Cell surface receptors for CCN proteins

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Abstract The CCN family (CYR61; CTGF; NOV; CCN1–6; WISP1–3) of matricellular proteins in mammals is comprised of six homologous members that play important roles in development, inflammation, tissue repair, and a broad range of pathological processes including fibrosis and cancer. Despite considerable effort to search for a high affinity CCN-specific receptor akin to growth factor receptors, no such receptor has been found. Rather, CCNs bind several groups of multi-ligand receptors as characteristic of other matricellular proteins. The most extensively documented among CCN-binding receptors are integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$, and $\alpha_D\beta_2$, which mediate diverse CCN functions in various cell types. CCNs also bind cell surface heparan sulfate proteoglycans (HSPGs), low density lipoprotein receptor-related proteins (LRPs), and the cation-independent mannose-6-phosphate (M6P) receptor, which are endocytic receptors that may also serve as co-receptors in cooperation with other cell surface receptors. CCNs have also been reported to bind FGFR-2, Notch, RANK, and TrkA, potentially altering the affinities of these receptors for their ligands. The ability of CCNs to bind a multitude of receptors in various cell types may account for the remarkable versatility of their functions, and underscore the diverse signaling pathways that mediate their activities.

Keywords CCN proteins · Matricellular proteins · Integrins · Receptors · Signaling

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It has been more than a quarter of a century since members of the CCN family were first described (O'Brien et al. 1990, Bradham et al. 1991, Joliot et al. 1992). Studies in the ensuing years have uncovered an impressive array of biological functions and pathological processes with which CCN proteins are associated. CCNs are important for embryonic and postnatal development; they regulate multiple aspects of cellular behavior, and play diverse roles in pathological conditions including inflammation, fibrosis, and cancer (Jun and Lau 2011, Kubota and Takigawa 2013, Wells et al. 2015). Through what mechanisms do CCN proteins exert their diverse functions? Research on this issue has been driven by two prevailing hypotheses: (1) CCN proteins may function as classical growth factors and interact with high affinity signaling receptors specific for CCN proteins; and (2) CCNs are matricellular proteins that bind a variety of multi-ligand receptors, primarily integrins, similar to many other proteins in the extracellular matrix (ECM). These hypotheses, while not mutually exclusive, imply distinct signaling mechanisms and may influence the experimental approaches employed for understanding CCN protein functions. Here I briefly summarize the various CCN receptors reported to date.

Historical perspective: early 90s

CCN1/CYR61, the first member of the CCN family described, was identified as a cysteine-rich (CYR) protein encoded by a serum-inducible immediate-early gene in fibroblasts (O'Brien et al. 1990). Although serum-inducible genes were initially thought to play a role in growth control, subsequent studies have indicated that many such genes are related to wound healing (Iyer et al. 1999). In contrast to classical growth factors that may be secreted into the cell culture media, CCN1 was found to be tightly associated with the ECM and

the cell surface upon secretion by binding heparan sulfate proteoglycans (HSPGs) with high affinity, suggesting that CCN1 may mediate cell-matrix communication (Yang and Lau 1991). However, no sequence homology with proteins of known functions was recognized at that time, and rigorous tests of this notion awaited purification and functional analysis of the CCN1 protein.

Independent efforts by Grotendorst and colleagues sought to identify a platelet-derived growth factor (PDGF)-related activity secreted by endothelial cells. By screening a cDNA expression library, a clone encoding a protein that reacted with an anti-PDGF antibody was identified and this protein was named connective tissue growth factor (CTGF; CCN2) (Bradham et al. 1991). CCN2/CTGF was found to be mitogenic and chemotactic for fibroblasts in this study, and was thought to represent a novel PDGF-related growth factor based on antibody cross-reactivity. However, there is no discernible protein sequence homology between PDGF and CCN2 (Bradham et al. 1991). Interestingly, blood platelets from which PDGF was purified for antibody preparation are now known to be a rich source of CCN2 (Kubota et al. 2004, Cicha et al. 2004). These initial studies, coming on the heels of many growth factors and cytokines being discovered in the 1980s, fueled considerable interest and supported the view that CTGF-related proteins may similarly act as classical growth factors. Recent studies have suggested that the effects of CCN2/CTGF on cell proliferation may be dependent on the cellular microenvironment and interaction with other growth regulatory factors (Guo et al. 2011, Lipson et al. 2012, Riley et al. 2015). For example, the ability of CCN2 to promote TGF- β -induced cell proliferation has been shown by several laboratories (Blalock et al. 2012, Lipson et al. 2012, Parada et al. 2013, Xu et al. 2015).

Searching for CCN receptors

Given the initial classification of CCN2/CTGF as a growth factor, significant effort was made by several laboratories to identify a specific, high affinity growth factor receptor. Cell surface cross-linking studies revealed a 280 kDa protein in close proximity with CCN2 in chondrocytes (Nishida et al. 1998), although no signaling function was associated with this protein and its identity was unknown. More recent work has similarly identified a 280 kDa CCN2-interacting protein in fibroblasts by cell surface cross-linking, and this protein was shown to be the cation-independent mannose-6-phosphate (M6P) receptor, also known as the insulin-like growth factor (IGF)-2 receptor (Blalock et al. 2012). The major function of M6P/IGF-2 receptor is to target M6P-tagged acid hydrolases from the trans-Golgi network to endosomes and ultimately to lysosomes, although ~10 % of this protein is found on the cell surface, where it captures and internalizes lysosomal enzymes

that are accidentally released from the Golgi through the secretory pathway (El-Shewy and Luttrell 2009). M6P/IGF-2 receptor may also contribute to cell signaling by binding and activating latent TGF- β , as well as binding the unglycosylated IGF-2 through a distinct site to regulate its bioavailability. It was observed that *Ccn2* knockdown or M6P/IGF-2 receptor knockout reduced TGF- β -induced proliferation in fibroblasts, suggesting that CCN2 may enhance TGF- β -induced proliferation through the M6P/IGF-2 receptor (Blalock et al. 2012). Whether the M6P/IGF-2 receptor mediates any other CCN2 functions remains to be determined.

The identification of CCN1 receptors took a different route based on the observation that CCN1 is associated with the ECM and may play a role in matrix signaling (Yang and Lau 1991). The purification of CCN1 to near homogeneity allowed direct demonstration that CCN1 does not by itself induce the proliferation of fibroblasts or endothelial cells, but rather, it enhances their mitogenesis induced by serum growth factors including fibroblast growth factor (FGF) and PDGF and supports cell adhesion (Kireeva et al. 1996). Soon thereafter, CCN1 was found to support endothelial cell adhesion through binding to integrin $\alpha_v\beta_3$, the first signaling receptor identified for a CCN protein (Kireeva et al. 1998). Direct physical interaction of purified CCNs and purified integrins has been observed in cell-free systems for CCN1- $\alpha_v\beta_3$ (Kireeva et al. 1998), CCN2- $\alpha_5\beta_1$ (Gao and Brigstock 2005), CCN3- $\alpha_v\beta_3$ and CCN3- $\alpha_5\beta_1$ (Lin et al. 2003). Since these initial findings, CCN-integrin interactions and integrin-mediated CCN functions have been documented by a number of laboratories in a broad range of cell types for all CCN proteins (Chen and Lau 2009, Jun and Lau 2011), including the more recently characterized CCN4, CCN5, and CCN6 (Batmunkh et al. 2011, Hou et al. 2013, Myers et al. 2014, Chuang et al. 2015, Stephens et al. 2015) (Fig. 1).

HSPGs are known to function as co-receptors for several signaling receptors and as endocytic receptors (Sarrazin et al. 2011). Two specific CCN1 binding sites for HSPGs were recognized in the CT domain, and CCN1 mutant proteins disrupted in each of these binding sites or both were constructed (Chen et al. 2000). CCN2 has homologous binding sites but with fewer positively charged residues, consistent with CCN2 binding to HSPGs with lower affinity than CCN1 (Kireeva et al. 1997). These studies led to the finding that HSPGs serve as co-receptors for $\alpha_6\beta_1$ in fibroblast adhesion to CCN1 (Chen et al. 2000), for $\alpha_v\beta_3$ in hepatic stellate cell adhesion to CCN2 (Gao and Brigstock 2004), and for $\alpha_5\beta_1$ in pancreatic stellate cell adhesion to CCN2 (Gao and Brigstock 2005). Subsequent studies showed that syndecan-4, a cell surface HSPG, is a co-receptor for CCN1/ $\alpha_6\beta_1$ -mediated induction of reactive oxygen species (ROS) in a Rac-1 dependent pathway, leading to apoptosis (Todorovic et al. 2005, Chen et al. 2007a) or cellular senescence (Jun and Lau 2010) in various contexts. Syndecan-4 is also a co-receptor for $\alpha_M\beta_2$ in CCN1-induced NF- κ B activation in macrophages (Bai

Fig. 1 Interaction of CCN proteins with their cell surface receptors. CCN proteins (with the exception of CCN5 which lacks the CT domain) are composed of four conserved structural domains with homologies to insulin-like growth factor binding proteins (IGFBPs), von Willebrand factor type C (VWC) repeat, thrombospondin type 1 repeat (TSR), and a carboxy-terminal domain (CT) with sequence similarity to Slit and

mucins. The locations of known binding sites for integrin receptors and heparan sulphate proteoglycans (HSPGs) are indicated in the schematic diagram. CCNs also bind endocytic receptors including HSPGs, low-density lipoprotein receptor-related proteins (LRPs), and the cation-independent mannose-6-phosphate receptor (M6P/IGF-2R), which may also serve as co-receptors or auxiliary receptors for other receptor systems

et al. 2010), and a co-receptor for CCN2-stimulated cell migration (Kennedy et al. 2007).

A cell surface cross-linking approach was used to identify a 620 kDa protein from bone marrow stromal cells that interacts with CCN2 as the low density lipoprotein receptor-related protein (LRP-1) (Segarini et al. 2001). LRP-1 binds more than 30 diverse extracellular ligands and functions as a co-receptor or auxiliary receptor for many growth factor receptors and integrins, facilitating endocytosis and signaling (Lillis et al. 2005). The LRP family members LRP-5 and LRP-6 are known to function as co-receptors for the Frizzled receptors to transduce Wnt signals (Joiner et al. 2012), and CCN1 or CCN2 binding to LRP-6 modulates Wnt signaling (Latinkic et al. 2003, Mercurio et al. 2004). LRP and HSPGs likely function as co-receptors with integrin $\alpha_v\beta_3$ as well, since these receptors are required for mediating hepatic stellate cell adhesion to CCN2 (Gao and Brigstock 2004). CCN1 has also been shown to form a physical complex with LRP-1, which is required for CCN1/ $\alpha_6\beta_1$ -induced ROS and apoptosis (Juric et al. 2012). Thus, CCNs may bind LRPs as co-receptors for integrin signaling and to modulate Wnt signaling.

Cell surface cross-linking studies have also found that CCN2/CTGF is in close proximity to the neurotrophin co-receptor p75^{NTR} and the 140 kDa protein TrkA (tropomyosin receptor kinase A), a tyrosine kinase receptor that binds nerve growth factor and related neurotrophins (Wahab et al. 2005). Pharmacological inhibition of TrkA blocked some aspects, but not all, of CCN2 activity in cardiomyocytes (Wang et al. 2009, Wang et al. 2010). More recent studies showed that TrkA is complexed to β_1 integrins in tumor initiating cells, and treatment of CCN2 in these cells results in the formation

of a CCN2-TrkA- β_1 -integrin complex (Edwards et al. 2011). Likewise, p75^{NTR} also forms a complex with β_1 integrins (Ventresca et al. 2015). These findings suggest that CCN2 may interact with TrkA and/or p75^{NTR} in a complex with β_1 integrins as co-receptors mediating CCN2 signals.

Recently, CCN2 has been found to bind fibroblast growth factor receptor 2 (FGFR2) to enhance FGF-induced expression of osteocalcin in an osteoblastic cell line (Aoyama et al. 2012). CCN2 has also been found to bind receptor activator of NF- κ B (RANK), a cell surface receptor for RANK ligand (RANKL) (Aoyama et al. 2015). The interactions of CCN2 with FGFR2 and RANK have been assessed in a solid phase binding assay and by surface plasmon resonance analysis. CCN2 also interacts with the dendritic cell-specific transmembrane protein (DC-STAMP), which may contribute to CCN2 function in osteoclast formation (Nishida et al. 2011). CCN3 has been shown to associate with the Notch1 receptor through its carboxyl-terminal domain (Sakamoto et al. 2002), and CCN3 suppresses vascular smooth muscle cell proliferation in part through Notch signaling (Shimoyama et al. 2010). Interestingly, CCN1 has been shown to regulate cholangiocyte proliferation through Notch signaling, but this effect is indirect. In this context, CCN1 binds to integrin $\alpha_v\beta_5$, which activates NF- κ B and *Jag1* expression, and *Jag1* in turn activates Notch-1 to control proliferation (Kim et al. 2015).

CCNs as matricellular proteins: if the shoe fits

In 1995, Bornstein proposed a new classification for a group of “modular, extracellular proteins whose functions are achieved

by binding to matrix proteins as well as to cell surface receptors, or to other molecules such as cytokines and proteases” as “matricellular proteins” (Bornstein 1995). These matricellular proteins, which at the time included thrombospondin-1, tenascin-C, SPARC/osteonectin, and osteopontin, are capable of an extensive repertoire of molecular and cellular interactions and are not directly involved in matrix integrity. In 1993, Bork recognized that the CCN proteins (CYR61, CTGF, and Nov) are comprised of four domains with homologies to insulin-like growth factor binding proteins (IGFBPs), von Willebrand factor type C (VWC) repeat, thrombospondin type 1 repeat (TSR), and a carboxy-terminal domain (CT) with sequence similarity to Slit and mucins (Bork 1993) (Fig. 1). Each of these homologous proteins, including IGFBP (Jones et al. 1993), is found in the ECM. These findings are consistent with the ECM localization of CCNs and highlight the sequence homology of CCNs to ECM proteins (Kireeva et al. 1997). The recognition that CCNs are present in the ECM, together with the discovery that CCN proteins bind directly to integrins, which are receptors mediating ECM signaling, led to the proposal that CCNs are similar to and may be considered as matricellular proteins (Lau and Lam 1999).

A common feature of matricellular proteins is their binding to soluble growth factors such as FGF, VEGF, and TGF- β , and their interaction with a diverse array of multi-ligand receptors (Murphy-Ullrich and Sage 2014). Most prominent among receptors that bind matricellular proteins are various integrins, LRP, and cell surface HSPGs. CCNs fulfill these attributes of matricellular proteins extremely well (Leask and Abraham 2006, Holbourn et al. 2008, Chen and Lau 2009, Lau 2011). In recent years, the matricellular family has been expanded to include CCN proteins, periostin, R-spondins, short fibulins, galectins, small leucine rich proteoglycans, autotaxin, pigment epithelium derived factor and plasminogen activator inhibitor-1 (Murphy-Ullrich and Sage 2014).

Integrins as primary signaling receptors of CCN proteins

Integrins were initially characterized as receptors that couple the ECM outside the cell to the cytoskeleton inside the cell, and play important roles in matrix signaling affecting diverse cellular functions, including cell adhesion, spreading, migration, proliferation, differentiation, and survival (Hynes 2002, Miranti and Brugge 2002). Integrins are versatile signal transducing receptors and there is significant cross-talk between integrins and other cell surface receptors, including growth factor receptors (Desgrosellier and Cheresch 2010). In mammals, 18 α and 8 β subunits comprise 24 known integrin heterodimers. Each integrin heterodimer is capable of binding multiple ligands, and many ligands can bind multiple

integrins. Immobilized CCN1 and CCN2 are shown to be bona fide cell adhesive substrates and support cell adhesion in fibroblasts through integrin $\alpha_6\beta_1$ with HSPGs, inducing activation of focal adhesion kinase (FAK), paxillin, Rac, and p42/p44 MAPKs, and formation of filopodia and lamellipodia (Chen et al. 2001). Subsequent studies have established a broad range of CCN functions mediated through distinct integrins in various cell types (Babic et al. 1998, Babic et al. 1999, Jedsadayanmata et al. 1999, Schober et al. 2002, Leu et al. 2002, Ellis et al. 2003, Chen et al. 2007b, Liu et al. 2012, Jun and Lau 2011, Tran et al. 2014, Liu et al. 2015). FAK plays a critical role in integrin signaling and mediates a variety of CCN activities (Todorovic et al. 2005, Batmunkh et al. 2011, Kiwanuka et al. 2013, Jun et al. 2015). Interestingly, *Ccn2* expression is also dependent on FAK activation (Chen et al. 2004b, Graness et al. 2006, Shi-wen et al. 2006), suggesting a regulatory loop in which FAK both promotes *Ccn2* expression and mediates its functions.

Biochemical, genetic, and functional evidence converges to provide compelling support for the notion that integrins are signaling receptors for CCN proteins in many biological contexts. First, the direct physical binding of CCNs to integrins has been established through the identification of specific binding sites. Since CCNs do not contain the canonical RGD-sequence motif that binds several integrins including $\alpha_v\beta_3$, a peptide screening approach was used to identify specific binding sites for $\alpha_v\beta_3$ within CCN1 (Chen et al. 2004a) and CCN2 (Gao and Brigstock 2004). Likewise, three binding sites for $\alpha_6\beta_1$ have been identified in CCN1 (Chen et al. 2000, Leu et al. 2003), and an $\alpha_5\beta_1$ binding site was found in CCN2 (Gao and Brigstock 2005). In each case, blockade of the integrins by either specific monoclonal antibodies or peptide mimetics abolished CCN protein functions. Moreover, siRNA knockdown of the cognate integrins eliminated CCN protein function in target cells (Kim et al. 2015). Further genetic and functional evidence came from the construction of CCN mutant proteins unable to bind specific integrins. A single amino acid change mutation (D125A) at the CCN1 binding site for $\alpha_v\beta_3/\alpha_v\beta_5$ that abolished CCN1 binding to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ specifically abrogated $\alpha_v\beta_3/\alpha_v\beta_5$ -dependent functions but had no effect on $\alpha_6\beta_1$ -mediated functions (Chen et al. 2004a). Likewise, mutant proteins that are disrupted in one, two, or all three $\alpha_6\beta_1$ binding sites in CCN1 either diminished or completely abolished $\alpha_6\beta_1$ -mediated functions specifically (Chen et al. 2000, Leu et al. 2004).

Ultimately, the biological function of CCN-integrin signaling is best assessed in vivo. To this end, knock-in mice in which the *Ccn1* genomic locus is replaced with alleles that encode mutants unable to bind $\alpha_v\beta_3/\alpha_v\beta_5$ (*Ccn1*^{D125A/D125A}) or $\alpha_6\beta_1$ (*Ccn1*^{DM/DM}) were generated (Chen et al. 2007a, Jun et al. 2015). Analyses of these knockin mice have identified critical integrin-specific functions in vivo, and these integrin-

mediated CCN1 functions can be replicated in relevant cell types in vitro. For example, CCN1 binding to $\alpha_6\beta_1$ is required for CCN1-induced apoptotic synergism with TNF α (Chen et al. 2007a, Juric et al. 2009), CCN1-induced cellular senescence (Jun and Lau 2010), and suppression of EGF-induced hepatocyte compensatory proliferation (Chen et al. 2015). Each of these activities is demonstrably $\alpha_6\beta_1$ -dependent in vitro, and is specifically eliminated in *Ccn1*^{DM/DM} mice, which express CCN1 unable to bind $\alpha_6\beta_1$. Likewise, *Ccn1*^{D125A/D125A} mice encoding CCN1 unable to bind $\alpha_v\beta_3/\alpha_v\beta_5$ showed defects in CCN1-induced efferocytosis of apoptotic cells (Jun et al. 2015) and cholangiocyte proliferation in biliary regeneration (Kim et al. 2015). Consistently, CCN1 induces efferocytosis in macrophages and cholangiocyte proliferation in vitro through $\alpha_v\beta_3/\alpha_v\beta_5$ -dependent mechanisms (Jun et al. 2015, Kim et al. 2015). Together, these multiple lines of evidence provide strong support for the integrin-mediated CCN1 functions in vitro and in vivo.

Summary and conclusions

Based on the proposal that CCN2/CTGF acts as a classical growth factor, considerable effort has been made to search for a classical growth factor receptor specific for CCN proteins. Currently there is no evidence for such receptors. Rather, all four structural domains in CCN proteins are homologous to ECM-associated proteins and CCNs can be recognized as matricellular proteins (Lau and Lam 1999, Murphy-Ullrich and Sage 2014). Consistent with this notion, CCNs bind to diverse groups of multi-ligand receptors. Among them, integrin receptors have been shown to interact with all six members of the CCN family and can mediate diverse CCN functions in various cell types. Compelling biochemical, genetic, and functional evidence supports the notion that integrins are critical signaling receptors mediating CCN functions in vitro and in vivo. In addition, CCNs bind to a number of endocytic receptors that may serve as co-receptors or auxiliary receptors for other receptor systems, thus enhancing or modifying the signaling outcome. These include HSPGs such as syndecan-4, LRP6, and the M6P/IGF-2 receptor. Recent findings of CCN2 interaction with FGFR-2, RANK, and TrkA are also of potential importance, although evaluation of the biological significance of these interactions, and whether these interactions involve engagement of CCN2 with integrins, requires further assessment in vivo.

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