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Functions and Mechanisms of Action of CCN Matricellular Proteins

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Abstract

Members of the CCN (CYR61/CTGF/NOV) family have emerged as dynamically expressed, extracellular matrix-associated proteins that play critical roles in cardiovascular and skeletal development, injury repair, fibrotic diseases and cancer. The synthesis of CCN proteins is highly inducible by serum growth factors, cytokines, and environmental stresses such as hypoxia, UV exposure, and mechanical stretch. Consisting of six secreted proteins in vertebrate species, CCNs are typically comprised of four conserved cysteine-rich modular domains. They function primarily through direct binding to specific integrin receptors and heparan sulfate proteoglycans, thereby triggering signal transduction events that culminate in the regulation of cell adhesion, migration, proliferation, gene expression, differentiation, and survival. CCN proteins can also modulate the activities of several growth factors and cytokines, including TGF-β, TNFα, VEGF, BMPs, and Wnt proteins, and may thereby regulate a broad array of biological processes. Recent studies have uncovered novel CCN activities unexpected for matricellular proteins, including their ability to induce apoptosis as cell adhesion substrates, to dictate the cytotoxicity of inflammatory cytokines such as TNFα, and to promote hematopoietic stem cell self-renewal. As potent regulators of angiogenesis and chondrogenesis, CCNs are essential for successful cardiovascular and skeletal development during embryogenesis. In the adult, the expression of CCN proteins is associated with injury repair and inflammation, and has been proposed as diagnostic or prognostic markers for diabetic nephropathy, hepatic fibrosis, systemic sclerosis, and several types of cancer. Targeting CCN signaling pathways may hold promise as a strategy of rational therapeutic design.

Keywords

angiogenesis; cancer; cardiovascular disease; chondrogenesis; fibrosis; integrin; TNFα; wound healing

INTRODUCTION

Far from being an inert scaffolding for the organization of cells into tissues, the extracellular matrix (ECM) is now recognized as a dynamic and multifunctional regulator of cell behavior (Aszodi et al., 2006). The ECM can bind and modulate the bioavailability and activity of growth factors, cytokines, chemokines, and extracellular enzymes. In addition, ECM proteins can

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directly interact with cell surface receptors to trigger the activation of signal transduction cascades, thereby regulating diverse cellular functions. A subset of ECM proteins, known as matricellular proteins, is dynamically expressed and does not serve obvious structural roles in the matrix (Bornstein and Sage, 2002). Rather, they function primarily to modulate cellular responses to other environmental factors. Known matricellular proteins include thrombospondins, SPARC, hevin, osteopontin, tenascin C and X, and members of the CCN family. Recent studies have shown that CCN proteins are essential regulators of embryonic development, and in the adult they play critical roles in inflammation, injury repair, fibrotic diseases, and cancer.

Members of the CCN family were first identified as secreted proteins whose synthesis was induced by mitogenic growth factors or oncogenes, or deregulated in transformed cells. The first three members described – CYR61 (cysteine-rich 61; CCN1)(O'Brien et al., 1990), CTGF (connective tissue growth factor; CCN2)(Bradham et al., 1991), and NOV (nephroblastoma overexpressed; CCN3)(Joliot et al., 1992) – provided the acronym for the CCN family. CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3) were subsequently identified as Wnt-inducible secreted proteins (Pennica et al., 1998), and together they comprise the family of six homologous, cysteine-rich proteins in vertebrates. 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 (TSP), and a carboxyl-terminal (CT) domain that contains a cysteine knot motif (Bork, 1993)(Fig. 1). Each structural module is encoded by a separate conserved exon, suggesting that CCN genes are products of exon shuffling (Brigstock, 1999; Lau and Lam, 1999). The N-terminal and C-terminal halves of the proteins are connected by a hinge region that is not conserved and is particularly sensitive to proteolysis (Kireeva et al., 1996; Dean et al., 2007). Since CCN proteins have acquired multiple names reflecting the various circumstances of their identification, a unified nomenclature has been proposed by international consensus to rename these proteins as CCN1-6 in order to minimize confusion (Brigstock et al., 2003). For example, the name CTGF (connective tissue growth factor) originally given to CCN2 implies activities and a mechanism of action akin to those of classical growth factors, a notion that has not been supported by experimental evidence to date.

Early studies on CCN proteins proceeded along two divergent paths: one advanced the idea that CCN proteins are polypeptide growth factors (Bradham et al., 1991; Frazier et al., 1996), while the other demonstrated their roles as ECM-associated cell adhesion molecules (Yang and Lau, 1991; Kireeva et al., 1996). The latter perspective envisions CCNs as matricellular proteins, which function primarily to modify cellular responses to other environmental factors and stimuli through interaction with cell adhesion receptors (Lau and Lam, 1999). The collective work from many laboratories in the CCN community now supports this view (Lau and Lam, 2005; Rachfal and Brigstock, 2005; Leask and Abraham, 2006; Yeger and Perbal, 2007). It should be noted that the purification of biologically active CCN proteins has presented a particular challenge, presumably due to the unusually high number of cysteine residues (~10%). The difficulty in purifying CCN proteins of high quality and the lack of unified biochemical and functional assays that define their specific activities have impeded progress in this field. It is possible that variabilities in results from different laboratories, where they exist, might be in part due to differences in methods of protein preparation.

Analyses of CCN functions are now extending the boundaries of known ECM functions. For example, CCN proteins can induce apoptosis as cell adhesion substrates, dictate the cytotoxicity of tumor necrosis factor α (TNFα), and play an essential role in hematopoietic stem cell self-renewal. In this review, we summarize the current information on CCN functions and action mechanisms, and endeavor to unravel the common threads that may underlie their

seemingly disparate roles in various contexts. A recent monograph on the CCN family provides an informative resource on research in this area (Perbal and Takigawa, 2005).

RECEPTORS AND CELLULAR FUNCTIONS OF CCN PROTEINS

Receptors of CCN proteins

Like some other ECM and matricellular proteins, CCN proteins regulate diverse cellular behavior including cell adhesion, migration, differentiation, proliferation, and survival. Extensive studies have focused on identifying the signaling receptors for CCN proteins, and results of these studies support the following conclusions. First, CCNs mediate their activities primarily through interaction with cell adhesion receptors, including integrins and heparan sulfate proteoglycans (HSPGs). The CCN-integrin connection was first demonstrated by the direct binding of CCN1 to integrin $\alpha_v\beta_3$ to mediate endothelial cell adhesion (Kireeva et al., 1998). At least seven other integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$, and $\alpha_D\beta_2$) have since been identified as signaling receptors mediating various CCN functions (Table I). HSPGs are known to serve as coreceptors with integrins in some contexts, and strong CCN-HSPG interaction has been documented (Yang and Lau, 1991). Indeed, CCN binding to the HSPG syndecan-4 is critical for several functions in fibroblasts (Chen et al., 2000; Chen et al., 2004b; Todorovic et al., 2005; Chen et al., 2007), whereas activities mediated through interaction with the chondroitin/dermatan sulfate proteoglycans decorin and biglycan are as yet undefined (Desnoyers et al., 2001).

Second, CCNs can also bind other receptors that do not typically interact with classical ECM proteins, such as the lipoprotein receptor-related proteins (LRPs)(Segarini et al., 2001). For instance, CCN2-mediated cell adhesion and modulation of Wnt signaling in some cell types depend on its binding to LRP-1 and LRP-6, respectively (Gao and Brigstock, 2003; Mercurio et al., 2004). Third, CCNs utilize distinct integrins depending on the target cell types and the activities mediated. In fibroblasts, CCN1 stimulates cell adhesion, migration, and DNA synthesis in fibroblasts through $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$, respectively (Table I). By contrast, it stimulates cell migration in endothelial cells and vascular smooth muscle cells (VSMCs) through binding to $\alpha_v\beta_3$ and $\alpha_6\beta_1$, respectively (Lau and Lam, 2005). Finally, the distinct integrin binding sites of CCN proteins can either function in concert with or independently of one another to induce distinct cellular responses. For example, CCN1 mutants that disrupt its $\alpha_6\beta_1$ binding sites specifically abrogate $\alpha_6\beta_1$-dependent CCN1 activities without affecting $\alpha_v\beta_3$-mediated angiogenic functions (Leu et al., 2004). Similarly, mutation that impairs the CCN1 $\alpha_v\beta_3$ binding site abolishes $\alpha_v\beta_3$- but not $\alpha_6\beta_1$-mediated functions, further establishing that these distinct integrin binding sites and their cognate signaling pathways can act independently of one another (Chen et al., 2004a). On the other hand, CCN1 must interact with both $\alpha_6\beta_1$ and $\alpha_v\beta_5$ to mediate its synergism with TNFα, indicating that different integrin pathways can also function in concert to elicit distinct CCN activities (Chen et al., 2007). The interaction of CCNs with multiple receptors may contribute to their unique activities and functions.

Cell adhesion, migration, and DNA synthesis

One of the most prominent and consistent functions of CCNs is their role as cell adhesive proteins. When immobilized on solid surfaces in cell culture, CCN proteins can support the adhesion of most adherent cell types through integrins and HSPGs and induce adhesive signaling. Mechanistically, adhesion of human skin fibroblasts to CCN1 and CCN2 occurs through $\alpha_6\beta_1$-HSPGs and rapidly induces the formation of $\alpha_6\beta_1$-containing focal adhesion complexes, activation of focal adhesion kinase (FAK), paxillin, Rac, actin cytoskeleton reorganization and formation of filopodia and lamellipodia (Chen et al., 2001a)(Fig. 2). These findings provide compelling evidence that CCN proteins induce adhesive signaling. Although

cell adhesion to ECM proteins is known to induce transient ERK1/2 activation, adhesion to CCN1 and CCN2 uniquely induces sustained ERK1/2 activation (Chen et al., 2001a), which may promote cell cycle progression by enhancing the stability of key regulatory proteins (Murphy and Blenis, 2006). A short peptide containing the $\alpha_6\beta_1$-HSPG binding sites of CCN1 is sufficient to support cell adhesion and activate sustained ERK activation (Leu et al., 2004). Additionally, CCN proteins can serve as adaptors to other ECM proteins to promote cell adhesion, as exemplified by the binding of CCN2 to fibronectin and perlecan (Nishida et al., 2003; Chen et al., 2004b).

In addition to supporting cell adhesion, one of the ubiquitous activities of CCN proteins is to regulate cell migration. Whereas CCN1, CCN2, and CCN3 proteins stimulate cell migration in many mesenchymal cell types (Grzeszkiewicz et al., 2001; Babic et al., 1999; Shimo et al., 1999; Lin et al., 2003; Gao and Brigstock, 2006), overexpression of CCN4 and CCN5 inhibits cell migration (Soon et al., 2003; Lake et al., 2003). Consistent with their angiogenic functions, CCN1, CCN2, and CCN3 are chemotactic (inducing directional cell migration) in microvascular endothelial cells, although CCN2 can also induce chemokinesis (random cell movement)(Babic et al., 1998; Babic et al., 1999; Lin et al., 2003).

The effect of CCN proteins on mitogenesis appears to be cell type-specific. Although CCN2 promotes DNA synthesis in chondrocytes and osteoblasts (Kubota and Takigawa, 2007b), its mitogenicity in fibroblasts has been controversial. Early studies reported that CCN2 is mitogenic in fibroblasts (Bradham et al., 1991; Frazier et al., 1996), whereas other studies showed that 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 through integrin $\alpha_v\beta_3$ (Kireeva et al., 1996; Kireeva et al., 1997; Grzeszkiewicz et al., 2001; Grotendorst et al., 2004). By contrast, the expression of CCN5, which lacks the CT domain, inhibits cell proliferation (Lake et al., 2003).

Cell survival and apoptosis

Cell adhesion to ECM molecules promotes cell survival, whereas detachment from the ECM induces rapid cell death by anoikis in many cell types. Remarkably, CCN proteins can promote apoptotic cell death while supporting cell adhesion in a cell type-specific manner (Todorovic et al., 2005). In fibroblasts, CCN1, CCN2, and CCN3 can induce apoptotic cell death as cell adhesion substrates. By contrast, endothelial cells adhered to CCNs are protected from apoptosis upon growth factor withdrawal through $\alpha_v\beta_3$, an integrin known to induce pro-survival signals (Babic et al., 1999; Leu et al., 2002). Mechanistically, CCN1 induces fibroblast apoptosis through binding to $\alpha_6\beta_1$ and syndecan-4, leading to the p53-dependent activation of Bax and cytochrome c release (Todorovic et al., 2005). Therefore, CCNs can either promote cell survival or induce apoptosis in a cell type and integrin-dependent manner, suggesting a potential role in tissue remodeling.

Angiogenesis

The angiogenic activity of CCNs was first described in CCN1 using the corneal micropocket implant assay (Babic et al., 1998). Subsequently, CCN1, CCN2, and CCN3 have been shown to induce angiogenesis in corneal implants (Babic et al., 1999; Lin et al., 2003), chick chorioallantoic membranes (Shimo et al., 1999), and rabbit ischemic hindlimbs (Fataccioli et al., 2002). Through direct binding to integrin $\alpha_v\beta_3$, CCN1, CCN2, and CCN3 can recapitulate angiogenic events *in vitro* by promoting endothelial cell adhesion, migration, proliferation, and tubule formation (Babic et al., 1999; Shimo et al., 1999; Leu et al., 2002; Lin et al., 2003). Furthermore, CCN1 stimulates integrin-dependent recruitment of $CD34^+$ progenitor cells to endothelial cells, thereby enhancing endothelial proliferation and neovascularization (Grote et al., 2007). Consistently, *Ccn1* knockout mice suffer cardiovascular defects, as discussed below

(Table II). In addition to direct effects, CCNs can also regulate the expression and activities of angiogenic factors such as VEGF-A and VEGF-C (Chen et al., 2001b; Ivkovic et al., 2003; Hashimoto et al., 2002; Dean et al., 2007). A significant decrease in CCN1 and CCN3 expression is found in human placentae associated with pre-eclampsia, consistent with a role of these proteins in placental angiogenesis (Gellhaus et al., 2006). CCN proteins may play important roles in embryonic development, inflammatory diseases and tumorigenesis in part through their potent angiogenic activities (reviewed by Kubota and Takigawa, 2007a).

Chondrogenesis and osteogenesis

CCN proteins exhibit both positive and negative regulatory roles in skeletal formation, as demonstrated in animal models and cell culture experiments (Table II). The most prominent phenotype of *CCN2* knockout mice is severe chondrodysplasia (Ivkovic et al., 2003), whereas *CCN3* mutant mice show enhanced chondrogenesis and osteogenesis (Heath et al., 2008). Several studies suggest that CCNs can regulate chondrogenic and osteogenic differentiation. The expression of CCNs is highly regulated in chondrogenic and osteogenic differentiation by mesenchymal stem cells (MSCs) isolated from adult bone marrow and cartilage (Luo et al., 2004;Schutze et al., 2005;Si et al., 2006). CCN1 enhances chondrogenic differentiation in mouse limb bud mesenchymal cells in micromass cultures and accelerates type II collagen expression (Wong et al., 1997). Similarly, CCN2 promotes chondrogenic differentiation in micromass cultures of branchial arch mesenchymal cells and proliferation of primary chondrocytes (Nakanishi et al., 2000;Shimo et al., 2004). By contrast, CCN3 appears to inhibit chondrogenic differentiation in micromass cultures (Heath et al., 2008). CCN1 stimulates osteoblast differentiation but inhibits osteoclastogenesis, suggesting a role as a bifunctional regulator that promotes osteogenesis (Crockett et al., 2007). Although CCN2 promotes cell proliferation and differentiation of primary osteoblast in cultures (Safadi et al., 2003), overexpression of CCN2 inhibits osteoblast function and leads to osteopenia in mice (Smerdel-Ramoya et al., 2008).

Stem cell self-renewal

An intriguing new study showed that CCN3 is expressed in $CD34^+$ pluripotent hematopoietic stem cells of human umbilical cord blood (Gupta et al., 2007). Both knockdown and add back experiments support the critical role of CCN3 in $CD34^+$ stem cell self renewal, suggesting potential utility of CCN3 in promoting stem cell engraftment.

FUNCTIONAL AND PHYSICAL INTERACTIONS OF CCN PROTEINS WITH GROWTH FACTORS AND CYTOKINES

CCNs unmask the cytotoxicity of TNFα

CCN proteins can profoundly modify the activities of some growth factors and cytokines through functional and/or physical interactions. A particularly dramatic example is the effect of CCNs on the cytotoxicity of TNFα, which functions mainly to regulate inflammation and immunity (Aggarwal, 2003). Although TNFα can activate a death receptor and is cytotoxic to certain tumor cells, it does not trigger cell death in normal cells. Instead, it promotes cell proliferation and survival through the activation of the pro-inflammatory transcription factor NFκB, which antagonizes the TNFα-induced apoptotic pathway (Aggarwal, 2003). Thus, TNFα can induce apoptosis in normal cells only when NFκB signaling is blocked or when protein synthesis is inhibited, typically by the addition of cycloheximide in cell culture systems. How TNFα induces apoptosis *in vivo* is not well understood. Remarkably, the presence of CCN1, CCN2, or CCN3 can unmask the cytotoxicity of TNFα without perturbation of NFκB signaling or inhibition of *de novo* protein synthesis, leading to rapid apoptosis in the otherwise resistant primary human fibroblasts (Chen et al., 2007). Thus, CCNs can profoundly modify

the activities of TNFα, converting it from a proliferation-enhancing factor into a potent apoptotic agent in fibroblasts. Mechanistically, CCN1 acts by binding to integrins $\alpha_v\beta_5$, $\alpha_6\beta_1$, and syndecan-4, leading to the generation of reactive oxygen species (ROS) via 5-lipoxygenase and the mitochondria through a RAC1-dependent pathway (Chen et al., 2007) (Fig. 2). The high and sustained level of ROS induced by CCN1/TNFα results in the biphasic activation of JNK necessary for apoptosis, most likely through oxidative inactivation of JNK phosphatases. Importantly, mice with the genomic *CCN1* locus replaced with an apoptosis-defective *CCN1* allele with mutations at the $\alpha_6\beta_1$/HSPG binding sites are substantially resistant to TNFα-induced apoptosis *in vivo* (Chen et al., 2007). These results establish CCN1 as a physiologic regulator of TNFα cytotoxicity, and suggest that CCN proteins may significantly affect the activity of TNFα during inflammatory responses. The requirement of multiple receptors for CCN1 action in this context may serve to specify the cell types targeted for elimination.

Physical interactions with growth factors and their functional consequences

CCN proteins may also modulate the bioavailability and signal transduction of growth factors. Substantial evidence indicates that CCN2 potentiates transforming growth factor-β (TGF-β) actions. CCN2 has been reported to bind TGF-β through the vWC domain and as a result enhance the binding of TGF-β to all three TGF-β receptors (Abreu et al., 2002)(Fig. 3). In addition to enhancing the effective concentration of TGF-β, CCN2 also modifies TGF-β signaling. Some 30% of the genes that are normally inducible by TGF-β are no longer responsive to TGF-β in *CCN2*-null or CCN2-knockdown cells, indicating that CCN2 is required for a subset of TGF-β responses (Wang et al., 2004; Shi-wen et al., 2006). *In vivo*, CCN2 cooperates with TGF-β to induce a sustained level of fibrotic response that is not achieved by either factor alone (Mori et al., 1999).

In contrast to promoting the functions of TGF-β, CCNs appear to inhibit the activities of bone morphogenetic proteins (BMPs). CCN2 binds BMP-4 through the vWC domain and in so doing inhibits BMP-4 binding to its receptors (Abreu et al., 2002). Microinjection of CCN1 mRNA into *Xenopus* embryos also inhibits BMP signaling (Latinkic et al., 2003; Mercurio et al., 2004), and injection of CCN6 RNA curtails the phenotypic effects of BMP-2b overexpression in zebrafish (Nakamura et al., 2007). Likewise, CCN3 binds and antagonizes BMP-2, inhibiting BMP-2 induced Smad signaling and osteogenic differentiation (Rydziel et al., 2007). Consistent with the idea that CCN2 and CCN3 can act as BMP antagonists, transgenic mice that overexpress CCN2 or CCN3 in osteoblasts develop osteopenia (Rydziel et al., 2007; Smerdel-Ramoya et al., 2008). Thus, CCN proteins may directly interact with and regulate the functions of TGF-β and BMPs.

In addition to the aforementioned interactions, CCN2 can bind $VEGF_{165}$ at two binding sites in the TSP and CT domains (Inoki et al., 2002). CCN2-VEGF interaction inhibits VEGF binding to its receptor VEGFR2 and the angiogenic activity of both molecules, whereas proteolysis of the complex by matrix metalloproteinases (MMPs) releases the bound VEGF in an active form (Hashimoto et al., 2002; Dean et al., 2007). Thus, CCN2 may act to fine tune the bioavailability of VEGF, releasing it for angiogenic action only in contexts where MMPs are being secreted and activated, such as during tissue remodeling and wound repair. Another example of bioavailability regulation is illustrated by the ability of CCN1 to displace ECM-bound bFGF, thereby enhancing bFGF-induced DNA synthesis (Kolesnikova and Lau, 1998).

Functional interaction with Wnt and Notch signaling

CCN1 and *CCN2* are transcriptionally activated by the Wnt3A/β-catenin signaling pathway during osteogenic differentiation, but have opposing effects on this pathway. Knockdown of

CCN1 inhibited Wnt3A-induced osteogenic differentiation (Si et al., 2006). Microinjection of *CCN1* mRNA into *Xenopus* embryos induces expression of Wnt/β-catenin transcriptional targets and the formation of a partial or complete secondary body axes, an effect similar to ectopic Wnt-signaling (Latinkic et al., 2003). However, injection of *CCN1* mRNA also inhibits secondary axes-induction by Xwnt8, suggesting that CCN1 can both mediate and antagonize Wnt signaling (Latinkic et al., 2003). By contrast, overexpression of CCN2 inhibited Wnt3A-induced osteogenic differentiation (Luo et al., 2004). CCN2 also inhibits Wnt signaling in *Xenopus*, most likely through direct interaction with the Wnt-coreceptor LRP-6 through the CT domain (Mercurio et al., 2004). Recent studies also showed that CCN6 antagonizes Wnt-signaling in zebrafish embryonic development (Nakamura et al., 2007). Thus, while CCN1 can mediate or activate Wnt-signaling in some instances, CCN2 and CCN6 can antagonize it in a context-dependent manner.

CCN3 interacts with Notch1 through its CT domain, and suppresses myogenic differentiation *in vitro* (Sakamoto et al., 2002). CCN3 and Notch1 are concomitantly expressed in presomitic mesoderm, suggesting that their interaction may contribute to Notch signaling and the resulting inhibition of myogenesis (Sakamoto et al., 2002).

REGULATION OF *CCN* GENE EXPRESSION BY GROWTH FACTORS, HORMONES, AND ENVIRONMENTAL STRESSES

The expression of *CCN* genes is exquisitely sensitive to environmental perturbations, including the availability of growth factors, hormones, and cytokines, and exposure to oxygen deprivation, UV, and mechanical forces. Both *CCN1* and *CCN2* were first identified in differential expression screens for immediate-early genes that are transcriptionally activated by serum (Lau and Lam, 1999) or TGF-β (*CCN1* and *CCN2* were named βIG-M1 and βIG-M2 in this study)(Brunner et al., 1991) without requiring *de novo* protein synthesis. These genes are also highly responsive to induction by a variety of mitogenic signals and external stimuli, including fibroblast growth factor, platelet-derived growth factor, phorbol esters, and cAMP (O'Brien et al., 1990). In the *CCN1* promoter, a serum response element (SRE) is essential for transcriptional activation by serum or platelet-derived growth factor in fibroblasts (Latinkic et al., 1991). A 2 kb fragment of the *CCN1* promoter that includes the SRE is sufficient to confer faithful developmental and pathological (wound healing) expression in transgenic mice, thereby defining the critical promoter for *CCN1* transcriptional regulation *in vivo* (Latinkic et al., 2001).

TGF-β exerts strong regulation of *CCN* gene expression, including transcriptional activation of *CCN1*, *CCN2*, *CCN4*, and *CCN5*, and repression of *CCN3* (Lafont et al., 2002; Parisi et al., 2006). The full activation of *CCN2* by TGF-β requires three *CCN2* promoter elements: a SMAD binding site, a tandem repeat of an ETS element, and an element important for basal transcriptional activity (Leask et al., 2003; Van Beek et al., 2006). Recently, a novel mechanism of regulation has been described in which the metalloproteinase MMP3 binds a *CCN2* enhancer element and augments its expression in a transcription factor-like manner in chondrocytes (Eguchi et al., 2008). Intracellular MMP3 synergizes with TGF-β to activate the *CCN2* promoter, suggesting an interaction between MMP3 and SMAD signaling.

Several hormones also upregulate *CCN* expression. Angiotensin II enhances *CCN1* expression in VSMCs *in vitro* and in rat aorta *ex vivo* (Hilfiker et al., 2002). *CCN2* is upregluated by endothelin-1 in fibroblasts, VSMCs, and cardiac myocytes, consistent with its roles in fibrotic and vascular functions (Kemp et al., 2004; Xu et al., 2004; Rodriguez-Vita et al., 2005). Estrogen is also a potent inducer of *CCN1* expression. In the mammary adenocarcinoma cell line MCF-7, which is dependent on estrogen for growth, blockade of CCN1 activity by neutralizing antibodies abrogated estrogen-dependent DNA synthesis (Sampath et al., 2001).

In addition, *CCN1* transcription is transiently induced by dihydroxy-vitamin D3 (1,25-$(OH)_2$D3), which promotes osteoblast differentiation (Schutze et al., 1998).

A striking aspect of *CCN* gene expression is its sensitivity to environmental stress. For example, *CCN1* is induced by exposure to UV light (Quan et al., 2006). *CCN1* and *CCN2* are transcriptionally induced under hypoxia, a condition that favors blood vessel growth by the induction of several angiogenic factors, including VEGF, through the action of hypoxia-inducible factor-1α (HIF-1α). HIF-1α interacts with c-Jun/AP-1 and thereby contribute to *CCN1* transcription in hypoxic conditions (Kunz et al., 2003). In *CCN2*, both HIF-1α-dependent transcription and enhanced mRNA stability through a sequence element in the 3′ UTR contribute to its expression under hypoxia (Higgins et al., 2004; Kondo et al., 2006). Mechanical force is another form of stress that induces *CCN* gene expression. *CCN1* and *CCN2* expression is rapidly up-regulated by tensile forces and mechanical stretch in fibroblasts, chondrocytes and VSMCs, hemodynamic forces in endothelial cells, and hydrostatic pressure in mesangial cells (reviewed by Chaqour and Goppelt-Struebe, 2006). Promoter analysis showed that a binding site for Egr-1 is critical for stretch-induced activation of *CCN1* in VSMCs (Grote et al., 2004). Mechanical stress also up-regulates *CCN1* and *CCN2* expression in both cardiac and skeletal muscles (Hilfiker-Kleiner et al., 2004; Kivela et al., 2007). In humans, a single bout of strenuous exercise is sufficient to enhance expression of *CCN1* and *CCN2* in skeletal muscles due to mechanical stretch rather than a temporary hypoxic condition after exercise (Kivela et al., 2007).

Inflammation and tissue injury constitute other forms of stress that induce *CCN* expression. In postnatal development and in the adult, CCN proteins generally expressed at a low level in most tissues, but become elevated again in sites of inflammation and injury repair (Igarashi et al., 1993; Chen et al., 2001b; Latinkic et al., 2001). Consistently, *CCN* expression is induced by inflammatory cytokines such as IL-1 and TNFα (Cooker et al., 2007; Gashaw et al., 2008), as well as by bacterial and viral infections (Kim et al., 2004; Wiedmaier et al., 2008).

CCN PROTEINS IN EMBRYONIC DEVELOPMENT

Targeted gene disruptions in mice have been accomplished for *CCN1*, *CCN2*, *CCN3*, and *CCN6*. With the exception of *CCN6*-null mice, which show no observable phenotypic change, the resulting phenotypes establish a critical role for CCNs in cardiovascular and skeletal development. Early studies showed that *CCN1* expression is tightly associated with the skeletal, cardiovascular, and neuronal systems during embryogenesis (O'Brien and Lau, 1992), suggesting a role for CCN1 in the development of these organ systems. These notions are supported by the finding that targeted disruption of *CCN1* in mice results in embryonic lethality with cardiovascular defects (Mo et al., 2002; Mo and Lau, 2006). Approximately 30% of *CCN1*-null embryos fail to form chorioallantoic fusion at E8.5 and die by E9.5, whereas the remaining embryos perish at mid-gestation from placental vascular insufficiency, loss of embryonic vessel integrity leading to hemorrhage, and cardiac atrioventricular septal defect (AVSD). The observed defect in chorioallantoic fusion, a process known to involve α_4 integrin and VCAM-1, implicates a role for CCN1 in cell adhesion events between the allantois and the chorion. *CCN1*-null embryos are defective in vessel bifurcation at the chorionic plate, leading to a paucity of sprouting vessels that penetrate into the labyrinth and thus an undervascularized placenta (Mo et al., 2002). Furthermore, the large vessels in *CCN1*-null embryos lack a discrete basement membrane, and the vascular cells are disorganized and apoptotic, leading to rupture and hemorrhage (Mo et al., 2002). *CCN1*-null mice perish too early in development to fully assess whether CCN1 deficiency may impair skeletal development, although no obvious skeletal defects were observed by the time of their embryonic deaths.

CCN1-null embryos exhibit severe defects in atrioventricular valvuloseptal morphogenesis, resulting in a common atrioventricular valve orifice that is the hallmark of complete AVSD (Mo and Lau, 2006). This phenotype is in part due to precocious apoptosis in mesenchymal cells of the endocardial cushion tissue, which must fuse with the atrial and ventricular septa to undergo extensive remodeling. Although $CCN1^{+/-}$ mice are largely viable, they display persistent ostium primum atrial septal defects in 20% of the adult. Human AVSD is a common group of congenital disorders that is frequently associated with Down's syndrome, whereas non-syndromic AVSDs are inherited with autosomal dominance (Sheffield et al., 1997). The atrial septal defects due to *CCN1* haploinsufficiency are similar to those observed in some human patients with mutations in *AVSD1*, a susceptibility gene for non-syndromic AVSD identified by linkage analysis (Sheffield et al., 1997). Remarkably, the human *CCN1* gene maps to chromosome 1p21-31 (Jay et al., 1997), precisely the same location as *AVSD1* (Sheffield et al., 1997). These findings suggest that *CCN1* may be a candidate gene for human AVSD.

As discussed above, CCN1 and CCN2 share many similarities in their activities and patterns of expression. Despite these similarities, targeted disruptions of *CCN1* and *CCN2* in mice show distinct phenotypes. While *CCN1* is essential for cardiovascular development, *CCN2*-null mice are neonatal lethal due to respiratory defects as a secondary consequence of severe skeletal malformations (Ivkovic et al., 2003). *CCN2* deficiency results in generalized chondrodysplasia throughout the appendicular and axial skeleton due to decreased growth plate angiogenesis, aberrant ECM metabolism, and defective endochondrial ossification (Ivkovic et al., 2003). These findings are consistent with a wealth of *in vitro* data showing the roles of CCN2 in chondrogenesis and endochondrial ossification (reviewed by (Kubota and Takigawa, 2007b). *CCN2*-null embryos also suffer from pulmonary hypoplasia, with reduced cell proliferation and increased apoptosis in the lung (Baguma-Nibasheka and Kablar, 2008). Loss of CCN2 perturbed differentiation of type II alveolar epithelial cells, resulting in excessive glycogen retention and diminished lamellar body and nuclear size, although surfactant synthesis was not affected. Although CCN2 is also highly expressed in the developing cardiovascular system, no prominent cardiovascular defects were observed in *CCN2*-null mice (Ivkovic et al., 2003; Chuva de Sousa Lopes SM et al., 2004).

Mice with targeted disruption of *CCN3* have been constructed in which exon 3 was replaced with a TK-neomycin cassette to generate mice that produce no full length CCN3 but express a very low level of mutant CCN3 that lacks the VWC domain (Heath et al., 2008). While <50% of homozygous *CCN3* mutant mice are viable, they show deficiencies in tissues also impaired in *CCN1* and *CCN2* null mice, including defects in the appendicular and axial skeleton, severe joint malformation, and abnormal remodeling of the endocardial cushions with associated cardiac septal defects. Premature tissue degeneration was also observed in the lens of *CCN3* mutant mice, with cataracts developing in adults older than 6 months.

Loss-of-function mutations in *CCN6* (*WISP3*) in humans cause the autosomal recessive skeletal disease progressive pseudorheumatoid dysplasia, a juvenile-onset degenerative disease of the joint (Hurvitz et al., 1999). However, the CCN6 mRNA is undetectable in mouse tissues by RNA blotting or *in situ* hybridization, and *CCN6*-null mice exhibit no observable phenotype (Kutz et al., 2005). Mice that over-express CCN6 are also normal. Therefore, CCN6 appears to play different roles in mice and in humans, and is not essential for skeletal growth or homeostasis in mice (Kutz et al., 2005).

Although CCN1, CCN2, and CCN3 are prominently expressed in the neuronal system during development, no neuronal phenotypes have been reported in mice with targeted disruptions of these genes to date. The specific functions of CCN protein in neuronal cells or neuronal development are currently unknown.

FUNCTIONS OF CCN PROTEINS IN WOUND HEALING AND DISEASE

Wound healing

Expression of CCNs is tightly regulated during injury repair in many organs, including the liver following partial hepatectomy (Ujike et al., 2000), the heart after myocardial infarction (Hilfiker-Kleiner et al., 2004; Chuva de Sousa Lopes SM et al., 2004), and in granulation tissue during cutaneous wound healing (Igarashi et al., 1993; Chen et al., 2001b; Lin et al., 2005b). In the initial phase of injury repair, an abundance of CCN2 is released from the α granules of platelets (Kubota et al., 2004; Cicha et al., 2004). CCN1 and CCN2 can both support the adhesion of activated platelets through direct binding to integrin $\alpha_{IIb}\beta_3$ (Jedsadayanmata et al., 1999), and serve as adhesion substrates to invading inflammatory cells such as monocytes through integrin $\alpha_M\beta_2$ (Schober et al., 2002). In later stages of wound healing, CCN proteins are highly induced in the granulation tissue during its remodeling. In this context, CCN proteins may synergize with TGF-β in matrix remodeling, and they may also interact with TNFα to trigger apoptosis of fibroblasts (Chen et al., 2007).

CCN1 and CCN2 expression is also elevated during fracture repair in the long bones throughout the reparative phase of the callus, notably in proliferating chondrocytes and osteoblasts (Hadjiargyrou et al., 2000; Nakata et al., 2002). Furthermore, blockade of CCN1 by antibodies inhibits bone fracture healing in mice (Athanasopoulos et al., 2007) and recombinant CCN2 protein promotes the repair of articular cartilage in a rat osteoarthritis model (Nishida et al., 2004). These studies suggest that CCN proteins may play important roles in the homeostasis of bone and cartilage tissues.

Fibrosis

CCN2 overexpression is strongly associated with fibrosis of various organs, and may potentiate the activity of TGF-β (Table II). For example, injection of either TGF-β or CCN2 alone in the skin induces only transient granulation tissue formation, whereas application of both TGF-β and CCN2 together produces a sustained fibrotic response (Mori et al., 1999). Likewise, mice resistant to bleomycin-induced lung fibrosis, a condition that is TGF-β-dependent, can be made susceptible by overexpression of *CCN2* concomitant with bleomycin treatment (Bonniaud et al., 2004). It has been hypothesized that TGF-β may initiate the fibrotic response, and CCN2 cooperates with TGF-β to maintain and exacerbate fibrosis (Takehara, 2003).

Genetic evidence has further endorsed the notion that CCN2 plays an important role in liver fibrosis and systemic sclerosis. Knockdown of CCN2 by siRNA prevents liver fibrosis induced by CCl_4 or N-nitrosodimethylamine in rats, showing that CCN2 plays a critical role (Li et al., 2006; George and Tsutsumi, 2007). Consistent with the role of CCN2 in fibrosis, its elevated expression has been noted in hepatic stellate cells of the fibrous septa in fibrotic and cirrhotic livers in experimental models and human patients (Rachfal and Brigstock, 2003). *In vitro*, CCN2 supports the adhesion of hepatic stellate cells and oval cells, and stimulate stellate cell proliferation and oval cell migration (Paradis et al., 2002; Pi et al., 2008). In addition, CCN2 is prominently overexpressed in systemic sclerosis or scleroderma, in which TGF-β and CCN2 both appear to play a role (Takehara, 2003). A polymorphism in the human *CCN2* promoter that relieves Sp3-mediated transcriptional repression has been found to be significantly associated with scleroderma (Fonseca et al., 2007). The N-terminal cleavage products of CCN2 are present at elevated levels in the plasma and dermal interstitial fluid of scleroderma patients, and serves as a marker for the disease (Dziadzio et al., 2005). Together, these studies underscore the important roles of CCN2 in fibrosis, and suggest that CCN2 may be a potential target for antifibrotic therapy (Leask, 2008).

Diabetic nephropathy

The critical role of CCN2 in renal disease is supported by the findings that its overexpression in podocytes worsens diabetic nephropathy in mice (Yokoi et al., 2008), whereas reduction of CCN2 expression by antisense oligonucleotides ameliorates renal tubulointerstitial fibrosis and attenuates nephropathy in mouse models of diabetes (Yokoi et al., 2004; Guha et al., 2007). *In vitro*, CCN2 promotes mesangial cell survival, stimulates matrix deposition, and inhibits high glucose effect on matrix degradation. Several recent studies have independently concluded that plasma or renal CCN2 level is a useful risk marker for diabetic nephropathy (Jaffa et al., 2008; Nguyen et al., 2008; Thomson et al., 2008).

Vascular diseases

Both CCN1 and CCN2 are over-expressed in VSMCs of atherosclerotic lesions, or in retenosis after balloon angioplasty (Hilfiker et al., 2002; Grzeszkiewicz et al., 2002; Schober et al., 2002). Suppression of *CCN1* expression by either siRNA or FOXO3a-mediated repression results in reduced neointimal hyperplasia after balloon angioplasty, an effect that is reversed by replenishment of CCN1 via gene transfer (Lee et al., 2007; Matsumae et al., 2008). These findings underscore a critical role for CCN1 in vascular injury repair, and suggest that inhibition of CCN1 may potentially prevent restenosis after vascular interventions (Matsumae et al., 2008).

Cancer

A large body of work on CCN proteins in cancer support the following observations: 1. CCNs are aberrantly expressed in cancers of a broad range of tissues; 2. ectopic expression of CCN genes can either enhance or suppress the tumorigenicity of tumor cells, depending on the specific cancer; 3. in some cases, anti-CCN therapy inhibits tumor growth or metastasis; and 4. CCN gene expression may serve as a diagnostic or prognostic marker for certain malignancies (Table II). Aberrant expression of CCNs is observed in cancers of numerous organs and tissues, including (but not limited to) breast, colorectal, gallbladder, gastric, ovarian, pancreatic, and prostate cancers, gliomas, hepatocellular carcinoma, non-small cell lung and squamous cell carcinoma, lymphoblastic leukemia, melanoma, and cartilaginous tumors (reviewed by Menendez et al., 2003;O'Kelly and Koeffler, 2005;Kleer et al., 2007;Yeger and Perbal, 2007).

One of the mechanisms by which CCNs may promote tumor growth is the enhancement of tumor angiogenesis. Consistent with the angiogenic activity of CCN1, its overexpression in gastric adenocarcinoma cells enhances the tumorigenicity of these cells in nude mice, resulting in tumors that are more vascularized than tumors of control cells (Babic et al., 1998). Likewise, increased tumorigenicity and tumor vascular density *in vivo* have been observed upon ectopic expression of *CCN1* in MCF7 breast cancer cells and ovarian cancer cells (Menendez et al., 2003; O'Kelly and Koeffler, 2005). Another pro-tumorigenic mechanism of CCNs is the enhancement of cell survival. Forced expression of *CCN1* in breast cancer cells confers resistance to apoptosis by upregulation of the anti-apoptotic protein XIAP (Lin et al., 2004), and promotes resistance to the pro-apoptotic anti-cancer drug Taxol through an integrin $\alpha_v\beta_3$-dependent mechanism (Menendez et al., 2005).

Recent studies have found that CCN2 is overexpressed in human pancreatic cancer, and CCN2-specific monoclonal antibody therapy inhibits pancreatic tumor growth, lymph node metastasis, and tumor angiogenesis in rodent models (Aikawa et al., 2006; Dornhofer et al., 2006). CCN2 is part of a gene signature that specifies osteolytic bone metastasis of breast cancer (Kang et al., 2003), and CCN2 neutralizing antibodies suppress breast cancer osteolytic bone metastasis and microvascularization (Shimo et al., 2006). These results support a critical

role for CCN2 in pancreatic cancer and metastatic breast cancer, and suggest targeting CCN2 as potential anti-cancer therapy.

Paradoxically, CCNs may also inhibit tumor growth. For example, CCN1 suppresses tumor growth of non-small cell lung carcinoma in nude mice (Tong et al., 2001). CCN2 inhibits metastasis and invasion of human lung adenocarcinoma through a CRMP-1 dependent mechanism (Chang et al., 2004), and suppresses metastasis of colorectal cancer (Lin et al., 2005a). CCN4 (Elm1) and CCN5 (COP1) were identified as genes that inhibited tumor growth of K-1175 murine melanoma cells and transformed fibroblasts in immunodeficient mice, respectively (Hashimoto et al., 1998; Zhang et al., 1998). Likewise, CCN6, which can modulate IGF signaling, suppresses inflammatory breast cancer tumor growth (Kleer et al., 2007).

While the angiogenic and apoptotic activities of CCN proteins may respectively promote or inhibit tumor growth, the roles of CCN proteins in cancer are likely complex and may involve multiple downstream effectors. Understanding how CCN proteins function to either promote or inhibit tumorigenesis will require further investigation. Nevertheless, the strong correlation of CCN expression in various cancers have underscored their prognostic values, and suggests that CCN signaling pathways may be useful targets for novel anti-cancer therapy.

CONCLUSIONS AND FUTURE PROSPECTS

The CCN family of proteins has emerged as ECM-associated, multifunctional regulators of development and injury repair. On the cellular level, CCN proteins regulate cell adhesion, migration, proliferation, differentiation, apoptosis, and survival, acting primarily through direct binding to integrins, with HSPGs and LRPs as coreceptors in some contexts. In addition, they also interact with and modulate the bioavailability and/or activity of growth factors and cytokines, including TGF-β, TNFα, VEGF, BMPs, and members of the Wnt family, thereby contributing to the seemingly bewildering array of processes that CCN proteins appear to influence. CCN proteins are potent regulators of angiogenesis and chondrogenesis, and play important roles in injury repair, fibrotic diseases, and cancer. Recent studies have underscored their unique ability to either promote cell death or survival as cell adhesion molecules, and to profoundly regulate the cytotoxicity of inflammatory cytokines such as TNFα. While CCN1 has been shown to be a physiologic regulator of TNFα, the full effects of CCN proteins on inflammation have yet to be thoroughly explored. Although the functional domains of CCN proteins have been delineated and several specific integrin binding sites have been identified, the three dimensional structures of CCN proteins are still unknown. Their unusually high number of conserved cysteine residues portends an interesting tertiary structure that may impact on their function.

Gene targeting studies to date have established the critical roles of *CCNs* in cardiovascular and skeletal development. However, since knockouts of *CCN1* and *CCN2* in mice result in embryonic or perinatal lethality, many aspects of their roles in later stages of development and in disease progression have yet to be uncovered. Therefore, tissue-specific and conditional ablations of CCN genes will be needed to unravel their roles in specific organs and tissues. In addition, analysis of mice with allelic replacements in which *CCN* genes are replaced by mutant alleles that abrogate specific binding sites for their receptors or other interacting proteins will likely illuminate CCN functions through specific signaling pathways (Chen et al., 2007). Given the association of CCN proteins with injury repair, it is likely that conditional ablation of CCNs will reveal defects in the physiological response to challenges of injury or stress. In this regard, RNAi-mediated knockdowns via viral vectors are already yielding useful information about the causal roles of CCNs in disease. Although aberrant expression of CCN proteins underlies pathologies such as fibrosis and cancer, their specific functions in these contexts are not well

understood. Further investigation on their mechanisms of action in disease will be necessary in contemplating the CCN signaling pathways as targets of rational therapeutic designs.

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ABBREVIATIONS

AVSD
atrioventricular septal defects

BMP
bone morphogenetic protein

ECM
extracellular matrix

FAK
focal adhesion kinase

HIF-1α
hypoxia-inducible factor-1α

HSPG
heparan sulfate proteoglycan

LRP
lipoprotein receptor-related protein

MMP
matrix metalloproteinase

MSC
mesenchymal stem cell

ROS
reactive oxygen species

SRE
serum response element

TGF-β
transforming growth factor β

TNFα
tumor necrosis factor α

TPA
12-O-tetradecanoyl-phorbol 13-acetate

VSMCs
vascular smooth muscle cells

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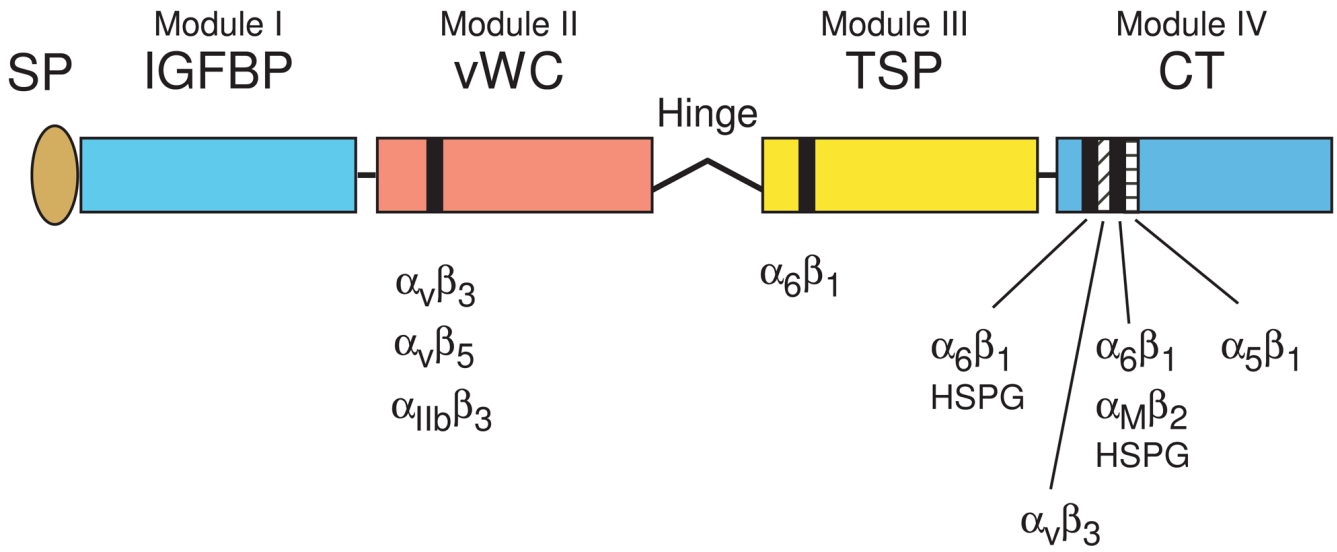

Figure 1.

Schematics of CCN protein structure and localization of their integrin binding sites. The six CCN proteins include CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1, ELM1), CCN5 (WISP-2, COP-1), and CCN6 (WISP-3). They share significant structural homology, including an N-terminal secretory signal peptide (SP), followed by modular domains (illustrated in different colors) with sequence homologies to insulin-like growth factor binding protein (IGFBP, module I), von Willebrand factor type C repeat (vWC, module II), thrombospondin type 1 repeat (TSP, module III), and a cysteine knot containing carboxyl domain (CT, module IV). Throughout the four modules are 38 cysteine residues that are highly conserved. CCN5 uniquely lacks the CT domain but conserves domains I–III. A protease-sensitive hinge region with no sequence homology among the CCN proteins separate domains II and III. Specific binding sites (black and hatched bars) for several integrins and HSPGs have been identified for CCN1 and CCN2 (Chen et al., 2000; Leu et al., 2003; Chen et al., 2004a; Leu et al., 2004; Gao and Brigstock, 2004; Gao and Brigstock, 2006).

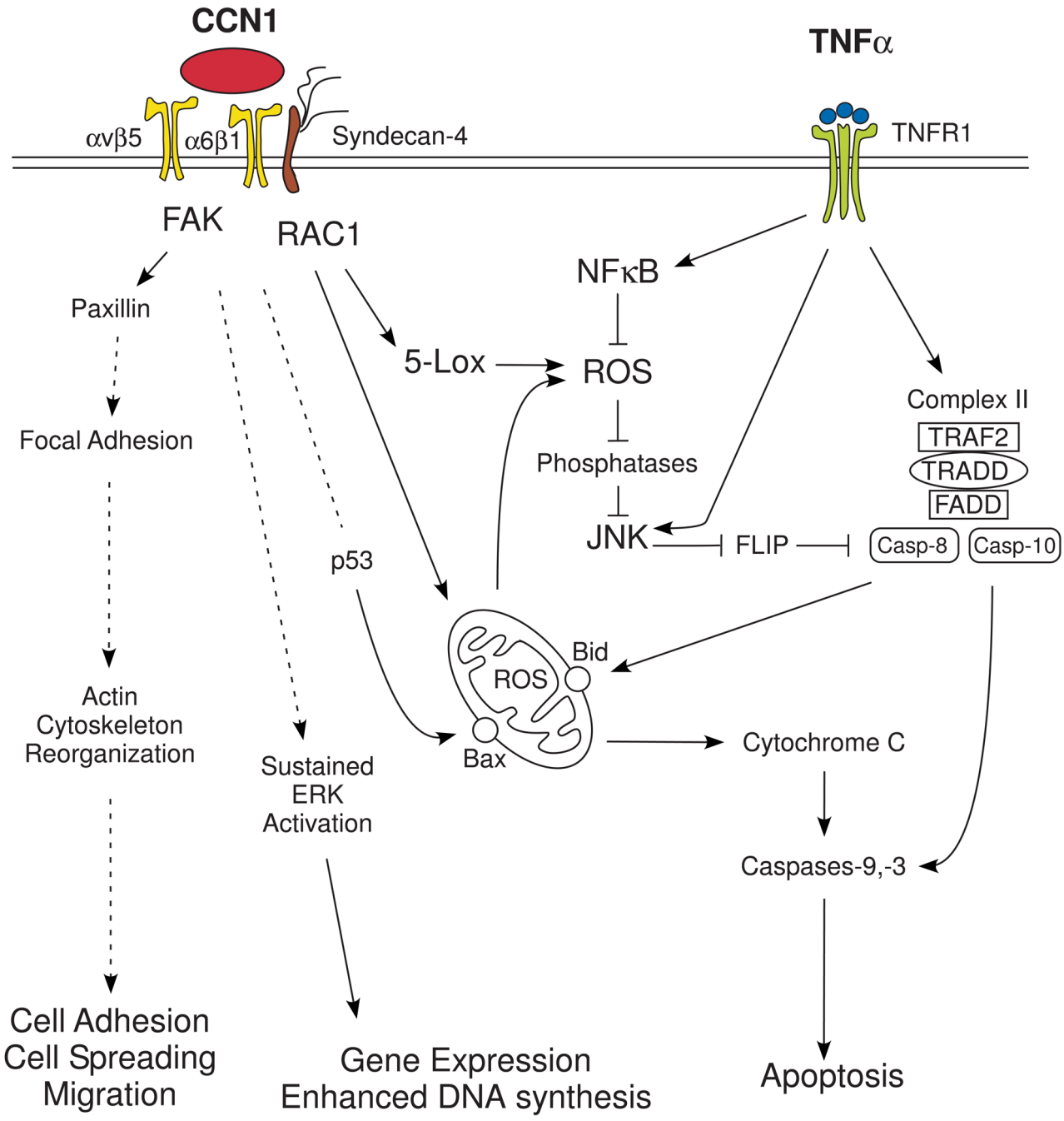

Figure 2.

CCN1 signaling and crosstalk with TNFα. Signal transduction initiated by CCN1, a prototypical member of the family, is mediated primarily through binding to $\alpha_6\beta_1$ and syndecan-4 in fibroblasts to support activities including cell adhesion, although $\alpha_v\beta_5$ is also necessary for fibroblast migration and crosstalk with TNFα (Grzeszkiewicz et al., 2001; Chen et al., 2007). Cell adhesion to CCN1 activates FAK, paxillin, and Rac1, leading to actin cytoskeleton reorganization, cell spreading, and formation of filopodia and lamellipodia (Chen et al., 2001a). Adhesion to CCN1 also induces sustained ERK activation, an activity that is mediated through binding to $\alpha_6\beta_1$-HSPG (Leu et al., 2004). CCN1 induces fibroblast apoptosis by activating p53 and Bax (Todorovic et al., 2005), and converts TNFα from a pro-mitogenic

factor into a potent apoptotic molecule through the Rac1-dependent generation of ROS via 5-lipoxygenase and the mitochondria (Chen et al., 2007). CCN1/TNFα-induced apoptosis occurs rapidly (within 4 hours of treatment) without requiring *de novo* protein synthesis, indicating that CCN1 activates this pathway directly.

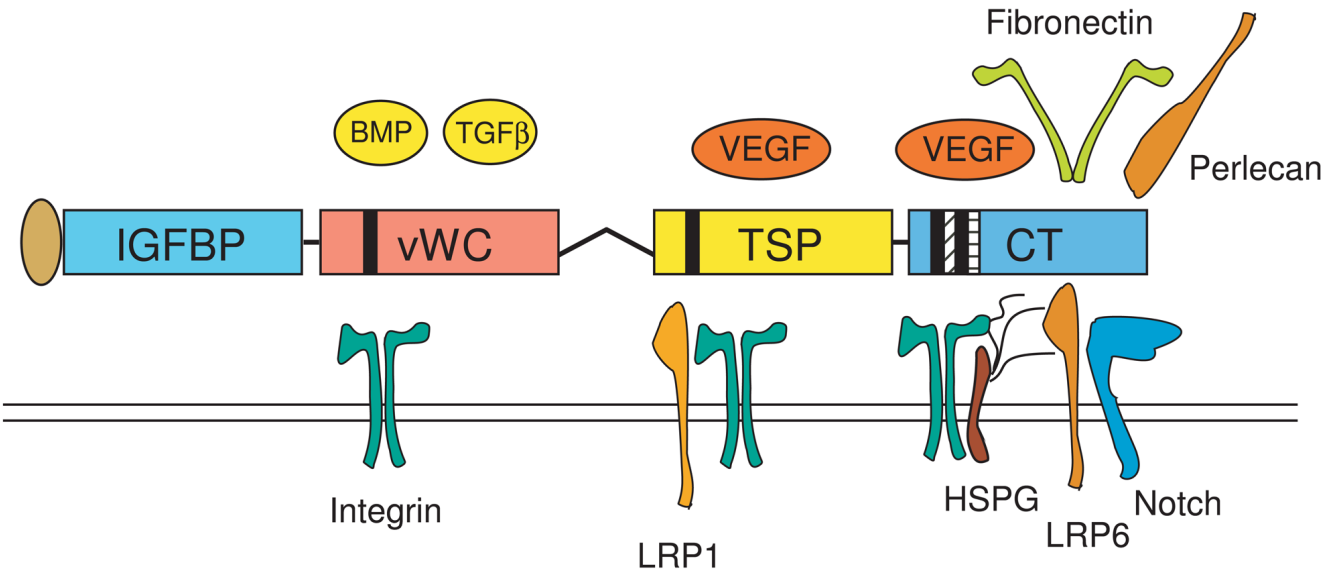

Figure 3.

Interaction of CCN proteins with other molecules. CCN proteins interact with a variety of cell surface receptors and extracellular ligands, including various integrins, HSPGs, and LRPs (Lau and Lam, 2005; Gao and Brigstock, 2003; Mercurio et al., 2004). Receptors that interact with CCN proteins are shown schematically below the four conserved CCN domains and extracellular proteins that bind CCNs are shown above, aligned with the interacting CCN domains. Whereas CCN3 binds the receptor Notch (Sakamoto et al., 2002), CCN2 has been shown to bind BMPs and TGF-β through the vWC domain and VEGF through the TSP and CT domains (Abreu et al., 2002; Inoki et al., 2002). CCN2 also binds ECM proteins such as fibronectin and perlecan through the CT domain (Nishida et al., 2003; Chen et al., 2004b).

Table I

Specific CCN-integrin interactions and activities they mediate

Integrins are cell adhesion receptors that also regulate other cellular functions. They serve as the principal receptors for CCN proteins.

Integrin	CCN protein	Activities	References
$\alpha_6\beta_1$	CCN1 CCN2 CCN3	cell adhesion, apoptosis in fibroblasts; adhesion, migration in VSMCs; synergism with TNFα cell adhesion in fibroblasts cell adhesion in fibroblasts, endothelial cells (ECs)	Chen et al., 2000; Chen et al., 2001a; Lin et al., 2003; Todorovic et al., 2005; Chen et al., 2007a
$\alpha_v\beta_3$	CCN1 CCN2 CCN3	cell adhesion, migration, DNA synthesis, cell survival in ECs; DNA synthesis in fibroblasts cell adhesion, migration, cell survival in ECs; hepatic stellate cell adhesion cell adhesion migration in ECs	Kireeva et al., 1998; Babic et al., 1999; Grzeszkiewicz et al., 2001; Leu et al., 2002; Gao and Brigstock, 2004; Ellis et al., 2003[a]
$\alpha_v\beta_5$	CCN1 CCN3	cell migration in fibroblasts, synergism with TNFα cell adhesion, migration in ECs; migration in fibroblasts	Grzeszkiewicz et al., 2002; Lin et al., 2005b; Chen et al., 2007a
$\alpha_5\beta_1$	CCN2 CCN3	cell adhesion in chondrocytes, adhesion and migration in pancreatic stellate cells cell adhesion, migration in ECs	Lin et al., 2003; Lin et al., 2005b; Ellis et al., 2003; Gao and Brigstock, 2006[b]; Hoshijima et al., 2006[c]
$\alpha_2\beta_1$	CCN1	cell adhesion	Lin et al., 2007
$\alpha_{IIb}\beta_3$	CCN1 CCN2	cell adhesion in platelets cell adhesion in platelets	Jedsadayanmata et al., 1999
$\alpha_M\beta_2$	CCN1 CCN2	cell adhesion in moncytes cell adhesion in moncytes	Schober et al., 2002
$\alpha_D\beta_2$	CCN1	cell adhesion	Yakubenko et al., 2006[d]

[a] Ellis, P. D., Metcalfe, J. C., Hyvonen, M., and Kemp, P. R. (2003) *J. Vasc. Res.* **40,** 234–243

[b] Gao, R. and Brigstock, D. R. (2006) *Gut* **55,** 856–862

[c] Hoshijima, M., Hattori, T., Inoue, M., Araki, D., Hanagata, H., Miyauchi, A., and Takigawa, M. (2006) *FEBS Lett.* **580,** 1376–1382

[d] Yakubenko, V. P., Yadav, S. P., and Ugarova, T. P. (2006) *Blood* **107,** 1643–1650

Table 2

Biological functions of CCN proteins

Functions of CCNs *in vivo* as demonstrated by knockout, knockin, knockdown, or forced expression studies are listed below, together with related activities observed *in vitro*.

Function	Effects of *CCN* gene alterations *in vivo*	Related activities *in vitro*	References
Angiogenesis and cardiovascular development	CCN1, CCN2, CCN3 stimulate blood vessel growth in corneal implants, or in ischemic hindlimb *CCN1*-null mice suffer embryonic lethality with placental vascular deficiency, embryonic vessel hemorrhage, and atrioventricular septal defects *CCN1*$^{+/-}$ mice are viable but exhibit ostium primum atrial septal defect *CCN3* mutant mice show cardiac septal defects	CCNs promote pro-angiogenic activity in microvascular endothelial cells: support cell adhesion, stimulate cell migration, enhance proliferation and survival, induce endothelial tubule formation	Mo et al., 2002; Mo and Lau, 2006; Heath et al., 2008 Babic et al., 1998; Babic et al., 1999; Fataccioli et al., 2002; Leu et al., 2002; Lin et al., 2003
Skeletal development	*CCN2*-null mice are perinatal lethal, showing severe chondrodisplasia, deficient ECM production in cartilage, impaired endochondrial ossification and reduced growth plate angiogenesis *CCN3* mutant mice show axial and appendicular skeletal defects and severe joint malformation *CCN2 and CCN3* overexpression in osteoblasts leads to osteopenia in mice	CCN1 promotes chondrogenesis in micromass cultures; CCN2 promotes chondrocyte proliferation and differentiation, synthesis of collagen and aggrecan, and osteogenic differentiation CCN2 binds BMP4 and inhibits its function CCN3 binds BMP2 and inhibits BMP2-induced osteogenic differentiation	Ivkovic et al., 2003; Rydziel et al., 2007; Heath et al., 2008; Smerdel-Ramoya et al., 2008 Abreu et al., 2002; Kubota and Takigawa, 2007b
Cell survival	*CCN1*-null mice show aberrant apoptosis in vascular cells of large arteries and mesenchymal cells of the cardiac cushion tissue	CCN proteins promote endothelial cell survival	Mo et al., 2002; Mo and Lau, 2006 Babic et al., 1999; Leu et al., 2002; Lin et al., 2003
Apoptosis	*CCN1* knockin mice expressing an apoptosis-defective allele in place of wild type *CCN1* are resistant to TNFα-induced apoptosis	CCN proteins can unmask the cytotoxicity of TNFα by inducing ROS accumulation through 5-lipoxygenase and the mitochondria	Chen et al., 2007
Fibrosis	Coinjection of both CCN2 and TGF-β, but not injection of either factor alone, induces sustained fibrosis *CCN2* knockdown in the liver prevents chemically-induced liver fibrosis; ectopic expression of *CCN2* in the lung render mice that are resistant to bleomycin-induced lung fibrosis to become susceptible	CCN2 potentiates TGFβ activity, promotes matrix protein synthesis CCN2 supports the adhesion of hepatic stellate cells and oval cells, and stimulate stellate cell proliferation and oval cell migration	Mori et al., 1999a; Paradis et al., 2002; Li et al., 2006; George and Tsutsumi, 2007; Pi et al., 2008
Diabetic Nephropathy	*CCN2* overexpression in podocytes in mice worsens diabetic nephropathy; *CCN2* knockdown in the kidney reduces diabetic nephropathy and renal fibrosis	CCN2 promotes cell survival, matrix deposition, and inhibits glucose effect on matrix degradation in mesangial cells	Yokoi et al., 2004; Guha et al., 2007; Yokoi et al., 2008
Restenosis	*CCN1* down-regulation by siRNA or FOXO3a reduces neointimal hyperplasia after balloon angioplasty, an effect that is reversed by replenishment of *CCN1* via gene transfer	CCN1 induced cell adhesion and migration in vascular smooth muscle cells	Lee et al., 2007; Matsumae et al., 2008; Grzeszkiewicz et al., 2002
Cancer	*CCN1* overexpression in gastric cancer cells, breast cancer cells and ovarian cancer cells enhances tumorigenicity in nude mice. Ectopic or stromal expression of *CCN2* promotes tumorigenicity of esophageal squamous cell carcinoma or prostate cancer cells, respectively	CCNs promote angiogenesis, enhance cancer cell proliferation and resistance to apoptosis	Babic et al., 1998; Hashimoto et al., 1998; Zhang et al., 1998; Xie et al., 2001; Tsai et al., 2002; Gery et al., 2005; Aikawa et al., 2006; Dornhofer et al., 2006; Shimo et al., 2006; Yang et al., 2005; Deng et al., 2007

Function	Effects of *CCN* gene alterations *in vivo*	Related activities *in vitro*	References
	CCN2 antibody treatment suppresses pancreatic tumor growth and breast cancer osteolytic bone metastasis Expression of *CCN1* inhibits tumorigenicity of non-small cell lung carcinoma cells in nude mice, and *CCN2* expression suppresses metastasis of human lung adenocarcinoma cells and colorectal cancer cells Expression of *CCN4* or *CCN5* inhibits tumorigenicity of murine melanoma cells or transformed fibroblasts, respectively		