Roles of heterotypic CCN2/CTGF-CCN3/NOV and homotypic CCN2-CCN2 interactions in expression of the differentiated phenotype of chondrocytes

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To identify proteins that regulate CCN2 activity, we carried out GAL4-based yeast two-hybrid screening using a cDNA library derived from a chondrocytic cell line, HCS-2/8. CCN2/CTGF and CCN3/NOV polypeptides were picked up as CCN2-binding proteins, and CCN2-CCN2 and CCN2-CCN3 binding domains were identified. Direct binding between CCN2 and CCN3 was confirmed by coimmunoprecipitation *in vitro* and *in vivo* and surface plasmon resonance, and the calculated dissociation constant (K_d) was 1.17×10^{-9} M between CCN2 and CCN2, and 1.95×10^{-9} M between CCN2 and CCN3. Ectopically overexpressed GFP-CCN2 and Halo-CCN3 in COS7 co-localized, as determined by direct fluorescence analysis. We present evidence that CCN2-CCN3 interactions modulated CCN2 activity such as enhancement of *aggrecan* and *col2a1* expression. Curiously, CCN2 enhanced, whereas CCN3 inhibited, the expression of *aggrecan* and *col2a1* mRNA in HCS-2/8 cells; and the combined treatment with CCN2 and CCN3 abolished the inhibitory effect by CCN3. These effects were neutralized with an antibody against the VWC domain of CCN2 (11H3). This antibody diminished the binding between CCN2 and CCN2, but enhanced that between CCN3 and CCN2. Our results suggest that CCN2 could form homotypic and heterotypic dimers with CCN2 and CCN3, respectively. Strengthening the binding between CCN2 and CCN3 with the 11H3 antibody had an enhancing effect on *aggrecan* expression in chondrocytes, suggesting that CCN2 had an antagonizing effect by binding to CCN3.

Introduction

CCN family protein 2/connective tissue growth factor (CCN2) is a major member of the CCN family of proteins, which consists of CCN1/Cyr61, CCN2/CTGF, CCN3/NOV, CCN4/WISP-1, CCN5/WISP-2/CTGF-L, and CCN6/WISP-3. CCN2 is expressed in various types of cells, such as fibroblasts, endothelial cells, vascular smooth muscle cells, osteoblasts, and chondrocytes [1-7]. Among those cell types, CCN2 is strongly expressed in growth-plate cartilage, especially in hypertrophic chondrocytes [1,4,5,7] during the developmental stages, and shows multiple cellular functions such as stimulation of cartilage-specific extracellular matrix (ECMs) synthesis as well as chondrocyte proliferation and maturation [1]. In CCN2 null mutant mice, bone formation is inhibited due to impairment of both chondrogenesis and growth-plate angiogenesis [8], which leads to neonatal respiratory failure and death within minutes of birth, thus indicating the essential role of CCN2 in developing chondrocytes. Other experiments also demonstrated that CCN2 is involved in the early cell-condensation step during the ectopic digit formation [9]. However, the ectopic overexpression in soft tissues is linked to various types of fibrosis, because of its strong enhancing effects on the production of extracellular matrices [1-4].

CCN2 consists of 4 modules, i.e., insulin-like growth factor binding protein-like (IGFBP), von

Willebrand factor type C (VWC), thrombospondin type 1 repeats (TSP-1), and C-terminal cystine knot (CT); and each of these modules has different binding partners and seems to regulate the multiple functions of CCN2 [1]. The CT domain of CCN2 interacts directly with fibronectin and enhances cell adhesion of chondrocytes through binding to integrin $\alpha5\beta1$ [10]. This domain also binds to integrin $\alpha5\beta1$ to promote adhesion and migration of pancreatic stellate cells [11]. Furthermore, it induces adhesion of hepatic cells by direct binding to the integrin receptor $\alpha v\beta3$ and to heparan sulfate proteoglycan through its C-terminal heparin-binding domain [12]. There are also reports indicating that CCN2 signaling occurs through low-density lipoprotein receptor-related protein-1 (LRP-1) [13-15]. Other studies show that CCN2 directly binds to bone morphogenetic protein-4 (BMP-4) and transforming growth factor-β (TGF-β) through its VWC module and prevents or enhances their binding to their own receptors [16]. Moreover, CCN2 binds vascular endothelial growth factor (VEGF) and inhibits its angiogenic effect [17]. There also is a report showing that CCN2 binds to aggrecan, which is a major marker of differentiated phenotype of chondrocytes, through its N-terminal IGFBP and VWC modules and that this binding may be related to the CCN2-enhanced production and secretion of aggrecan by chondrocytes [18].

In order to identify additional extracellular or cell-surface targets for CCN2 that may be involved in the regulatory functions of CCN2 in chondrocytes, we searched for CCN2-binding proteins by using the yeast two-hybrid screening assay. A cDNA library derived from human chondrosarcoma-derived chondrocytic cell line HCS-2/8 was screened for products binding to CCN2, since this cell line has a differentiated phenotype similar to that of normal chondrocytes in terms of aggrecan and cartilage collagen secretion and integrin expression profiles [19]. We found CCN2 and CCN3 as binding partners of CCN2, indicating that CCN2 forms homotypic and heterotypic dimers with CCN2 and CCN3, respectively.

CCN3, also a CCN family protein, is highly expressed in the nervous system, blood vessels, and musculoskeletal system as well as in pre- and early hypertrophic chondrocytes and osteoblasts [1,20,21]. The functions of CCN3 protein among these different tissues are not yet well defined. Although CCN3 was originally described as being antiproliferative [22] and its expression associated with differentiation and growth arrest in Wilm's tumor [23], chondrosarcomas [24], and rhabdomyosarcomas [25], more recent data correlate CCN3 with an increased proliferative index of 3T3 fibroblasts [26] and prostate tissue samples [27]. Both CCN2 and CCN3 are expressed in the early hypertrophic to pre-hypertrophic zone of the growth plate in cartilage; and CCN2 enhances the expression of ECMs components, whereas CCN3 suppresses it in cultured growth plate chondrocytes [20].

There seems to be some correlation between CCN2 and CCN3; however, the functional and physiological interaction between these 2 proteins had not been elucidated at all. In the present study, we show for the first time that (1) CCN2 bound to CCN2 through its IGFBP, VWC, and CT domains,

but not TSP-1 domain, and to CCN3 only via its VWC and CT domains; and (2) CCN2 rescued chondrocytes from CCN3-induced suppression of *aggrecan* and *col2a1* expression. Inhibition of CCN2-CCN2 binding suppressed the expression of *aggrecan*, whereas promotion of CCN2-CCN3 binding enhanced it.

Results

Identification of CCN2 and CCN3 as CCN2 dimerization partners

Using yeast two-hybrid screening to search for proteins that directly interacted with full-length CCN2 (amino acid residues 27-349), we obtained several clones from a HCS-2/8 human chondrocytic cell cDNA library that encoded CCN2 and CCN3, indicating homotypic and heterotypic dimerization of CCN2. To confirm direct interactions between CCN2 and CCN2 or between CCN2 and CCN3, we used several methods. First, to identify the CCN2 interaction sites in CCN2 or CCN3, full-length, N-terminal, C-terminal, and individual domains of CCN2 or CCN3 were expressed in AH109 yeast cells as GAL4-transactivation domain (GAL4-AD)-fusion proteins, whereas full-length CCN2 was co-expressed as a GAL4-DNA binding domain (GAL4-BD)-fusion protein (Fig.1 A). Interactions between CCN2 or CCN3 fragments and full-length CCN2 were monitored as growth of yeast transformants in selection media (SD/-Ade, -His, -Leu, -Trp). Self-interaction with full-length CCN2 was observed after co-transformation with CCN2 vectors expressing IGFBP, VWC, and/or CT, but not when the TSP-1 domain alone was tested. Interaction with CCN3 fragments was seen with vectors expressing the VWC or CT domain, but not with those expressing the IGFBP or TSP-1 domain (Fig.1 A).

The difference in binding strength between full-length CCN2 (CCN2$_{full}$) and IGFBP domain of CCN2 (CCN2$_I$), and between CCN2$_{full}$ and IGFBP domain of CCN3 (CCN3$_I$), was estimated by measuring the β-galactosidase activity of the reporter gene in yeast expressing full-length CCN2 (GAL-BD/CCN2$_{full}$), standardizing it to the protein concentration, and comparing it with the β-galactosidase activity obtained after co-transformation with the pGADT7 empty vector (mock, Fig.1 B). CCN2$_I$ showed almost the same strength of binding to CCN2$_{full}$ as CCN2$_{full}$, whereas the binding of CCN3$_I$ to CCN2$_{full}$ was significantly lower than that of CCN3$_{full}$ or CCN2$_I$ to CCN2$_{full}$ (Fig.1 B). These results indicate that among the 4 domains in CCN2, the IGFBP, VWC, and CT domains exhibited direct homotypic interaction with CCN2, whereas only the VWC and CT domains of CCN3 showed direct interaction with full-length CCN2.

CCN2 directly interacts with CCN2 and CCN3 *in vitro*.

For *in vitro* pull-down experiments using glutathione-Sepharose resin, recombinant His$_6$-tagged

CCN2 (His-CCN2) and GST-fused CCN3 (GST-CCN3) were prepared as *E.coli* cell lysates. Immunoblotting of the precipitates with anti-His and anti-GST antibody largely confirmed the yeast two-hybrid assay results (Fig.2 A): His-CCN2 was effectively pulled down in the presence of GST-CCN3. We further confirmed the binding between CCN2 and CCN2 or between CCN3 and CCN2 by performing a solid-phase binding assay (Fig.2 B). Purified CCN2 or GST-CCN3 was immobilized on the plates, and increased amounts of biotinylated recombinant CCN2 (rCCN2) were used for binding. Biotinylated rCCN2 bound to immobilized rCCN2 or GST-CCN3 in a dose-dependent manner (Fig.2 B). Finally, the dissociation constant of CCN2 to CCN2 or to CCN3 was determined by using surface plasmon resonance (SPR) analysis (Fig.2 C). rCCN2 protein was immobilized on a sensor tip as a ligand and rCCN2 and rCCN3 were used as analytes at concentrations of 4, 8, 16, 32, and 64 nM. Calculated from sensorgram analysis, the K_d value was 1.17×10^{-9} M for the CCN2-CCN2 interaction and 1.95×10^{-9} M for the CCN2-CCN3 one (Fig.2 C). These data indicate that CCN2 underwent a homotypic interaction and a heterotypic one with CCN3 with a similar binding affinity.

Endogenous CCN2 and CCN3 interact and co-localize in chondrocytic HCS-2/8 cells.
To investigate the endogenous interaction between CCN2 and CCN3, we coimmunoprecipitated endogenous CCN2 with CCN3 from HCS-2/8 cell lysates using anti CCN2 antibody. CCN3 was effectively immunoprecipitated with an anti-CCN2 antibody (Fig.3 A), but not with control IgG.. Indirect immunostaining of endogenous CCN2 (red) and CCN3 (green) with spcific antibodies showed subcellular colocalization inside of the permeabilized cells with TritonX-100 and on cell surface without permeabilization treatment (Fig.3 B).

Ectopically overexpressed CCN2 and CCN3 interact and co-localize in COS7 cells.
    To confirm the interaction between CCN2 and CCN3 *in vivo*, we co-expressed GFP-fused CCN2 and Halo-tagged CCN3 in COS7 cells. GFP or GFP-CCN2 was immunoprecipitated with anti-GFP antibody from cell lysates with binding proteins, and the precipitated proteins were analyzed by using anti-Halo antibody. Halo-CCN3 (arrow in lane 8 of Fig.4 A) was pulled down effectively only after co-expression with GFP-CCN2 (Fig.4 A). The subcellular localization of GFP-CCN2 and Halo-CCN3 largely overlapped when the cells were directly analyzed by GFP (green) fluorescence and the fluorescence (red) from the dye binding to Halo (Fig.4 B).

CCN2 and CCN3 cooperatively regulate gene expression of *cartilagenous matrix genes* in HCS-2/8 cells.
CCN2 enhances *aggrecan* and *col2a1* expression in chondrocytes. To elucidate the role of CCN3 interaction with CCN2 on this activity of CCN2 in chondrocytes, we treated cells of the

chondrocytic cell line HCS-2/8 with CCN2 and/or CCN3, and then monitored the expression of the *aggrecan* and *col2a1* mRNA after 12 h (*aggrecan*) or 24 h (*col2a1*) by real-time PCR. The addition of rCCN2 (1.25 nM) enhanced mRNA expression of *aggrecan* and *col2a1* by approximately 20%, whereas the addition of rCCN3 (1.25 nM) inhibited it by around 15%. The combination of rCCN2 and rCCN3 abolished the repression caused by rCCN3 (Fig.5 A), and this abolishment occurred in a CCN2 dose-dependent manner (Fig.5 B). Similar results were obtained in the mouse primary chondrocytes isolated from rib cartilage (data not shown). These data suggest that CCN2 and CCN3 cooperatively regulated the gene expression of extracellular matrices such as *aggrecan* and *col2a1* mRNA in chondrocytes.

Addition of 11H3 changes *aggrecan* gene expression of HCS-2/8 cells treated with CCN2 and/or CCN3

To modify the binding between CCN2 and CCN2 or between CCN2 and CCN3, we prepared several antibodies against the VWC domain of CCN2. One of the antibodies, 11H3, modulated the binding strength between CCN2-CCN2 and between CCN2-CCN3. In solid-phase binding studies using biotinylated CCN2 and immobilized CCN2 or GST-CCN3, the addition of 11H3 reduced the binding of CCN2 to CCN2, but accelerated that to GST-CCN3 (Fig.5 A). Furthermore, HCS-2/8 cells were treated with rCCN2 (1.25 nM) and/or rCCN3 (0.625 nM) in the presence of mouse IgG or 11H3 antibody, and gene expression of *aggrecan* was monitored 12 h later. The addition of 11H3 antibody to CCN2-treated HCS-2/8 cells cancelled the enhanced expression of the *aggrecan* gene, whereas the 11H3 antibody further enhanced the gene expression in the presence of CCN3 with or without CCN2 (Fig.5 B). These results suggest that when CCN2 formed heterotypic dimers with CCN3, these dimers show rescuing the inhibited effect of *aggrecan* expression by CCN3 in chondrocytes. Those interactions could modulate CCN2 activity such as enhancement of *aggrecan* expression.

Discussion

CCN2 is a secreted protein and accumulates at the cell surface, binding various extracellular matrix components such as aggrecan [18] and fibronectin [10]. CCN2 also interacts with several growth factors including IGF [28], TGF-*β*, and BMP-4 [16] and thereby modulates their activities. Among the CCN family members, CCN3 acts as a negative regulator of CCN2 in the fibrotic pathway of model of renal disease [29,30]; and the addition of recombinant CCN3 slightly decreases the expression of extracellular matrix genes in the growth-plate chondrocytes, in contrast to CCN2, which strongly up-regulates those genes [20]. Furthermore, CCN2 down-regulates CCN3 expression

in growth-plate chondrocytes [20]. In epiphyseal chondrocytes, however, CCN3 promotes the expression of matrix genes and stabilizes the phenotype of articular cartilage [31]. Here we show for the first time homotypic and heterotypic interactions of CCN2 with itself and CCN3, respectively, and provide evidence that homotypic and heterotypic dimers of CCN2 may regulate the production of chondrocytic extracellular matrices in a different manner.

A previous study indicated that the CT domain of CCN3 interacted weakly with full length CCN3 and CCN2 in their yeast two-hybrid study [32]. In our present study, not only the CT domain of CCN3, but also the VWC domain had the ability to bind to full-length CCN2. Furthermore, the specificity of the homotypic and heterotypic interactions of CCN2 was substantiated by the yeast two-hybrid assay performed to determine the binding domain in CCN2 and CCN3. CCN2 self-dimerized through its IGFBP, VWC, and CT domains; whereas only VWC and CT domains, but not the IGFBP domain of CCN3 bound to full-length CCN2. The homotypic dimerization sites of CCN2 to CCN2 were different from the heterotypic dimerization sites for the binding of CCN2 to CCN3. Our data suggest that this difference in binding sites between CCN2-CCN3 and CCN2-CCN2 may be also used as a molecular system for recognition of different cellular surface binding partners such as fibronectin [10], fibulin [32], integrins [33], and aggrecan [18].

Specific CCN2-CCN2 and CCN2-CCN3 interactions were confirmed by performing several binding assays *in vitro* and *in vivo*, including *in vitro* pull-down assays, solid-phase binding assays, and SPR analysis. The relative binding strengths of these domains to CCN2 was also confirmed by measuring the β-galactosidase reporter gene activity in the yeast two-hybrid assay. Interestingly, the binding strength of homotypic and heterotypic bindings of CCN2 measured by SPR analysis was very similar, indicating that the binding partner of CCN2 would be dependent on the expression levels of CCN2 and CCN3 in the specific tissues.

Further evidence for interactions between CCN2 and CCN3 was obtained from immunofluorescence and co-immunoprecipitation experiments. Not only in the case of endogenous CCN2 and CCN3 in chondorcytic cell line, HCS-2/8, but also in the case of ectopically overexpressed GFP-CCN2 and Halo-CCN3 in COS7 cells, CCN2 and CCN3 were co-immunoprecipitated and the subcellular localization of these proteins largely overlapped (Fig. 3 and 4), confirming that CCN2 and CCN3 interacted *in vitro* and *in vivo*. A recent report on CCN family members demonstrated the possibility that CCN5 may regulate by acting as a transcriptional factor [34], and our yeast two-hybrid screening identified several intracellular proteins [Hoshijima et al, unpublished data]. Those interactions may have important roles for cellular functions.

The physiological effects of homomeric and heteromeric interaction between CCN2 and CCN2, and CCN2 and CCN3 on the production of extracellular matrix of chondrocytes were monitored in human chondrocytic cell line, HCS-2/8 by performing real-time PCR, since CCN2 is an enhancer of *aggrecan* and *col2a1* gene expression in chondrocytes [1,8]. In contrast, CCN3 down-regulates the

expression of extracellular matrix genes in growth-plate chondrocytes [20], TGF-β-stimulated Col1 promoter activity in a renal fibrosis model [29], and BMP-2-induced osteocalcin production through inhibition of the Notch signaling cascade [35]. On the other hand, CCN3 enhances matrix production in articular chondrocytes [31], and stimulates BMP-4 gene expression and bone mineralization in osteoblasts [36]. According to our results, however, CCN2 enhanced, whereas CCN3 down-regulated, *aggrecan* expression. This down-regulation was partially reversed by CCN2 treatment. CCN2 dose-dependently increased the amount of *aggrecan* and *col2a1* gene expression that was down-regulated by CCN3. To modify the binding between CCN2 and CCN3, we used antibody 11H3, raised against the VWC domain of CCN2, which antibody attenuated the binding between CCN2 and CCN2, but accelerated the binding between CCN2 and CCN3 as measured by the immobilization binding assay. The addition of 11H3 antibody antagonized CCN2-induced *aggrecan* expression, whereas it further enhanced that in the case of combined treatment with CCN2 and CCN3, indicating that the interaction between CCN2 and CCN3 stimulated *aggrecan* expression in HCS-2/8 cells. Interestingly, 11H3 also accelerated *aggrecan* gene expression in CCN3-treated HCS-2/8 cells, suggesting the existence of endogenous CCN2 in HCS-2/8 cells. This is the first demonstration of homotypic CCN2 and heterotypic CCN2-CCN3 interactions and the data suggest that CCN2 seems to act as a negative regulator of CCN3. This could explain the varied effect of CCN3, since the activity of CCN3 largely depends on CCN2.

Our results indicate that it is the IGFBP domains of CCN2 and CCN3 that make the difference between the homotypic or heterotypic complex formation. CCN2 interacts with many extracellular signaling molecules; e.g., it binds to TGF-β, TGF-βRI and II [37], BMP-2 [38], and BMP-4 [16]. Furthermore, CCN2 modulates Wnt signal pathways [39], as well as the activity of a Wnt inhibitory protein (Wif-1) [40]. CCN3 may also have a role in the modulation of those signaling pathways by interacting with CCN2, but the mechanisms remain to be elucidated.

In CCN2-deficient mice, which show incomplete endochondral ossification, the expression level of CCN3 is strongly enhanced [20]. Two different lines of CCN3-deficient mice were developed. One of them, the *nov*$^{del3-/-}$ mutant mice, which produce no full-length CCN3 protein and express CCN3 without its VWC domain at a barely detectable level, shows abnormal skeletal and cardiac development [41]. The other line, which is completely CCN3 null mice, show enhanced neointimal thickening compared with the wild type, confirming that CCN3 suppresses angiogenesis and fibrosis. These results indicate that CCN2 and CCN3 seem to mutually regulate their activities and expression levels. However, in our experiments the expression level of CCN2 did not alter after addition of CCN2 or CCN3 in HCS-2/8 cells (data not shown). Since HCS-2/8 cells produce higher level of CCN2 compare to primary chondrocytes, further addition of CCN2 may not have effect on the expression level of CCN3. Regulatory mechanism of CCN2 and CCN3 gene expression is currently being elucidated.

Experimental Procedures

Yeast two-hybrid cDNA library screening

Yeast two-hybrid cDNA library screening was performed as described previously [10,18]. Briefly, full-length CCN2 cDNA was expressed as a GAL4BD (GAL4 DNA-binding domain)-fusion protein. cDNA library genes from HCS-2/8 cells were expressed as GAL4AD (GAL4 transactivation domain)-fusion proteins for a two-hybrid assay in yeast AH109 cells, and the cells were screened on selection medium [SD/-Ade/-His/-Leu/-Trp (synthetic dropout medium lacking Ade, His, Leu and Trp)]. Positive clones were analyzed by DNA sequencing. For analysis of the CCN2 or CCN3 domain binding to full-length CCN2, expression plasmids of full-length CCN2, which were expressed as GAL4BD-fusion proteins, and CCN2 or CCN3 cDNA fragments, which were expressed as a GAL4AD-fusion protein, were used to transform yeast cells. The binding was monitored by growth in SD/-Ade/-His/-Leu/-Trp selection medium. The primers used for amplification of full-length and truncated forms of *ccn2* and *ccn3* were the following: $CCN2_{full}$ (27-349), 5'-ATCCGAATTCCAGAACTGCAGCGGGCCGTGCCGGTGCCCG-3' and 5'-ATACGGATCCCTCATGCCATGTCTCCGTACATCTTCCTGT-3'; $CCN2_{IGFBP}$ (27-101): 5'-ATCCGAATTCCAGAACTGCAGCGGGCCGTGCCGGTGCCCG-3' and 5'-ATACGGATCCGAGCACCATCTTTGGCGGTGCACACGCCGA-3'; $CCN2_{VWC}$ (94-198): 5'-ATCCGAATTCGTGTGCACCGCCAAAGATGGTGCTCCCTGC-3' and 5'-ATACGGATCCAGTTGGCTCTAATCATAGTTGGGTCTGGGC-3'; $CCN2_{TSP}$ (193-258): 5'-ATCCGAATTCACTATGATTAGAGCCAACTGCCTGGTCCAGA-3' and 5'-ATACGGATCCGGATGCACTTTTTGCCCTTCTTAATGTTCT-3'; $CCN2_{CT}$ (249-349): 5'-ATCCGAATTCAACATTAAGAAGGGCAAAAAGTGCATCCGT-3' and 5'-ATACGGATCCCTCATGCCATGTCTCCGTACATCTTCCTGT-3'. $CCN3_{full}$ (32-357): 5'-ATCCGAATTCACTCAGCGCTGCCCTCCCCAGTG-3' and 5'-ATACGGATCCCATTTTCCCTCTGGTAGTCTTCAGC-3'; $CCN3_{IGFBP}$ (32-108): 5'-ATCCGAATTCACTCAGCGCTGCCCTCCCCAGTG-3' and 5'-ATACGGATCCATCTCCCTCTACCGCCGTGCAGATG-3'; $CCN3_{VWC}$ (106-192): 5'-ATCCGAATTCGAGGGAGATAACTGTGTGTTCGATG-3' and 5'-ATACGGATCCTTCTGGCCTGTAAGCTGCAAGGGTAAG-3'; $CCN3_{TSP}$ (185-256): 5'-ATCCGAATTCACCCTTGCAGCTTACAGGCCAGAAG-3' and 5'-ATACGGATCCTGGCTGCTCTGGCTCTTGTTCACAG-3'; $CCN3_{CT}$ (253-357): 5'-ATCCGAATTCCCAGAGCAGCCAACAGATAAGAAAG-3' and 5'-ATACGGATCCCATTTTCCCTCTGGTAGTCTTCAGC-3'.

β-galactosidase assays

To confirm the binding specificity of CCN2 and CCN3 peptides, we re-transformed a pGADT7 vector expressing full-length *cnn2* (pGBKT7/*ccn2*$_{full}$) in the AH109 yeast strain together with the pGADT7 vector expressing either full-length *ccn2* or *ccn3* or their IGFBP domain (pGADT7/*ccn2*$_{full}$, *ccn2*$_I$, *ccn3*$_{full}$ or *ccn3*$_I$). The empty pGBKT7 vector was also used for re-transformation together with the same series of CCN2 or CCN3 truncations as a control. All double transformants were cultured in SD/-Trp/-Leu selection medium, equal numbers of cells were collected, and cellular proteins were prepared by 3 cycles of freezing and thawing in yeast Z buffer (0.1 M Na-phosphate, pH7.0, 20 mM KCl, 1 mM MgSO$_4$). To measure the binding strength between full- length CCN2 and either full-length or truncated forms of CCN2 or CCN3, we measured the *β*-galactosidase activity of the reporter gene was measured [42], standardized it to the protein concentration, and subtracted the *β*-galactosidase activity obtained after co-transformation of the pGADT7 empty vector with the same series of polypeptides.

Expression and purification of GST-CCN3 and rhCCN2

For preparation of purified recombinant GST-CCN3 protein, the *ccn3* gene was amplified without its signal peptide by PCR with primers 5'-ATACGGATCCACTCAGCGCTGCCCTCCCCAGTG-3' and 5'-ATCCGAATTCTTACATTTTCCCTCTGGTAGTCTTC-3', and subcloned into the pGEX-2TK vector (GE Healthcare), which carries a GST-tag at its *BamH*I/*EcoR*I sites. The recombinant plasmid was sequenced to ensure the absence of mutations. BL21(DE3)pLysS *Escherichia coli* cells (Novagen) were then transfected with the plasmid for expression of recombinant proteins. The expressed GST-CCN3 protein was purified with Glutathione-Sepharose 4B agarose (GE Healthcare) as described previously [43]. The purified protein was dialyzed against phosphate-buffered saline (PBS). In some experiments, rCCN3 (Abnova) was used. Preparation and purification of CCN2 carrying a His$_6$-tag were performed as described previously [18]. In some experiments, rhCCN2 (BioVendor Laboratory Medicine) was used.

In vitro binding assay

E.coli expressing rhCCN2 or GST-CCN3 were harvested and sonicated in co-immunoprecipitation (co-IP) buffer [20 mM Tris/HCl (pH 8.0), 25 mM NaCl, 1mM MgCl$_2$, 1 mM EGTA, 1% Triton X-100, 1 mM DTT and 1 mM PMSF]. After removal of cell debris, both cell lysates were mixed at 4°C to form CCN2-CCN3 complex. The complexes were then incubated with Glutathione-Sepharose 4B for 1 h at 4°C. The beads were subsequently washed with co-IP buffer with 10% glycerol, after which the bead-protein complexes were subjected to SDS-PAGE and analyzed by Western blotting using an anti-His tag antibody (BETHYL) and GST-tag antibody (GE Healthcare).

Solid-phase binding assay and antibody against CCN2

Wells of an ELISA plate were coated with 100 μl of 500 ng/ml recombinant CCN2 (BioVendor Laboratory Medicine) or GST-CCN3 in 50 mM NaHCO$_3$ buffer (pH 9.6) at 4°C overnight, and blocked with 200 μl of binding buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% BSA, 0.05% Tween20] for 3 h at 37°C. Biotinylated CCN2 with or without 11H3 antibody was added to the wells in a total volume of 100 μl of binding buffer and incubated for 6 h at 37°C. Next, the wells were washed with binding buffer and then incubated with 100 μl of streptavidin-HRP (R&D Systems, MN). Bound HRP was monitored by using a TMD peroxidase substrate kit (Bio-Rad, CA). As control experiments, wells were coated with BSA or GST. The mouse monoclonal antibody used in these experiments was raised against the VWC domain of CCN2 and kindly supplied by Dr. Y. Seto (Nippon Flour Mills Co., Ltd., Japan).

Surface plasmon resonance analysis (BIAcore)

Binding of CCN2 to CCN2 or CCN3 was analyzed by surface plasmon resonance with the BIAcore X instrument (GE Healthcare). rhCCN2 (1.25 μg) was immobilized on a C1 sensor chip by amine coupling according to the manufacturer's instructions. The amount of immobilized CCN2 was in the range of 5000-7000 resonance units (RU). Binding studies were performed in HBS-P [Hepes-buffered saline containing surfactant P: 10 mM Hepes/HCl, 150 mM NaCl and 0.005% surfactant P-20 (pH 7.5) at 25°C] with analyte concentrations of 4, 8, 16, 32, and 64 nM. The dissociation constant (K_d) values were calculated by using BIA evaluation software version 4.1 (GE Healthcare) as previously reported [18].

Expression vectors

pEGFP/*ccn2* vector expressing CCN2 protein with green fluorescent protein at the C-terminus of CCN2 was prepared by amplification of *ccn2* cDNA with signal peptide by using primers 5'-CTTCGAATTCCCATGACCGCCAGTATGGGCCCCGTC-3' and 5'-CGGTGGATCCCGTGCCATGTCTCCGTACATCTTCCTGTA-3', and insertion into *EcoR*I and *BamH*I sites of the pEGFP-N1 vector. pFlag-CMV/*ccn3-halo* expressing CCN3 protein with a Halo-tag at the C-terminus of CCN3 was prepared by amplification of *ccn3* cDNA with signal peptide by using primers 5'-ATCCAAGCTTATGCAGAGTGTGCAGAGCAC-3' and 5'-ATACGAATTCATTTTCCCTCTGGTAGTCTTC-3' and by amplication of *halo* with primers 5'-ATACGAATTCAATGGCAGAAATCGGTACTGG-3' and 5'-ATACGGATCCTTAGCCGGAAATCTCGAGCGTC-3' and pFN21AB3946, which expresses Sox9-Halo protein [44], as a template. The PCR fragments were cloned into the pFlag-CMV-2 vector at *Hind*III/*EcoR*I/*BamH*I sites.

Cell culture and RNA extraction

COS7 monkey kidney cells and HCS-2/8 human chondrosarcoma-derived chondrocytic cells [45] were maintained in Dullbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described before [42,46]. In some experiments, total RNA was isolated from HCS-2/8 cells by using a RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) after the cells had been treated with the desired factors.

In vivo **binding assay**

COS7 cells were co-transfected with pEGFP/*ccn2* and pFlag-CMV/*ccn3-halo* by use of Fugene 6 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions and incubated for 24 hours. The co-transfected COS7 cells or HCS-2/8 cells were then harvested in lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 0.5% protease inhibitor cocktail (Sigma)] and incubated with anti-GFP antibody (Roche) or anti-CCN2 antibody AF660 (R&D) or normal control IgG (Sigma). The complex including GFP-CCN2 or endogenous CCN2 was precipitated by use of HaloLink™ Resin (Promega) or Protein G Sepharose™ (GE Healthcare). After the precipitated proteins had been washed with wash buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100], they were analyzed by Western blotting using an anti-Halo antibody (Promega) or anti-CCN3 antibody.

Fluorescence imaging

COS7 cells transiently transfected with different expression vectors were treated with the fluorescent ligand TMR, which recognize Halo-Tag (Promega), for 15 min at 37°C. After having been washed with DMEM, the cells were fixed with 4% formaldehyde/PBS for 15 min at room temperature; and Halo-tagged proteins and GFP-tagged proteins were directly monitored by fluorescence microscopy using an IX70 Microscope (Olympus Corporation, Japan). The images were analyzed with Axiovision software (Zeiss, Oberkochen, Germany).

Indirect Immunofluorescence and Fluorescence Deconvolution Microscopy

HCS-2/8 cells were first fixed with 3.7% formaldehyde/PBS for 15 min at room temperature. The cells were then either permeabilized with 0.25% TritonX-100/PBS or without permeabilization for 8 min and blocked with 5% skim milk/PBS for 1 h and incubated with primary antibodies for 90 min at room temperature. After washing, the cells were incubated with secondary antibodies. Images were obtained with an Olympus fluorescence microscope.

Reverse transcription and quantitative real-time PCR

For quantification of mRNA expression levels, 0.5 μg of total RNA of each sample was reverse-transcribed with avian myeloblastosis virus (AMV) reverse transcriptase (Takara Bio, Japan) at 42°C for 60 min. Quantitative real-time PCR reaction using SYBR Green Realtime PCR Master Mix (Toyobo, Japan) was done with StepOnePlus (Applied Biosystems, USA). Primer sequences used for *aggrecan* were 5'-GAGGAGAGAACTGGAGAAG-3' and 5'-GCCGATAGTGGAATACAAC-3'; and those for *col2a1* 5'-TGGTCCTGGCATCGACATG-3' and 5'-GGCTGCGGATGCTCTCAAT-3'; and those for *gapdh,* 5'-CAATGACCCCTTCATTGACC-3' and 5'-GACAAGCTTCCCGTTCTCAG-3'.

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Figure legends

Fig. 1 Interaction between CCN2 and CCN2 and between CCN2 and CCN3.

(**A**) Vector constructs of CCN2 expressed as a GAL4-DNA binding domain (BD)-fusion protein and those of CCN2 fragments or CCN3 fragments expressed as GAL4-transactivation domain (AD)-fusion proteins for use in the two-hybrid assay in yeast AH109. Numbers are positions of amino acids from the start codon. Growth test of yeast transformants for self-interaction between CCN2 and CCN2 (upper panel) and for CCN2 and CCN3 fragments (lower panel) in selection media (SD/-Ade, -His, -Leu, -Trp). GAL4-DNA AD-fusion fragments cloned into the pGADT7 expression vector were co-expressed with GAL4-DNA BD/CCN2$_{full}$, which was encoded by pGBKT7/*ccn2*$_{full}$ (*ccn2*$_{full}$), or pGBKT7 (mock). Cell growth was observed to be strong in transformants containing CCN2$_{full}$ with most of the CCN2 and CCN3 fusion proteins except for CCN2$_T$, CCN3$_I$, and CCN3$_T$. (**B**) The binding between CCN2 and CCN3 fragments was monitored by performing the β-galactosidase reporter gene activity in yeast. Full-length CCN2 and CCN2$_{full}$ or CCN3$_{full}$ showed increased binding activity compared to the empty expression vector. Full-length CCN2 bound to the IGFBP domain of CCN2 and full-length CCN3, but not to the IGFBP domain of CCN3.

Fig. 2 Direct interaction between rCCN2 and rCCN3 *in vitro*.

(**A**) Western blot analysis of recombinant His-fused CCN2 (His-CCN2) binding to recombinant GST-fused CCN3 (GST-CCN3) after immunoprecipitation with anti-His. The cell lysates of *E. coli* expressing His-CCN2 were collected and incubated on ice for 10 min with or without the cell lysates of *E.coli* expressing GST-CCN3. Then, pull-down was performed with glutathione-Sepharose beads at 4°C. The beads were washed, and the bound complexes were subjected to SDS-PAGE and Western blotting with anti-His antibody. Representative results for total cell lysate (input) and treatment without GST-CCN3 (-) and with GST-CCN3 (+) are shown. The arrows indicate the signal for His-CCN2 and GST-CCN3. (**B**) Solid-phase binding between biotinylated CCN2 and immobilized CCN2 (left) or GST-CCN3 (right). Microplate wells coated with 0.5 μg/ml of BSA or recombinant CCN2 or GST or GST-CCN3 were incubated with biotinylated recombinant CCN2 (rCCN2) at the concentrations indicated. Binding of biotinylated rCCN2 was quantified by performing a colorimetric assay at Abs$_{655}$ using streptavidin-HRP. (**C**) rCCN2 protein was immobilized on a sensor tip C1 as a ligand, and rCCN2 (left) and rCCN3 (right) were used as analytes at the concentration of 4, 8, 16, 32 and 64 nM in HBS-P. The sensorgrams were corrected for non-specific binding to the reference cell (non-treated). From sensorgram analysis the CCN2-CCN2 and CCN2-CCN3 dissociation constants were calculated to be $K_d \fallingdotseq 1.17 \times 10^{-9}$ and $K_d \fallingdotseq 1.95 \times 10^{-9}$ M, respectively. [Resonance Units]

Fig. 3 Expression and subcellular localization of endogenous CCN2 and CCN3 proteins in chondrocytic HCS-2/8 cells. (**A**) An immunoprecipation (IP) of endogenous CCN2-CCN3 complex from HCS-2/8 cell lysates by using CCN3 antibody or normal IgG, were detected by anti-CCN2 antibody and anti-CCN3 antibody. Ten percent of total input proteins and immunoprecipitated proteins were loaded onto SDS-PAGE gels and electrophoresed. Endogenous CCN3 was effectively pulled down by anti-CCN2 antibody compared to the control IgG. (**B**) Subcellular localization of endogenous CCN2 and CCN3 were detected by indirect immunofluorescence with each specific antibody on HCS-2/8 cell surface and inside of the cells when the cells were permeabilized by TritonX-100. Images were analyzed by fluorescence microscopy. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Scale bars, 20 μm.

Fig. 4 Expression, subcellular localization, and interaction of CCN2 and CCN3 proteins in COS7 cells.

(**A**) Overexpressed GFP-fused CCN2 and binding proteins were precipitated from COS7 cell lysates by using GFP antibody. Among the precipitated CCN2-binding proteins, Halo-fused CCN3 was detected by using anti-Halo antibody. Ten percent of total input proteins and proteins immunoprecipitated with anti-GFP antibody were loaded onto SDS-PAGE gels and electrophoresed. In the presence of GFP-fused CCN2, Halo-fused CCN3 was pulled down effectively by anti-GFP antibody as compared with the control. (**B**) Subcellular localization of CCN2 and CCN3. GFP-fused CCN2 and Halo-fused CCN3 were detected by their fluorescence in living COS7 cells. GFP-fused CCN2 and Halo-fused CCN3, but not the other indicated combinations show co-localization in the cell. Scale bars, 20 μm.

Fig. 5 CCN2 and CCN3 cooperatively regulate gene expression of *aggrecan* and *col2a1* mRNA in HCS-2/8 cells.

After 12 h (*aggrecan*) or 24 h (*col2a1*) treatment of chondrocytic HCS-2/8 cells with rCCN2 and/or rCCN3, gene expression of *aggrecan* or *col2a1* was monitored by real-time PCR. (**A, B**) The addition of rCCN2 enhanced the expression of *aggrecan* and *col2a1* mRNA, whereas the addition of rCCN3 inhibited it. The combination of rCCN2 and rCCN3 abolished the expression of *aggrecan* and *col2a1* repressed by rCCN3 (rCCN2: 1.25 nM, rCCN3: 1.25 nM). (**C, D**) Addition of rCCN2 in conjunction with rCCN3 of a fixed concentration (rCCN3: 0.625 nM) abolished the repression of *aggrecan* and *col2a1* mRNA by rCCN3 in rCCN2 dose-dependent manner. *Aggrecan* gene expression (A and C) was determined by the mean±SD value of triplicate samples. Similar results were obtained three times. *Col2a1* gene expression (B and D) was determined by the mean±SD of 9 samples from three times different experiments performed in triplicate.

Fig. 6 Modulation of CCN2-CCN3 binding by anti-CCN2 antibody (11H3) changes the *aggrecan* **gene expression in HCS-2/8 cells.**

(**A**) Solid-phase binding analysis between biotinylated CCN2 and immobilized CCN2 (left) or GST-CCN3 (right). The addition of 11H3 antibody partially abolished the binding of CCN2 to CCN2 (left), but accelerated the binding to GST-CCN3 (right). (**B**) HCS-2/8 cells were treated with rCCN2 (1.25 nM) and/or rCCN3 (0.625 nM) in the presence of mouse IgG or 11H3 antibody, and gene expression of *aggrecan* was monitored by real-time PCR 12 h later. The addition of 11H3 antibody to CCN2-treated HCS-2/8 cells cancelled the enhanced expression of the *aggrecan* gene, whereas the 11H3 antibody further enhanced gene expression in the presence of CCN3 with or without CCN2, suggesting that enhanced binding of CCN2 and CCN3 led to stronger stimulation of *aggrecan* expression. Data are presented as the mean±SD of triplicate samples.

(A)

pGBKT7/*ccn2* — IGFBP — VWC — TSP-1 — CT —

pGADT7/		pGBKT7/	*ccn2*	mock
mock				
*ccn2*_{full} (27-349)	IGFBP — VWC — TSP-1 — CT			
*ccn2*_{VTC} (94-349)	VWC — TSP-1 — CT			
*ccn2*_{TC} (193-349)	TSP-1 — CT			
*ccn2*_C (249-349)	CT			
*ccn2*_{IVT} (27-258)	IGFBP — VWC — TSP-1			
*ccn2*_{IV} (27-198)	IGFBP — VWC			
*ccn2*_I (27-101)	IGFBP			
*ccn2*_V (94-198)	VWC			
*ccn2*_T (193-258)	TSP-1			

pGADT7/		pGBKT7/	*ccn2*	mock
mock				
*ccn3*_{full} (32-357)	IGFBP ⋯ VWC ⋯ TSP-1 ⋯ CT			
*ccn3*_{VTC} (106-357)	VWC ⋯ TSP-1 ⋯ CT			
*ccn3*_{TC} (185-357)	TSP-1 ⋯ CT			
*ccn3*_C (253-357)	CT			
*ccn3*_{IVT} (32-256)	IGFBP ⋯ VWC ⋯ TSP-1			
*ccn3*_{IV} (32-192)	IGFBP ⋯ VWC			
*ccn3*_I (32-108)	IGFBP			
*ccn3*_V (106-192)	VWC			
*ccn3*_T (185-256)	TSP-1			

Fig. 1 (continued)

(B)

Fig. 1

(A)

WB：anti-His

10% input　GST pull-down

(kDa)

56 —

38 —　　　　　　　　　　　← His-CCN2

His-CCN2　＋　＋　＋　＋
GST-CCN3　－　＋　－　＋

WB：anti-GST

10% input　GST pull-down

← GST-CCN3

56 —

38 —

His-CCN2　＋　＋　＋　＋
GST-CCN3　－　＋　－　＋

(B)

- - o - - BSA
—o— CCN2　] immobilized

Abs_655

0.15

0.1

0.05

0

0　　1　　2　　3
biotinylated CCN2 (μg/mL)

- - o - - GST
—o— GST-CCN3　] immobilized

Abs_655

0.3

0.2

0.1

0

0　　1　　2　　3
biotinylated CCN2 (μg/mL)

(C)

ligand：rCCN2
analyte：rCCN2

Resonance Units

45

35

25

15

5

-5

64 nM

32 nM

16 nM

8 nM

4 nM

$K_d \fallingdotseq 1.17 \times 10^{-9}$ M

0　200　400　600　800

ligand：rCCN2
analyte：rCCN3

Resonance Units

95

75

55

35

15

-5

64 nM

32 nM

16 nM

8 nM

4 nM

$K_d \fallingdotseq 1.95 \times 10^{-9}$ M

0　200　400　600　800

Fig. 2

(A)

(B)

Fig. 3

(A)

10% input

(kDa)

WB：anti-Halo — 76 — Halo-CCN3

WB：anti-CCN2 — 52 — GFP-CCN2

| GFP-CCN2 | - | - | + | + |
| Halo-CCN3 | - | + | - | + |

IP：anti-GFP

76 — Halo-CCN3 / IgG

52 — GFP-CCN2

| GFP-CCN2 | - | - | + | + |
| Halo-CCN3 | - | + | - | + |

(B)

GFP　　Halo　　Merge

GFP-mock ＋Halo-mock

GFP-CCN2 ＋Halo-mock

GFP-mock ＋Halo-CCN3

GFP-CCN2 ＋Halo-CCN3

Fig. 4

Fig. 5

Fig. 6