the pathobiology of many inflammatory diseases, as discussed below.

Stem cell differentiation and self-renewal—A recent study shows that CCN2 is sufficient to drive the differentiation of human bone marrow mesenchymal stem cells (MSCs) into α-smooth muscle actin (SMA) negative fibroblasts, which are primed for differentiation into α-SMA$^+$ myofibroblasts when subsequently treated with TGF-β1[49]. Both CCN1 and CCN2 are targets of the canonical Wnt/β-catenin signaling pathway in MSCs and regulate their differentiation into osteoblasts[50, 51]. CCN3 is essential for the self-renewal of CD34$^+$ hematopoietic stem cells from umbilical cord blood, suggesting its potential utility for promoting stem cell engraftment[52]. Consistent with a function in stem cell self-renewal, CCN3 inhibits both myogenic and osteoblastic differentiation of MSCs[53].

CCN FUNCTIONS IN EMBRYONIC DEVELOPMENT

Targeted disruptions of *Ccn* genes in mice have been accomplished with the exception of *Ccn4*, and phenotypes of the knockout mice are listed in Table II. Although the phenotypes of each knockout are distinct, skeletal and vascular defects are among the most commonly

observed. Given the importance of several CCNs in ovulation, placentation, and development, the potential risks of targeting their expression or function in women expecting or during pregnancy should be carefully evaluated. *Ccn1*-null mice are embryonic lethal due to impaired angiogenesis, placental insufficiency, and severe atrioventricular septal defects (AVSD)[33, 54]. Although *Ccn1*$^{+/-}$ mice are largely viable, they display persistent ostium primum atrial septal defects in 20% of the adult[54]. These defects resemble those of human patients with mutations in *AVSD1*, a susceptibility locus for non-syndromic AVSD identified by linkage analysis[55]. Since the human *CCN1* gene and *AVSD1* map to the same chromosomal location, these findings suggest that CCN1 may be a candidate gene for human AVSD[37].

Ccn2-null mice are perinatal lethal due to respiratory defects as a secondary consequence of severe skeletal malformations[56], consistent with a wealth of data showing the roles of CCN2 in chondrogenesis and endochondrial ossification[38]. *Ccn2*-null embryos also exhibit pulmonary hypoplasia[57] and decreased β cells in the pancreas[58]. Cell type-specific deletion of *Ccn2* in the ovary and uterus resulted in disrupted follicle development and steroidogenesis, decreased ovulation, and increased numbers of corpus luteum[59]. By contrast, *Ccn3* knockout mice are viable and largely normal, exhibiting only modest and transient sexually dimorphic skeletal abnormalities[60]. Whereas *Ccn5*-null mice suffer early embryonic death[61], *Ccn6* knockout mice display no observable phenotype[62].

CCNs IN DISEASES AND TARGETING STUDIES

As CCN proteins are highly expressed and play important roles at sites of inflammation and tissue injury repair, they are often deregulated when these processes become chronic and progress to pathological conditions. For this reason, numerous gene profiling studies have found CCNs aberrantly expressed in a plethora of diseases, many of which are associated with chronic inflammation and tissue repair. Studies in animal models have established that deregulated CCNs indeed contribute to the pathologies of many of these diseases, although their mechanisms of action are not completely understood (Table III). Nevertheless, test-of-principal studies have shown that targeting CCNs can yield significant benefits in animal models. Moreover, the levels of CCNs in biological fluids may serve as non-invasive diagnostic or prognostic markers for certain pathologies. Clinical trials targeting CCN2 have already begun and initial results are encouraging as discussed in a later section, reinforcing the notion that CCNs are emerging as attractive therapeutic targets for a broad spectrum of diseases.

Wound healing and fibrotic diseases

Mammalian wound healing and tissue repair occur similarly in virtually all organ systems in three distinct but overlapping phases, initiating with inflammation, followed by granulation tissue formation and ECM deposition, and concluding with matrix remodeling and resolution of the granulation tissue[63]. At the site of injury, activated resident fibroblasts and recruited fibrocytes differentiate into α-SMA^{+} myofibroblasts, which proliferate and deposit ECM proteins to promote healing and support tissue integrity while the damaged tissue is being repaired and remodeled. However, excessive ECM deposition may occur in wound repair, leading to fibrosis, scarring, and loss of tissue function. Fibrosis is greatly exacerbated when inflammation or tissue injury becomes chronic and contributes to the pathologies of chronic diseases in many organ systems, for example in the liver as a result of viral infections or alcoholism, in the kidney as a result of diabetes, or in the heart as a result of tissue remodeling following myocardial infarctions[63, 64]. These pathologies can lead to organ failure and death, yet currently there is no FDA-approved drug available for treating fibrosis. Emerging studies indicate that various CCN proteins are involved in promoting or

inhibiting fibrosis in association with injury repair, and provide unique opportunities for the development of novel therapeutics and diagnostics for this pathology.

Upon wounding, platelet α granules release abundant amounts of CCN2[65, 66] and other growth factors that can induce the expression of CCNs at the site of injury. CCN2 acts synergistically with TGF-β1, a potent profibrotic regulator, to promote matrix protein deposition and fibrogenesis both *in vitro* and *in vivo*[67]. Subcutaneous or intraperitoneal injections of either TGF-β or CCN2 individually does not induce persistent fibrosis, whereas coinjection of both proteins together results in sustained fibrosis[68]. CCN2 is overexpressed in human fibrotic diseases of virtually every organ or tissue. Correspondingly, serum and biofluid levels of CCN2 correlate with the severity of systemic fibrosis (scleroderma) and fibrosis of the heart, kidney, liver, and lung, suggesting that CCN2 levels in biofluids may be useful as a non-invasive biomarker for many fibrotic diseases[69, 70]. Despite this close association with fibrosis, overexpression of CCN2 *per se* in parenchymal cells of most organs does not induce fibrosis in mice, but exacerbates the fibrotic response when challenged with injuries[67]. However, overexpression of CCN2 specifically in fibroblasts, the major cell type responsible for matrix deposition, is sufficient to drive fibrosis in lung, skin, kidney and arteries[71], and mice deleted for *Ccn2* in fibroblasts are resistant to bleomycin-induced skin fibrosis[72]. These observations suggest that CCN2 action in fibroblastic cells may be strongly autocrine in nature. Consistent with the pro-fibrotic function of CCN2, inhibition or downregulation of CCN2 ameliorates fibrosis in many organ systems. Knockdown of CCN2 by anti-sense oligonucleotides or siRNAs reduces CCl_4[73, 74]- and N-nitrosodimethylamine[75]-induced liver fibrosis, ureteral obstruction-induced renal fibrosis[76], fibrotic scarring in cutaneous wounds[77], and renal interstitial fibrogenesis following partial nephrectomy[78]. Although less is known about CCN4 actions, it has been shown that CCN4 is upregulated in human patients with idiopathic pulmonary fibrosis and in a mouse model of bleomycin-induced lung fibrosis, and recombinant CCN4 enhances ECM deposition in human fibroblasts[79]. Correspondingly, orotracheal application of CCN4 neutralizing antibodies to the lung ameliorates bleomycin-induced lung fibrosis[79].

In contrast to the pro-fibrotic CCN2 and CCN4, CCN1 functions to limit fibrosis through a novel mechanism that invokes the induction of cellular senescence in myofibroblasts[8]. Accumulation of senescent cells is an integral part of the normal wound healing response that functions to curb fibrosis, and this process is controlled by CCN1[30] (Fig. 4). CCN1 gradually accumulates in the granulation tissue in excisional cutaneous wounds, reaching a level sufficient to trigger senescence in myofibroblasts as the healing process enters the resolution phase, whereupon the senescent cells express the SASP that includes the secretion of MMPs and down-regulation of collagen I and TGF-β1[8]. Thus, CCN1 acts as an anti-fibrotic senescence switch, converting the ECM-synthesizing myofibroblasts into ECM-degrading senescent cells. This mechanism of fibrosis control achieves molecular parsimony by at once halting further ECM production in myofibroblasts, accelerating ECM degradation, and facilitating granulation tissue resolution as senescent cells are cleared by natural killer cells[30, 80]. In knockin mice expressing senescence-defective CCN1, senescent cells do not accumulate in the granulation tissue and wound healing proceeds with exacerbated fibrosis[8]. Topical treatment of these wounds with purified CCN1 protein reverses this defect and reduces fibrosis by increasing the number of senescent cells, suggesting a therapeutic potential for CCN1 in the treatment of fibrosis. Interestingly, hepatic stellate cells also undergo senescence to limit fibrosis in chemically-induced liver injury[80], indicating that this mechanism of fibrotic control may be general to wound repair in different organs and diverse modes of injuries.

Whereas CCN1 inhibits fibrosis by inducing cellular senescence[8], CCN3 and CCN5 also appear to have antifibrotic activities, although their antifibrotic mechanism may potentially

be distinct. CCN3 suppresses CCN2 and collagen I expression in mesangial cells *in vitro*[81], whereas overexpression of CCN5 reduces cardiac hypertrophy and fibrosis in transgenic mice, possibly by inhibiting TGF-β/SMAD signaling[82]. Since CCN2 and CCN4 are pro-fibrotic whereas CCN1, CCN3, and CCN5 are anti-fibrotic at least in certain biological contexts, it is critical that reagents targeting members of the CCN family have a high level of specificity. Approaches in this regard are discussed in a later section.

CCN proteins in cancer—It has long been recognized that chronic injuries and deregulated wound healing are risk factors in the development of cancer[83]. Recent studies have further underscored the importance of inflammation in tumorigenesis and tumor progression[84]. Inflammation occurring at early stages of neoplastic transformation can accelerate the development of incipient neoplasias into full-blown cancer by releasing mutagenic chemicals such as ROS. Moreover, inflammation is associated with most if not all tumors and contributes to cancer progression by providing a rich arsenal of bioactive molecules to the tumor microenvironment, including factors that promote cell growth, survival, motility, invasion, angiogenesis, and matrix degradation. Aberrant expression of CCNs has been identified in a broad range of tumor types[85]. With some notable exceptions[86, 87], CCN1, CCN2, and CCN4 have been generally associated with promotion of cell proliferation and tumor growth, whereas CCN3, CCN5 and CCN6 have been associated with inhibition of cell proliferation and tumor growth (Table IV).

CCN1 and CCN2 may enhance tumor growth through their potent angiogenic activity[31, 88]. Accordingly, forced expression of *CCN1* in breast cancer cells promotes tumor growth in xenografts with increased vascularization[89, 90] and expression of *CCN2* in tumor-reactive stroma enhances microvessel density of xenograft tumor in nude mice[91, 92]. Consistently, xenograft tumor growth is inhibited by silencing either *CCN1* or *CCN2* expression in cancer cells of the prostate[93], pancreas[94, 95], and ovary[96]. Both CCN1 and CCN2 can promote epithelial-to-mesenchymal transition (EMT)[95, 97], enhance survival of some cancer cells through the induction of anti-apoptotic proteins[98, 99], and mediate taxol resistance in breast cancer[100]. Direct intratumoral delivery of *CCN1* siRNA into pre-established glioma xenografts decreases tumor growth by up to 40% in a dose-dependent manner, suggesting that targeting CCN1 has therapeutic potential[101]. Furthermore, *CCN2* is a key component of the gene signature that facilitates osteolytic bone metastasis in breast cancer cells[102], and treatment with a murine anti-CCN2 monoclonal antibody markedly decreased osteolytic bone metastasis of human breast cancer cell xenografts in nude mice[103]. Notably, administration of FG-3019, a humanized anti-CCN2 monoclonal antibody, inhibits tumor growth and metastases in xenograft and orthotopic models of pancreatic cancer in mice[104, 105]. As human pancreatic cancers are largely recalcitrant to therapy, the potential of treatment through targeting CCN2 is particularly encouraging.

Overexpression of CCN4 in normal rat kidney fibroblasts confers the ability to form tumors in nude mice[106], and high CCN4 expression is noted in advanced breast and colon cancers[107, 108] and adenosquamous cell carcinoma of the lung[109]. However, CCN4 appears to inhibit metastasis and is preferentially expressed in melanoma cell lines with low metastastic potential[110]. Lung cancer cells overexpressing CCN4 are less invasive and exhibit reduced metastasis in nude mice, possibly due to its integrin-mediated inhibition of the small GTPase Rac[111]. A splicing variant of CCN4 lacking the VWC domain has been detected in scirrhous gastric carcinomas, and transfectants expressing this variant enhance the invasive characteristic of co-cultured gastric carcinoma cells[112]. Therefore, it is possible that CCN4 isoforms expressed as a result of differential splicing might underlie the pro- and anti-invasive activities of CCN4, although this possibility has not been thoroughly investigated.

CCN3, CCN5 and CCN6 have a negative effect on cell proliferation. Although CCN3 inhibits proliferation of cancer cells[113], it appears to promote metastasis[20]. Overexpression of *CCN3* results in reduced tumor size in glioma cells xenografts[114], but enhances metastatic potential in xenotransplanted melanoma cells[115]. CCN3 expression is associated with a higher risk of metastasis and worse prognosis in patients with cancers such as Ewing's sarcoma[116], melanoma[115], and breast cancer[117]. In addition to solid tumors, CCN3 is down regulated in chronic myeloid leukemia (CML) as a consequence of the kinase activity of BCR-ABL, a chimeric protein generated through the chromosomal translocation between chromosome 9 and 22[118]. Patients responding to Imatinib (Gleevec) therapy show an increase in *CCN3* expression. Forced expression of *CCN3* inhibits proliferation and restores growth control in CML cells, and sensitizes them to Imatinib-induced apoptosis, suggesting that CCN3 may be an alternate targets for novel therapeutics against CML[119].

CCN5 has been shown to inhibit the growth of vascular smooth muscle cells, uterine myometrial cells, leiomyoma cells, and estrogen receptor-negative breast cancer cells[61]. CCN5 expression is significantly decreased in leiomyomas, pancreatic cancers, and salivary gland tumors. *CCN6* is expressed in the normal mammary epithelium, but expression is lost or downregulated in 60% of inflammatory breast carcinomas, and restoration of *CCN6* expression reduced tumor growth in nude mice[120]. It has been suggested that CCN6 acts by interfering with the IGF-1 signaling pathway, which promotes the proliferation, survival and metastatic ability of breast cancer cells[121].

The picture that emerges is that CCNs can act both positively and negatively in tumorigenesis and tumor progression, depending on the tumor type. Although CCN1 and CCN2 are known angiogenic inducers and may promote the growth of some tumor types, they can suppress tumor growth in some cancers[86, 87]. CCN1, CCn2, and CCN3 can synergize with cytokines of the TNF family to induce apoptosis, particularly TRAIL, which preferentially kills tumor cells[27], and CCN1 can induce cellular senescence[8]. Both apoptosis and senescence are well-established mechanisms of tumor suppression if triggered in damaged cells at risk of neoplastic transformation[122], suggesting that CCNs may inhibit the development of incipient tumors by promoting apoptosis or senescence in damaged cells. It is important to note that most experimental studies on CCN functions in cancer have employed xenograft models and established tumor cell lines, and thus focus on tumor growth and progression rather than the initial stages of tumorigenesis. Whether CCNs exert a positive or negative effect on tumor growth may depend on which integrin is preferentially expressed in the tumor cells (e.g., $\alpha_v\beta_3$ is growth promoting and $\alpha_6\beta_1$ is growth inhibitory with CCN1 as ligand[37]), whether angiogenic factors are limiting, and whether conditions that favor apoptosis or senescence (i.e., ROS accumulation) prevail. Despite these uncertainties on how CCNs act in tumorigenesis, targeting CCNs has shown therapeutic promise in specific cancer as described above and these approaches warrant further translational studies.

Diabetic nephropathy and retinopathy—Diabetes is associated with a multitude of metabolic and homeostatic abnormalities, including hyperglycemia and systemic and intraglomerular hypertension, which contribute to microvascular pathologies such as diabetic nephropathy and retinopathy. Substantial evidence from recent studies support the involvement of chronic inflammation in the pathogenesis of diabetic nephropathy and retinopathy[123, 124]. Chronic hyperglycemia, the hallmark of diabetes, is a pro-inflammatory condition that leads to multiple biochemical alterations including the formation of advanced glycation endproducts (AGEs), which are thought to trigger or amplify many aspects of diabetic pathology[125]. Exposure to high glucose stimulates *CCN2* expression in human mesangial cells[126], and both *CCN1* and *CCN2* are induced by AGEs *in vitro* and *in vivo*[127, 128]. In addition, mechanical stretch and hemodynamic forces simulating

hypertension have been shown to induce the expression of *CCN1* and *CCN2* in vascular endothelial cells and smooth muscle cells, and *CCN2* in renal mesangial cells[129]. Thus, hyperglycemia and hypertension are both factors that contribute to the progression of diabetic disease with associated induction of *CCN1* and *CCN2*.

Diabetic nephropathy is characterized by hypertrophy of glomeruli and tubules, thickening of their associated basement membrane, and glomerular and interstitial fibrosis, all of which compromise kidney function[130]. Microalbuminuria at the incipient stage of diabetic nephropathy progresses to overt proteinuria as glomerular filtration deteriorates. In diabetic patients, *CCN2* expression is increased in both glomerular podocytes and mesangial cells[130], whereas *CCN1* expression in podocytes is downregulated[131]. CCN2 induces pro-fibrotic responses in cultured human mesangial cells and promotes the infiltration of inflammatory cells into the renal interstitium[130, 132]. Treatment of diabetic rats with aminoguanidine to inhibit AGE formation concomitantly block the induction of *Ccn2* and albuminuria[127]. Knockdown of *Ccn2* expression by antisense oligonucleotides attenuates progression of nephropathy in mouse models of diabetes[133], whereas transgenic overexpression of CCN2 in podocytes enhances urinary albuminuria[134]. Current treatment of diabetic nephropathy focuses on the control of renal hypertension using angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs)[135], thus targeting CCN2 may provide an alternative or complementary therapy. Moreover, plasma and urinary levels of CCN2 correlate with clinical parameters associated with the severity of diabetic nephropathy, suggesting that CCN2 can be a useful biomarker for this disease[136, 137].

Retinopathy is a common microvascular complication of diabetes caused by enhanced angiogenesis and fibrosis in the vitreous cavity of the eye, resulting in loss of vision. CCN1 appears to be involved in retinal angiogenesis in streptozotocin (STZ)-induced diabetes or oxygen-induced retinopathy (OIR) in rodents, and in proliferative diabetic retinopathy in humans[128, 138]. Intravitreal injection of a neutralizing anti-CCN1 antibody in the OIR model significantly reduces retinal neovascularization with no apparent toxicity or inflammation[138]. CCN2 is highly expressed in microvascular pericytes of the human diabetic retina, and its levels correlate with the degree of fibrosis in vitroretinal disorders[139]. Heterozygous *Ccn2*$^{+/-}$ mice subjected to STZ-induced diabetes exhibit significantly lower basal membrane thickening of retinal capillaries compared to wild type mice[140]. These results are consistent with the notion that targeting CCNs may help ameliorate the pathology of diabetic retinopathy.

Cardiovascular diseases

CCN proteins have been implicated in such vascular pathologies as atherosclerosis, restenosis, thrombosis, and hypertension. Atherosclerosis is a chronic inflammatory disease of the arterial wall triggered by the prolonged elevation of lipids in the bloodstream (hyperlipidemia), whereas proliferative restenosis is a common response to vascular injury after balloon angioplasty. CCN1 and CCN2 promote neointimal hyperplasia after vascular injury, whereas CCN3 and CCN5 inhibit it. Both CCN1 and CCN2 are detected at high levels in rupture-prone atherosclerotic plaques, particularly in areas of neovascularization or areas that are infiltrated with inflammatory cells[141, 142], and in the neointima in restenosis after balloon injuries[22, 143]. Inhibiting *CCN1* expression by either its negative regulator FOXO3a[144] or siRNA[145] effectively reduced neoinitimal hyperplasia following balloon injury in the rat carotid artery. As neoinitimal hyperplasia is a common complication in the treatment of atherosclerosis, targeting CCN proteins may be beneficial. By contrast, *CCN3* expression is substantially reduced following balloon injury. CCN3 inhibits smooth muscle cell proliferation in culture, and *Ccn3*-null mice suffer enhanced neointimal thickening when challenged with vascular injury, indicating that CCN3 inhibits neoinitimal hyperplasia[24].

Likewise, CCN5 inhibits vascular smooth muscle cell proliferation and motility, and its expression is greatly reduced in arteries after balloon injury[23].

CCN1 is upregulated in inflammatory cardiomyopathy in humans, and *CCN1* gene transfer in animal models attenuates autoimmune myocarditis by inhibiting the infiltration of spleen macrophages and lymphocytes[47]. The effects of CCN1 on immune cells are partially mimicked by cyclic RGD peptides, which bind α_v integrins and are in clinical trials as cancer therapeutics[146]. Both CCN1 and CCN2 are highly induced in cardiomyocytes of patients with ischemic cardiomyopathy and in cardiac remodeling after myocardial infarction[147, 148]. Cardiomyocyte-specific expression of *CCN2* in transgenic mice did not by itself induce fibrosis, but exacerbates pressure overload-induced cardiac fibrosis[82] and leads to cardiomyocyte hypertrophy by 7 months of age[149]. By contrast, CCN5 inhibits the CCN2-induced hypertrophic responses in cardiomyocytes, and transgenic expression of CCN5 in cardiomyocytes reduces pressure overload-induced cardiac fibrosis[82]. These results indicate that CCN2 and CCN5 play opposing roles in cardiac hypertrophy and fibrosis. CCN5 is the only member of the family that lacks the CT domain. Deleting the CT domain in CCN2 renders it CCN5-like in inhibiting cardiomyocyte hypertrophic responses *in vitro*, and fusing the CT domain from CCN2 to CCN5 transforms it into a CCN2-like pro-hypertrophic molecule[82], indicating a pro-fibrotic function in the CCN2 CT domain.

Arthritis and other inflammatory diseases

Osteoarthritis (OA) is a common and debilitating degenerative joint disease for which the principal forms of treatment provide only temporary pain management, and surgery may be required if the joint degeneration is severe. Both CCN2 and CCN4 are strongly upregulated in cartilage of human OA patients[150, 151]. CCN4 contributes to cartilage damage by inducing the production of MMPs and aggrecanase in macrophages and chondrocytes[151]. CCN2 promotes the proliferation and differentiation of chondrocytes without inducing calcification of articular cartilage[38]. Direct injection of CCN2 into the joint cavity stimulates cartilage repair in an experimental OA model in rats, suggesting that CCN2 may have therapeutic value in treating OA[152]. CCN1 and CCN2 are highly expressed in hypertrophic and proliferative chondrocytes during fracture healing, and blockade of CCN1 by neutralizing antibodies inhibits bone fracture healing in mice[153, 154].

CCN1 and CCN2 are both highly expressed in rheumatoid arthritis (RA), an inflammatory joint disorder that leads to the destruction of articular cartilage[155, 156]. CCN1 may contribute to hyperplasia of the synovial lining, resulting in joint destruction in RA. CCN2 synergizes with M-CSF/sRANKL to enhance osteoclastogenesis and contributes to bone destruction in RA[155]. Current treatment for recalcitrant RA includes monoclonal antibodies (infliximab, golimumab, adalimumab, and certolizumb pegol) against TNFα, which can regulate the expression of *CCN* genes and functionally interact with CCN proteins[7, 36]. In this regard, it is interesting to note that TNFα antagonists are also effective therapies in inflammatory bowel disease and CCN1 is upregulated 10–20 fold in patients with Crohn's disease or ulcerative colitis[157], suggesting that CCN1 and TNFα may functionally interact in these diseases as well.

In the central nervous system (CNS), chronic inflammation-like glial responses cause neurodegenerative events including plaque formation, dystrophic neurite growth, and excessive tau phosphorylation. Thus, neuroinflammation has been implicated in pathological conditions of the CNS such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, or multiple sclerosis. Elevated CCN2 expression is observed in AD brain neurons and astrocytes, and its expression level correlates with the progression of clinical dementia and the deposition of neuritic plaques and neurofibrillary tangles[158]. Moreover, CCN2 promotes amyloid precursor protein (APP) processing and subsequent amyloid-β-

peptide (Aβ) generation *in vitro*[159], suggesting that CCN2 may contribute to AD progression by enhancing β-amyloidosis. However, currently little is known about the normal functions of CCN proteins in the CNS.

Genetic mutations and polymorphisms in human diseases

Of the six members of the CCN gene family, only *CCN6* has been associated with a human disease with Mendelian inheritance. Mutations in *CCN6* cause the autosomal recessive skeletal disease progressive pseudorheumatoid dysplasia (PPD), a juvenile-onset degenerative disease of the joint[160]. Alleles leading to PPD harbor loss-of-function mutations that include deletion, frameshift, nonsense, and missense mutations[160]. The development of DNA-based prenatal diagnosis targeting *CCN6* may be useful for at-risk families, as symptoms of PPD are not evident until years after birth. Gene disruption of *Ccn6* in mice fails to recapitulate the human disease and presents no discernible phenotype[62], although in zebra fish *Ccn6* is shown to modulates the canonical BMP and Wnt signaling pathways critical for cartilage homeostasis[161].

DNA sequence polymporphisms in CCN genes associated with various diseases have been identified (Table V). Notably, polymorphisms in the human *CCN2* gene are found to be significantly associated with scleroderma[162–164], although some of these results may be controversial[165]. Other polymorphisms of *CCN* genes have been associated with susceptibility to hypertension, type I diabetes, colorectal cancer, hepatic fibrosis, etc. How these polymorphisms affect *CCN* gene expression or function is largely unknown and warrant further studies.

THERAPEUTIC APPROACHES AND CLINICAL TRIALS

Much of the clinical potential of targeting CCNs in therapy is still largely unexplored, as translational studies are still in early stages. Since CCNs are secreted proteins, humanized monoclonal antibodies are particularly well suited as blocking agents for their activities and thus hold promise as potential therapeutics. In this regard, the hinge region encoded by the same exon as the VWC domain (Box 1) may be a good choice of antigen, since this region is unique to each CCN family member and contains an abundance of charged amino acids, enhancing its antigenicity. Other targeting strategies such as the use of siRNA or antisense oligonucleotides to down-regulate the expression of specific CCN genes have been successful in animal models[166]. These approaches can also afford a high degree of target gene specificity if possible off-target effects are minimized through careful oligonucleotide sequence selection. The use of synthetic peptides as targeting agents for CCNs is less well explored but nevertheless has some potential. For example, recent studies show that decorin can bind CCN2 directly through its leucine-rich repeat and inhibit the pro-fibrotic activity of CCN2[167], suggesting the possibility of targeting CCN2 using a binding peptide. However, decorin is also known to bind other members of the CCN family and may not act specifically on CCN2[168]. Since CCNs interact with integrin $\alpha_v\beta_3$ to mediate angiogenic functions[31, 169], peptides that bind and inhibit $\alpha_v\beta_3$ signaling may thwart CCN1-promoted angiogensis in tumor growth. However, this strategy of targeting $\alpha_v\beta_3$ and is not specific for CCN proteins. Other peptides have been identified to block CCN binding to integrin $\alpha_6\beta_1$-HSPGs and inhibit activities mediated through these receptors[170, 171], although the utility of these peptides in blocking CCN functions have not been investigated *in vivo*.

A number of clinical trials have been conducted or are underway targeting CCN2. A phase 1 study to evaluate the safety and tolerability of FG-3019[172], a humanized monoclonal antibody directed against the VWC domain, was performed in patients with mild to moderate idiopathic pulmonary fibrosis found it safe and well tolerated[173]. A follow up phase 2 study is currently in progress (NCT01262001). Another phase 1b study showed that

treatment of patients with type I or type II diabetes and microalbuminuria using FG-3019 was well tolerated[6]. Particularly encouraging is the observation that the urinary albumin/creatinine ratio (ACR) was reduced by more than 50% by the end of the treatment, suggesting that blockade of CCN2 is associated with a decrease in albuminuria. A similar phase 1 study on diabetic patients with more severe macroalbuminuria with a background of treatment for hypertension has also been completed (NCT00754143). On the strength of these observations, a phase 2 study was initiated in patients with type 2 diabetes and advanced kidney disease to evaluate the effect of FG-3019 on renal function and associated cardiovascular co-morbidities (NCT00913393). Another ongoing phase 1 study is evaluating FG-3019 therapy in combination with gemcitabine (nucleoside analog chemotherapeutic drug) and erlotinib (tyrosine kinase inhibitor) for patients with locally advanced or metastatic pancreatic cancer (NCT01181245). In addition, a phase 2 study has just begun in patients with liver fibrosis due to chronic hepatitis B infection in which the efficacy of FG-3019 for reversing fibrosis is being evaluated (NCT01217632).

Aside from monoclonal antibodies, a 2-O-methoxyethyl (2-MOE) modified antisense oligonucleotide (EXC-001) designed to inhibit CCN2 expression has been used in several phase II clinical trials to study its effects in controlling scarring after breast surgery (NCT01037413) or abdominoplasty surgery (NCT01037985; NCT01038297). Results show a reduction in scarring with no significant drug-related adverse effects (http://www.excaliard.com/news/PressReleaseExcaliardJanuary2011.pdf). Additionally, RXI-109, a self-delivering RNAi compound that reduces CCN2 expression, is being prepared for potential clinical trial (RXi Pharmaceuticals).

It is encouraging that no side effects have been observed in phase 1 trials using humanized monoclonal antibody or antisense oligonucleotide targeting CCN2. Since CCN proteins are generally downregulated in many tissues in the adult and are highly expressed only at sites of inflammation and injury repair, it is likely that therapeutic targeting of CCN proteins will preferentially affect the sites of pathology and have minimal impact on functions of normal organs and tissues. An exception might be pregnancy, since systemic targeting of certain CCN proteins may increase the risk of preeclampsia[174] or inhibit placentation[33].

CONCLUSIONS AND FUTURE DIRECTIONS

The CCN family of matricellular proteins plays important roles in modulating inflammation and tissue injury repair, and is often deregulated in a broad spectrum of pathologies that develop when these processes become chronic. Emerging studies have identified opportunities in which levels of CCN proteins in biofluids may serve as non-invasive diagnostic or prognostic tools, including systemic fibrosis[69, 175], diabetic nephropathy[137] and renal hypertension[136], and some forms of cancer[87, 116, 176]. The potential of monitoring CCNs as a method of non-invasive assessment of fibrosis is of particular interest clinically. Studies in animal models of disease have also demonstrated therapeutic value in targeting relevant CCN genes in such diseases as cancer, diabetic nephropathy, and fibrosis of the liver, kidney, and skin. Clinical trials targeting CCN2 have begun and initial results are encouraging. We surmise that as studies on CCNs progress, more pathologies will be identified for which CCNs may serve as potential diagnostic or therapeutic targets.

Among the key challenges in future research is to elucidate the specific role of CCNs in disease etiology. In only a few cases have there been limited functional insight into how CCNs might be triggering or contributing to disease pathology. Understanding the critical pathological role of CCNs and their action mechanisms will help identify relevant targets for therapeutic intervention as the key signaling steps are identified. Since some members of the CCN family often play opposing roles, expression of a specific CCN protein may be either

deleterious or beneficial in a particular pathological context. Therefore, the specificity of reagents targeting members of the CCN family is of paramount importance. Furthermore, how various CCN proteins act to provoke antithetical effects in some contexts is currently not well understood. Future studies that clarify how opposing CCN proteins function mechanistically may present further prospects for combinatorial therapies that simultaneously downregulate and upregulate the expression or function of rival CCN members, potentially magnifying the therapeutic impact.

Although CCNs represent unusual opportunities as therapeutic targets in a broad spectrum of diseases, their potential has remained relatively underexplored to date. Currently clinical trials are being conducted and planned targeting only CCN2, whereas other members of the family have not been investigated despite their apparent roles in many of the same pathologies in which CCN2 plays a role. This may be due in part to the lack of coordinated efforts to develop humanized neutralizing monoclonal antibodies against other members of the CCN family, although studies using other targeting strategies can be initiated. As the relative efficacies of targeting distinct members of the CCN family in the same pathologies remain to be determined, future studies that compare the full range of CCNs as therapeutic targets will be of considerable interest.

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GLOSSARY

Matricellular proteins	Dynamically expressed extracellular matrix proteins with a modular structure that serve regulatory roles and are often involved in wound healing.
Integrins	A family of heterodimeric cell surface signaling receptors that mediate cell-cell and cell-matrix interactions, and are comprised of 18 α and 8 β subunits to constitute 24 known integrin heterodimers in mammals.
LRP	Members of the low density lipoprotein receptor-related protein family are cell surface receptors that bind a broad spectrum of ligands, facilitating endocytosis and signaling of associated receptors.
Immediate-early genes	Genes that are transcriptionally activated with rapid kinetics without requiring *de novo* protein synthesis by a broad range of stimuli, including viral infection or various extracellular signals.
Cellular senescence	A state of irreversible cell cycle arrest induced by various forms of cellular stresses including DNA damage, oncogene activation, oxidative stress, chromatin disruption, and telomere erosion.
SASP	One of the characteristics of senescent cells is expression of the senescence-associated secretory phenotype, which includes increased secretion of inflammatory cytokines and matrix degrading enzymes, and downregulation of ECM proteins such as collagen.
MMPs	Matrix metalloproteinases comprise a family of >20 zinc-dependent endopeptidases involved in the degradation of ECM

	proteins, cleavage of cell surface receptors, release of apoptotic ligands and activation of chemokines/cytokines.
Platelet α granules	Cytoplasmic granules containing many proteins factors involved in inflammation and wound repair synthesized predominantly in megakaryocytes, including cell adhesive proteins, growth and coagulation factors, and protease inhibitors, that are released upon platelet activation at sites of vessel injury.
Epithelial-to-mesenchymal transition	A process in which epithelial cells lose their cell adhesive properties and gain cell mobility, typically involving repression of E-cadherin expression. EMT is essential for many developmental processes and occurs in oncogenic transformation.
Microalbuminuria	A condition in which small amounts of albumin leaks into the urine upon injury or damage to the kidney, indicating abnormal permeability in the renal glomerulus.
Balloon angioplasty	A method of mechanically widening an obstructed blood vessel, typically a result of atherosclerosis, by passing a collapsed balloon catheter through the obstructed area and inflating the balloon to open up the blood vessel as the catheter is withdrawn.

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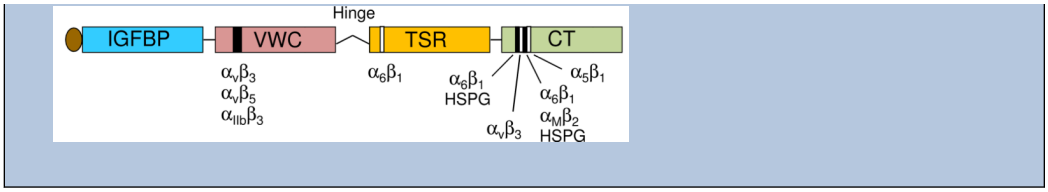
Box 1. The CCN family of matricellular proteins

CCN proteins (except CCN5) are comprised of an N-terminal secretary peptide and four conserved modular domains – IGFBPs, VWC, TSR, and CT – each encoded by a separate exon. A non-conserved central hinge region divides the protein into two halves with different binding capabilities to extracellular proteins and cell surface receptors (see Fig. 2). The locations of identified binding sites for integrin receptors and HSPGs are indicated in the schematic diagram[2]. CCN proteins contain 38 conserved cysteines located throughout the polypeptide, with the exception of CCN5, which lacks precisely the CT domain but otherwise conserves all cysteines in the remaining three domains, and CCN6, in which four cysteines in the VWC domain are not conserved.

CCN genes are exquisitely sensitive to regulation by mitogenic signals and a wide range of environmental perturbations, including exposure to growth factors, inflammatory cytokines, steroid hormones, oxygen deprivation, UV irradiation, and mechanical forces (Fig. 1). During embryonic development, CCNs are broadly expressed in many organs and tissues, especially in endothelial cells throughout the embryo and in the developing cardiovascular, skeletal, renal, and neuronal systems[32, 201]. In the adult, expression of CCNs is downregulated in many tissues but becomes redeployed at sites of inflammation, wound healing, and injury repair[2, 36].

Identified as immediate-early genes, *CCN1* and *CCN2* are expressed at a very low level in quiescent fibroblasts, but are transcriptionally activated without requiring *de novo* protein synthesis within minutes of stimulation by mitogenic growth factors such as platelet-derived growth factor, fibroblast growth factor 2, and transforming growth factor β1 (TGF-β1). Analysis of the *Ccn1* and *Ccn2* promoters have revealed critical regulatory elements and transcription factors governing their expression[2, 3], and identified the essential promoter for apposite regulation of *Ccn1* in transgenic mice[37]. *CCN1* and *CCN2* are targets of the YAP/TAZ transcriptional co-activators, which interact with the TEAD family of transcription factors to regulate genes related to cell proliferation and survival[100] and in response to mechanotransduction[202] (see Fig. 2). By contrast, *CCN3* and *CCN5* are downregulated by mitogenic signals and upregulated under conditions of growth arrest in several cell types[61, 113]. Additionally, *CCN* genes are downregulated by specific microRNAs in various cell types, for example *CCN2* is regulated by miR-133 and miR-30 in cardiomyocytes and miR-18a in chondrocytes [203, 204], whereas downregulation of *CCN1* by miR-155 may contribute to preeclampsia[174]. The presence of internal ribosome entry sites facilitates the preferential translation of the *CCN1* mRNA under conditions of cellular stress, for example after viral infections[205, 206].

Structural variants or isoforms of CCN proteins, some of which have been detected in biofluids and may have different activities, can be generated by differential splicing[207, 208], proteolysis[35, 209], and post-translational modifications[210]. Alternative splicing typically results in the production of truncated proteins or proteins with either the VWC or TSR domains deleted. These structural variants may potentially greatly expand the functional complexity of CCNs, although how they are regulated and how they impact the biological functions of CCNs *in vivo* are largely unknown. Whereas CCNs are secreted proteins with N-terminal signal peptides, they may be endocytosed after receptor binding. Several CCNs have been detected in the nucleus and although their nuclear function is unknown, recent studies have suggested the intriguing possibility that CCN1 and CCN5 might regulate transcription[211, 212].



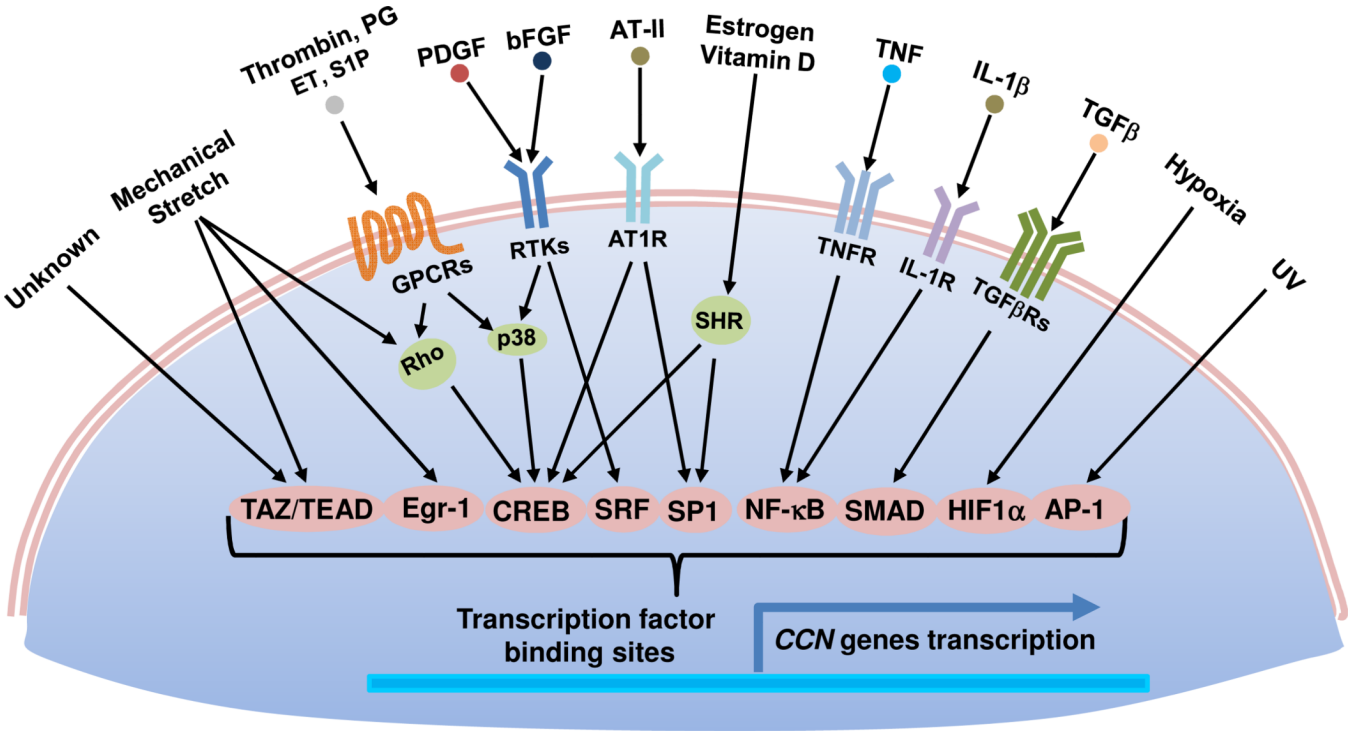


Figure 1. Transcriptional regulation of *CCN* genes

Multiple extracellular and environmental stimuli rapidly induce *CCN* genes through activation of various transcription factors, usually without requiring *de novo* protein synthesis. These include PDGF, FGF2, TGF-β, IL1β, TNFα, angiotensin II (AT-II)[2, 3], agonists of G protein-coupled receptors (GPCRs)[177] such as thrombin, prostaglandins (PE), endothelin-1 (ET), and sphingosine-1-phosphate (S1P), hormones (estrogen and vitamin D) that which bind steroid hormone receptors (SHR), hypoxia, UV, and mechanical stretch[37, 129]. The locations of various transcription factor bindings sites vary for each *CCN* gene.

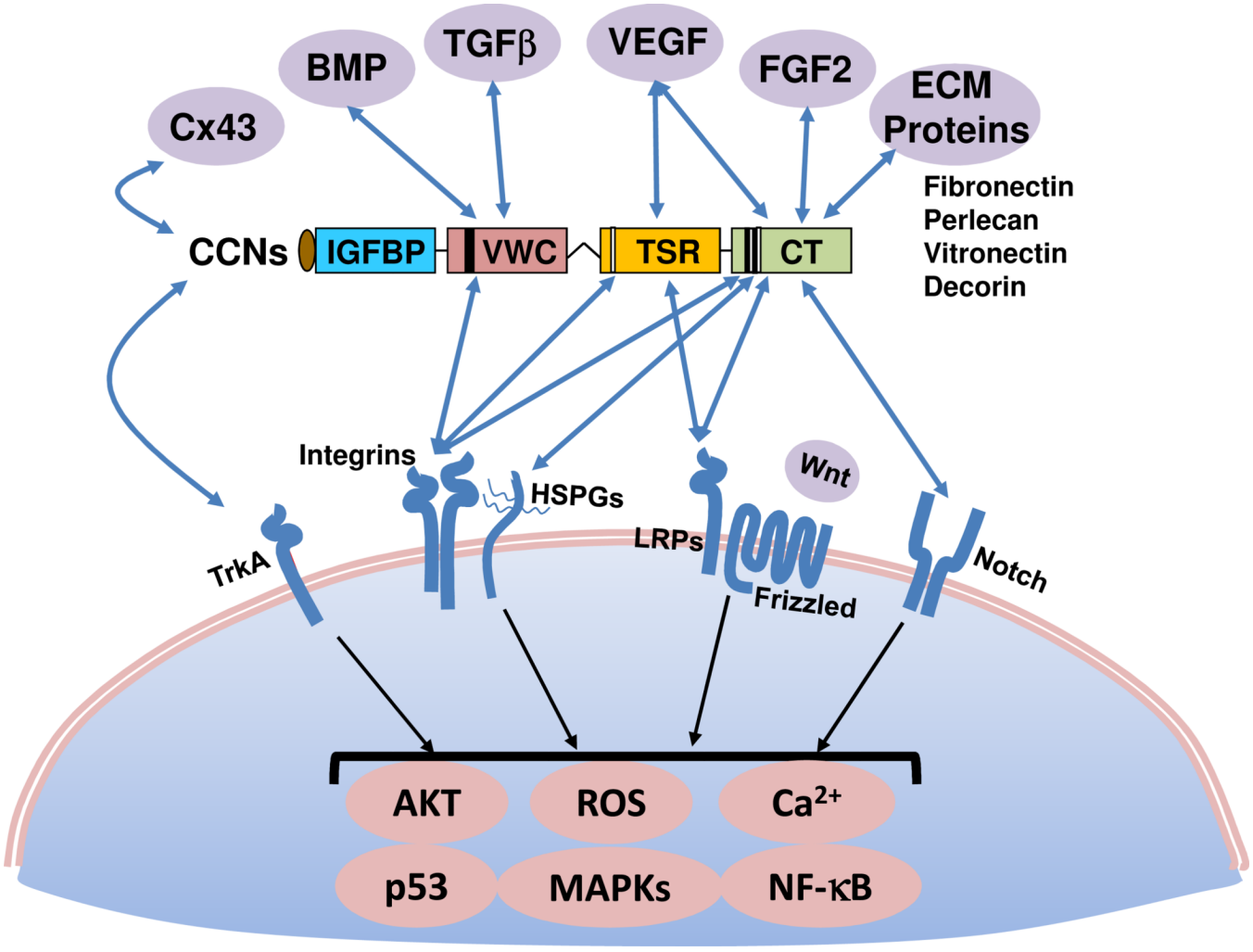


Figure 2. Molecular interactions through modular domains of CCN proteins

CCNs physically interact with a number of ECM proteins (including fibronectin[213], perlecan[214], vitronectin[215], decorin[168]), growth factors (including such as VEGF[34], FGF2[216], TGF-β[39], and BMPs[39]), and the gap junction protein connexin43[217, 218]. The specific modular domains mediating the interactions, where elucidated, are indicated. Whereas fibronectin and perlecan bind the CT domain, whether decorin and vitronectin also bind CT is not as clear. CCNs also bind to and signal through a number of cell surface receptors including several integrins[2, 37], which function in concert with HSPGs or LRPs as coreceptors in some contexts. CCN2 also binds TrkA in a complex with β_1 integrins[13] as co-receptors, and CCN3 can bind Notch[14]. CCNs can modulate Wnt signaling, in part through binding to the Wnt coreceptor, LRP-6[11]. The activities of the CCN modular domains may interact in a combinatorial manner to induce unique activities and functions[4]. For example, CCN1 synergism with TNFα requires its binding to both $\alpha_v\beta_5$ and $\alpha_6\beta_1$ integrins through the VWC and CT domains, respectively, to induce signals from both integrins that converge within the cell[7]; activation of either integrin alone is insufficient.

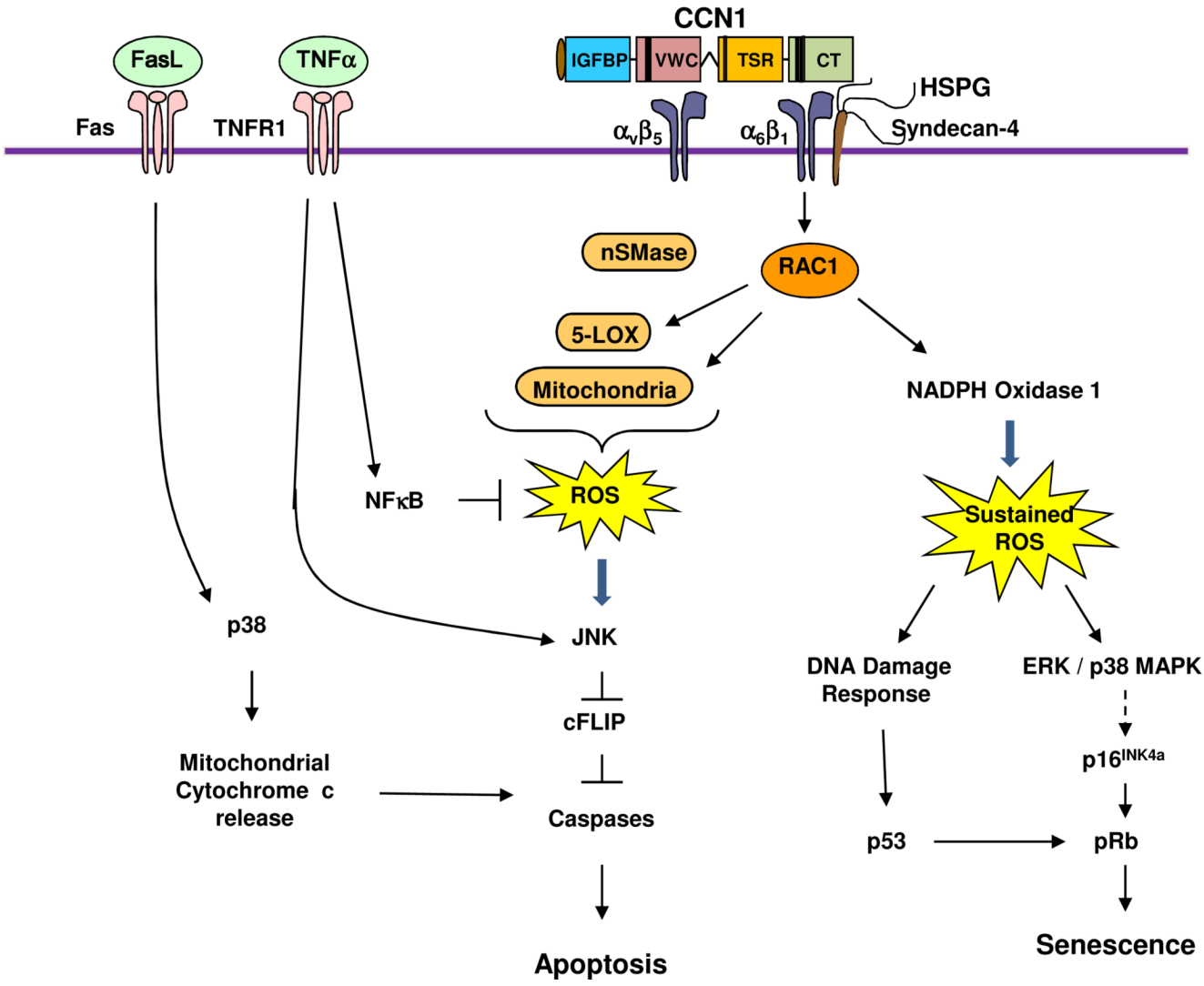


Figure 3. Signaling mechanism of CCN1-induced senescence and crosstalk with TNFα and FasL

The binding of CCN1 to integrin $\alpha_6\beta_1$-HSPGs (syndecan 4) triggers the activation of RAC-1 and NADPH oxidase 1, leading to a much more robust and sustained level of ROS compared to cell adhesion to other ECM proteins. The sustained ROS induces a DNA damage response and the activation of p53, and triggers the activation of ERK and p38 MAPK, which in turn induces $p16^{INK4a}$ and activates pRb[8]. Activated p53 and pRb contribute to the induction of cellular senescence. If integrin $\alpha_v\beta_5$ is also engaged by CCN1, RAC1-dependent ROS accumulation includes contribution from 5-lipoxygenase (5-LOX) and the mitochondria[7]; neutral sphingomyelinase (nSMase) also contributes to CCN1-induced ROS[26]. CCN1-induced ROS counteracts the effect of NFκB, which is strongly activated by TNFα and induces the expression of antioxidant proteins. The high level of ROS inhibits cysteine phosphatases that can inactivate MAPKs such as JNK, ERK, and p38 MAPK, leading to a hyperactivation of these kinases[219]. Activated JNK targets the proteosomal degradation of cFLIP[220], an inhibitor of caspase activation, allowing the activation of caspases 8/10 by TNFα to induce apoptosis without blocking *de novo* protein synthesis or NFκB signaling[7]. In addition to CCN1, CCN2 and CCN3 also synergize with

TNFα to induce apoptosis, presumably through a similar mechanism[25]. In the presence of FasL, which can trigger apoptosis on its own, CCN1 or CCN2-induced ROS leads to the hyperactivation of p38 MAPK, which enhances cytochrome c release from the mitochondria and thereby increases apoptosis[26].

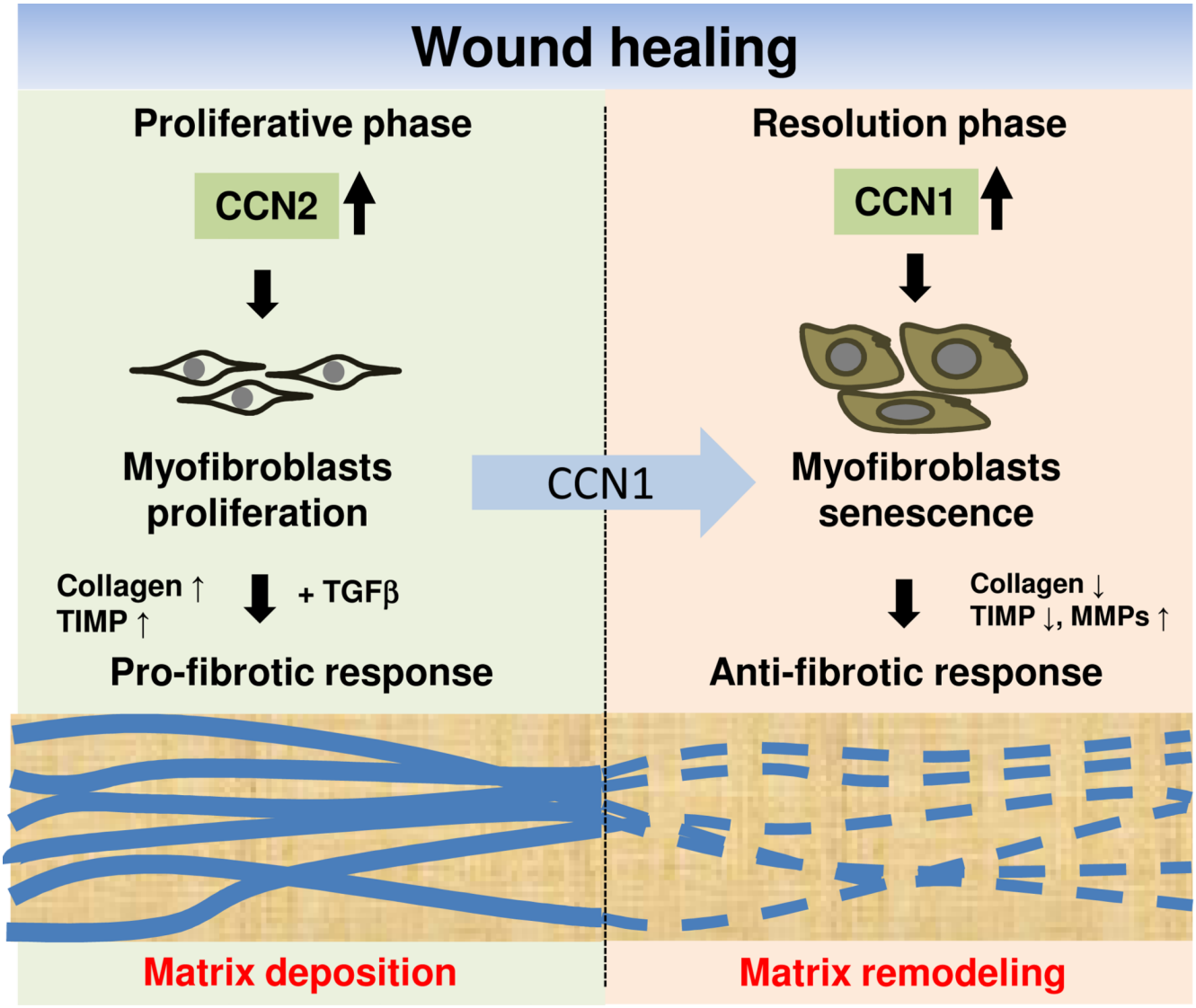


Figure 4. Role of CCNs in wound healing

CCN1 and CCN2 have distinct roles in wound healing. CCN2 functions in the granulation tissue during the proliferative phase and acts with TGF-β to promote the synthesis ECM, leading to a pro-fibrotic response. As wound healing progresses, CCN1 accumulates to a sufficiently high level to induce an anti-fibrotic senescence switch in myofibroblasts, thereby limiting fibrosis by converting the ECM-synthesizing myofibroblasts into ECM-degrading senescent cells.

Table I

Selected cellular processes stimulated (+) or inhibited (−) by CCN proteins in specific cell types and the cell surface receptors involved

Cellular responses	CCN Protein	Cells types and cell-surface receptors involved	Ref
Cell adhesion	CCN1, CCN2 (+)	• Fibroblasts – $\alpha_6\beta_1$ and HSPGs	2
	CCN1, CCN2 (+)	• Activated endothelial cells – $\alpha_v\beta_3$	2
	CCN1 (+)	• Smooth muscle cells – $\alpha_6\beta_1$ and HSPGs	2
	CCN1, CCN2 (+)	• Platelets – $\alpha_{IIb}\beta_3$	2
	CCN1, CCN2 (+)	• Monocytes – $\alpha_M\beta_2$,	2
	CCN1, CCN2 (+)	• Macrophages – $\alpha_M\beta_2$	46
	CCN2 (+)	• Hepatic stellate cells – $\alpha_v\beta_3$ and LRP	2
	CCN2 (+)	• Pancreatic stellate cells – $\alpha_5\beta_1$ and HSPGs	2
	CCN3 (+)	• Endothelial cells – $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and HSPGs	2
Migration/Invasiveness	CCN1 (+)	• Fibroblasts –$\alpha_v\beta_5$	2
	CCN1, CCN2, CCN3 (+)	• Microvascular endothelial cell chemotaxis – $\alpha_v\beta_3$	2
	CCN1 (+)	• Smooth muscle cell chemotaxis – $\alpha_6\beta_1$ and HSPGs	2
	CCN3 (+)	• Endothelial cells – $\alpha_v\beta_3$, $\alpha_5\beta_1$	2
	CCN3, CCN5 (−)	• Inhibits vascular smooth muscle cell migration	23, 24
	CCN4 (−)	• Lung cancer cell invasiveness	111
DNA synthesis/Proliferation	CCN1 (+)	• Fibroblasts – $\alpha_v\beta_3$	2
	CCN1 (+)	• Astrocytoma cells – α_5, α_6, and β_1	177
	CCN3, CCN5 (−)	• Smooth muscle cells	23
Survival	CCN1, CCN2, CCN3 (+)	• Endothelial cells – $\alpha_v\beta_3$	2
	CCN1 (+)	• Breast cancer cells – $\alpha_v\beta_3$	98
Apoptosis	CCN1 (+)	• Fibroblasts – $\alpha_6\beta_1$ and syndecan 4	9
	CCN1, CCN2, CCN3 (+)	• Synergism with TNF-α in fibroblasts – $\alpha_6\beta_1$, $\alpha_v\beta_5$, and syndecan-4	7
	CCN1, CCN2 (+)	• Synergism with FasL in fibroblasts – $\alpha_6\beta_1$ and HSPGs	26
Differentiation	CCN1 (+)	• Osteoblastic differentiation – $\alpha_v\beta_3$	178
Angiogenesis	CCN1, CCN2, CCN3 (+)	• Endothelial tubule formation – $\alpha_v\beta_3$	2
Inflammation	CCN1 (+)	• Macrophages, NFκB activation and M1 polarization – $\alpha_M\beta_2$	46
	CCN1 (+)	• MCF7 breast cancer cells, NFκB activation – $\alpha_v\beta_3$	98
	CCN1 (−)	•Inhibits immune cell infiltration in autoimmune myocarditis	47
	CCN2 (+)	• Mesangial cells chemokines expression – TrkA and HSPGs	179
	CCN3 (+)	•Endothelial cells, NFκB activation	48
Senescence	CCN1 (+)	• Fibroblasts – $\alpha_6\beta_1$ and HSPGs	8

Table II

Phenotypes of Knockout (KO) and knockin (KI) mice

Mouse Model	Phenotypes	Other Information	Refs.
Ccn1 KO	• Embryonic lethality • Failure in chorioallantoic fusion in 30% of embryos • Placental insufficiency; deficient vessel bifurcation at the chorioallantoic junction • Hemorrhage; loss of large vessel integrity • Severe atrioventricular septal defects	• Aberrant apoptosis in vascular cells and cardiac cushion mesenchymal cells • Haploinsufficiency leads to persistent ostium primum atrial septal defects in 20% of adults	33, 54
Ccn2 KO	• Perinatal lethality • Severe skeletal dysplasia, enlarged hypertrophic zones, impaired endochondral ossification • decreased β-cells and increased glucagon^{+} cells in pancreas • Pulmonary hypoplasia	• Premature exit of chondrocytes from the cell cycle • Delayed replacement with osteoblast • Decreased expression of VEGF in the hypertrophic zones	56–58
Ccn3 KO	• Viable and appear largely normal • Modest, transient, and sexually dimorphic skeletal abnormalities	• Enhanced neoinitimal thickening after photochemically induced thrombosis	24, 60
Ccn5 KO	• Early embryonic lethality		61
Ccn6 KO	• No phenotype in mice • No *Ccn6* mRNA detected in mice by Northern blotting or in situ hybridization	• Mutations in human CCN6 cause the autosomal recessive disorder progressive pseudorheumatoid dysplasia	62, 160
Ccn1 KI (*Ccn1*$^{dm/dm}$)	• Viable, fertile, and developmentally normal • Significantly resistant to TNFα or Fas-mediated hepatocyte apoptosis • Lack senescent cells in skin wound healing, leading to exacerbated fibrosis	• Express CCN1 unable to bind fibroblast receptors ($\alpha_6\beta_1$-HSPG)	7, 8, 26
Ccn2 floxed	• Deletion in early limb bud mesemchyme show transient osteopenia at 1 month • Deletion in osteoblasts results in osteopenia in 6 month male mice		180
	• Deletion in fibroblasts renders resistant to bleomycin-induced skin fibrosis		72
	• Deletion in ovary and uterus results in impaired follicle development, steroidogenesis, and ovulation		59
Ccn2 high to low expression	• 9-fold overexpression causes embryonic death at E10–12 • 30% of normal expression are viable and normal		181

Table III

Targeting CCNs in animal models of disease

Disease/ pathology	CCN proteins	Animal model	Phenotypes/intervention	Refs.
Fibrosis	CCN1	• Wound healing in $\alpha_6\beta_1$-binding defective *Ccn1* knockin mice	• Enhanced fibrosis in cutaneous wounds; topical application of CCN1 reverses effect	8
	CCN2	• Transgenic mice overexpressing CCN2 in hepatocytes	• Exacerbated liver fibrosis induced by CCl_4 or bile duct ligation	182
		• Mouse liver fibrosis (CCl_4)	• CCN2 siRNAs reduce fibrosis	74
		• N-nitrosodimethylamine-induced liver fibrosis	• CCN2 siRNAs reduce fibrosis	75
		• Streptozotocin (STZ)-induced diabetes in podocyte-specific *Ccn2* transgenic mice	• Exacerbated renal hypertrophy, proteinuria, and mesangial matrix expansion	134
		• Tubulointerstitial fibrosis induced by unilateral ureteral obstruction	• Reduction of ECM induction by CCN2 AS-ODN	76
		• Subtotal nephrotectomy in TGF-β1 transgenic mice	• CCN2 AS-ODN blocked interstitial fibrosis in remnant kidney	78
		• Cardiomyocyte-specific *Ccn2* transgenic rodents; angiotensin II-induced pressure overload heart failure	• No cardiac fibrosis; promotes cardiac hypertrophy and protects against pressure-overload heart failure	149
		• Deletion of *Ccn2* in fibroblasts and smooth muscle cells	• Resistant to bleomycin-induced skin fibrosis	72
		• Fibroblast-specific *Ccn2* transgenic mice	• Fibrosis in skin, lung, kidney, and vasculature	71
	CCN4	• Bleomycin-induced pulmonary fibrosis	• Neutralizing antibodies attenuates lung fibrosis	79
	CCN5	• Transverse aortic constriction in *Ccn5* transgenic mice	• Inhibition of pressure overload-induced cardiac hypertrophy and fibrosis	82
Cancer	CCN1	• Glioma xenografts	• siRNA inhibited pre-established glioma growth	101
		• Pancreatic cancer cells	• shRNA reverses characteristics of EMT and reduced tumorigenicity	95 94
	CCN2	• Pancreatic cancer xenografts		
		• Orthotopic and xenografted pancreatic cancer	• Decreased tumor growth with shCCN2 or siCCN2	104
			• FG-3019 attenuates tumor growth and metastasis	105
Cardio-vascular disease	CCN1	• Rat carotid artery balloon injury	• CCN1 siRNAs suppress neointimal hyperplasia	145
	CCN1	• Mouse autoimmune myocarditis	• Adenoviral expression of CCN1 attenuates myocarditis	47
	CCN2	• Rat carotid artery angioplasty with viral perfusion	• Recombinant CCN2 application increased neointimal thickening	143
Nephropathy/Retinopathy	CCN1	• Mouse oxygen-induced retinopathy (OIR)	• Intravetreal injection of anti-CCN1 antibody reduced retinal neovascularization	138
	CCN2	• Streptozotocin (STZ)-induced diabetic mice	• Subcutaneous CCN2 AS-ODN decreased proteinuria and albuminuria	133
			• Reduced basal lamina thickening of retinal capillaries in *Ccn2*+/− background	140

Disease/ pathology	CCN proteins	Animal model	Phenotypes/intervention	Refs.
Wound healing	CCN1	• Bone fracture (transverse osteotomy)	• Intraperitoneal injection of anti-CCN1 antibody impaired fracture healing	154
	CCN2	• Rabbit punch wound at ears	• Intradermal CCN2 AS-ODN injection reduced hyperptrophic scarring	77

AS-ODN, antisense oligonucleotide

Table IV

CCN proteins in cancers

Tumors (Cancers)	CCN proteins	Expression level	Clinical characterization/experimental observations	Refs.
Breast cancer	CCN1	Higher	• Associated with poor prognosis, tumor stage, metastasis, and mortality	107
			• Overexpression of CCN1 promotes tumorigenesis in xenografts	90
			• Mediates taxol resistance	100
	CCN2	Higher	•Associated with poor prognosis and metastasis, mediates taxol resistance	100, 107
			• Mediates metastasis to bone	102
	CCN3	Higher	• Associated with therapy resistant ER-positive breast cancer	117
	CCN4	Higher	• Associated with older patients with advanced tumor	107
	CCN5	Higher	• Associated with more differentiated breast cancers with low metastatic potential; inversely correlated with E2-independency and invasive phenotype	61
	CCN6	Lower	• Associated with poor prognosis in inflammatory breast cancer	120
Endometrial cancer	CCN1	Higher	• Poor survival of patients with endometrioid subtype	183
			• CCN1 inhibits endometrial cancer cell growth	184
Ovarian cancer	CCN1	Higher	• Associated with advanced tumor stage; enhanced tumorigenesis in xenografts of CCN1 overexpressing cells	96
	CCN2	Lower	• Promoter hypermethylation; associated with advanced tumor stage	185
Prostate cancer	CCN1	Higher	• Associated with high grade prostate cancer and lower risk of recurrence after surgery	176
			• Decreased xenografted tumors of DU145 cells with decreased CCN1	93
	CCN2		• Increased microvessel density and tumor growth in xenografts of CCN2 overexpressing prostate cancer cells	91
Glioma	CCN1& CCN2	Higher	• Associated with tumor grade, pathology, gender and age at diagnosis	101
	CCN3		• Reduced tumorgenicity in CCN3 overexpressing cells	114
Lung cancer	CCN1& CCN2	Lower	• Associated with tumor stage, tumor histology, metastasis, smoking, and family history; expression level correlated with patients' survival	109
			• Overexpression of CCN1 decreased tumor growth in xenografts	186
	CCN4		• Higher expression in adenosquamous cell carcinoma	109
Pancreatic cancer	CCN1	Higher	• Expression increases with pancreatic adenocarcinoma progression	95
	CCN2	Higher	• Highly expressed in pancreatic cancer	104
	CCN5	Lower	• Inversely correlated with tumor grade and p53 status	187
Colon cancer	CCN1,2	Higher	• Associated with tumor stage	188
	CCN4	Higher	• Associated with tumor stage, histologic grade, and lymph node status	108
Musculo-skeletal tumor	CCN2	Higher	• Associated with survival of rhabdomyosarcoma cells	189
	CCN3	Higher	• Higher risk of metastasis and worse prognosis in Ewing's sarcoma	116

Tumors (Cancers)	CCN proteins	Expression level	Clinical characterization/experimental observations	Refs.
Melanoma	CCN3	Higher	• Associated with higher metastasis of xenografted CCN3 overexpressing cells	115
Leukemia	CCN2	Higher	•Associated with pre-B lineage in ALL	190
	CCN3	Lower	•Restoring CCN3 expressing sensitizes CML cells to imatimib therapy	118

Table V

Mutation and polymorphism associated with human diseases

CCN	Identified mutation & polymorphism	Associated human diseases	Refs.
CCN1	• rs3753794, rs3753793 and rs2297140, rs2297141 and IVS 2+50	• Plasma HDL-cholesterol levels in obese individuals	191
	• rs954353	• Colorectal cancer (CRC)	192
CCN2	• −945 (G to C) substitution	• Susceptibility to systemic sclerosis	162
	• rs9399005	• Susceptibility to systemic sclerosis	164
	• rs6918698	• Susceptibility to systemic sclerosis	163
	• rs9402373 and rs12526196	• Severity of hepatic fibrosis (HF)	193
	• −945 GG genotype	• Cardiovascular events of stroke and myocardial infarction (MI) in surviving hemodialysis (HD) patients	194
	• −447C	• Higher degree of calcification in patients with symptomatic aortic stenosis	195
	• −20 GG genotype	• Susceptibility to nephropathy in type I diabetes	196
CCN3	N/A		
CCN4	• 2364 (A to G) substitution	• Prevalence of hypertension	197
	• 2365 AA genotype	• Spinal osteoarthritis with high endplate sclerosis	198
CCN5	N/A		
CCN6	• Germline mutations (likely loss of function)	• Autosomal recessive disorder progressive pseudorheumatoid dyaplasia (PPD)	160
	• Somatic mutation (frameshifts at a polyadenosine tract)	• Gastrointestinal tumors from patients with mutation in the mismatch repair pathway	199
	• 84 AA genotype	• Susceptibility to juvenile idiopathic arthritis (JIA)	200