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Review

Molecular control of vascular development by the matricellular proteins *CCN1* (*Cyr61*) and *CCN2* (*CTGF*)

Brahim Chaqour*

Department of Cell Biology and Ophthalmology, State University of New York (SUNY), Eye Institute-Downstate Medical Center, Brooklyn, New York 11203, USA

ABSTRACT

The circulatory system is the first hierarchically ordered network to form during the development of vertebrates as it is an indispensable means of adequate oxygen and nutrient delivery to developing organs. During the initial phase of vascular development, endothelial lineage-committed cells differentiate, migrate, and coalesce to form the central large axial vessels and their branches. The subsequent phase of vessel expansion (i.e., angiogenesis) involves a cascade of events including endothelial cell migration, proliferation, formation of an immature capillary structure, recruitment of mural cells and deposition of a basement membrane to yield a functional vasculature. These series of events are tightly regulated by the coordinated expression of several angiogenic, morphogenic and guidance factors. The extracellular matrix (ECM) is synthesized and secreted by embryonic cells at the earliest stages of development and forms a pericellular network of bioactive stimulatory and inhibitory angiogenesis regulatory factors. Here we describe the role of a subset of inducible immediate-early gene-encoded, ECM-associated integrin- and heparin-binding proteins referred to as *CCN1* (or Cyr61) and *CCN2* (or *CTGF*) and their function in the development of the vascular system. Gene-targeting experiments in mice have identified *CCN1* and *CCN2* as critical rate-limiting determinants of endothelial cell differentiation and quiescence, mural cell recruitment and basement membrane formation during embryonic vascular development. Emphasis will be placed on the regulation and function of these molecules and their contextual mode of action during vascular development. Further understanding of the mechanisms of *CCN1*- and *CCN2*-mediated blood vessel expansion and remodeling would enhance the prospects that these molecules provide for the development of new treatments for vascular diseases.

KEYWORDS: extracellular matrix, *CCN1*, *CCN2*, blood vessels, development, angiogenesis

INTRODUCTION

"*CCN*" is an acronym that refers to the initials of the first three members of a subset of extracellular matrix (ECM) proteins namely cysteine-rich 61 (*Cyr61* or *CCN1*), connective tissue growth factor (*CTGF* or *CCN2*), and nephroblastoma overexpressed (Nov or *CCN3*) [1]. There are three other members of this family labeled *Wnt*-inducible secreted proteins (WISP-1 or *CCN4*; *WISP-2* or *CCN5*; and *WISP-3* or *CCN6*) based on their induction by *Wnt* ligands. The six members of this family of proteins share structural features but they all are functionally distinct. By far, the first 2 members, *CCN1* and *CCN2*, exhibit more critical functions in vascular tissue development and diseases, and they will be the main focus of this review [2].

CCN1 and *CCN2* are non-structural bioactive ECM molecules which bridge the functional divide between structural macromolecules and growth factors, cytokines, proteases, and other related proteins [3]. As such, these molecules have been classified

*bchaqour@downstate.edu

as members of the matricellular protein family which also includes diverse ECM proteins such as thrombospondins, tenascins, osteopentin and osteonectin. Overall, matricellular proteins do not subserve a physical role in the extracellular environment like the collagens, proteoglycans and glycoproteins which essentially provide the mechanical scaffolding within which cells and tissues are built [4]. Instead, they influence the cell fate and function and modulate signals emanating from the extracellular environment. Different functions and roles in vascular development and/or pathology have been attributed to each matricellular protein. Experimental perturbations in *in vitro* and *in vivo* systems modeling cardiovascular diseases and cancer have shown that the altered matricellular gene expression affected organ susceptibility/vulnerability to pathogenic factors, which underscored their roles in myocardium and vascular system remodeling, angiogenesis, developmental synaptogenesis, connective tissue organization and dynamics of wound repair [5, 6].

However, *CCN1* and *CCN2* exhibit several distinctive characteristics. First, while other matricellular proteins are ubiquitously and constitutively expressed, *CCN1* and *CCN2* proteins are encoded by inducible immediate-early genes whose expression is associated with developmental or pathological events only [3]. Second, most matricellular proteins function as direct modulators of specific ECM fibril systems regulating the organization or higher order assembly of basement membranes and collagen fibrils [7, 8, 9]. Instead, *CCN* proteins seem to act as adaptors or scaffolds that can bring cytokines and growth factors into close proximity to the cell surface by binding integrins, heparane sulfate proteoglycans (HSPGs) and receptor tyrosine kinases [10, 11, 12]. However, the molecular status of CCN1 and CCN2 *in situ* is still unknown. Third, the *CCN1* and *CCN2* genes are essential for viability as either *CCN1*- or *CCN2*-deficient mice exhibited severe deficiencies in new blood vessel formation and/or skeletogenesis causing embryonic lethality [13]. Conversely, deficiency in mice of matricellular genes other than *CCN1* and *CCN2* resulted in superficially mild phenotypes [4]. The lack of lethal phenotypes in those cases is probably a consequence of gene family expansion that led to a balance of functional redundancy and specialization. As extracellular proteins that interact with and regulate the expression of other ECM proteins, proteases and cytokines, *CCN1* and *CCN2* are necessary for the creation of a balanced ECM environment, the disruption of which can affect fundamental aspects of cell differentiation and tissue growth and development.

Structurally, *CCN1* and *CCN2* are organized as multimodular molecules composed of four distinct individual cysteine-rich motifs [14, 15], each of which is encoded by a separate exon (Fig. 1). These multimodlular proteins consist of an N-terminal secretory peptide followed by (1) an insulin-like growth factor-binding protein (*IGFBP*) homology domain, (2) a von Willebrand factor type C domain (*vWC*), (3) a thrombospondin type 1 repeat homology domain (*TSP1*), and (4) a C-terminal domain (*CT*) containing oligomerization and heparin binding motifs. The primary sequence contains 38 cysteines accounting for 17 disulphide bonds spread throughout the 4 domains. *CCN* proteins may have been formed during evolution by exon shuffling or other recombination mechanisms from ancestral domains. Each domain consists of a consensus sequence that has evolved independently from the ancestral motif following its transfer gradually changing its structure and acquiring new functions.

CCN1 and *CCN2* gene expression is regulated at the transcriptional, post-transcriptional, translational and post-translational levels in response to mitogenic stimuli such as vascular endothelial growth factor (*VEGF*), fibroblast growth factor (*FGF*), platelet-derived growth factor (*PDGF*), transforming growth factor (TGF)-β, sphingosine 1-phosphate, endothelin-1, angiotensin II and lipopolysaccharides as well as physical and environmental inputs such as shear stress, mechanical stretch and oxygen deprivation [16, 3, 17]. The transcriptional requirements of the *CCN1* and *CCN2* genes do not involve new protein synthesis but only transcriptional activators with a *PDZ*-binding motif such as *Yap1* and serum response factor-*CarG box* tertiary complexes [18]. This is consistent with the immediate early-gene expression pattern of these genes [18, 19]. Conversely, *CCN1* expression has been found to be downregulated during tissue involution, in avascular tissues and under conditions associated with vasoobliteration which is consistent with a potential role of this protein in vessel formation, stabilization and integrity [20, 21]. The transcription factor, FOXO3a, is a negative transcriptional regulator of the *CCN1*

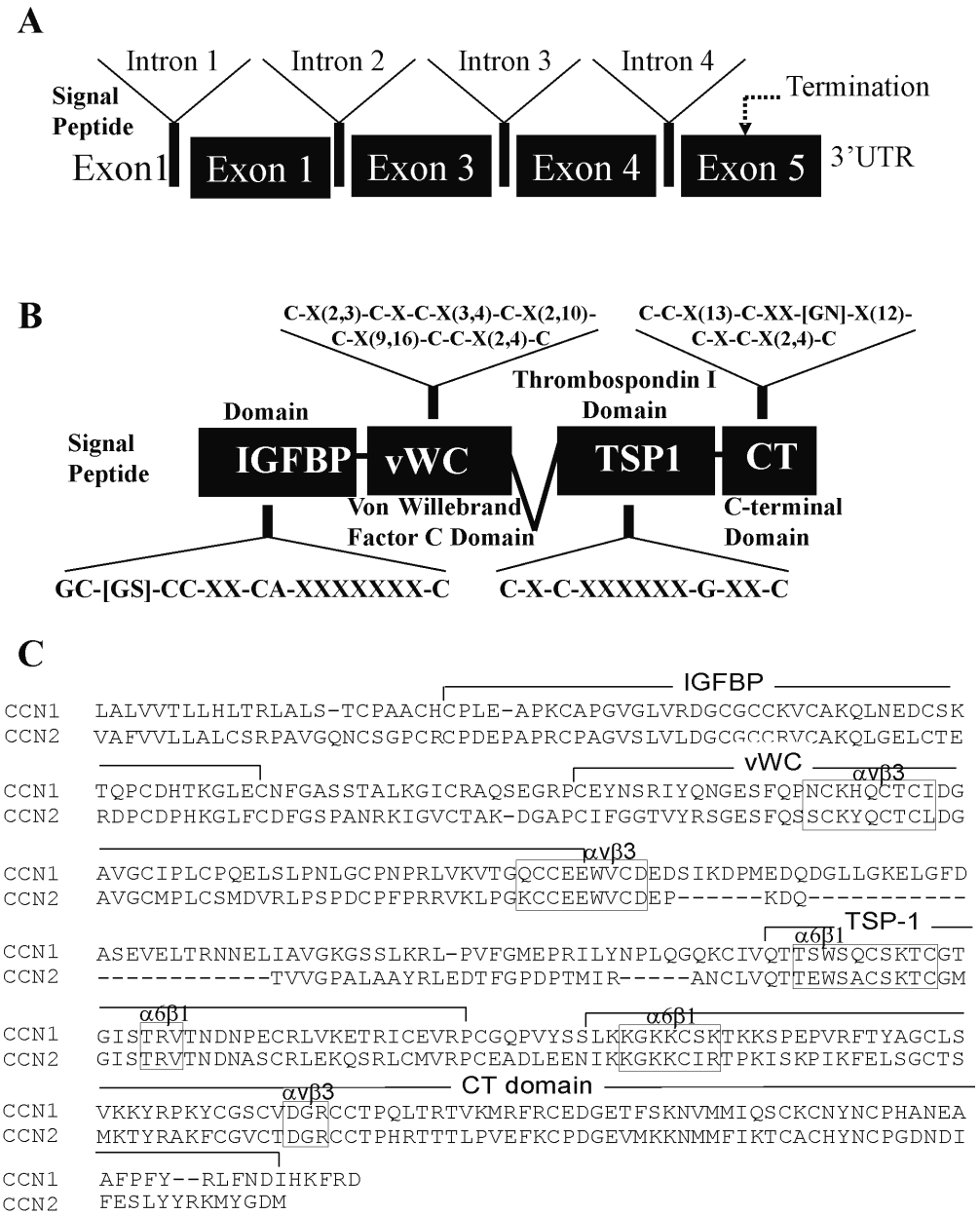

Fig. 1. Gene and modular domain structure of *CCN1* and *CCN2*. A and B: diagrams of *CCN1* and *CCN2* gene structure and modular domains of the encoded proteins. The consensus motifs found in each modular domain are indicated. C: aligned amino acid sequences of the *CCN1* and *CCN2* proteins. The conserved consensus sequences are framed within rectangles.

gene and the suppression of *CCN1* is among several mechanisms by which FOXO3a inhibits vascular smooth muscle cell proliferation [22].

Like *CCN1*, the *CCN2* gene is transcriptionally activated in vascular cells in response to mitogenic and environmental stimuli [17, 23]. Under hypoxic conditions, the expression of the *CCN2* gene is induced both transcriptionally through hypoxia-inducible factor (*HIF)-1α* activation of the *CCN2* promoter and post-transcriptionally through enhanced mRNA stability through an AUA-rich 3'UTR sequence [24, 25]. *CCN2* is also a predicted and

validated target of the miR-17-92 microRNA cluster which regulates tumor neovascularization as well [26]. However, the half life of the *CCN2* protein is relatively low (half of that of *CCN1*) because its affinity for heparin is much lower than that of *CCN1*, significantly reducing its interactivity with the ECM proteins [27].

Functionally, the *CCN1* protein regulates many cellular activities such as cell adhesion, migration, proliferation, differentiation, survival, senescence and apoptosis while the primary biological roles of *CCN2* appear to be in the formation of connective tissues during embryonic development and their maintenance and homeostasis in the adult, and in coordinating tissue repair following injury [28, 29, 30]. As such, both proteins have emerged as critical regulators of developmental vessel formation as demonstrated by the lethal angiogenic and vascular deficiencies upon their targeted deletions in mice [31]. We will use the implications of *CCN1* and *CCN2* knockout mice as a springboard to analyze the role of these molecules in vascular development.

Formation and developmental control of blood vessels

Formation of the earliest primitive vascular tubes is achieved by *in situ* differentiation of mesenchymal cell-derived haemangiogenic stem cells that further differentiate into angioblasts, the progenitors of endothelial cells (ECs) and to haemangioblasts, the progenitors of haematopoietic cells [32, 33]. Upon their differentiation, endothelial precursors coalesce into cords and then form a lumen. This vasculogenic process is the major mechanism of initial formation of blood island vessels, dorsal aorta, endocardium, and vitelline vessels in the embryo [34]. On the other hand, the predominant means of vessel expansion in all organ systems is angiogenesis, which is based on endothelial sprouting or intussusceptive microvascular growth [35, 36]. The formation of the vasculature by vasculogenesis or angiogenesis depends on the proportions of the angioblast progenitors in each developing organ. However, both processes are not mutually exclusive in as much as angioblasts can be incorporated into expanding pre-existing blood vessels [37, 38]. During vascular repair, circulating endothelial precursors populate a growing vascular network from within blood vessels [37].

The first vascular structures (i.e., endothelial cells) appear in the extraembryonic yolk sac at the gastrulation stage as blood islands/angiogenic clusters lined by ECs and perfused by early erythrocytes [39]. An immature vascular network then forms by fusion of blood islands and a subsequent vascular remodeling leads to the formation of the complex yolk sac vasculature. The intraembryonic vasculature proper forms further as angioblasts differentiate into ECs followed by an active angiogenic sprouting of blood vessels [40, 41]. As the embryonic blood vessels become established and various tissues differentiate, ECs continue to undergo numerous changes, generating functionally distinct, tissue specific vascular beds including the complex vascular structures of the dorsal aorta and vitelline vessels, and primary plexuses of lungs, spleen, and heart regions. The dorsal aorta develops at the same time as the early cardiac structure and produces branches that supply blood to the rest of the embryo. For venous circulation, three paired veins form to drain into the sinus venosus which later becomes incorporated into the right atrium [42]. These include (1) the vitelline veins which return poorly oxygenated blood from the yolk sac, (2) the umbilical vein which carries well oxygenated blood from the primordial placenta and (3) the common cardinal veins which return poorly oxygenated blood from the embryo's body.

The newly formed embryonic vessels further specialize into arteries, veins, and capillaries, which have distinct characteristics based on the presence and amount of smooth muscle cells and specific ECM. Capillaries, the business end of the cardiorespiratory system, are the microvessels responsible for most of the peripheral exchange. These structures consist of a single layer of ECs surrounded by a basement membrane within which mural cells (e.g., pericytes) are embedded. The appropriate guidance of vessels and development of tissue-specific differences is critical for proper tissue function. Specific sprout guidance factors are spacially organized within certain tissues (e.g., nervous system including retina) and yield highly stereotypical blood vessel networks. In other tissues (e.g., mouse yolk sac), the absence of guidance cues leads to a freely branched vessel network [43]. The loss of these regulatory factors often leads to vascular lethal defects during fetal development or an abnormal neovascularization and a variety of

pathological conditions in the adult [44, 45]. Many excellent reviews cover vascular development more globally and the mechanisms involved and the readers are referred to them for more detailed information [46, 47, 48].

There is some information on the cellular and molecular processes that control where and when blood vessels form. The primary stimulus for angiogenesis is often a mismatch between the growing metabolic demand of the developing tissue and substrate delivery [49, 50]. A complex network of cytokines of the *VEGF*, *FGF*, *PDGF*, *TGF-β*, insulin-like growth factor (*IGF*) and angiopoietin systems has been identified as the ultimate responsive factors that palliate the metabolic and oxygen demands in tissues [51, 52, 53]. In avian models for instance, *FGF* is involved in the induction of angioblasts while *VEGF* supports the assembly and patterning of vessels [54, 55]. The relevant *VEGF* receptors, the tyrosine kinases *VEGF*R2 and *VEGF*R3, are activated on EC membrane surface upon ligand binding, which triggers downstream signaling including activation of the mitogen-activated kinase (MAPK) pathway, phosphoinositide kinase-3 (*PI3K*), *Akt*, phospholipase C and small GTPases such as *Rac1* [56, 57]. How these signals or their selective activation translates into different biological responses as diverse as EC differentiation or proliferation, or angiogenesis is not fully understood. The *Notch* pathway, which is well known for its roles in cell fate determination and differentiation processes, is upregulated by *VEGF* and is responsible for EC specification in sprouting vessels [58, 59]. Sprouts are headed by migrating endothelial tip cells, which dynamically extend long filopodial protrusions in a polarized way, migrate into the ECM, and sense their environment for attractive and repulsive signals for guidance. In the embryonic spinal cord and the retina, the heparane sulfate anchored isoform of *VEGF*-A (*VEGF*^{164} in mice, *VEGF*^{165} in humans) promotes the polarization of tip cells and the directional extension of filopodia [60, 61]. The Delta-like 4 (*Dll4*)/*Notch* pathway establishes an adequate ratio between stalk and tip cell populations by restricting tip cell formation through "lateral inhibition" in response to a *VEGF* gradient. Thus, specificity may be provided by the spaciotemporal expression patterns of *VEGF* and its receptors.

Following this angiogenic remodeling, the endothelium secretes *PDGF*, which induces the recruitment and differentiation of vascular smooth muscle cells [62, 63]. Subsequently, the vascular smooth muscle cells secrete angiopoietins, which ensure proper interaction between endothelial and mural cells [64, 65]. Finally, the vascular smooth muscle cells deposit matrix proteins, such as elastin, that inhibit vascular smooth muscle cell proliferation thereby stabilizing the mature vessel [66]. Modulation of the activity of these and other molecular intermediates of vessel formation may represent one of the underlying molecular mechanism(s) leading to the CCN protein-dependent regulation of vascular development.

Expression and molecular control of vascular development by *CCN1*

CCN1 is most highly and dynamically expressed in the second and third trimesters of pregnancy, especially in non-proliferating interstitial extravillous trophoblastic giant cells, vascular ECs, and mesenchymal and stromal cells of the placental villi [67]. *CCN1* was found to be continuously expressed during the formation of and in the fully developed placenta in 12.5-day *post coitum* mouse embryos particularly in angiogenic cell types such as the giant trophoblasts and the overlying compact deciduas [68]. These angiogenic structures produce the essential factors (e.g., *VEGF*, basic (b) *FGF* and *PDGF*) required for maternal vessel growth towards the developing embryo [69]. Meanwhile, a prominent cardiac expression of *CCN1* was seen as early as E8.5 in mice expressing lacZ under the control of the endogenous *CCN1* promoter [70]. CCN1 expression continued at E10.5 especially in the trunk arteriosus, which later divides to form the aorta and pulmonary trunks [71]. *CCN1* expression was also found in all major arteries branching from the heart of the developing fetal circulatory system. These include the aortic and pulmonary trunks which are branches of the aortic arches that are of neural crest origin and in the dorsal aorta and umbilical artery which are of mesodermal origin. Mesodermal cells ultimately differentiate into smooth muscle. Of interest is that structures originating from the neural crest also give rise to cartilaginous tissue; therefore, all regions of mouse embryo involving cartilage formation expressed *CCN1* as well [72]. *CCN1* was shown to function as a soluble

chemotactic signal in human mesenchymal stem cells and induce a site-directed cell recruitment *in vivo* [20, 73]. Whether *CCN1* affects the linear commitment of mesenchymal stem cells and/or regulates cell and/or further cell differentiation is yet to be determined.

Our group has examined the vascular localization of *CCN1* during development of the retinal vasculature, as the morphology of the primitive plexus at the edge of the developing retinal vasculature is highly similar to some of the first blood vessels in early vertebrate embryos [13]. In the mouse, the retina is avascular at birth and vessels grow concentrically from the center toward the periphery following an existing network of astrocytes [74, 75, 76]. Once a primary plexus is established, sprouts dive vertically deep into the retina and form intermediary and deeper vascular layers. Using transgenic mice expressing the green fluorescent protein (GFP) gene under the control of the *CCN1* promoter, we found that *CCN1* was dynamically expressed postnatally in the mouse eye and that its expression was transient and largely confined to the advancing vascular front of the developing vasculature. *CCN1* appeared to be dynamically expressed in both endothelial tip and stalk cells suggesting its potential role in the phenotypic plasticity of ECs. Interestingly, *CCN1* expression is also associated with hyaloid vessels as well, especially in macrophages and shifts to the retinal vasculature emerging from the optic disc [13]. Hyaloid vessels form a complex of intraocular vessels that penetrate the retina at the optic disc and branch anteriorly through the vitreous to the lens. Hyaloid vessels then regress progressively by apoptosis as the retinal vasculature develops by angiogenesis in a synchronized manner suggesting that overlapping *CCN1* signals may control both processes simultaneously.

The generation of *CCN1* mutant mice provided a unique animal model for addressing the role of *CCN1* in vascular development. A large majority of *CCN1* null embryos died of hemorrhage and/or placental defects between E11.5 and E14.5 while a fraction of embryos died earlier as a result of defects in chorioallantoic fusion [77]. Both cardiac and vascular defects were the most prominent alterations observed. They seemed to originate at the chorioallantoic junction, where the allantois failed to fuse with the placenta, resulting in severe undervascularization in the placental labyrinth or from defects of large vessel bifurcation resulting in a poor vascular coverage of the chorionic plate. Large fetal vessels such as the aorta appeared dilated as in large aneurysm. Vascular cells such as ECs and smooth muscle cells were not confined within their layers as ECs were mislocalized in the media layer of the aorta. Subsequently, *CCN1*-deficient embryos suffer from compromised integrity and hemorrhage of arterial vessels in which the most expression of *CCN1* occurs during development.

Meanwhile, severe atrioventricular septal defects (AVSD) have also been observed in *CCN1*-deficient embryos [71]. AVSDs are characterized by the complete or partial absence of partitioning of the atrioventricular valve. These are common genetic disorders that cause congenital heart disease in humans.

Regulation of vascular cell function and behavior by *CCN1*

Once secreted, the *CCN1* protein regulates many aspects of vascular cell function including adhesion, migration, proliferation, differentiation and survival [10, 78]. Many of these activities derive from *CCN1* interaction with integrin receptors localized in the *vWC* domain which contains binding sites bearing structural homologies to those involved in the interaction with $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_2\beta_3$ integrins [78, 79, 80, 81]. *CCN1* signaling requires HSPG as a co-receptor and involves either interaction with a single integrin type or the concerted binding to several integrins [82]. The interaction between $\alpha_v\beta_3$ integrins and the *vWC* domain was shown to be largely responsible for *CCN1*-mediated endothelial cell adhesion, migration and survival while *CCN1* binding to integrin $\alpha_6\beta_1$ mediates capillary-like tubule formation of non-activated ECs [83, 84]. Interestingly, *CCN1*-deficient mutants phenocopied chorioallantoic and vascular defects previously reported in α4 and αv integrin-deficient mice respectively, which underscores the importance of *CCN1*-integrin signaling [85]. Defective signaling through *CCN1*-integrin interaction may, at least in part, account for the chorioallantoic and vascular defects associated with *CCN1* deletion in mice.

Fig. 2 provides a simplified pathway map of *CCN1*- (and *CCN2*)-dependent intracellular signaling

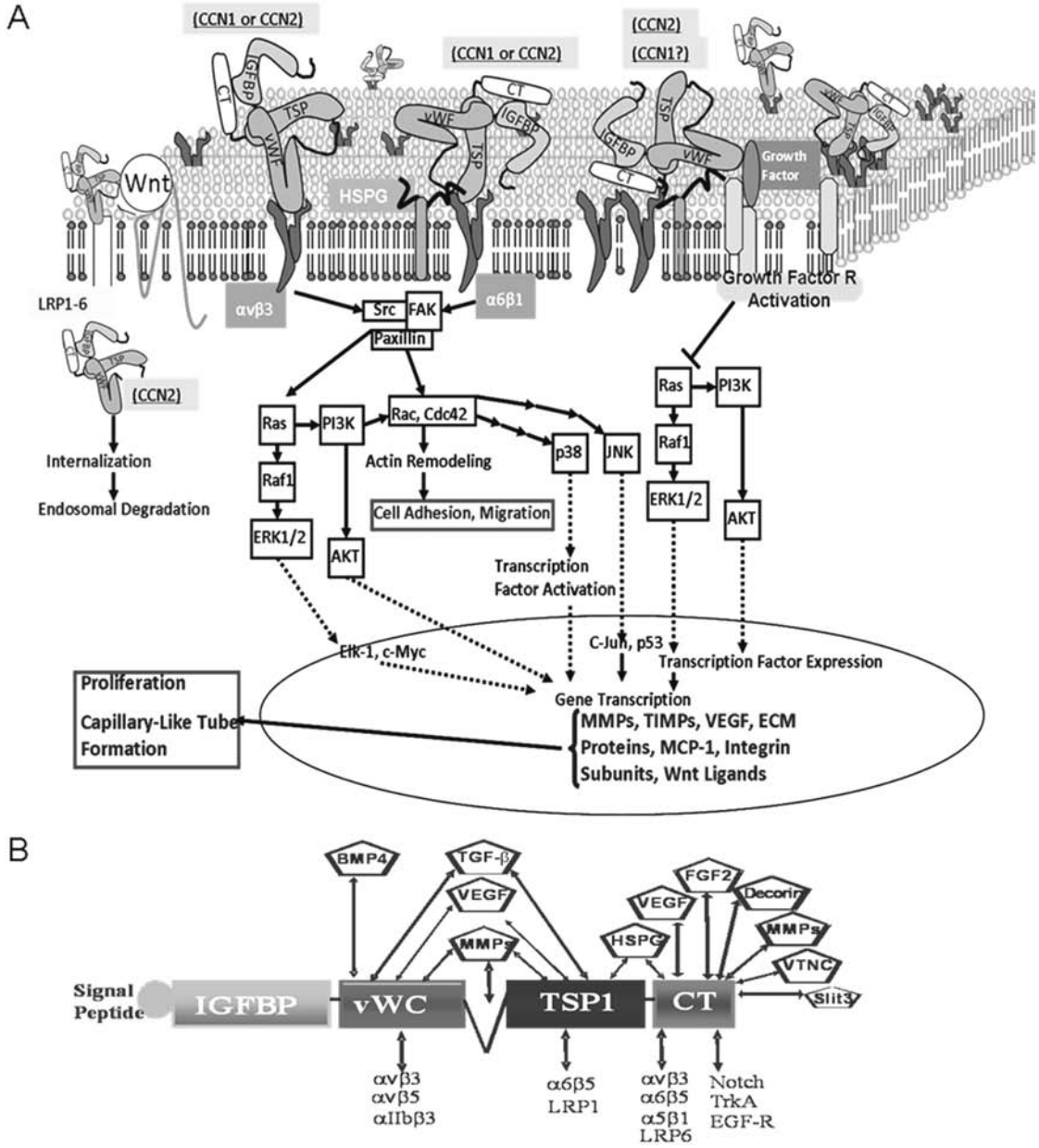

Fig. 2. A: Simplified pathway map of *CCN1* and *CCN2*-dependent outside-in signaling pathways regulating endothelial cell function and behavior. B: Interaction of *CCN1* and *CCN2* proteins via their constituent domains with extracellular ligands and transmembrane proteins.

and their putative interacting partners. *CCN1*-integrin interaction culminates into the activation of small GTPases such as *RhoA* GTPases, *Rac* and *Cdc42*, which leads to cytoskeletal actin reorganization, formation of lamelilpodia/filipodia and cell migration [86, 87]. *CCN1* also activates the *Ras* pathway axis through signaling molecules in focal complexes, MAPK (*ERK*1/2), and/or *Akt*/*PKB* signaling [88, 28], and enhances the expression and/or stability of key regulatory proteins that promote cell cycle progression, adhesion and capillary-like tube formation [89]. A broad spectrum of genes that are responsive to EC stimulation by *CCN1* includes but is not limited to matrix metalloproteinases (*MMPs)*, tissue inhibitor of metalloproteinases (*TIMPs)*, integrins and *VEGF* [90, 91, 92]. In particular, *VEGF*-C, which is

predominantly expressed by embryonic endothelium and macrophages, was downregulated in *CCN1*-null mouse embryo [77]. The spatial distribution and temporal qualities of the dynamics of *CCN1* targets vary with the cell context, enabling the cell to program an appropriate biological response. The adhesive and chemotactic activities of *CCN1* on endothelial and smooth-muscle cells may underlie the loss of vessel integrity upon *CCN1* gene deletion in mice.

Not only does *CCN1* initiate its own intracellular signaling, it also is able to modulate the activity of other unrelated molecules (e.g., growth factors, cytokines, ECM proteins) by potentiating their crosstalk with cell membrane and growth factor receptors (Fig. 2B). For instance, in *Xenopus* embryos, *CCN1* inhibits bone morphogenic protein (*BMP)* signaling through interference with *BMP* interaction with its receptor [93, 94]. *CCN1* may constitute one of the extracellular fine-tuning modulators of BMP signaling during vascular development essentially by shaping gradients of *BMP* activity in areas of uniform ligand expression.

CCN1 also directly interacts via its *CT* domain with fibronectin and vitronectin and facilitates the activation of αv integrins by these proteins [95, 96, 93]. *CCN1* physically binds to the *somatomedin B* domain of vitronectin which is also the functional region that mediates interactions with plasminogen activator inhibitor-1, *uPAR*, and integrins αvβ3/αvβ5. Such interaction may affect the angiogenic tonus conveyed by those molecules. Along these lines, binding of *CCN1* to ECM proteins displaces b*FGF* from the matrix and increases both its availability to interact with its receptor on ECs and its angiogenic properties [79]. Similarly, *VEGF* has been shown to bind to fibronectin via its fibronectin-like domain and promotes EC growth and migration [97]. As the *vWC* domain of *CCN1* bears similarities with fibronectin-like domain, and may have evolutionarily preceded it (i.e., the latter has evolved as a truncation of the *vWC* domain), raises the possibility that functional similarities may, at least in part, exist between these two types of modules. Therefore, the angiogenic activity of *CCN1* may depend on the interaction with and/or modulation of *VEGF* activity. Our group has shown that both the *vWC* and *TSP1* domains of *CCN1* containing stretches of amino acid residues involved in integrin and growth factors binding form a synergy region located on the same face of the molecule [98]. This suggests that the simultaneous interaction of growth factors and integrins with this region may be sterically hindered and thus, mutually exclusive.

Expression and molecular control of vascular development by *CCN2*

Since its initial discovery, *CCN2* has been considered an important determinant factor of extracellular matrix synthesis and remodeling controlling the formation of connective tissue-rich compartments [99, 100, 29]. However, direct tissue-specific overexpression of the *CCN2* transgene in fibrosis-prone organs (e.g., heart, liver, kidney) failed to induce any fibrotic reaction in the target tissues [101, 102, 103]. Interestingly, lung-specific *CCN2* transgene overexpression showed evidence of an impaired formation of the alveolar vascular network characterized by fibrosis in and around blood vessels, alveolar septa and bronchioles [104]. This clearly suggests that *CCN2* affects blood vessel formation but only at specific stages of their development.

During mouse embryogenesis, *CCN2* is widely but dynamically expressed especially within embryonic tissues that give rise to the cardiovascular, musculoskeletal and nervous systems [105]. Precisely, *CCN2* was detected as early as E7.5 in the parietal endoderm and crypt cells. It further continued in the notochord, cephalic mesenchymal cells, first bronchial pouch and in the roof and floor palate of the neural tube. At E.8, *CCN2* expression was prominent in the heart myocardium and in the common cardinal vein, bronchial arch arteries, and the dorsal aorta. Later during fetal development, *CCN2* levels increased in all large blood vessels including the aorta, pulmonary artery, and particularly in the ductus arteriosus and pulmonary trunks. *CCN2* localized at the abluminal surface of the endothelium and in mural cells in blood vessels and capillaries. Although *CCN2* expression decreased postnatally, it remained detectable in all large blood vessels. The persistence of basal *CCN2* gene expression during adulthood suggests that *CCN2* may be involved in vessel stability and/or maintenance.

CCN2 localized pericellularly in arteries, veins and capillaries. ECs and pericytes were the major sources of *CCN2*. Interestingly, *CCN2* is also highly expressed in skeletal tissues during endochondral

ossification primarily in the cartilage, within maturing chondrocytes and in the developing ribs and long bones [106]. *CCN2*-null mice die shortly after birth from respiratory failure due to disruption of basic lung development (i.e., lungs were hypoplastic with reduced cell proliferation and increased apoptosis) and failed thoracic expansion [107]. The mutant phenotype essentially revealed a role for *CCN2* in growth plate angiogenesis [108]. Hypertrophic zones of CCN2 mutant growth plates were expanded, and endochondral ossification was impaired. A dramatic decrease of the capillary network in the growth plate was evident in *CCN2* mutants which accounted for defective replacement of cartilage by bone during endochondral ossification. No obvious vascular defects during the initial formation of the large vasculature from E9.5 to E13.5 were observed probably due to compensatory pathways mediated by the related *CCN1* protein [109]. However, after E14.5, the large vessels in *CCN2*-null embryos were enlarged with localized edema. There was no deficiency in mural cell recruitment to these vessels but the cells in the media layer appeared heterogeneous in size and dysmorphic. Conversely, poor coverage of microvessels by pericytes was evident in *CCN2* mutant mice. Surprisingly, arterial-venous identity appeared to be maintained in *CCN2* mutants suggesting that *CCN2* has no effect on arteriovenous specification markers.

Regulation of vascular cell function and behavior by *CCN2*

CCN2 was characterized as a growth factor inducible, immediate early gene-encoded matricellular protein sharing the multimodular structure of *CCN1* and a 52% identity with its primary sequence [15]. The *CCN2* protein elicits a strong angiogenic response comparable to that of either *CCN1* or b*FGF* when tested in the chorioallantoic membrane assay of chick embryos or when injected subcutaneously in mice [110, 27, 111]. Exogenously added *CCN2* to cultured ECs effectively activated mitogenic signaling cascades (e.g., *ERK*1/2, *p38* MAPK) and promoted cell growth, clearly indicating that *CCN2* has a strong intrinsic angiogenic activity [112].

Like *CCN1*, the function of *CCN2* is contextual and depends on the availability of membrane receptors and/or interacting partners in the cellular microenvironment (Fig. 2). Through direct binding to integrin αvβ3, *CCN2* promotes endothelial cell adhesion, migration, proliferation, and tubule formation, thus, recapitulating typical angiogenic events [80, 113, 114]. *CCN2* also promotes survival of ECs deprived of growth factors, a condition that otherwise induces apoptosis [83]. *CCN2*-integrin interaction activates *FAK* and other intracellular signaling molecules such as MAPK, PI 3-kinase and *Rho* GTPases [115, 116, 81, 117]. The latter signaling pathway culminates in changes in cytoskeletal actin organization, filopodia and lamellipodia formation and ECM protein deposition (e.g., fibronectin, fibrillar collagens, proteoglycans). *CCN2* binds to a variety of other integrins expressed by different cells, e.g., αIIbβ3 on activated platelets [118], α6β1 on skin fibroblasts [116] and αMβ2 in blood monocytes [114]. In fibroblasts, *CCN2* binds to integrins α5β1 and α4β1, and syndecan 4 [116]. Such interaction is critical for cell spreading and adhesion. *CCN2* also interacts with the low-density lipoprotein receptor proteins (LRP) although such interaction was shown to only lead to *CCN2* internalization and degradation in the endosomes [119].

CCN2 signaling was shown to modulate growth factor and ECM protein gene expression as well [120, 121, 122]. *CCN2* induced *PDGF*-B expression in ECs, and potentiated *PDGF*-B-mediated *Akt* signaling in mural (e.g., vascular smooth muscle/ pericytes) cells [123, 124]. *PDGF* signaling and the establishment of the endothelial basement membrane are required for pericyte recruitment and retention [125, 126]. These processes appeared to be defective in *CCN2*-deficient mice [109]. In addition, *CCN2* induced the production of endothelial basement membrane components *in vitro*, and was required for their expression *in vivo* [106, 127]. Blood vessel leakage and altered basement membrane integrity were attributed to *CCN2* deletion *in vivo* while basement membrane thickening was linked to excessive production of *CCN2* in glomerular capillaries of transgenic animal models [108, 128]. Thus, *CCN2* is required for the normal production of the vascular basement membrane.

In addition to the aforementioned interactions, *CCN2* may exert its effects through direct physical interaction via one of its domains with bioactive molecules and/or one or more cell surface tyrosine kinase receptors. In particular, Inoki *et al*. have shown that *CCN2* directly interacts with *VEGF*[164] both

in vitro and *in vivo* via its modular domain, *TSP1*, which is highly conserved between *CCN1* and *CCN2* [129]. Such interaction hinders *VEGF*[164] binding to its receptor (*VEGF*R2) and reduced *VEGF*-mediated angiogenesis. Proteolysis of the complex *CCN2-VEGF* by MMPs released the bound *VEGF* in an active form and enhanced its angiogenic potential [113, 130]. Thus, *CCN2* may act by fine-tuning the bioavailability of *VEGF*, releasing it for angiogenic action only in contexts where *MMP*s are being secreted and activated. Meanwhile, a recent study has shown that *CCN2* physically interacts with the epidermal growth factor receptor (EGFR) and *tropomyosin receptor kinase A* (*TrkA*) via its carboxy terminal domain leading to MAPK activation and proinflammatory factor gene upregulation [131]. *CCN2* also has broad affinity to a series of other angiogenic regulators with common CT motifs including *Slit3*, *von Willebrand factor*, *PDGF-B* and *FGF* [132, 133]. Mutation of the CT domain of *CCN2* abolished its interactivity with bioactive molecules with common *CT* motifs and reduced their proangiogenic properties *in vitro*. However, whether these *in vitro* findings have physiological significance is still unknown and warrant further investigations.

CONCLUSION

Overall, *CCN1* and *CCN2* share many similarities in their activities and patterns of expression, and exhibit overlapping and non overlapping functions. With so many potential modes of action and interacting partners it is conceivable that *CCN1* and *CCN2* influence different vascular developmental events. Given their context- and cell type-dependent biological activities that vary based on the presence of specific receptors and interacting partners in the cell microenvironment, the function of these proteins may vary under physiological and disease conditions.

Despite significant advances in recent years, many questions concerning the mechanisms whereby *CCN1* and *CCN2* regulate vascular development remain to be addressed. Vascular growth and morphogenesis are initiated, at least in part, by *VEGF*-derived signals and ECM-integrin-cytoskeletal signaling axis which causes simultaneous sprouting (i.e., cord formation) and lumen formation events that eventually lead to interconnecting networks of EC-lined tubes [134]. These and other processes appeared to be defective in *CCN1*- and/or *CCN2*-deficient mouse embryo despite the presence of *VEGF*. In both normal and pathological angiogenesis, sprouts are headed by migrating endothelial tip cells, which dynamically extend long filopodial protrusions in a polarized way, migrate into the ECM, and sense their environment for attractive and repulsive signals for guidance [135]. Therefore, are *CCN1* and *CCN2*-derived signals important for the control of endothelial tip and stalk cell specification and endothelial tip cell protrusive activity? As *Notch* and *Wnt* signaling pathways control multiple aspects of endothelial cell biology, are *CCN1* and *CCN2* important in engaging these signaling pathways at various stages of vascular development? Are they indispensable in rearrangement and migration of ECs and mural cells to ensure a coordinated outgrowth of vascular sprouts while maintaining integrity of nascent vessels? How do *CCN1* and *CCN2* help tissues cope with or succumb to hypoxic stress? A deeper understanding of these fundamental principles will undoubtedly aid in the development of new treatments for blood vessel-related pathologies.

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CONFLICT OF INTEREST STATEMENT

The author has no conflict of interest to disclose.

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