All in the CCN family: essential matricellular signaling modulators emerge from the bunker

Andrew Leask[1,*] and David J. Abraham[2]

[1]CIHR Group in Skeletal Development and Remodeling, Division of Oral Biology, and Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, Dental Sciences Building, London, ON N6A 5C1, Canada
[2]Centre for Rheumatology and Connective Tissue Diseases, Royal Free and University College Medical School, University College London (Royal Free Campus), Rowland Hill Street, London, NW3 2PF, UK
*Author for correspondence (e-mail: Andrew.Leask@schulich.uwo.ca)

Accepted 20 September 2006
Journal of Cell Science 119, 4803-4810 Published by The Company of Biologists 2006
doi:10.1242/jcs.03270

Summary

The CCN family is a group of six secreted proteins that specifically associate with the extracellular matrix. Structurally, CCN proteins are modular, containing up to four distinct functional domains. CCN family members are induced by growth factors and cytokines such as TGFβ and endothelin 1 and cellular stress such as hypoxia, and are overexpressed in pathological conditions that affect connective tissues, including scarring, fibrosis and cancer. Although CCN family members were discovered over a decade ago, the precise biological role, mechanism of action and physiological function of these proteins has remained elusive until recently, when several key mechanistic insights into the CCN family emerged. The CCNs have been shown to have key roles as matricellular proteins, serving as adaptor molecules connecting the cell surface and extracellular matrix (ECM). Although they appear not to have specific high-affinity receptors, they signal through integrins and proteoglycans. Furthermore, in addition to having inherent adhesive abilities that modulate focal adhesions and control cell attachment and migration, they execute their functions by modulating the activity of a variety of different growth factors, such as TGFβ. CCN proteins not only regulate crucial biological processes including cell differentiation, proliferation, adhesion, migration, apoptosis, ECM production, chondrogenesis and angiogenesis, but also have more sinister roles promoting conditions such as fibrogenesis.

Key words: CCN1, CCN3, Connective tissue growth factor, Integrins, Signal transduction, CCN2

Introduction

Named after three prototypical members, cysteine-rich protein 61 (Cyr61; also known as CCN1), connective tissue growth factor (CTGF; also known as CCN2) and nephroblastoma overexpressed protein (Nov; also known as CCN3), the CCN family comprises six secreted proteins grouped together on the basis of a similar predicted modular secondary structure (Fig. 1) (Bork, 1993; Perbal, 2004)[1]. CCN proteins comprise four modules: an insulin-like growth factor binding protein (IGFBP) domain (module I), a Von Willebrand factor domain (module II), a thrombospondin-homology domain (module III), and a cysteine knot, heparin-binding domain (module IV) (Bork, 1993; Perbal, 2004). Befitting secreted proteins, each also possesses a signal sequence (Lechner et al., 2000; Chen, Y. et al., 2001) (Fig. 1). Between modules II and III is the 'hinge region', which is susceptible to proteinase cleavage. Indeed, in biological fluids, CCN2 can be found as fragments, including N-terminal and C-terminal halves cleaved in the 'hinge region', as well as the individual 10-12 kDa C-terminal heparin-binding domain (module IV) (Brigstock et al., 1997). These variations in structure may have a direct effect on CCN function.

[1]Although each of the six members was initially given their own distinct name, the meeting in 2000 of the International CCN Society in St Malo, France, unified the nomenclature to the CCN family members 1-6 in order to reflect their structural similarity (Brigstock et al., 2003).

The three prototypical members of the CCN family were originally identified ~15 years ago (O'Brien et al., 1990; Bradham et al., 1991; Joliot et al., 1992). Since their discovery, >800 papers on CCN2, ~200 papers on CCN1 and ~100 papers on CCN3 have been published. Substantially fewer papers have examined CCN4, CCN5 and CCN6. In part, progress in this field has been hampered by a lack of unrestricted, readily commercially available reagents, such as 'gold standard' recombinant material and neutralizing antibodies. Consequently in vitro studies have used a variety of different protein sources, purification procedures and antibodies. Indeed, a major difficulty in the CCN field has been the purification of active proteins, presumably because of the presence of repeated cysteine residues, which require the use of mammalian expression systems such as baculovirus or stable cell lines.

Initially, it was believed that these proteins were classical growth factors, and that simple application of recombinant material to cells was sufficient to recapitulate the entire range of CCN-dependent activities. The collective work of many laboratories, and especially the recent development of transgenic and knockout mice, has resulted in a greater appreciation of the range and complexity of CCN action. Indeed, it is now established that the CCN proteins are not growth factors and thus should not be referred to as such. This fact was a principal driving force within the CCN community

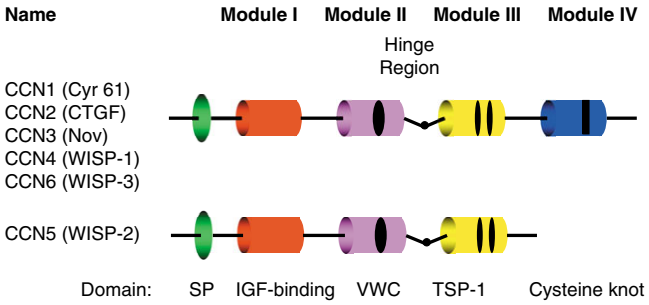

Fig. 1. Structure of CCN family members. The CCN family members, CCN1 (Cyr61), CCN2 (CTGF), CCN3 (nov), CCN4 (WISP-1), CCN5 (WISP-2) and CCN6 (WISP-3) have a shared structure, consisting of a secretory signal peptide (SP), an IGF-binding domain (Module I), a von Willebrand type C domain (VWC, Module II), a thrombospondin-1 domain (TSP-1, Module III) and a cysteine knot (Module IV) domain. Domains are linked by hinge regions, susceptible to protease cleavage.

to rename each family member. We now appreciate that, although the CCN proteins indeed have some independent activity, they principally modify signaling of other molecules, the identity of which can widely vary, depending on the particular biological system. CCN proteins stimulate mitosis, adhesion, apoptosis, extracellular matrix production, growth arrest and migration of multiple cell types. As a result, they play essential roles in development, wound healing and disease. The reader is referred to a recent comprehensive book on this subject (Perbal and Takigawa, 2005). Here, we summarize current knowledge of role and function of CCN family members in these processes.

CCN receptors

Much effort has been made to identify specific receptors mediating the effects of CCN family members. CCN family members appear not to rely upon a unique, specific signaling receptor. Rather, CCN1, CCN2 and CCN3 all directly bind to specific integrins through discrete and often separate domains, several of which have been mapped by peptide inhibition studies (e.g. Ellis et al., 2003; Schober et al., 2003; Leu et al., 2003; Leu et al., 2004; Gao and Brigstock, 2004; Chen, N. et al., 2004) (Fig. 3). That CCN proteins bind integrins was first shown by Kireeva et al. (Kireeva et al., 1998), who showed CCN1 binds to integrin $\alpha v\beta 3$. The integrins bound include the principal integrins mediating angiogenesis and matrix attachment, such as $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ (Chen et al., 2000; Ellis et al., 2003; Leu et al., 2003; Gao and Brigstock, 2004; Chen, Y. et al., 2004; Lau et al., 2005); however, it is not known whether CCN proteins display preferential binding to α or β integrin subunits. Both CCN1 and CCN2 are ligands of integrins $\alpha IIb\beta 3$ in platelets and $\alpha M\beta 2$ in monocytes (Jedsadayanmata et al., 1999; Schober et al., 2003).

CCN1 and CCN2 also interact with heparan-sulfate-containing proteoglycans (HSPGs), including syndecan 4 and perlecan, through a heparin-binding domain in module IV (Chen, Y. et al., 2004; Nishida et al., 2003; Todorovicc et al., 2005) (Fig. 3). CCN4 binds the HSPGs decorin and biglycan (Desnoyers et al., 2001). As discussed below, integrins and HSPGs are essential for the adhesive and mitogenic function of CCN proteins and should be properly considered to be the functional receptors for this family (Chen, Y. et al., 2004; Nishida et al., 2003; Todorovicc et al., 2005). In addition, syndecan 4 and integrin $\alpha 6\beta 1$ are required for CCN1 to induce apoptosis in fibroblasts (Todorovicc et al., 2005). CCN2 also binds to the low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) (Segarini et al., 2001), in a heparin-dependent fashion, through module III (Gao and Brigstock, 2003). Furthermore, it can bind to the Wnt co-receptor LDL-receptor-related protein 6 (LRP6) through the C-terminal (CT) domain (LRP6) (Mercurio et al., 2004). Finally, CCN2 can bind to the tyrosine kinase receptor TrkA (Wahab et al., 2005). Therefore multiple biological activities can be elicited through the interactions of discrete 'functional' domains of the CCN family members and different cell surface receptors.

CCN proteins: role in adhesion, migration and signaling

As might be expected from proteins that bind integrins and proteoglycans, CCN family members are independently active in standard adhesion assays identical to those used for type I collagen and fibronectin. CCN proteins promote adhesion through both integrins and HSPGs, although the identity of the integrins and HSPGs differ, depending on the system and cell type examined (Chen et al., 2000; Ellis et al., 2003; Leu et al., 2003; Gao and Brigstock, 2004; Chen, Y. et al., 2004). This presumably reflects the relative importance of the particular integrin to the adhesive ability of fibroblast compared with endothelial cells rather than an inherent specificity for CCN family members toward any particular integrins or HSPG. In addition to integrins and proteoglycans, CCN2-dependent adhesion can also involve LRP1 (Gao and Brigstock, 2003).

Fibroblasts plated on CCN2 activate the ERK pathway, which is required for their ability to attach to CCN2 (Chen, Y. et al., 2004). Unlike cells adhering to fibronectin, fibroblasts adhering to CCN1 or CCN2 need not spread properly or generate actin fiber networks and phosphorylate focal adhesion kinase (FAK) (Chen, Y. et al., 2004; Latinkic et al., 2003). However, in some systems, such mature cell spreading can occur, and FAK phosphorylation occurs in response to CCN1, CCN2 and CCN3 (Li et al., 2002; Chen, C. C. et al., 2001). Intriguingly, immobilized recombinant CCN2 regulates migration of mesangial cells (Blom et al., 2001) by promoting dephosphorylation of FAK (Crean et al., 2004). Furthermore, CCN2-deficient mouse embryonic fibroblasts (MEFs) show defects in cell adhesion to fibronectin, including a significant reduction in FAK and ERK phosphorylation and delays in cell spreading and formation of actin stress fibers (Chen, Y. et al., 2004).

In normal mouse embryo fibroblasts CCN2 is located within a complex composed of fibronectin and the fibronectin receptors, integrin $\alpha 4\beta 1$ and integrin $\alpha 5\beta 1$ and syndecan 4 (Chen, Y. et al., 2004). Although likely, it is not clear whether other CCN molecules are also present in a complex of matrix and matrix receptors. One important physiological function of CCN proteins therefore appears to be to integrate cellular adhesion responses with the extracellular matrix environment, which is consistent with the proposed crucial role of CCN family members as adapter molecules integrating signaling between extracellular ligands and their receptors (Perbal, 2004) (Fig. 2). Note, however, that although the observations discussed above suggest that CCN proteins have some inherent

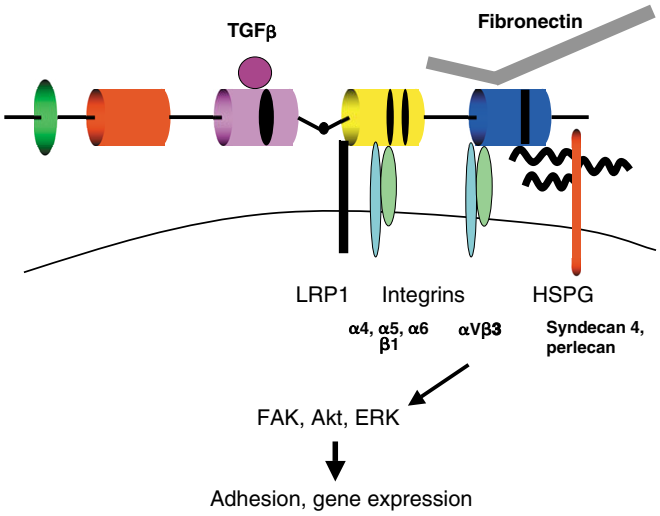

Fig. 2. Signaling by CCN family members. CCN1, CCN2 and CCN3 bind TGFβ, fibronectin, integrins, LRP1 and HSPGs as indicated. CCN proteins appear to signal principally through the C-terminal quarter (domain IV) to activate adhesive signaling pathways and hence amplify responses to TGFβ or fibronectin.

adhesive ability, their physiological function may be to modify, rather than be the immediate cause of, cellular adhesive responses.

CCNs as co-factors for the ECM, growth factors and cytokines

In addition to their ability to bind integrins and HSPGs, CCN proteins can also bind growth factors and cytokines. For example, CCN2 binds TGFβ, through the N-terminal Von-Willebrand factor domain (module II) of CCN2 (Abreu et al., 2002) (Fig. 2), and this may play an important role in augmenting TGFβ activity (see below). It also binds to fibronectin, through module IV (Chen, Y. et al., 2004; Hoshijima et al., 2006) (Fig. 2) and to VEGF (Inoki et al., 2002). Similarly, CCN3 binds connexin 43, although the domain of CCN3 mediating this action is unknown (Fu et al., 2004). CCN3 also associates with the epidermal-growth-factor-like repeats of Notch1 through its C-terminal cysteine knot domain (Sakamoto et al., 2002).

Consistent with the notion that CCN proteins promote signaling from growth factors is the observation that CCN1 does not possess mitogenic activity on its own, but rather enhances that of FGF and PDGF (Kireeva et al., 1996). Similarly, CCN2 also enhances FGF- or EGF-induced DNA synthesis, but lacks mitogenic activity alone (Kireeva et al., 1997; Grotendorst and Duncan, 2005). CCN2 induces differentiation of myofibroblasts and increased collagen synthesis in concert with EGF, insulin-like growth factor 2 or insulin (Grotendorst et al., 2004; Gore-Hyer et al., 2003). Moreover, although not mitogenic on its own, CCN3 enhances basic-FGF-induced DNA synthesis and upregulates matrix metalloproteinase (MMP)-1 and PAI-1 expression (Lafont et al., 2005a; Lin, C. et al., 2005). In some studies, however, CCN2 has been shown to possess independent yet modest proliferative activity (~20% above control) (Asano et al., 2005). The proliferative activity of CCN2 resides in domain IV and acts through Ras/MEK/ERK signaling (Gao et al., 2004).

Significantly, CCN3 promotes FGF- and PDGF-mediated proliferation of C2C12 myoblast cells in an integrin-dependent fashion (Lafont et al., 2005a). Through integrins, CCN proteins thus probably modify signaling responses to other proteins and, in principle, CCN molecules could modify a wide range of signal transduction pathways. A corollary of this is that CCN proteins need not necessarily have independent activity; their physiological effects may depend on the action of the particular partner with which the CCN proteins interact.

CCN proteins in tissue repair and fibrosis

Possibly the best insight into the physiological relevance of the interactions between CCN and extracellular ligands comes from observations concerning the contribution of CCN2 to tissue repair following injury and in scarring or fibrotic responses. In adults, CCN1, CCN2 and CCN3 are induced during tissue repair (Igarashi et al., 1993; Latinkic et al., 2001; Lin, C. et al., 2005). Elevated, constitutive CCN2 expression is a hallmark of fibrosis (Blom et al., 2002; Leask and Abraham, 2003). Transient overexpression of CCN2 results only in a minimal fibrotic response, however (Mori et al., 1999; Bonniaud et al., 2003). Moreover, CCN2-deficient MEFs express α-smooth muscle actin (α-SMA) and type I collagen (Shi-wen et al., 2006a), which indicates that CCN2 is not required for the basal expression of these proteins in embryonic fibroblasts.

What inappropriate overexpression of CCN2 appears to do is to create an environment permissive for other stimuli to induce potent fibrotic responses. For example, overexpression of CCN2 results in fibrosis in mice that are otherwise resistant to developing pulmonary fibrosis in response to bleomycin (Bonniaud et al., 2004), a model that is TGFβ dependent (Zhao et al., 2002). Simultaneous co-injection of CCN2 and TGFβ causes sustained fibrotic responses in vivo, in contrast to application of TGFβ alone, which causes only a transient fibrotic response that depends on the constant injection of ligand (Mori et al., 1999). Indeed, although CCN2 is induced by TGFβ and has long been hypothesized to be a downstream mediator of at least some of the effects of TGFβ (Grotendorst, 1997), recent evidence suggests that, in fact, CCN2 is an essential co-factor for and augments TGFβ activity. In cultured *Xenopus* cells, CCN2 binds TGFβ and enhances the ability of TGFβ to bind TGFβ receptors at low TGFβ concentrations and hence indirectly affects Smad-responsive promoters (Abreu et al., 2002).

CCN2 is constitutively expressed in fibrotic and embryonic fibroblasts independently of TGFβ (Holmes et al., 2001; Holmes et al., 2003; Chen et al., 2006). Experiments using *Ccn2*$^{-/-}$ MEFs have shown that loss of CCN2 results in an inability of TGFβ to induce expression of approximately one-third of those mRNAs induced in *Ccn2*$^{+/+}$ MEFs (Shi-wen et al., 2006a). Consistent with the fact that CCN2 is required only for a subset of TGFβ responses, *Ccn2*$^{-/-}$ MEFs show no impairment of the generic Smad pathway, emphasizing the relative selectivity of CCN2-dependent action (Shi-wen et al., 2006a). In contrast to the lack of effect of loss of CCN2 expression on basal type I collagen and α-SMA expression, the ability of TGFβ to induce these proteins is impaired in *Ccn2*$^{-/-}$ MEFs (Shi-wen et al., 2006a). Intriguingly, the ability of

TGFβ to activate adhesive FAK/PI3kinase/Akt signaling is significantly impaired in *Ccn2*$^{-/-}$ fibroblasts, and this pathway is necessary for optimal induction of CCN2-dependent genes in wild-type MEFs (Shi-wen et al., 2006a) (Fig. 2). Induction of α-SMA by TGFβ has been shown to be FAK-, adhesion- and integrin-dependent (Thannickal et al., 2003), which supports the notion that integrins are functional receptors for CCN2. Real-time PCR analysis has revealed that CCN2-dependent transcripts require CCN2 even at the extremely early time-points examined, before de novo induction of CCN2 protein (Shi-wen et al., 2006a). Thus, in MEFs, CCN2 appears to be an essential cofactor required for TGFβ to induce adhesive signaling responses and the correct signals for the formation of myofibroblasts. Indeed, the ability of TGFβ to induce adhesion to the matrix is impaired in *Ccn2*$^{-/-}$ MEFs (Shi-wen et al., 2006a). The results of these in vivo studies are therefore consistent with the in vivo experiments described above and confirm that CCN2 is required for maximal adhesive signaling in fibroblasts undergoing active tissue remodeling, such as in embryogenesis, fibrotic cells or tumor stroma (Blom et al., 2002; Yang et al., 2005). These results also suggest that CCN proteins act by enhancing signals not only from the extracellular matrix but also from growth factors through integrin-dependent pathways.

CCN proteins play crucial roles in bone formation

Integrins and adhesive signaling are essential for the tissue remodeling necessary for embryonic development (Thiery, 2003). CCN family members might therefore play key roles in this process. Indeed, substantial evidence suggests that CCN proteins are essential for development. In cell culture systems, CCN proteins have long been known to promote differentiation and proliferation of chondrocytes and osteoblasts (for a review, see Takigawa et al., 2003). CCN2 promotes proliferation and differentiation of osteoblast and chondrocyte cell lines (Nishida et al., 2000; Nakanishi et al., 2000). Similarly, CCN1, CCN3 and CCN6 induce expression of chondrogenic markers (Wong et al., 1997; Lafont et al., 2005b; Sen et al., 2004). CCN4 promotes mesenchymal cell proliferation and osteoblastic differentiation while repressing chondrocytic differentiation (French et al., 2004).

All CCN family members are expressed in chondrocytes and osteoblasts and are induced during fracture repair (Nakata et al., 2002; French et al., 2004; Schutze et al., 2005; Parisi et al., 2006), and each family member appears to respond differently to stimuli. For example, CCN1 expression and CCN6 expression decrease during chondrogenic differentiation, but only CCN6 RNA expression is reduced during osteogenic differentiation (Schutze et al., 2005). In osteoblasts, TGFβ enhances CCN1, CCN2 and CCN5 expression but decreases CCN4 expression (Parisi et al., 2006). Intriguingly, CCN1 is activated by Wnt3A, and RNAi-mediated knockdown of CCN1 diminishes Wnt3A-induced osteogenic differentiation (Si et al., 2006). This indicates that CCN1 is an essential mediator of Wnt signaling (Si et al., 2006). Thus, although CCN proteins appear to have similar functions in vitro, each member may have different physiological effects in vivo depending on their different regulation and expression profiles.

Perhaps the most significant recent insights into the specific physiological roles of the CCN family have come from the generation of mutant mice lacking CCN2 (Ivkovic et al., 2003) or CCN1 (Mo et al., 2002). *Ccn2*$^{-/-}$ mice display severely malformed ribcages and die soon after birth owing to a failure to breathe (Ivkovic et al., 2003). These mice exhibit impaired chondrocyte proliferation and proteoglycan production within the hypertrophic zone (Ivkovic et al., 2003). Excessive chondrocytic hypertrophy and a concomitant reduction in endochondral ossification are also observed (Ivkovic et al., 2003). Further support for the idea that CCN2 regulates bone formation in development comes from studies of transgenic mice that overproduce CCN2 under the control of the mouse type XI collagen promoter. These mice develop normally but show dwarfism within a few months of birth owing to a reduced bone density (Nakanishi et al., 2001). The molecular basis for this deformity has not yet been explored; however, a possible explanation is that CCN2 overexpression results in abnormally premature ossification, before proper chondrocyte maturation. Point mutations in CCN6 have been linked to the autosomal recessive skeletal disease progressive pseudorheumatoid dysplasia (PPD), a human disease (Hurvitz et al., 1999), resulting in juvenile-onset cartilage degeneration. Conversely, mice in which exons III, IV and V of CCN6 were deleted, resulting in absence of CCN6 expression, showed no apparent phenotype (Kutz et al., 2005). In this study CCN6 expression was not detected anywhere in the mouse (Kutz et al., 2005). Although it is possible that complete loss of CCN6 expression in *ccn6*$^{-/-}$ mice may have resulted in the compensatory overexpression of other CCN family members, it is also plausible that additional factors than CCN6 may be the cause of PPD.

CCN family members contribute to angiogenesis and cancer

CCN proteins probably also have a key role during angiogenesis in development. Recombinant CCN1, CCN2 and CCN3 promote angiogenesis in vivo, when applied alone subcutaneously into corneas or in a chick chorioallantoic membrane assay (Babic et al., 1998; Babic et al., 1999; Shimo et al., 1999; Lin et al., 2003). Angiogenic activity of CCN1 has been confirmed in a rabbit ischemic hindlimb assay (Fataccioli et al., 2002). CCN1 promotes endothelial tubule formation in vitro through integrin αvβ3 (Leu et al., 2002). At much higher doses than those that promote angiogenesis, CCN2 can suppress the angiogenic activity of VEGF (Inoki et al., 2002). MMPs can cleave CCN2, neutralizing this effect (Inoki et al., 2002). Individual CCN family members may thus have different effects based on their doses or level of expression. The CCN1-null mutation is embryonic lethal: ~30% of those mice that die exhibit a complete failure in chorioallantoic fusion, whereas the remainder perish as a result of placental vascular insufficiency and compromised vessel integrity (Mo et al., 2002). These observations provide clear evidence for a key physiological role for the protein in angiogenesis. Although CCN2-null mutants do not exhibit defects in vessel integrity or placental vasculature, they display angiogenic deficiency in the growth plates during endochondral bone formation (Ivkovic et al., 2003). These results suggest that, although recombinant CCN1 and CCN2 have similar effects on cells, they have non-overlapping functions in vivo.

Aberrant expression of the CCNs is associated with cancer and vascular disease (Pennica et al., 1998; Gupta et al., 2001; Rachfal et al., 2004; Holloway et al., 2005; Zhang et al., 2005).

For informative, focused reviews on the role of CCNs in cancer, the reader is referred elsewhere (Mendenez et al., 2003; Rachfal and Brigstock, 2005). Confirming a role for CCN1 in cancer, ectopic expression of CCN1 enhances the growth of ovarian cancer cells in liquid culture and increases tumorigenicity in nude mice, whereas inhibition of CCN1 expression decreases proliferation and increases apoptosis in these cells (Gery et al., 2005). Similarly, patients with gastric adenocarcinomas display levels of CCN1 that correlate well with aggressive lymph node metastasis, more advanced tumor stage, histologically diffuse type, and early recurrence (Lin, M. et al., 2005).

Treatment of mice with a CCN2-neutralizing antibody greatly decreases osteolytic bone metastasis, the appearance of microvasculature, and suppresses the growth of subcutaneous tumors (Shimo et al., 2006). Similarly, in a recent study, anti-CCN2 antibody decreased tumor growth and metastasis and attenuated tumor angiogenesis and cancer cell proliferation in vitro and in vivo models of pancreatic cancer (Aikawa et al., 2006). Conversely, CCN5, which lacks the pro-proliferative module IV (Fig. 1), suppresses proliferation and its expression is reduced in cancers (Mason et al., 2004). Intriguingly, expression of CCN3, although it possesses module IV, inversely correlates with tumorigenicity (Gupta et al., 2001); since CCN3 binds connexin, it might promote the formation of gap junctions, which would reduce metastasis (Gellhaus et al., 2004; Fu et al., 2004). RNAi-mediated CCN6 inhibition promotes neoplastic progression, as visualized by increased anchorage-independent growth of human mammary epithelial cells, and elevated responsiveness to the mitogen insulin-like growth factor 1 (Zhang et al., 2005). These results suggest that, although CCN family members share similar structures, their activities differ, presumably because of differences in their amino acid sequences and the proteins with which they interact. Furthermore, they suggest that altering the relative expression levels of individual CCN family members may have profound physiological and pathological effects.

CCN gene regulation

As discussed above, although CCN proteins may have similar activities in vitro, the net effect of their contribution to physiology may be based on their abilities to respond to different stimuli. Of the CCN family, the only member whose gene regulation has been characterized in detail is CCN2. CCN2 is primarily regulated at the level of transcription (Grotendorst et al., 1996; Holmes et al., 2001; Holmes et al., 2003; Chen et al., 2002; Leask et al., 2003). Similarly, faithful expression of a reporter gene in transgenic mice, including in development and in response to wound healing, can be achieved if a 2 kb fragment of the *CCN1* promoter is used, which indicates that this gene is also regulated at the level of transcription (Latinkic et al., 2001). Synthesis of CCN2 protein and mRNA is stimulated by specific growth factors, such as endothelin 1 and TGFβ, in addition to environment changes such as hypoxia and biomechanical stimuli (Grotendorst et al., 1996; Holmes et al., 2001; Ott et al., 2003; Leask et al., 2003; Shi-wen et al., 2004; Higgins et al., 2004) (Fig. 2). TGFβ also induces CCN1, CCN4 and CCN5, but not CCN6, and reduces CCN3 expression (Lafont et al., 2002; Sakamoto et al., 2004; Parisi et al., 2006); data obtained regarding the control of CCN2 gene expression by TGFβ are therefore likely to be applicable for CCN1, CCN4 and CCN5. Indeed, the CCN1 promoter has been shown to contain a 'classical' Smad-binding motif (Bartholin et al., 2006). TGFβ-mediated induction of CCN2 mRNA in fibroblasts occurs within 30 minutes of TGFβ treatment, without involving de novo protein synthesis (Grotendorst et al., 1996). It is severely impaired in fibroblasts lacking Smad3 (Holmes et al., 2001). Indeed, a functional Smad element resides within the *CCN2* promoter; Smad 3 and 4 potently activates, whereas Smad 7 suppresses, the *CCN2* promoter through this motif (Holmes et al., 2001) (Fig. 3).

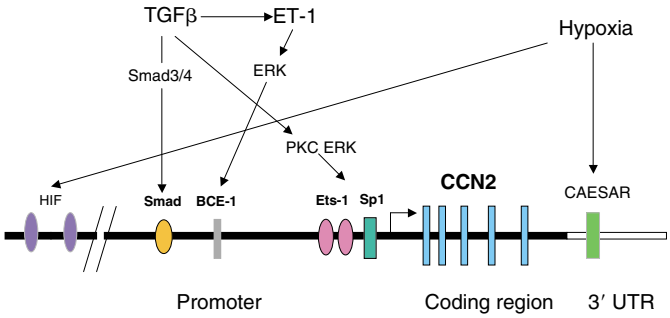

Fig. 3. Regulation of the *CCN2* promoter and 3′ untranslated region (3′ UTR). The *CCN2* promoter contains recognition sequences for HIF, Smad, BCE-1, Ets-1 and Sp1, as indicated. The 3′ UTR of the gene (white rectangle) contains a cis-acting element of structure-anchored repression (CAESAR). Hypoxia, TGFβ and endothelin-1 induce *CCN2* as indicated.

The ability of TGFβ to induce CCN2 also requires protein kinase C and the Ras/MEK/ERK MAP kinase cascade (Chen et al., 2002; Stratton et al., 2002; Leask et al., 2003) (Fig. 3). As in the case of other TGFβ-responsive promoters that do not require the transcription factor AP-1, the induction of CCN2 by TGFβ is antagonized by hyperactive AP-1 or Jun N-terminal kinase (JNK) (Leask et al., 2003), because of the ability of active Jun to bind to Smads off DNA and inhibit Smads from interacting with the target DNA sequences (Verrecchia et al., 2001). Intriguingly, the TGFβ-mediated suppression of CCN3 expression is Smad independent but JNK dependent (Lafont et al., 2002). The Smad element of the *CCN2* promoter acts in concert with a tandem repeat of an ETS element (Leask et al., 2001; Leask et al., 2003). Endogenous Ets-1 transcription factor binds this element and is required for the induction of *CCN2* by TGFβ (van Beek et al., 2006) (Fig. 2). Additional elements that contribute primarily to the basal transcriptional activity of the *CCN2* promoter include a BCE-1 (basal control element 1) site and an Sp1 site (Holmes et al., 2001; Holmes et al., 2003; Chen et al., 2002). The BCE-1 site also mediates the Ras/MEK/ERK-dependent response of the *CCN2* promoter to endothelin 1 (Shi-wen et al., 2004). Because endothelin is induced by TGFβ (Rodriguez-Pascual et al., 2003; Shi-wen et al., 2006b), BCE-1 is probably indirectly involved in the induction of *CCN2* by TGFβ. Befitting a gene associated with angiogenesis, *CCN2* is also induced by hypoxia through a hypoxia-inducible factor (HIF)-response element (Higgins et al., 2004) (Fig. 3). In addition, *CCN2* mRNA displays a degree of post-transcriptional regulation, primarily concerned with message stability. Thus there is a minimal 84-nucleotide element in the CCN2 mRNA 3′-UTR, which acts as a cis-acting element of structure-anchored

repression (CAESAR) in a chondrocyte cell line (Kubota et al., 2000) and also contributes to hypoxia-mediated CCN2 mRNA stabilization (Kondo et al., 2006) (Fig. 3). Post-transcriptional regulatory mechanisms might similarly control the expression of other CCN family members.

CCN2 overexpression is a fairly robust biological marker for fibrotic diseases (Blom et al., 2002). An ELISA that detects CCN2 in biological fluids is a very useful marker of the severity of fibrosis in diseases such as scleroderma and diabetic nephropathy (Dziadzio et al., 2005; Nguyen et al., 2006). CCN2 overexpression in the fibrotic disease scleroderma is independent of TGFβ and Smads but dependent on BCE-1 and Sp1 (Abraham et al., 2000; Holmes et al., 2001; Holmes et al., 2003). It is likely therefore to depend in part on the expression of endothelin 1. Indeed, antagonism of the A and B endothelin receptors significantly reduces the overexpression of CCN2 in scleroderma (X. Shi-wen, A.L. and D.J.A., unpublished results); aberrant endothelin-1-dependent overexpression of CCN2 thus appears to be an important hallmark of fibrogenesis (Leask and Abraham, 2004). Whether endothelin induces expression of other CCN family members is not known.

Conclusion

CCN family members provide independent adhesive functions by acting through integrins and HSPGs and facilitating interactions with the ECM. However, a wide body of evidence suggests that alone they are not capable of recapitulating the range of CCN-dependent activities either in vitro or in vivo. Rather, CCN proteins exert their influences by modifying, through integrin- and/or adhesion-mediated signaling pathways, the function of extracellular ligands such as extracellular matrix components and growth factors. It is conceivable that CCN proteins interact with different ligands and thereby have different biological effects. Undoubtedly the entire range of ligands with which they interact has not been elucidated, making establishment of in vitro assays recapitulating the range of CCN actions problematic. Biochemical and two-hybrid analyses are necessary to identify additional ligands of CCN proteins. To gain greater molecular insights into the precise mechanisms of CCN action, genetic analysis of the roles of CCN family members is therefore essential. In particular, conditional knockout or gene-replacement strategies, or examination of the effects of loss of protein expression in particular cell types, are warranted. These models could test the effect of ablating CCN action specifically in certain tissues, or the functional equivalence of CCN proteins by replacing one family member with another. The latter approach could also test the extent to which differential control of CCN family gene expression is responsible for the net physiological effect of these proteins. Similarly, although much work has resulted in an appreciation of the control of CCN2 gene expression, a greater understanding of the mechanisms underlying the control of other CCN proteins is warranted.

References

Abraham, D. J., Shiwen, X., Black, C. M., Sa, S., Xu, Y. and Leask, A. (2000). Tumor necrosis factor alpha suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. *J. Biol. Chem.* **275**, 15220-15225.

Abreu, J. G., Ketpura, N. I., Reversade, B. and De Robertis, E. M. (2002). Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat. Cell Biol*. **4**, 599-604.

Aikawa, T., Gunn, J., Spong, S. M., Klaus, S. J. and Korc, M. (2006). Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Mol. Cancer Ther*. **5**, 1108-1116.

Asano, M., Kubota, S., Nakanishi, T., Nishida, T., Yamaai, T., Yosimichi, G., Ohyama, K., Sugimoto, T., Murayama, Y. and Takigawa, M. (2005). Effect of connective tissue growth factor (CCN2/CTGF) on proliferation and differentiation of mouse periodontal ligament-derived cells. *Cell Commun. Signal*. **3**, 11.

Babic, A. M., Kireeva, M. L., Kolesnikova, T. V. and Lau, L. F. (1998). CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc. Natl. Acad. Sci. USA* **95**, 6355-6360.

Babic, A. M., Chen, C. C. and Lau, L. F. (1999). Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol. Cell. Biol.* **19**, 2958-2966.

Bartholin, L., Wessner, L. L., Chirgwin, J. M. and Guise, T. A. (2006). The human Cyr61 gene is a transcriptional target of transforming growth factor beta in cancer cells. *Cancer Lett.* doi:10.1016/j.canlet.2006.02.019

Blom, I. E., van Dijk, A. J., Wieten, L., Duran, K., Ito, Y., Kleij, L., deNichilo, M., Rabelink, T. J., Weening, J. J., Aten, J. et al. (2001). In vitro evidence for differential involvement of CTGF, TGFbeta, and PDGF-BB in mesangial response to injury. *Nephrol. Dial. Transplant*. **16**, 1139-1148.

Blom, I. E., Goldschmeding, R. and Leask, A. (2002). Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? *Matrix Biol.* **21**, 473-482.

Bonniaud, P., Margetts, P. J., Kolb, M., Haberberger, T., Kelly, M., Robertson, J. and Gauldie, J. (2003). Adenoviral gene transfer of connective tissue growth factor in the lung induces transient fibrosis. *Am. J. Respir. Crit. Care Med*. **168**, 770-778.

Bonniaud, P., Martin, G., Margetts, P. J., Ask, K., Robertson, J., Gauldie, J. and Kolb, M. (2004). Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. *Am. J. Respir. Cell Mol. Biol.* **31**, 510-516.

Bork, P. (1993). The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* **327**, 125-130.

Bradham, D. M., Igarashi, A., Potter, R. L. and Grotendorst, G. R. (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J. Cell Biol.* **114**, 1285-1294.

Brigstock, D. R., Steffen, C. L., Kim, G. Y., Vegunta, R. K., Diehl, J. R. and Harding, P. A. (1997). Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin-regulated Mr 10,000 forms of connective tissue growth factor. *J. Biol. Chem.* **272**, 20275-20282.

Brigstock, D. R., Goldschmeding, R., Katsube, K. I., Lam, S. C., Lau, L. F., Lyons, K., Naus, C., Perbal, B., Riser, B., Takigawa, M. et al. (2003). Proposal for a unified CCN nomenclature. *Mol. Pathol*. **56**, 127-128.

Chen, C. C., Mo, F. E. and Lau, L. F. (2001). The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. *J. Biol. Chem*. **276**, 47329-47337.

Chen, N., Chen, C. C. and Lau, L. F. (2000). Adhesion of human skin fibroblasts to Cyr61 is mediated through integrin alpha 6beta 1 and cell surface heparan sulfate proteoglycans. *J. Biol. Chem*. **275**, 24953-24961.

Chen, N., Leu, S. J., Todorovic, V., Lam, S. C. and Lau, L. F. (2004). Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. *J. Biol. Chem.* **279**, 44166-44176.

Chen, Y., Segarini, P., Raoufi, F., Bradham, D. and Leask, A. (2001). Connective tissue growth factor is secreted through the Golgi and is degraded in the endosome *Exp. Cell Res*. **271**, 109-117.

Chen, Y., Blom, I. E., Sa, S., Goldschmeding, R., Abraham, D. J. and Leask, A. (2002). CTGF expression in mesangial cells: involvement of SMADs, MAP kinase, and PKC. *Kidney Int*. **62**, 1149-1159.

Chen, Y., Abraham, D. J., Shi-Wen, X., Pearson, J. D., Black, C. M., Lyons, K. M. and Leask, A. (2004). CCN2 (connective tissue growth factor) promotes fibroblast adhesion to fibronectin. *Mol. Biol. Cell* **15**, 5635-5646.

Chen, Y., Shi-wen, X., Eastwood, M., Black, C. M., Denton, C. P., Leask, A. and Abraham, D. J. (2006). Contribution of activin receptor-like kinase 5 (transforming growth factor betareceptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. *Arthritis Rheum.* **54**, 1309-1316.

Crean, J. K., Furlong, F., Finlay, D., Mitchell, D., Murphy, M., Conway, B., Brady, H. R., Godson, C. and Martin, F. (2004). Connective tissue growth factor [CTGF]/CCN2 stimulates mesangial cell migration through integrated dissolution of focal adhesion complexes and activation of cell polarization. *FASEB J.* **18**, 1541-1543.

Desnoyers, L., Arnott, D. and Pennica, D. (2001). WISP-1 binds to decorin and biglycan. *J. Biol. Chem*. **276**, 47599-47607.

Dziadzio, M., Usinger, W., Leask, A., Abraham, D., Black, C. M., Denton, D. and Stratton, R. (2005). N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *QJM* **98**, 485-492.

Ellis, P. D., Metcalfe, J. C., Hyvonen, M. and Kemp, P. R. (2003). Adhesion of endothelial cells to NOV is mediated by the integrins alphavbeta3 and alpha5beta1. *J. Vasc. Res.* **40**, 234-243.

Fataccioli, V., Abergel, V., Wingertsmann, L., Neuville, P., Spitz, E., Adnot, S., Calenda, V. and Teiger, E. (2002). Stimulation of angiogenesis by Cyr61 gene: a new therapeutic candidate. *Hum. Gene Ther*. **13**, 1461-1470.

French, D. M., Kaul, R. J., D'Souza, A. L., Crowley, C. W., Bao, M. Frantz, G. D., Filaroff, E. H. and Desnoyers, L. (2004). WISP-1 is an osteoblastic regulator

expressed during skeletal development and fracture repair. *Am. J. Pathol.* **165**, 855-867.

Fu, C. T., Bechberger, J. F., Ozog, M. A., Perbal, B. and Naus, C. C. (2004). CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression. *J. Biol. Chem.* **279**, 36943-36950.

Gao, R. and Brigstock, D. R. (2003). Low density lipoprotein receptor-related protein (LRP) is a heparin-dependent adhesion receptor for connective tissue growth factor (CTGF) in rat activated hepatic stellate cells. *Hepatol. Res.* **27**, 214-220.

Gao, R. and Brigstock, D. R. (2004). Connective tissue growth factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin alpha(v)beta(3) and heparan sulfate proteoglycan. *J. Biol. Chem.* **279**, 8848-8855.

Gao, R., Ball, D. K., Perbal, B. and Brigstock, D. R. (2004). Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells. *J. Hepatol.* **40**, 431-438.

Gellhaus, A., Dong, X., Propson, S., Maass, K., Klein-Hitpass, L., Kibschull, M., Traub, O., Willecke, K., Perbal, B., Lye, S. J. et al. (2004). Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells. *J. Biol. Chem.* **279**, 36931-36942.

Gery, S., Xie, D., Yin, D., Gabra, H., Miller, C., Wang, H., Scott, D., Yi, W. S., Popoviciu, M. L., Said, J. W. et al. (2005). Ovarian carcinomas: CCN genes are aberrantly expressed and CCN1 promotes proliferation of these cells. *Clin. Cancer Res.* **11**, 7243-7254.

Gore-Hyer, E., Pannu, J., Smith, E. A., Grotendorst, G. and Trojanowska, M. (2003). Selective stimulation of collagen synthesis in the presence of costimulatory insulin signaling by connective tissue growth factor in scleroderma fibroblasts. *Arthritis Rheum.* **48**, 798-806.

Grotendorst, G. R. (1997). Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev.* **8**, 171-179.

Grotendorst, G. R. and Duncan, M. R. (2005). Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J.* **19**, 729-738.

Grotendorst, G. R., Okochi, H. and Hayashi, N. (1996). A novel transforming growth factor beta response element controls the expressionof the connective tissue growth factor gene. *Cell Growth Differ.* **7**, 469-480.

Grotendorst, G. R., Rahmanie, H. and Duncan, M. R. (2004). Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. *FASEB J.* **18**, 469-479.

Gupta, N., Wang, H., McLeod, T. L., Naus, C. C., Kyurkchiev, S., Advani, S., Yu, J., Perbal, B. and Weichselbaum, R. R. (2001). Inhibition of glioma cell growth and tumorigenic potential by CCN3 (NOV). *Mol. Pathol.* **54**, 293-299.

Higgins, D. F., Biju, M. P., Akai, Y., Wutz, A., Johnson, R. S. and Haase, V. H. (2004). Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am. J. Physiol. Renal Physiol.* **287**, F1223-F1232.

Holloway, S. E., Beck, A. W., Girard, L., Jaber, M. R., Barnett, C. C., Jr, Brekken, R. A. and Fleming, J. B. (2005). Increased expression of Cyr61 (CCN1) identified in peritoneal metastases from human pancreatic cancer. *J. Am. Coll. Surg.* **200**, 371-377.

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M. and Leask, A. (2001). CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J. Biol. Chem.* **276**, 10594-10601.

Holmes, A., Abraham, D. J., Chen, Y., Denton, C., Shi-wen, X., Black, C. M. and Leask, A. (2003). Constitutive connective tissue growth factor expression in scleroderma fibroblasts is dependent on Sp1. *J. Biol. Chem.* **278**, 41728-41733.

Hoshijima, M., Hattori, T., Inoue, M., Araki, D., Hanagata, H., Miyauchi, A. and Takigawa, M. (2006). CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin alpha5beta1. *FEBS Lett.* **580**, 1376-1382.

Hurvitz, J. R., Suwairi, W. M., Van Hul, W., El-Shanti, H., Superti-Furga, A., Roudier, J., Holderbaum, D., Pauli, R. M., Herd, J. K., Van Hul, E. V. et al. (1999). Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat. Genet.* **23**, 94-98.

Igarashi, A., Okochi, H., Bradham, D. M. and Grotendorst, G. R. (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol. Biol. Cell* **4**, 637-645.

Inoki, I., Shiomi, T., Hashimoto, G., Enomoto, H., Nakamura, H., Makino, K., Ikeda, E., Takata, S., Kobayashi, K. and Okada, Y. (2002). Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J.* **16**, 219-221.

Ivkovic, S., Yoon, B. S., Popoff, S. N., Safadi, F. F., Libuda de Stephenson, R. C., Daluiski, A. and Lyons, K. M. (2003). Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* **130**, 2779-2791.

Jedsadayanmata, A., Chen, C. C., Kireeva, M. L., Lau, L. F. and Lam, S.C. (1999). Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue growth factor is mediated through integrin alpha(IIb)beta(3). *J. Biol. Chem.* **274**, 24321-24327.

Joliot, V., Martinerie, C., Dambrine, G., Plassiart, G., Brisac, M., Crochet, J. and Perbal, B. (1992). Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol. Cell. Biol.* **12**, 10-21.

Kireeva, M. L., Mo, F. E., Yang, G. P. and Lau, L. F. (1996). Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Mol. Cell. Biol.* **16**, 1326-1334.

Kireeva, M. L., Latinkic, B. V., Kolesnikova, T. V., Chen, C. C., Yang, G. P., Abler, A. S. and Lau, L. F. (1997). Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. *Exp. Cell Res.* **233**, 63-77.

Kireeva, M. L., Lam, S. C. and Lau, L. F. (1998). Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin alphavbeta3. *J. Biol. Chem.* **273**, 3090-3096.

Kondo, S., Kubota, S., Mukudai, Y., Moritani, N., Nishida, T., Matsushita, H., Matsumoto, S., Sugahara, T. and Takigawa, M. (2006). Hypoxic regulation of stability of connective tissue growth factor/CCN2 mRNA by 3′-untranslated region interacting with a cellular protein in human chondrosarcoma cells. *Oncogene* **25**, 1099-1110.

Kubota, S., Kondo, S., Eguchi, T., Hattori, T., Nakanishi, T., Pomerantz, R. J. and Takigawa, M. (2000). Identification of an RNA element that confers post-transcriptional repression of connective tissue growth factor/hypertrophic chondrocyte specific 24 ctgf/hcs24) gene: similarities to retroviral RNA-protein interactions. *Oncogene* **19**, 4773-4786.

Kutz, W. E., Gong, Y. and Warman, M. L. (2005). WISP3, the gene responsible for the human skeletal disease progressive pseudorheumatoid dysplasia, is not essential for skeletal function in mice. *Mol. Cell. Biol.* **25**, 414-421.

Lafont, J., Laurent, M., Thibout, H., Lallemand, F., Le Bouc, Y., Atfi, A. and Martinerie, C. T. (2002). The expression of novH in adrenocortical cells is down-regulated by TGFbeta 1 through c-Jun in a Smad-independent manner. *J. Biol. Chem.* **277**, 41220-41229.

Lafont, J., Thibout, H., Dubois, C., Laurent, M. and Martinerie, C. (2005a). NOV/CCN3 induces adhesion of muscle skeletal cells and cooperates with FGF2 and IGF-1 to promote proliferation and survival. *Cell Commun. Adhes.* **12**, 41-57.

Lafont, J., Jacques, C., Le Dreau, G., Calhabeu, F., Thibout, H., Dubois, C. F., Laurent, M. and Martinerie, C. (2005b). New target genes for NOV/CCN3 in chondrocytes: TGF-beta2 and type X collagen. *J. Bone Miner. Res.* **20**, 2213-2223.

Latinkic, B. V., Mo, F. E., Greenspan, J. A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Ross, S. R. and Lau, L. F. (2001). Promoter function of the angiogenic inducer Cyr61gene in transgenic mice: tissue specificity, inducibility during wound healing, and role of the serum response element. *Endocrinology* **142**, 2549-2557.

Latinkic, B. V., Mercurio, S., Bennett, B., Hirst, E. M., Xu, Q., Lau, L. F., Mohun, T. J. and Smith, J. C. (2003). Xenopus Cyr61 regulates gastrulation movements and modulates Wnt signaling. *Development* **130**, 2429-2441.

Leask, A. and Abraham, D. J. (2003). The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochem. Cell Biol.* **81**, 355-363.

Leask, A. and Abraham, D. J. (2004). TGF-beta signaling and the fibrotic response. *FASEB J.* **18**, 816-827.

Leask, A., Sa, S., Holmes, A., Shiwen, X., Black, C. M. and Abraham, D. J. (2001). The control of ccn2 (ctgf) gene expression in normal and scleroderma fibroblasts. *Mol. Pathol.* **54**, 180-183.

Leask, A., Holmes, A., Black, C. M. and Abraham, D. J. (2003). Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J. Biol. Chem.* **278**, 13008-13015.

Lechner, A., Schutze, N., Siggelkow, H., Seufert, J. and Jakob, F. (2000). The immediate early gene product hCYR61 localizes to the secretory pathway in human osteoblasts. *Bone* **27**, 53-60.

Leu, S. J., Lam, S. C. and Lau, L. F. (2002). Pro-angiogenic activities of CYR61 (CCN1) mediated through integrins alphavbeta3 and alpha6beta1 in human umbilical vein endothelial cells. *J. Biol. Chem.* **277**, 46248-46255.

Leu, S. J., Liu, Y., Chen, N., Chen, C. C., Lam, S. C. and Lau, L. F. (2003). Identification of a novel integrin alpha 6 beta 1 binding site in the angiogenic inducer CCN1 (CYR61). *J. Biol. Chem.* **278**, 33801-33808.

Leu, S. J., Chen, N., Chen, C. C., Todorovic, V., Bai, T., Juric, V., Liu, Y., Yan, G., Lam, S. C. and Lau, L. F. (2004). Targeted mutagenesis of the angiogenic protein CCN1 (CYR61). Selective inactivation of integrin alpha6beta1-heparan sulfate proteoglycan coreceptor-mediated cellular functions. *J. Biol. Chem.* **279**, 44177-44187.

Li, C. L., Martinez, V., He, B., Lombet, A. and Perbal, B. (2002). A role for CCN3 (NOV) in calcium signaling. *Mol. Pathol.* **55**, 250-261.

Lin, C. G., Leu, S. J., Chen, N., Tebeau, C. M., Lin, S. X., Yeung, C. Y. and Lau, L. F. (2003). CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family. *J. Biol. Chem.* **278**, 24200-24208.

Lin, C. G., Chen, C. C., Leu, S. J., Grzeszkiewicz, T. M. and Lau, L. F. (2005). Integrin-dependent functions of the angiogenic inducer NOV (CCN3): implication in wound healing. *J. Biol. Chem.* **280**, 8229-8237.

Lin, M. T., Zuon, C. Y., Chang, C. C., Chen, S. T., Chen, C. P., Lin, B. R., Wang, M. Y., Jeng, Y. M., Chang, K. J., Lee, P. H. et al. (2005b). Cyr61 induces gastric cancer cell motility/invasion via activation of the integrin/nuclear factor kappaB/cyclooxygenase-2 signaling pathway. *Clin. Cancer Res.* **11**, 5809-5820.

Mason, H. R., Lake, A. C., Wubben, J. E., Nowak, R. A. and Castellot, J. J., Jr (2004). The growth arrest-specific gene CCN5 is deficient in human leiomyomas and inhibits the proliferation and motility of cultured human uterine smooth muscle cells. *Mol. Hum. Reprod.* **10**, 181-187.

Menendez, J. A., Mehmi, I., Griggs, D. W. and Lupu, R. (2003). The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives. *Endocr. Relat. Cancer* **10**, 141-152.

Mercurio, S., Latinkic, B., Itasaki, N., Krumlauf, R. and Smith, J. C. (2004). Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development* **131**, 2137-2147.

Mo, F. E., Muntean, A. G., Chen, C. C., Stolz, D. B., Watkins, S. C. and Lau, L. F. (2002). CYR61 (CCN1) is essential for placental development and vascular integrity *Mol. Cell. Biol*. **22**, 8709-8720.

Mori, T., Kawara, S., Shinozaki, M., Hayashi, N., Kakinuma, T., Igarashi, A., Takigawa, M., Nakanishi, T. and Takehara, K. J. (1999). Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: a mouse fibrosis model. *J. Cell. Physiol*. **181**, 153-159.

Nakanishi, T., Nishida, T., Shimo, T., Kobayashi, K., Kubo, T., Tamatani, T., Tezuka, K. and Takigawa, M. (2000). Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* **141**, 264-273.

Nakanishi, T., Yamaai, T., Asano, M., Nawachi, K., Suzuki, M., Sugimoto, T. and Takigawa, M. (2001). Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. *Biochem. Biophys. Res. Commun*. **281**, 678-681.

Nakata, E., Nakanishi, T., Kawai, A., Asaumi, K., Yamaai, T., Asano, M., Nishida, T., Mitani, S., Inoue, H. and Takigawa, M. (2002). Expression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 (CTGF/Hcs24) during fracture healing. *Bone* **31**, 441-447.

Nguyen, T. Q., Tarnow, L., Andersen, S., Hovind, P., Parving, H. H., Goldschmeding, R. and van Nieuwenhoven, F. A. (2006). Urinary connective tissue growth factor excretion correlates with clinical markers of renal disease in a large population of type 1 diabetic patients with diabetic nephropathy. *Diabetes Care* **29**, 83-88.

Nishida, T., Nakanishi, T., Asano, M., Shimo, T. and Takigawa, M. (2000). Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *J. Cell. Physiol*. **184**, 197-206.

Nishida, T., Kubota, S., Fukunaga, T., Kondo, S., Yosimichi, G., Nakanishi, T., Takano-Yamamoto, T. and Takigawa, M. (2003). CTGF/Hcs24, hypertrophic chondrocyte-specific gene product, interacts with perlecan in regulating the proliferation and differentiation of chondrocytes. *J. Cell. Physiol*. **196**, 265-275.

O'Brien, T. P., Yang, G. P., Sanders, L. and Lau, L. F. (1990). Expression of cyr61, a growth factor-inducible immediate-early gene. *Mol. Cell. Biol*. **10**, 3569-3577.

Ott, C., Iwanciw, D., Graness, A., Giehl, K. and Goppelt-Struebe, M. (2003). Modulation of the expression of connective tissue growth factor by alterations of the cytoskeleton. *J. Biol. Chem*. **278**, 44305-44311.

Parisi, M. S., Gazzerro, E., Rydziel, S. and Canalis, E. (2006). Expression and regulation of CCN genes in murine osteoblasts. *Bone* **38**, 671-677.

Pennica, D., Swanson, T. A., Welsh, J. W., Roy, M. A., Lawrence, D. A., Lee, J., Brush, J., Taneyhill, L. A., Deuel, B., Lew, M. et al. (1998). WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc. Natl. Acad. Sci. USA* **95**, 14717-14722.

Perbal, B. (2004). CCN proteins: multifunctional signalling regulators. *Lancet* **363**, 62-64.

Perbal, B. and Takigawa, M. (ed.) (2005). *CCN Proteins: A New Family of Cell Growth And Differentiation Regulators*. London: World Scientific Publishers.

Rachfal, A. W., Luquette, M. H. and Brigstock, D. R. (2004). Expression of connective tissue growth factor (CCN2) in desmoplastic small round cell tumour. *J. Clin. Pathol*. **57**, 422-425.

Rodriguez-Pascual, F., Redondo-Horcajo, M. and Lamas, S. (2003). Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-beta-mediated induction of endothelin-1 expression. *Circ. Res*. **92**, 1288-1295.

Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kabasawa, Y., Takagi, M., Li, C. L., Perbal, B. and Katsube, K. (2002). The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J. Biol. Chem*. **277**, 29399-29405.

Sakamoto, S., Yokoyama, M., Aoki, M., Suzuki, K., Kakehi, Y. and Saito, Y. (2004). Induction and function of CYR61 (CCN1) in prostatic stromal and epithelial cells: CYR61 is required for prostatic cell proliferation. *Prostate* **61**, 305-317.

Schober, J. M., Lau, L. F., Ugarova, T. P. and Lam, S. C. (2003). Identification of a novel integrin alphaMbeta2 binding site in CCN1 (CYR61), a matricellular protein expressed in healing wounds and atherosclerotic lesions. *J. Biol. Chem*. **278**, 25808-25815.

Schutze, N., Noth, U., Schneidereit, J., Hendrich, C. and Jakob, F. (2005). Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation *Cell Commun. Signal*. **3**, 5.

Segarini, P. R., Nesbitt, J. E., Li, D., Hays, L. G., Yates, J. R, 3rd and Carmichael, D. F. (2001). The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. *J. Biol. Chem*. **276**, 40659-40667.

Sen, M., Cheng, Y. H., Goldring, M. B., Lotz, M. K. and Carson, D. A. (2004). AWISP3-dependent regulation of type II collagen and aggrecan production in chondrocytes. *Arthritis Rheum*. **50**, 488-497.

Shimo, T., Nakanishi, T., Nishida, T., Asano, M., Kanyama, M., Kuboki, T., Tamatani, T., Tezuka, K., Takemura, M., Matsumura, T. et al. (1999). Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. *J. Biochem*. **126**, 137-145.

Shimo, T., Kubota, S., Yoshioka, N., Ibaragi, S., Isowa, S., Eguchi, T., Sasaki, A. and Takigawa, M. (2006). Pathogenic role of Connective Tissue Growth Factor (CTGF/CCN2) in osteolytic metastasis of breast cancer. *J. Bone Miner. Res*. **21**, 1045-1059.

Shi-wen, X., Howat, S. L., Renzoni, E. A., Holmes, A., Pearson, J. D., Dashwood, M. R., Bou-Gharios, G., Denton, C. P., du Bois, R. M., Black, C. M. et al. (2004). Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. *J. Biol. Chem*. **279**, 23098-23103.

Shi-wen, X., Stanton, L., Kennedy, L., Chen, Y., Pala, D., Chen, Y., Howat, S. L., Renzoni, E. A., Carter de Bou-Gharios, G., Stratton, R. J. et al. (2006a). CCN2 is necessary for adhesive responses to TGFß1 in embryonic fibroblasts. *J. Biol. Chem*. **281**, 10715-10726.

Shi-wen, X., Rodrigues-Pascual, F., Lamas, S., Holmes, A., Howat, S., Pearson, J. D., Dashwood, M. R., du Bois, R. M., Denton, C. P., Black, C. M. et al. (2006b). Constitutive ALK5-indepenent JNK activation contributes to endothelin-1 over-expression in pulmonary fibrosis. *Mol. Cell. Biol*. **26**, 5518-5527.

Si, W., Kang, Q., Luu, H. H., Park, J. K., Luo, Q., Song, W. X., Jiang, W., Luo, X., Li, X., Yin, H. et al. (2006). CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. *Mol. Cell. Biol*. **26**, 2955-2964.

Stratton, R., Rajkumar, V., Ponticos, M., Nichols, B., Shiwen, X., Black, C. M., Abraham, D. J. and Leask, A. (2002). Prostacyclin derivatives prevent the fibrotic response to TGF-beta by inhibiting the Ras/MEK/ERK pathway. *FASEB J*. **16**, 1949-1951.

Takigawa, M., Nakanishi, T., Kubota, S. and Nishida, T. (2003). Role of CTGF/HCS24/ecogenin in skeletal growth control. *J. Cell. Physiol*. **194**, 256-266.

Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., Horowitz, J. C., Day, R. M. and Thomas, P. E. (2003). Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J. Biol. Chem*. **278**, 12384-12389.

Thiery, J. P. (2003). Cell adhesion in development: a complex signaling network. *Curr. Opin. Genet. Dev*. **13**, 365-371.

Todorovicc, V., Chen, C. C., Hay, N. and Lau, L. F. (2005). The matrix protein CCN1 (CYR61) induces apoptosis in fibroblasts. *J. Cell Biol*. **171**, 559-568.

van Beek, J., Kennedy, L., Rockel, J. S., Bernier, S. M. and Leask, A. (2006). The induction of CCN2 by TGFbeta1 involves Ets-1. *Arthritis Res. Ther.* **8**, R36.

Verrecchia, F., Vindevoghel, L., Lechleider, R. J., Uitto, J., Roberts, A. B. and Mauviel, A. (2001). Smad3/AP-1 interactions control transcriptional responses to TGF-beta in a promoter-specific manner. *Oncogene* **20**, 3332-3340.

Wahab, N. A., Weston, B. S. and Mason, R. M. (2005). Connective tissue growth factor CCN2 interacts with and activates the tyrosine kinase receptor TrkA. *J. Am. Soc. Nephrol*. **16**, 340-351.

Wong, M., Kireeva, M. L., Kolesnikova, T. V. and Lau, L. F. (1997). Cyr61, product of a growth factor-inducible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. *Dev. Biol.* **192**, 492-508.

Yang, F., Tuxhorn, J. A., Ressler, S. J., McAlhany, S. J., Dang, T. D. and Rowley, D. R. (2005). Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res*. **65**, 8887-8895.

Zhang, Y., Pan, Q., Zhong, H., Merajver, S. D. and Kleer, C. G. (2005). Inhibition of CCN6 (WISP3) expression promotes neoplastic progression and enhances the effects of insulin-like growth factor-1 on breast epithelial cells. *Breast Cancer Res*. **7**, R1080-R1089.

Zhao, J., Shi, W., Wang, Y. L., Chen, H., Bringas, P., Jr, Datto, M. B., Frederick, J. P., Wang, X. F. and Warburton, D. (2002). Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol*. **282**, L585-L593.