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The CCN family acting throughout the body: recent research developments

Abstract: The animal body is composed of a variety of cells and extracellular matrices that are organized and orchestrated in a harmonized manner to support life. Therefore, the critical importance of a comprehensive understanding of the molecular network surrounding and integrating the cells is now emphasized. The CCN family is a novel group of matricellular proteins that interact with and orchestrate a number of extracellular signaling and matrix molecules to construct and maintain living tissues. This family comprises six distinct members in mammals, which are characterized by a unique and conserved modular structure. These proteins are not targeted to limited and specific receptors to execute specific missions, but manipulate a vast number of biomolecules in the network by serving as a molecular hub at the center. The unified nomenclature, CCN, originates from a simple acronym of the three classical members, which helps us to avoid having any preconception about their pleiotropic and anonymous functional nature. In this review, after a brief summary of the general molecular concepts regarding the CCN family, new aspects of each member uncovered by recent research are introduced, which represent, nevertheless, only the tip of the iceberg of the profound functionality of these molecules.

Keywords: CCN family; fibrosis; gene regulation; malignancy; matricellular protein.

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Introduction

More than two decades have passed since the discovery of the first CCN family member, CCN1. Initially, this gene product was identified as a cysteine-rich protein that was induced by growth factors and, thus, designated Cyr61. The second and most well-known member, CCN2, was isolated as a platelet-derived growth factor-related protein with mitogenic activity. The original name of this molecule, connective tissue growth factor (CTGF), was given based on this functional property. Thereafter, a gene structurally related to the two described above was found to be overexpressed as a truncated form in nephroblastoma, which resulted in the designation of this protein as nephroblastoma-overexpressed (NOV). Thus, the name of this family of three genes/proteins was proposed based on the names of these classical members (1–5). Although the discovery of the other three members occurred after this proposal, proper names other than CCN family-based ones were given as well. Among the different names given for these 'nonclassical' members, WISP1, 2, and 3 (2) have been widely accepted and are being preferred by several research communities even now.

In the early days of the investigation into each CCN family member, major progress was made under the bondage of the original name. As typically represented by the classical name of CCN2, researchers tend to regard these extracellular moiety molecules as growth factors or related proteins and analyzed CCN family members from the viewpoint of being extracellular messengers. Thus, a number of researchers spent a vast amount of time in determining the unique receptor for each.

However, as the research progresses, we have become aware that CCN family members are not messengers with highly specified missions, but rather novel molecules that manipulate a number of counterparts to regulate the whole information network. Nowadays, most investigators recognize that CCN family member interacts with multiple molecules in their microenvironment rather than only with a unique receptor on a target cell. Along with the understanding of such molecular behavior of the CCN family, it has gradually become clear that CCN family members may be playing critical roles in multiple organs and tissues. For example, the function and role of CCN2 in connective tissues and malignancies were extensively characterized, and a vast number of publications appeared in these categories (1–5). However, recent studies are now revealing the role of CCN2 in the development of Alzheimer's disease (5). As such, in this review, following the introduction of the molecular and genetic characteristics of the CCN family, recent knowledge about each member playing significant

roles throughout the body will be summarized in an organ/tissue-wise manner. This review concludes with our personal outlook and opinion about future research into this intriguing family of biomolecules.

Structure and molecular properties

The basic structure of the CCN family proteins is strictly conserved among the members and among various species. These proteins are constructed to have four distinct modules, which are the most prominent structural characteristics of this family, with minor exceptions (1–5). Following the signal peptide for secretion through the endoplasmic reticulum, the insulin-like growth factor binding protein-like (IGFBP) module is located near the amino-terminus. Then, von Willebrand factor type C repeat (VWC) and thrombospondin 1 type I repeat (TSP1) modules are connected in this order to be concluded by the carboxy-terminal cystine knot (CT) module (Figure 1). Interestingly, this order, IGFBP-VWC-TSP1-CT, has been strictly conserved during evolution and, thus, has never been shuffled. However, dimodular or trimodular variants are occasionally formed through alternative splicing of the mRNAs or proteolytic cleavage of the full-length molecule by matrix metalloproteinases (MMPs) and other proteases (6–8; Figure 1). Even single modular variants have been suggested in the case of human CCN2 and CCN4 (4, 9).

During the course of animal evolution, the CCN family is thought to have been born after the branching of animals into the bilateria lineage. At present, a single distinct CCN prototype is found in amphioxus, sharing closest homology to our CCN2 (10). As the modules constituting CCN family proteins are encoded in independent exons and can be found in other related protein families, such as SPARC and IGFBPs, CCN family genes are suspected to have been formed through exon shuffling (10). After the birth of the CCN2 prototype, the other members probably evolved through gene duplication and rearrangement. As a result, four CCN family members became established in the urochordates, all of which members retain homology with human CCN1 and CCN2. Finally, we mammals possess six CCN family members with four modules aligned in this order, only one of which lacks the CT module. The fact that no variation is allowed in the order of the module alignment suggests its critical importance for these molecules; nevertheless, the structural and functional significances of this IGFBP-VWC-TSP1-CT order still remain to be clarified. At present, little is known about the tertiary structure of the CCN family proteins. One previous report indicated that CCN3 and CCN5 are rich in β-strands and random coils, as evaluated by circular dichroism spectrum analysis and that both form extended, rather than globular, shapes in solution as determined by X-ray scattering analysis and *in silico* modeling (11). Further structural characterization may clarify the functional role of such novel structural features of CCN family proteins in the future.

From the viewpoint of function, one should note that all of these modules are highly interactive with a number

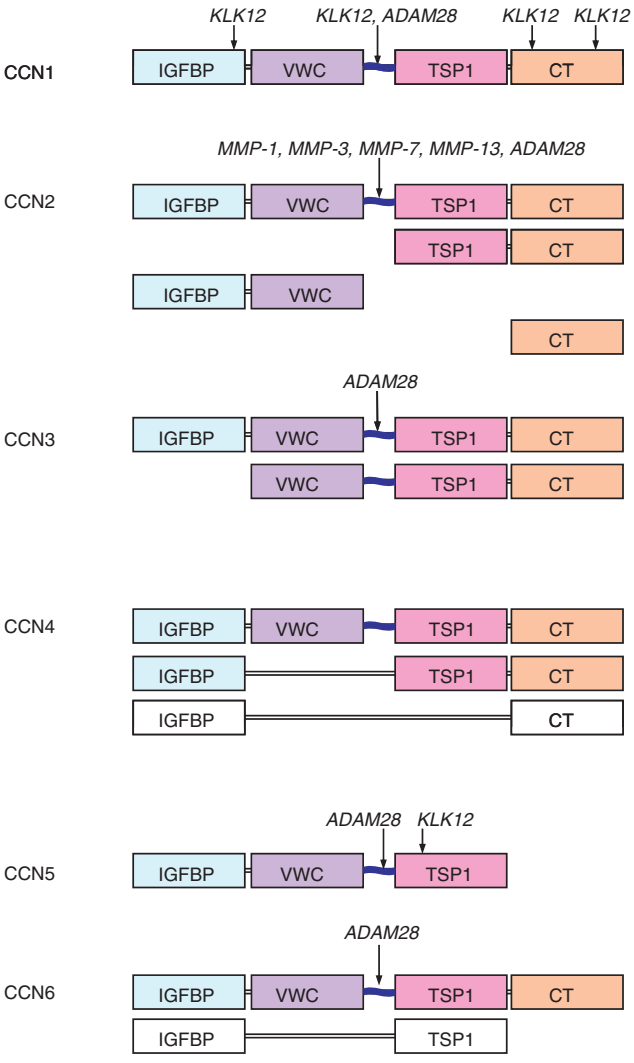

Figure 1 Structure of CCN family proteins.
Each CCN family protein comprises four conserved modules known as insulin-like growth factor binding protein-like (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin 1 type 1 repeat (TSP1), and carboxyl-terminal cystine knot (CT) modules. The amino-terminal and carboxyl-terminal halves are linked by hinge regions (wavy lines) that are susceptible to proteolytic processing. Molecules with a colored background were confirmed at protein levels, whereas those without color were detected only at the mRNA level. Approximate positions of *in vitro* cleavage sites attacked by endoproteases are indicated by arrows and the names of the corresponding enzymes.

of other molecules (4). These molecular counterparts include, as detailed in the next section, growth factors, extracellular matrix (ECM) components, cell-surface receptors, and structural proteins, i.e., almost all kinds of biomolecules. Using these four 'hands', CCN family proteins orchestrate the molecular network by manipulating these molecular counterparts (Figure 2). Even direct interaction between two CCN family members, CCN2 and CCN3, has been recently reported (12). As CCN family proteins, themselves, may interact with signal-transducing, cell-surface receptors, they may initiate intracellular signal transduction *per se*. However, on the other hand, they may be modulating the activity of other molecules at the same time. Consequently, the biological outcome as a consequence of such multiple actions of CCN family proteins is quite diverse, highly depends upon the microenvironment, and thus is sometimes unpredictable or unexpected.

Multiple molecular counterparts

CCN1

The interaction between the CCN family of proteins and integrins constitutes a major part of the functionality of these proteins (Figure 2). Particularly, the CCN1-integrin connection was discovered in the early days of CCN family research and has been extensively investigated. In fact, CCN1 binds to a number of integrins including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_M\beta_2$, and $\alpha_6\beta_1$ to mediate adhesion of various types of the cells and other cell biological responses, such as cytokine production (5, 13). Heparan sulfate proteoglycans are the other major family of the CCN1 molecular counterparts, representing the molecular property of CCN1 as a matricellular protein to facilitate cell signaling (5, 14). As an accessory ligand, CCN1 interacts with a huge cell-surface receptor known as low-density lipoprotein (LDL) receptor-related protein (LRP)-1, contributing to apoptotic signaling (15). The binding of CCN1 to vitronectin also suggests the involvement of this ECM molecule in the matricellular action of CCN1 (16). Of note, sclerostin, which is a Wnt signal inhibitor produced by osteocytes, was found to regulate CCN1 activity by direct interaction, indicating the presence and possible role of CCN1 in bone metabolism (17).

CCN2

CCN2 is the most extensively investigated one among the six members. During the history of CCN2 research, it has been revealed that CCN2 shares common functionality with CCN1 under the interaction with common biomolecules. The most prominent examples of such molecules

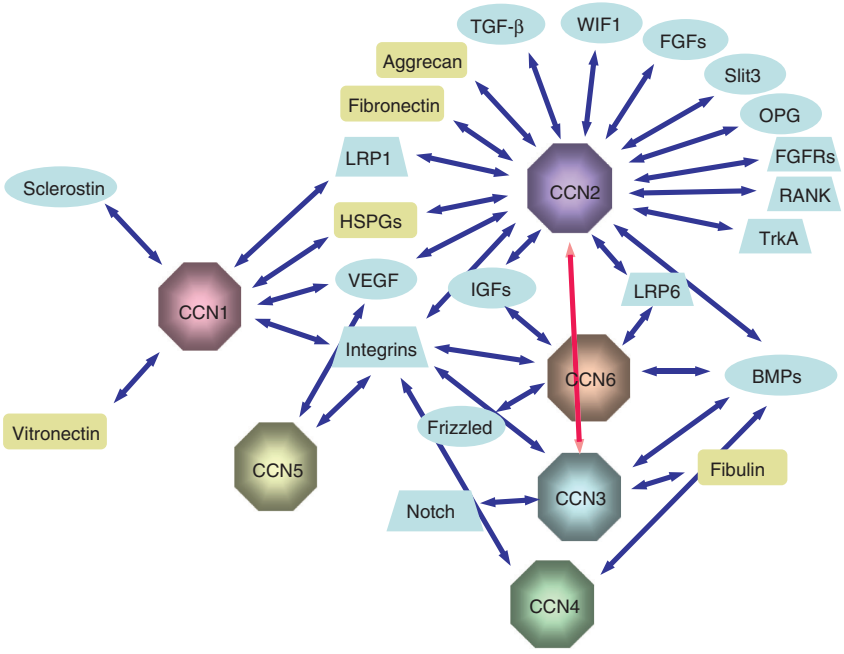

Figure 2 Interactomic representation of CCN family and cofactors.
Direct binding between two molecules, as confirmed experimentally, is indicated by bidirectional arrows. Interaction between CCN2 and CCN3 is indicated as a red arrow to avoid confusion.

are the integrins and HSPGs. In fact, CCN2 exerts effects comparable to those of CCN1 by binding to exactly the same integrins on the same cells (5, 13, 18). In addition, LRP1 is another example of a common cofactor, although the target cell and biological outcome are totally different between CCN1 and CCN2 (15, 19). However, it is a matter of course that CCN2 also interacts with its proper partners in exerting CCN2-specific functions. As a matricellular protein, CCN2 binds to fibronectin and aggrecan, which are major components of the ECM (20, 21). Other counterparts include cell-surface receptors, such as receptor activator of NF-κB (RANK; 22), LRP6 (23, 24), TrkA (25), fibroblast growth factor (FGF) receptor (FGFR) 2, and FGFR3 (26). It should be particularly noted that CCN2 interacts with a number of growth factors, which include transforming growth factor β (TGF-β), bone morphogenetic protein (BMP) 2, BMP4, FGF2, vascular endothelial cell growth factor (VEGF) and Slit 3, a molecule that activates Rho family members to promote cell migration (21, 27–29). In most cases, CCN2 was found to modulate the signal emitted from these molecules via direct interaction. Additionally, CCN2 manipulates modifiers of extracellular signaling. For example, Wif1, an inhibitory molecule of Wnt signaling (30) and osteoprotegerin (OPG; 22), which is a decoy receptor of RANK, recently joined the community of CCN2 partners. Obviously, multiple interactions with these molecules are providing the basis for CCN2 to exert diverse biological effects in various organs and tissues, which are described in other sections of this review.

CCN3

Binding not only to integrins, CCN3 was found also to bind to Notch (5), fibulin (2), and BMPs (5). In particular, the interaction between CCN3 and BMP2 was suggested to play a critical role in the negative regulation of bone formation (5). Integrins were found to support the angiogenic effect of CCN3 (4). As already stated elsewhere, CCN3 is currently the only CCN family member that has been shown to form a heterodimer with CCN2, although heteromer formation has not yet been examined with other combinations of distinct CCN family members (12).

CCN4–6

Compared to the three prototypic members discussed above, not so many molecular counterparts have been discovered for the other three members identified later. Nevertheless, all of these members were found to interact with integrins as well. Interaction with integrins supports the IL-6 production stimulated by CCN4 (31), neurite formation by CCN5 (32), and chondrocyte migration by CCN6 (33). BMPs were also described as cofactors for CCN4 (34) and CCN6 (35). Recent advances in CCN6 research identified novel cofactors for this last member of the CCN family. These molecules include BMPs (35), LRP6 (36), frizzled (36), and IGF1 (37), suggesting the role of CCN6 as a modifier of Wnt and insulin/IGF signaling.

Gene structure and regulation

CCN family in the genome

As already mentioned above, we mammals possess the genes of six CCN family members in our genome. Among them, those of CCN1 and 2 are characterized by their compact structures with minimal introns, giving total genomic sizes of approximately 3 kbp (38). Conversely, *CCN4*, *5*, and *6* that joined the CCN family after its establishment, exceed 10 kbp in total size due to larger introns. Especially, *CCN4*, the largest member, occupies a locus as long as 40 kbp on chromosome 8 of the human genome. *CCN3* is intermediate between the two groups in size (38). The compact structure of *CCN1* and *2* suggests that these members are the prototypes of this family, which notion is supported by the early emergence of these members along the course of evolution. However, it is not clear whether *CCN3* appeared next, or not, as *CCN3* is not necessarily the third ortholog predicted in early chordata. For example, the lamprey genome contains four CCN family members, among which three are homologous to *CCN1*, *2*, and *6*, whereas the other one is not homologous to any specific member.

In the viewpoint of gene function, structural characteristics of these members are closely related to the formation of transcripts. No splicing variants that yield different species have been reported or predicted in the case of *CCN1*, *2*, and *3* with concise genetic structures. By contrast, a number of *CCN4*, *5*, and *6* mRNA variants encoding different combinations of modules have been predicted and experimentally shown to be present in certain human cells, probably because of the large introns (4, 9; Figure 1). As such, the bulky structures of *CCN4*, *5*, and *6*, themselves, supposedly contributes to the complexity of their gene function.

Every mRNA contains untranslated regions (UTRs) at both the 5′ and 3′ ends. It should be noted that all of

the CCN family members except for *CCN6* are commonly characterized by the retention of their 3′-UTRs of considerable lengths (38). As described in the next section, these 3′-UTR have been shown to be fundamental regulators of the fate of the mRNA.

CCN regulators and their action

Extracellular CCN regulators and relevant signaling molecules

The functionality of CCN family members highly depends upon the microenvironment, indicating that the function of a CCN family protein, itself, is determined by the temporal and spatial regulation of gene expression. Up to today, a number of biomolecules have been found to regulate CCN family gene expression. As positive regulators of CCN family gene expression, TGF-β is probably the best known, whereas inflammatory cytokines, such as TNF-α, are generally recognized as being negative regulators of several CCN family genes (39, 40). The involvement of these molecules is of great interest, as some of the CCN family members have recently been suggested to be associated with inflammatory processes. Particularly, the mechanism and biological significance of the CCN2 induction by TGF-β have been extensively investigated. CCN2 is transcriptionally induced by TGF-β through multiple pathways (3). Indeed, TGF-β physically interacts with CCN2, induces CCN2, and collaborates with CCN2 in certain biopathological situations, such as fibrogenesis (5). TGF-β is attracting the interest of medical scientists also as a mediator of the epithelial-mesenchymal transition (EMT), enhancing *CCN1*, *CCN2*, and *CCN4* expression, while repressing *CCN3* expression (39, 40). As another multiple *CCN* regulator, glucocorticoids enhance the gene expression of CCN1, CCN2, and CCN5 (39, 41). However, in mice, the induction of CCN2 by dexamethasone is strain dependent (42), and CCN5 induction in humans has been confirmed, so far, only in estrogen receptor (ER)-negative breast cancer cells.

In addition to these molecules, an earlier investigation revealed platelet-derived growth factor (PDGF) and epithelial growth factor (EGF) to be a potent inducer of CCN1 (43). Later on, substance P (44) was also found to enhance CCN1 gene expression through mitogen-activated protein kinase (MAPK) pathways and a histone deacetylase complex (HDAC)-dependent pathway, respectively. Clinically, the effect of zoledronate, a drug that represses bone metastasis of tumors, on *CCN1* via FOXO 3a is worthy of note in relation to breast cancer chemotherapy (45). The induction of CCN1 in skin fibroblasts by cigarette smoke through Egr1 also suggests a possible role in CCN1 in skin aging due to smoking (46). Other classical CCN2 inducers include angiotensin II and endothelin, the latter of which mediates, in part, the TGF-β-induced enhancement of CCN2 gene expression (3, 21, 47). Recent advances in CCN2 research also found secreted frizzled-like protein 2 (sFRP2) and an antiproliferative factor as CCN2-inducing proteins (48, 49). Significant roles of signal transducers and activators of transcription (STATs) in CCN2 induction have been suggested in the up- and downregulation of CCN2 by thrombin and IFN-γ, respectively (50, 51). The natural compound curcumin inhibits thrombin and TGF-β-induced *CCN2* expression through multiple signaling pathways (52, 53). Nicotine is also known to induce CCN2 production in periodontal and pulmonary artery smooth muscle cells, suggesting the contribution of CCN2 to nicotine-induced tissue remodeling in response to smoking (54, 55). Interestingly, matrix metalloproteinase (MMP)-3, which is widely recognized as an extracellular proteinase, was found to regulate *CCN2* by acting as a transcription factor (56, 57). The story of such transcription factors follows this subsection.

Novel factors that drive the expression of other *CCN* members are being discovered as well. According to a recent study, *CCN3* is under the regulation of interleukin (IL)-3 and plays a critical role in the hematopoietic system (58). CCN4 was found to be induced by Notch activation, nitric oxide production, and a nephrotoxin in dermal cells, colitis, and renal cells, respectively (59–61), indicating pathophysical roles of CCN4 in these tissues. In addition, enhancement of *CCN5* expression by IGF-1 is reported to occur in ER-positive breast cancer cells (62).

Apart from biomolecules, hypoxia, mechanical stress, and UV irradiation also significantly affect CCN family gene expression (5). Hypoxia triggers the production of both CCN1 and CCN2 (63). Of note, a vast number of reports have described the induction of CCN2 by various forms of mechanical stresses in a variety of cells, where signals through Rho family members play a central part (63, 64). Recent progress in molecular medicine is revealing the utility of low-intensity pulsed ultrasound (LIPUS) as a physical therapeutic to accelerate tissue regeneration, which is accompanied by enhanced CCN2 production (65).

Transcriptional and posttranscriptional regulation of CCN family members

After intracellular signal transduction has occurred, gene expression of CCN members is performed at the

transcriptional or posttranscriptional level. Such regulatory systems of gene expression inside cells have been extensively investigated in the case of CCN2 (Figure 3), whereas, relatively, little is known for the others. As classical transcription factors that drive *CCN2* transcription, Ets1 (66), p53 (67), Hif1α, Smads, SP1 (3), NF-κB (63), FoxO3a (68), TAZ (69), and STATs (50, 51) may be listed (5, 21). Among them, p53 is suggested to be involved in *CCN5* expression as well (70). The most interesting molecule that mediates the transcriptional regulation of *CCN2* is MMP-3. This particular extracellular protease can be internalized into cells and become localized in the nucleus (56, 57). Furthermore, nuclear MMP-3 was found to interact with the CCN2 gene in the chromatin structure and actually to activate the transcription of the gene. Activation of *CCN2* by MMP-3 was reported to occur in two different types of the cells by independent research groups (56, 57). For the expression of CCN3 in rhabdomyosarcomas, PAK3-FKHR is the only indicated transcription factor so far (71). Further research on the transcriptional regulation of other CCN members is to be desired.

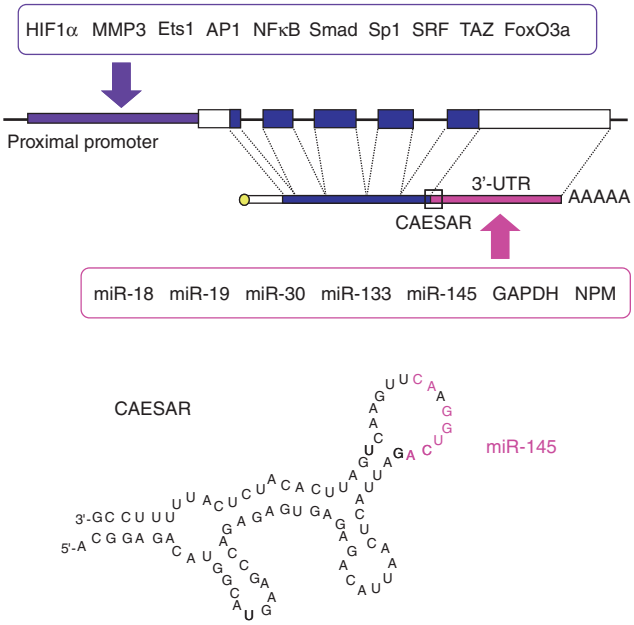

Figure 3 Transcription and posttranscriptional factors regulating the CCN2 gene.
Transcription factors that directly regulate the transcription of the CCN2 genes are summarized in a box over the schema representing the genomic structure of the CCN2 gene. Open and blue boxes indicate untranslated and coding exons, respectively, and introns are represented by black lines. Posttranscriptional factors including miRNAs are similarly summarized in the box below the illustration of the *CCN2* mRNA. The approximate location of the *cis*-acting element of structure-anchored repression (CAESAR) is indicated by a box, together with the secondary structure of this element at the bottom. Note that the predicted target of miR-145 (shown in pink) is located within the accessible major loop therein.

Concerning posttranscriptional gene regulation, significant knowledge is available for CCN2, most of which is asserting the critical importance of the 3′-UTR of its mRNA. After the discovery of a *cis*-acting element for structure-anchored repression (CASEAR) in human and murine *CCN2* mRNAs (72–74), another RNA element was discovered in chicken *CCN2* mRNA, one that regulates the stability of *CCN2* mRNA via direct interaction with a nucleocytoplasmic shuttling protein, nucleophosmin (75). It is widely recognized that regulation of gene expression by microRNAs (miRNAs) is mostly performed via the 3′-UTRs. Consistent with this notion, several miRNAs have been described to target *CCN2* mRNA at its 3′-UTR, which seem to play proper roles in corresponding organs and tissues (76, 77). Interestingly, one such target of miRNAs is located on the major loop of CAESAR, suggesting common regulatory components shared between the regulatory systems mediated by these two elements (Figure 3). Considering that a few reports showing the repressive regulatory function of the *CCN1* 3′-UTR are being published (78), related information may be available shortly on the other members as well.

Of note, posttranscriptional regulatory systems mediated by regions other than the 3′-UTR have been reported for *CCN1*. In chicken *CCN1*, a coding segment on the mRNA supports the expression of *CCN1* by facilitating ribosomal recruitment (79). Another report suggests that miR-181a represses *CCN1* at a posttranscriptional stage of gene expression, which is mediated by a region outside of the 3′-UTR (80). Progress in similar research on other CCN family members will clarify the solid contribution of miRNAs to the regulation of the CCN family genes.

CCN family proteins as transcription factors

Despite their properties as secretory proteins with signal peptides, several reports suggest possible functionality of certain CCN family members as transcription factors. Surprisingly, CCN5 is actually recruited to the promoter of a TGF-β receptor gene in the nucleus to suppress TGF-β signaling (81). Additionally, a few other CCN members are also found in the nucleus (5), suggesting their intracrine function, possibly for gene regulation.

The CCN family in development and growth

Skeletal system

Our skeletal system is formed through a combinatory developmental system composed of two modes of bone formation: one is intramembraneous ossification, whereas the other is endochondral ossification. The former process was established early in the history of animal evolution to arm the body with an exoskeleton by direct ossification, when only a CCN prototype was present at that time (82). The latter process is initiated by the formation of cartilage anlagen of bone parts, followed by their growth and subsequent ossification after the terminal differentiation of chondrocytes, designated hypertrophy. On the way to the establishment of this endoskeletal system, the members of CCN family appear to have increased in importance, suggesting the critical role of this family in this system (2, 82, 83). First of all, it should be noted that all members of the CCN family are produced during particular stages of endochondral ossification (82–84). Consistent with this notion, the profound role of this family in the skeletal development of vertebrates has been extensively characterized and reported. The initial report describing the involvement of a particular CCN family in skeletal development appeared in 1997. In that study, CCN2 was rediscovered as a factor that was overexpressed in hypertrophic chondrocytes in the growth-plate cartilage of mouse bones and, thus, was designated as hypertrophic chondrocyte-specific gene product 24 (Hcs24; 21, 82, 83). Thereafter, a vast number of studies revealed the critical role of CCN2 in skeletal development. Indeed, CCN2 was found to promote both the proliferation and differentiation of chondrocytes, osteoblasts, and vascular endothelial cells, which are central players of both endochondral and intramembranous ossification. These findings were further confirmed by *in vivo* studies on *Ccn2*-null mice and rat models of cartilage/bone regeneration (21, 84, 85). Nowadays, CCN2 is recognized as a potential therapeutic material for cartilage/bone regeneration (86, 87).

The contribution of CCN1 to chondrogenesis, which is indispensable for the early events of endochondral ossification, has been clearly demonstrated (5). Nevertheless, the role of CCN1 in later events in this process is still unclear. In contrast, CCN4 was reported to promote the ossification process by enhancing BMP function (34), whereas its effect on chondrogenesis has not been described. At the time of its initial discovery, CCN5 was described as a factor that promotes osteoblast differentiation *in vitro* (82); however, no additional information is available to date, except for one report describing a limited osteogenic effect *in vitro* (88).

Recent advances in CCN family research are unveiling an interesting aspect of CCN3 function in skeletal development. In the early days, CCN3 was found to promote angiogenesis *in vitro* (4) and was also defined as a positive regulator of chondrogenesis (89), but a number of later studies indicated that CCN3 rather counteracts bone formation when interacting with BMP-2 (5, 90). The fact that endochondral ossification is delayed in *Ccn2*-null mice, in which animals display elevated CCN3 expression, also indicates the inhibitory action of CCN3 toward skeletal growth (84). The apparent contradiction observed between the chondrogenic and anti-osteogenic effects of CCN3 was recently found explainable based on the findings described by Janune et al. (91). In their report, CCN3 was shown to direct the maturation of chondrocytes toward articular chondrocytes, while inhibiting the terminal differentiation toward ossification (91). Thus, CCN3 is now believed to play a significant role in constructing and maintaining articular cartilage, which connects skeletal parts in joints, and this role is consistent with the phenotypic change observed in CCN3 mutant mice (92).

In view of joint development, CCN6 has been attracting the interest of medical scientists because of its association with an inheritable joint disease known as progressive pseudorheumatoid dysplasia (PPD; 5). A variety of mutations in human CCN6 gene were found in PPD patients, and such mutations affect both BMP and Wnt signaling (36, 93), which may account for the pathogenesis of PPD at least in part. As CCN6 is also present in normal articular cartilage and seems to prevent hypertrophy by interacting with insulin growth factors (IGFs), this particular CCN family member is probably involved in normal joint formation (94, 95).

Cardiovascular system and angiogenesis

Through the characterization of knockout mice, only one CCN family member, CCN1, is known to be indispensable for cardiac development (10, 96). The other CCN family members are not a critical determinant of cardiovascular development, as cardiogenesis and vasculogenesis, themselves, can be carried out, even in the absence of one such member. Nevertheless, all CCN members have been shown to modulate the development and maintenance of cardiovascular tissues under proper biological and pathological situations. Therefore, CCN family members are anticipated to collaborate with each other in the

developing cardiovascular system. A deficiency in a single member, except for CCN1, can be efficiently compensated by the other members in most events occurring *in vivo*.

Angiogenic activities of all three of the classical members, CCN1, CCN2, and CCN3, were confirmed both *in vivo* and *in vitro*, where integrins are required as their partners (4, 5). Among these classical members, CCN1 promotes all types of vascular developmental processes; hence, this factor is indispensable for the integrated development of the fetal vascular system. Such properties of CCN1 are firmly confirmed by the phenotype of *Ccn1*-null mice, which have severe defects in chorioallantoic fusion and in the placental blood vessel system (5, 97). Furthermore, physical defects are also found in both atrial and ventricular septa in such mice (96). Albeit at a milder level, vascular development in *Ccn2*-null mice is also impaired. This impairment is typically represented by delayed vascular invasion into the bone tissues, which delay leads to skeletal defects causing respiratory failure (85). In addition to the vasculature-forming activity under experimental conditions (4), the angiogenic activity of CCN2 is further emphasized in the context of angiogenesis in skeletogenesis and tumor angiogenesis (4). CCN2 is induced by hypoxia and promotes bone metastasis of breast cancer cells (4, 98, 99). Here, it should be noted that CCN6 is known to contrarily repress breast cancer invasion through multiple pathways (100, 101). In contrast to that of CCN1 and CCN2, the role of CCN3 in vascular development has not yet been clearly established, in spite of its angiogenic property.

After development, several CCN family members are expressed in cardiovascular tissues under pathological conditions. CCN3 was found to counteract neointimal hyperplasia by repressing the proliferation of vascular smooth muscle cells (102). A similar effect on vascular smooth muscle cells was also observed for CCN5 (4). Finally, both CCN2 and CCN4 have been regarded as a promoter and biomarker of cardiac hypertrophy and fibrosis (4, 103, 104); but a recent report contrarily describes the cardioprotective effects of CCN2 against chronic cardiac stresses (105). This finding also appears contradictory to the fact that CCN5 exerts similar cardioprotective effects in the transgenic mice (5).

Hematopoietic system

In light of the heavy trafficking of growth factors and cytokines, CCN family members, except for CCN3, are currently anticipated to play relatively minor roles in the development of cells of the hematopoietic lineage. CCN2 is found in abundance in platelets (82), but this platelet-encapsulated CCN2 does not seem to be produced by the platelet producer, i.e., the megakaryocyte. Instead, nascent platelets are thought to incorporate CCN2 that is supplied by mesenchymal cells in the bone marrow (106). CCN2 is one of the important components of platelets, which initiate and promote the wound healing process, as the critical role of CCN2 in wound healing has been emphasized in a number of previous reports (3–5). Thus, if the CCN2-containing ones are defined as mature platelets, the mesenchymal-hematopoietic interaction to supply CCN2 would be the final step of thrombopoiesis. In addition, the inclusion of the other CCN family members into platelets has not yet been assessed, and thus, this possibility ought to be examined.

Compared to CCN2, the contribution of CCN3 to the hematopoietic system is more profound and fundamental. In general, CCN3 function is closely related to the 'stem cell-ness' of cellular progenitors, rather than to the lineage development. In normal bone marrow, CCN3 is secreted by hematopoietic progenitor cells and is also present in human serum. This molecule is required for the stem cell renewal and the maintenance of naïve hematopoietic progenitors, in which interaction with Notch plays a major role. In addition, CCN3 is required for the development of cells of the myelomonocytic and erythroid lineages, and for facilitating B-cell development as well (107, 108). Such CCN3 functions appear to be highly specific to CCN3, as no functional redundancy or compensation by another CCN family member has been observed.

Nervous system

For a significant period of time, investigation into the relationship between the CCN family and the nervous system had been mostly ignored. This was probable because both representative members, CCN1 and CCN2, were identified and characterized mainly in the context of mesenchymal tissues. Additionally, it may be pointed out that, because of the embryonic lethality of *Ccn1* and *Ccn2* null mice, loss-of-function studies on the central nervous system were difficult. Therefore, although a few early studies implied a possible function of CCN2 in the nervous system, as indicated by immunohistochemical localization (109) and molecular interaction of CCN2 with neuronal factors (110), a decade was spent thereafter to find out the actual behavior and functionality of CCN family members.

One of the most striking reports concerning CCN1 describes the requirement of this protein for dendritic growth of hippocampal neurons (111). Of note, this process is also dependent upon the interaction of CCN1 with integrins. In spite of the absence of distinct evidence indicating

a physiological role of CCN2 in neuronal tissue development, the role of CCN2 in the development of Alzheimer's disease has been indicated and discussed (39, 112). Consistent with the expression of CCN3 during the development of the central nervous system, CCN3 was recently found to be produced by Purkinje cells and to promote the maturation of cerebellar granule neurons by acting in a paracrine manner through integrins (113). In relation to Alzheimer's disease and other neurodegenerative disorders, CCN4 was described as a cytoprotective agent against oxidative stress and β-amyloid-induced neuronal cell death (114). CCN5 expression is upregulated in the central nervous system of ganglioside-deficient mice, and in these animals, there are positive effects of CCN5 on neurite formation, which action is mediated by the integrin-AKT signaling pathway (32). These findings suggest that these two CCN family members are able to protect the central nervous system upon possible disorder, even if they are not definitely required for the normal development of this system.

Other organs

The pancreas is not only a major exocrine producer of digestive enzymes but also an endocrine producer of critical hormones including insulin. The expression and functionality of CCN2 during the development of islet morphogenesis and the proliferation of β-cells during embryonic development have been unveiled by recent studies (115). Concerning the eyes, vascular network formation is a critical step in both physiological development and establishment of major eye disorders. Although its precise molecular function is unclear, dynamic expression of *CCN1*, *CCN2*, and *CCN3* is observed during mammalian eye development with overlapping spatiotemporal expression patterns (116, 117). Expression and a possible role of CCN2 in the development of tooth germs were indicated as well (118). In these organs, however, the roles of the other CCN family members still remain to be investigated.

The CCN family in biological responses and diseases

Fibrosis

The CCN family is probably the best known in the context of the fibrogenic role of CCN2. In fact, overexpression of *CCN2* is observed in most fibrotic disorders in a number of organs and tissues (5, 82). Conversely, overexpression of *CCN2* successfully produces experimental fibrosis in several *in vivo* systems (5). Therefore, it had once been believed that CCN2 could be the sole developer of fibrosis. Nevertheless, recent advance in CCN research is illustrating that fibrosis is an outcome of an imbalance in the CCN family network during the tissue regeneration process.

Fibrosis is the outcome of a continuous failure of tissue regeneration. According to recent studies, not only CCN2 but also CCN4, exerts profibrotic effects in cardiac fibrosis (104). CCN3 counteracts these effects by downregulating these molecules at gene expression and molecular function levels and through other unknown mechanisms (119). As described elsewhere, TGF-β induces the expression of *CCN1*, *CCN2*, and *CCN4* while repressing that of *CCN3*. TGF-β is known as an enhancer of ECM production, or a profibrotic factor; hence, the behavior of CCN2, CCN4, and CCN3 downstream of this molecule is quite understandable. However, CCN1 does not act as a profibrotic factor. What is the role of CCN1 herein? A possible answer was given by studies by Jun and Lau (5, 120). In those studies, CCN1 was found to induce dermal fibroblast senescence by binding to integrin and HSPG through the induction of DNA damage response pathways and the generation of reactive oxygen species (ROS). As a result, fibroblasts undergo senescence and express antifibrotic genes. Therefore, CCN1 is integrating the formation of a proper ECM in collaboration with fibrotic CCN family members (5).

In the kidney, chronic fibrosis is incurred by several etiological factors including hyperglycemia and its secondary products, in which the balance between CCN1 and CCN2 plays a pivotal role. During the development of diabetic nephropathy, both CCN1 and CCN2 are induced, following different time courses with different missions in an attempt to repair the damaged glomeruli. However, along with the fading out of CCN1 induction, fibrotic changes progress at later stages (63, 121). Consistent with these findings, CCN1 inhibits the migration of mesangial fibroblasts, whereas CCN2 promotes fibrotic changes in the kidney (63, 122, 123). Of note, CCN3 is reported to be involved in the regulation of renal fibrosis, counteracting CCN2 gene expression (124). Collectively, these findings clearly represent the scenario of fibrosis regulation via the CCN family network, although the behavior of CCN4 remains to be investigated.

The lung is another major organ in which CCN2-mediated fibrosis is commonly reported (1, 3, 5, 82). The involvement of CCN4 in a few types of pulmonary fibrosis

is also recognized (125). Lung fibrosis can be induced by a number of endogenous and exogenous factors including iatrogenic ones. Interestingly, CCN6 was found to be induced in a pulmonary fibrosis model of mice treated with bleomycin (126). Subsequent investigation *in vitro* revealed relatively weak fibrogenic activity of CCN6. Although further study to verify its biological significance is necessary, CCN6 may also join the CCN family network for fibrosis regulation as a profibrotic member (126).

The antifibrotic function of CCN1 via integrin and HSPG-mediated provocation of cellular senescence is indicated in the fibrotic remodeling of liver tissue (127). This process is, as found in dermal fibroblasts, mediated by ROS formation. As a fibrosis-associated inheritable disease, skeletal muscle dystrophy was also found to be mediated by CCN2, as overexpression of *CCN2* is able to mimic this clinical entity (128).

Inflammation

The profibrotic property of certain CCN family members strongly suggested their involvement in inflammatory processes, especially in chronic inflammation. This notion is also emphasized by the anigiogenic properties of certain CCN family members. As such, inflammatory aspects of CCN family function were uncovered subsequently by later studies (39).

The facts that the gene expression of CCN family members is under the regulation of chemical mediators of inflammation, and these members also regulate the expression of genes of proinflammatory proteins, indicate that these members are firmly built into the network of the biodefense system. As already stated elsewhere, *CCN1*, *CCN2*, and *CCN4* are all upregulated by TGF-β and downregulated by TNF-α, whereas *CCN3* is regulated by the same cytokines in the opposite manners (39). The induction of *CCN1*, *CCN2*, and *CCN3* by substance P (45), MCP-1 (129), and IL-10 (39), respectively, has also been reported. Multiple regulation of *CCN2* by small chemical mediators such as nitric oxide (NO), histamine, serotonin, and prostaglandins, is also known (39). As classical downstream mediators of the CCN family members during inflammation, MMPs that play major roles in inflammation and tissue remodeling are widely known. CCN1 was found to induce MMP-13 (130), and CCN2, to induce a variety of MMPs via multiple regulatory systems (131). The induction of MMP-1 and MMP-3 by CCN3 was also reported (132). In contrast, CCN4 and CCN5 downregulate MMP1 and MMP-2, respectively (133, 134). These findings, again, assert the role of CCN members as the principal coordinators of ECM remodeling.

A few recent studies revealed a novel mode of cytokine induction by the CCN family (Figure 4). Clinically, the level of CCN1 or CCN2 is enhanced in rheumatoid arthritis (RA) joints (13, 18, 67), whereas in osteoarthritis (OA), increased levels of CCN2 and CCN4 are observed in the synovial fluid of inflamed joints (18, 31). Interestingly, all three members stimulate inflamed synovial fibroblasts to produce IL-6, which may promote the differentiation of T cells into those of a Th17 subpopulation (13). Surprisingly, CCN1, CCN2, and CCN4 share integrin $\alpha_v\beta_5$ as a common receptor and NF-κB as a common transcription factor to provoke IL-6 gene expression (4, 13, 21). Nevertheless, the intracellular signaling pathways they use are not exactly identical; i.e., CCN1 and CCN4 eventually activate NF-κB through the PI3K-AKT pathway, whereas CCN2 initiates ASK activation under integrin $\alpha_v\beta_5$, leading to NF-κB and AP-1-mediated enhancement of IL-6 gene expression. The mechanism by which different signaling pathways can be differentially activated through the same receptor remains to be clarified, but probably can be ascribed to the difference in the types of collaborators acting together with integrin $\alpha_v\beta_5$ and these CCN family members.

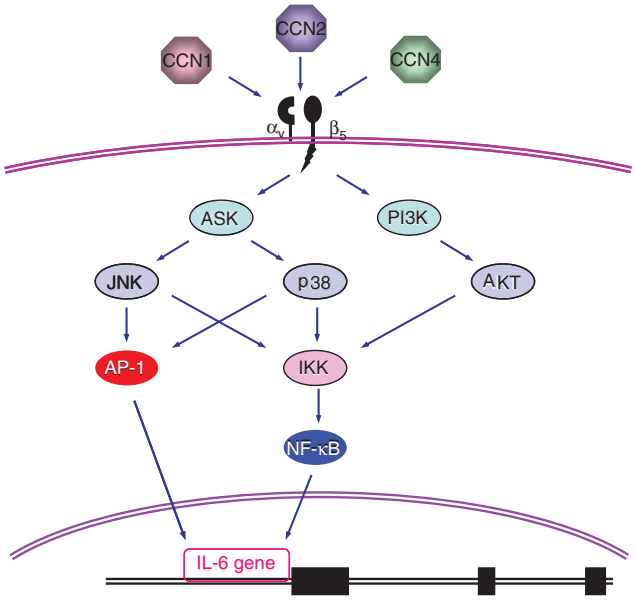

Figure 4 Overview of the signaling for IL-6 induction in inflamed synoviocytes by CCN family members.
Upper and lower arcs denote plasma and nuclear membranes, respectively. The objects depicted on the plasma membrane represent integrin subunits. CCN1 and CCN4 principally use the PI3K-AKT pathway at the right, whereas CCN2 is reported to induce IL-6 via the ASK1-dependent dual transcriptional induction illustrated on the left.

Malignancies

An association of CCN family members with malignancies has been recognized in a vast variety of human tumors (5, 38). Although elevated expression of particular CCN family members is observed in the tumors, there is significant controversy regarding their pathological roles. For example, CCN2 promotes pancreatic tumor growth (115), whereas the same molecule suppresses the growth of oral squamous cell cancer by promoting epithelial differentiation (135). Considering the molecular nature of the CCN family, such context-dependent effects in view of tumor development is understandable.

Nevertheless, by viewing the association between the six CCN family members and relevant malignancies (Figure 5), several intriguing features may be pointed out (5, 136). First, all of the CCN-associated malignancies are related to either CCN1 or CCN2. In other words, no tumors that are associated with CCN3, CCN4, CCN5, or CCN6 alone have been specified so far. Notably, all malignancies that have association with CCN4, CCN5, or CCN6 are also related to both CCN1 and CCN2, emphasizing the major roles of these classical members in tumor biology as well. Second, only negative effects against malignant phenotype has been reported in the case of CCN5 and CCN6 up to now. Finally, it should be noted that breast cancer is now known to be associated with all of the CCN family members (5).

Breast cancer is one of the major clinical entities that cause the death of adult females in advanced countries. A positive or negative association with poor prognosis was reported for CCN1, CCN2, and CCN4, or CCN5 and CCN6, respectively (5). In the case of CCN3, a significant relationship between low gene expression and poor prognosis is observed in clinical cases (137), whereas CCN3 favors metastasis to bone by promoting bone resorption (138).

The promotion of bone metastasis by CCN2 was experimentally confirmed in animal models. Also, in the case of this particular type of tumor, CCN1 always acts to enhance tumorigenesis, tumor growth, metastasis, and even drug resistance (5). Therefore, CCN1 is now recognized as one of the promising targets for the chemotherapy against breast cancers.

Ocular diseases

The recurrence of angiogenic events after ocular development occasionally leads to neovascularization-related disorders in the eye (116). These diseases include proliferative diabetic retinopathy, age-related macular degeneration, and experimental oxygen-induced retinopathy (OIR), mimicking the retinopathy of prematurity in humans. In spite of the clear involvement of these CCN family members in these diseases, the pathological roles of CCN1

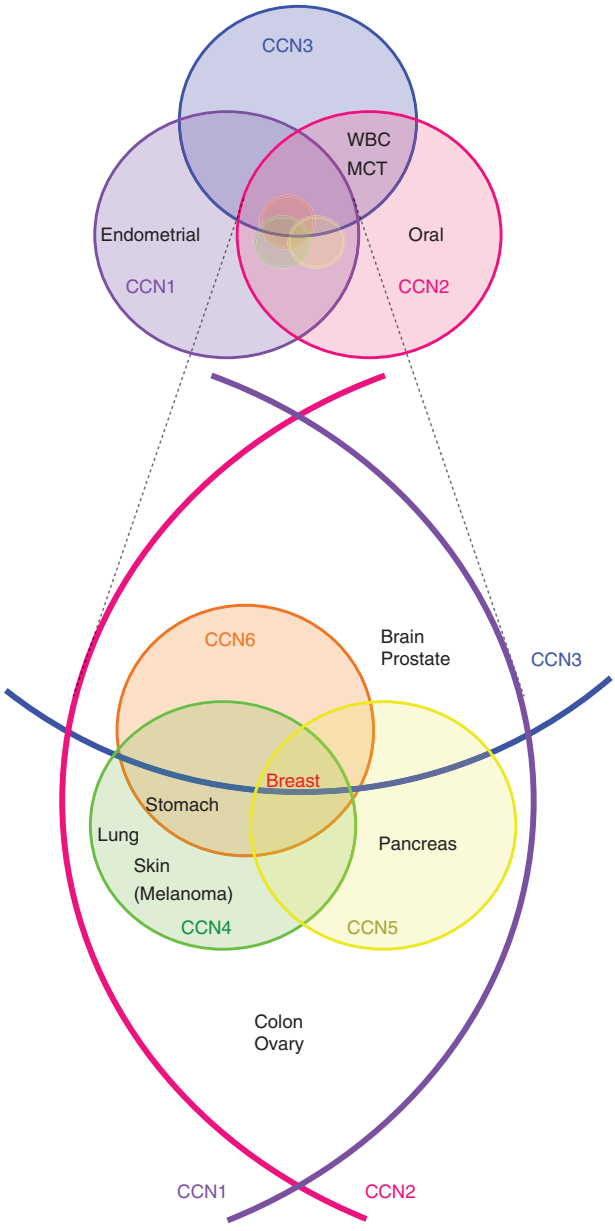

Figure 5 Comprehensive illustration of the organ-wise tumor-CCN family connection.
An organ or tissue name within a circle labeled by a CCN family member indicates the relevance between tumors originating from that tissue and the CCN family member. The area with both CCN1 and CCN2 connections in the upper panel is enlarged in the lower panel. WBC and MCT denote white blood cell malignancies (leukemia) and mesenchymal tumors (chondrosarcoma and osteosarcoma). Note that breast tissue, shown in red, is the only tissue in which all of the CCN family members are involved upon tumorigenesis or metastasis.

and CCN2 are, again, controversial. However, according to a recent study, the roles of these molecules in OIR development are reminiscent of those in nephropathy (116). Namely, additive expression of CCN1 reduced pathological angiogenesis without adverse effects, whereas silencing CCN2 exerted comparable effects in an OIR model (123, 139). These complicated effects by CCN1 and CCN2 are anticipated to result from their molecular interaction with VEGF and integrins. It is widely recognized that VEGF is the major growth factor that directs angiogenesis. First, both CCN1 and CCN2 directly bind to VEGF, which may control the availability and net angiogenic activity of these molecules, in collaboration with particular proteases that digest CCN1 and CCN2 (7, 8, 140). Second, the interaction between VEGF and integrins, which plays a significant role in the aberrant growth of blood vessels, may be affected by the CCN-integrin connection (5). Interestingly, a previous report showed that CCN2 levels correlate positively, but VEGF levels, negatively, with the degree of fibrosis in progressive diabetic retinopathy patients (141). CCN2-VEGF interaction may direct the pathogenic tissue remodeling of the retina toward either neovascularization or fibrosis. From this point of view, it is of particular interest that an amino-terminal dimodular fragment of CCN2 accumulates in the vitreous of patients with proliferative diabetic retinopathy (142).

Apart from these neovascularization-related diseases, the level of CCN2 is also increased in the case of glaucoma. Although *CCN2* is expressed in the normal trabecular network to regulate aqueous humor outflow, an elevated level of CCN2 is found in glaucoma, together with TGF-β (143). As the expression of both *CCN1* and *CCN2* is responsive to mechanical stimuli, elevated expression of these genes induced by increased intraocular pressure may mediate the pathological changes constituting glaucoma (63). In addition, targeted deletion of a CCN3 module was found to cause cataract in mice (92). The involvement of CCN2 in this ocular disorder is also suspected, but still remains to be clarified.

Expert opinion

As introduced in this review, it is occasionally observed that more than one CCN family member play significant roles in the development and pathogenesis of the same organ or tissue. In typical cases, three out of six family members present in the same tissue exert almost identical effects on the same cells, as found in the case of those of CCN1, CCN2, and CCN4 on IL-6 production by synovial fibroblasts. In contrast, counteraction between two coexisting members, such as the combination of CCN2 and CCN3, is widely known under a variety of biological situations. Therefore, in order to uncover the molecular networks extending out from CCN family members in any biological microenvironment, comprehensive analysis taking all of the CCN family members into consideration is indispensable. Mutual direct molecular interaction, which was clarified by recent studies, and genetic regulation among the CCN family members further strengthen this critical notion.

Outlook

Life is the outcome of the integration of continuous interactions among multiple biomolecules, rather than the assemblage of biomaterials. In other words, the molecular interaction is as important as the molecule itself in understanding how life works, and the function of a particular molecule is variable depending upon its microenvironment. Therefore, both defining the proper function of and the function-based terminology for a molecule may be meaningless. CCN family members have taught us this critical concept. Nowadays, biologists are starting to reevaluate the ‘proper’ function of a number of molecules comprising the living body. After a few decades into the future, a significant number of extracellular signaling molecules may need to be renamed, just as CTGF metamorphosed into CCN2 in the past.

Highlights

- The matricellular CCN family is a novel group of proteins that exert multiple functionalities by manipulating extracellular molecular networks.
- The prototypic CCN is anticipated to be CCN2 with its compact gene structure, which has expanded up to six members in mammals during the evolution of vertebrates.
- Typical members consist of four conserved modules that interact with a variety of growth factors, ECM components, and cell-surface receptors including integrins.
- The function of CCN family proteins highly depends upon the cofactors in their microenvironment and, hence, is determined by spatiotemporal gene regulation.

- Recent research has revealed that the CCN family is critically involved not only in the development of skeletal, cardiovascular, hematopoietic, and nervous systems, but also in that of the eyes and pancreas.
- In addition to the well-known role of CCN2 in a number of fibrotic disorders, the involvement of most family members in inflammatory processes has also been uncovered.
- Among various CCN family-associated malignancies, the contribution of all six family members is now recognized in the development of breast cancers.
- Because of the copresence of multiple CCN members throughout the body, comprehensive investigation on all members is required for the total understanding of the extracellular information network that supports life.

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