Connective tissue growth factor (CTGF; now often referred to as CCN2) is a secreted protein predominantly expressed during development, in various pathological conditions that involve enhanced fibrogenesis and tissue fibrosis, and in several cancers and is currently an emerging target in several early-phase clinical trials. Tissues containing high CCN2 activities often display smaller degradation products of full-length CCN2 (FL-CCN2). Interpretation of these observations is complicated by the fact that a uniform protein structure that defines biologically active CCN2 has not yet been resolved. Here, using DG44 CHO cells engineered to produce and secrete FL-CCN2 and cell signaling and cell physiological activity assays, we demonstrate that FL-CCN2 is itself an inactive precursor and that a proteolytic fragment comprising domains III (thrombospondin type 1 repeat) and IV (cystine knot) appears to convey all biologically relevant activities of CCN2. In congruence with these findings, purified FL-CCN2 could be cleaved and activated following incubation with matrix metalloproteinase activities. Furthermore, the C-terminal fragment of CCN2 (domains III and IV) also formed homodimers that were ~20-fold more potent than the monomeric form in activating intracellular phosphokinase cascades. The homodimer elicited activation of fibroblast migration, stimulated assembly of focal adhesion complexes, enhanced RANKL-induced osteoclast differentiation of RAW264.7 cells, and promoted mammosphere formation of MCF-7 mammary cancer cells. In conclusion, CCN2 is synthesized and secreted as a preproprotein that is autoinhibited by its two N-terminal domains and requires proteolytic processing and homodimerization to become fully biologically active.

CCN2 is the most studied member of a family of secreted glycoproteins termed CCN proteins (acronym of Cyr61/CTGF/Nov). CCN2 is implicated in several diseases (1), in particular diseases in which enhanced fibrogenesis and tissue fibrosis are a characteristic pathophysiological feature. In this context, CCN2 has been reported to be among the 10 most abundant transcripts in primary human fibroblasts and thus plays an important role in defining the phenotypic characteristics of the fibroblast (2, 3). Although the designation “connective tissue growth factor” (CTGF) implies that CCN2 may act as a growth factor, the prevailing opinion in the field is that the actions of CCN2 are not limited to that of growth factors per se. The CCN proteins contain up to four structural domains that are highly conserved among the family. Following the second structural domain is a nonconserved unstructured “hinge” region of variable length that connects the third domain (a schematic of modular organization is depicted in Fig. 1a). Although the role of the modular structure of CCN proteins is poorly understood, this structural organization has fostered the hypothesis that CCN proteins are matricellular proteins (4–7) to refer to a group of secreted proteins with diverse regulatory roles at the interface of the extracellular matrix and the cell surface. According to this paradigm the complex regulatory actions of CCN2 are made possible by diverse protein–protein interactions involving the different domains of CCN2 (4–7). However, the categorization of CCN proteins as matricellular proteins (4–7) may have dissuaded studies on the structure-activity relationships of CCN2 as a signaling molecule. In several reports on mice genetically engineered to overexpress FL-CCN2, rather limited phenotypes have been observed in the absence of experimentally induced disease (8, 9). One interpretation of these findings could be that CCN2 must undergo further processing to become biologically active. In this respect, smaller fragments of CCN2 from 10 to 20 kDa have also been observed in some

4 The abbreviations used are: CTGF, connective tissue growth factor; FL-CCN2, full-length CCN2; CHO, Chinese hamster ovary; RANKL, receptor activator of nuclear factor κB ligand; TGFβ, transforming growth factor β; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; S6K, p70 S6 kinase; RSK, p90 ribosomal S6 kinase; Cl, confidence interval; aa, amino acids; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloproteinase; vWF, von Willebrand type C repeat; BMP, bone morphogenetic protein; DHRF, dihydrofolate reductase; SUMO, small ubiquitin-like modifier; DMEM, Dulbecco’s modified Eagle’s medium; PVDF, polyvinylidene difluoride; AUC, area under the curve; 4SBb, four SMAD-binding response elements; DAPI, 4′,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRAP, tartrate-resistant acid phosphatase; ANOVA, analysis of variance; SNAIL, zinc finger protein SNAI1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Received for publication, June 21, 2018, and in revised form, August 23, 2018. Published, Papers in Press, September 27, 2018, DOI 10.1074/jbc.RA118.004559
tissues that display high activities of CCN2 (10–12) (see also Fig. S1, a and c). Indeed, some of these fragments have previously been shown to display biologic activity (11, 13–17). For example, the 10-kDa C-terminal fragment of CCN2 (comprising the cystine knot domain) isolated by Brigstock and co-workers (14, 15) and produced by recombinant DNA technology is widely used and has reported capacity for eliciting cell signaling responses (18–20). However, as previously reported from our laboratory, the potency of the 10-kDa fragment in eliciting rapid cell signaling responses is very low compared with the presumptive full-length 37-kDa CCN2 (19). Interestingly, a recent report by Mokalled et al. (21) showed that genetic overexpression of an N-terminally truncated form of CCN2 containing only domains III and IV was sufficient and even appeared to be more effective than full-length CCN2 in mediating spinal cord regeneration in a zebrafish model of injury to the spinal cord. Thus, the time has come to assess the efficiencies and potencies of full-length CCN2 and the various fragments of CCN2 that can be isolated following secretion of CCN2. In this report, we address the fundamental question of to what extent CCN2 is secreted as a preproprotein that needs to undergo proteolytic processing to become a biologically active signaling molecule. Although this issue is not an imminent research question in the field of CCN proteins, release of fragments with signaling capacity is certainly well established for many other autocrine/paracrine factors (22), structural extracellular matrix proteins (matrikines) (23), and even some matricellular proteins (24–26). Thus, the major goal of this study was to resolve the structure-activity relationships of fully active CCN2. Finally, we investigated to what extent the structure-activity relationships of bioactive CCN2 may also apply for CCN1 and CCN3.

Results

Characterization of CCN2 entities

Western blot analysis of the cell culture medium of DG4 CHO cells engineered to produce and secrete FL-CCN2 revealed several bands immunoreactive to anti-CCN2 antibodies. Based on the presumptive hypothesis that CCN2 undergoes proteolytic activation following secretion, the different entities of CCN2 were separated through several chromatographic steps and subsequently subjected to structural analysis and investigation of biologic activity. Separation of different entities of CCN2 was complicated by the dispersed isoelectric points attributed to the varying glycosylation pattern of CCN2 (27) (Fig. S1, a and b). However, the following CCN2 entities were purified to apparent homogeneity (Fig. 1 for Western blotting analyses and Fig. S2 for purification scheme and MS data) and designated as follows. 1) FL-CCN2-M is the 37-kDa full-length CCN2 monomer. 2) FL-CCN2-D is a 60-kDa entity immunoreactive to anti-CCN2 antibodies and migrating as 37-kDa in the presence of β-mercaptoethanol (confirmed by MS analysis to represent a homodimer of full-length CCN2). 3) CT-CCN2 is a band migrating at 18 kDa under reducing conditions, confirmed by Western blot analysis and MS analysis to be a C-terminal fragment of CCN2 comprising domains III (TSP1) and IV (cystine knot) with N-terminal identity 181AYRLED186. This C-terminal fragment of CCN2 generated by endopeptidase cleavage of the amide bond between Ala180 and Ala181 is conserved with previously reported endopeptidase cleavage fragments of CCN2 (28). 4) NT-CCN2 is the N-terminal 17-kDa fragment of CCN2. In our experience, commercially available antibodies against NT-CCN2 displayed poor avidity and specificity; hence, the identity of NT-CCN2 was determined by MS analysis to constitute domains I and II (Fig. S2e).

During the initial activity testing of the purified CCN2 entities, it became clear that for some of the CCN2 entities (for example the 37-kDa CCN2 entity) signaling activity was diminished or eventually lost as progressively purer CCN2 entities were obtained. Also, the concentrations of the 18-kDa CT-CCN2 fragment required to elicit CCN2 signaling responses were substantially higher than what we observed at earlier stages of the purification. Thus, we attempted to purify CCN2 entities based on specific activities (activity/mg of protein) from sequential chromatographic steps to uncover whether even less prominent CCN2 entities with higher specific activities could be present. To expedite screening of the signaling activity of the various CCN2 fractions eluted from the different chromatographic steps, we modified a FRET-based AKT kinase-activity biosensor (29) by exchanging the FRET chromophores with fragments of NanoLuc luciferase to generate a biosensor based on enzyme complementation of NanoLuc luciferase activity (30) (Fig. 2a), yielding an assay with substantially improved signal-to-noise ratio compared with the FRET readout (29). The resulting biosensor assay, henceforth referred to as Nano-iAKT, was stably transfected into CCN2-responsive Rat2 cells (31) and validated with AKT inhibitors (see Fig. S3 for biosensor validations). The eluted fractions of each chromatographic step were then assessed for the ability to stimulate AKT kinase activity. Fractions that stimulated AKT were subsequently subjected to Western blot analysis of CCN2 immunoreactivities using antibodies directed at various domains of CCN2. Western blot analyses of the resulting fractions were performed under both reducing and nonreducing conditions as the C-terminal cystine knot domain of CCN2 is postulated to engage in protein dimer formation (32). This strategy made it possible to separate an immunoreactive entity comigrating with the far more abundant FL-CCN2-M around 37 kDa in the absence of β-mercaptoethanol and migrating at 18 kDa in the presence of β-mercaptoethanol (F16 in Fig. S1e), suggesting that this entity might represent a homodimer of the 18-kDa fragment. Fractions containing the latter CCN2 entity displayed the highest specific activities among the various CCN2 entities assessed by the Nano-iAKT assay (e.g. fraction 16 in Fig. S1f).

Western blot analysis of myocardial tissue extracts subjected to electrophoresis under nonreducing conditions also revealed a band migrating at ~60 kDa that was immunoreactive to anti-CCN2 IgG (Fig. S1c). However, under reducing conditions, the immunoreactivity at 60 kDa disappeared, and the predominant immunoreactivity was observed around 37 kDa (Fig. S1c). In congruence with the fragments isolated and purified from the DG4 CHO cells expressing full-length CCN2, anti-CCN2 (C-terminal) immunoreactivity migrating at ~18 kDa appeared in extracts of granulation tissue from infarcted hearts separated
in the presence of β-mercaptoethanol. However, it was not possible to discern immunoreactivity under nonreducing conditions that might represent a putative dimer of the 18-kDa band from the principal immunoreactivity migrating at 37 kDa (Fig. S1c).

However, as it was not possible to separate the potential dimer of the 18-kDa fragment in a substantially enriched form from the far more abundant full-length CCN2 as well as from the monomer of the 18-kDa fragment present in the cell culture medium of the FL-CCN2–producing cell line, we engineered another stable CHO cell line producing and secreting the recombinant C-terminal fragment of CCN2 comprising domains III-IV of CCN2 (d3-4-CCN2). Thus, from the cell culture medium of this CCN2–expressing cell line secreted both monomeric and dimeric d3-4-CCN2. Notably, this d3-4-CCN2-D preparation migrates at approximately 40 kDa under nonreducing conditions, whereas immunoreactivities appear between 17 and 20 kDa under reducing conditions. The d3-4-CCN2 preparation was readily detected too, as the hinge epitopes were included in the d3-4-CCN2-D construct to enable the utilization of the hinge antibody for detection of nonreduced d3-4-CCN2 protein preparations. The FL-CCN2-D preparation displays immunoreactivity that migrates at 60 kDa under nonreducing conditions and shifts to 37 kDa under reducing conditions. The d3-4-CCN2 preparations are readily detected too, as the hinge epitopes were included in the d3-4-CCN2-D preparation of different entities by MS (Fig. S2).

Figure 1. Characterization of purified CCN2 entities. a, schematic illustrating the primary structure of the CCN2 entities purified from the FL-CCN2–producing cell line based on immunoblots and immunoblot detection of both nonreduced and reduced CCN2. The d3-4-CCN2 preparations are readily detected too, as the hinge epitopes were included in the d3-4-CCN2-D preparation of different entities by MS (Fig. S2): black, signal peptide; red, NT-CCN2; blue, CT-CCN2, with bold letters indicating the N-terminal part of CT-CCN2 as identified by Edman sequencing. The cysteine residues highlighted in purple underlining show identified disulfide bridges in the d3-4-CCN2 preparations. FL-CCN2-M, full-length CCN2 monomer; CT-CCN2, C-terminal CCN2 (purified from FL-CCN2–producing cell line); NT-CCN2, N-terminal CCN2 (purified from FL-CCN2–producing cell line); FL-CCN2-D, full-length CCN2 dimer; d3-4-CCN2-M, domains III-IV CCN2 monomer (purified from domains III-IV CCN2–producing cell line); d3-4-CCN2-D, domains III-IV CCN2 dimer (purified from domains III-IV CCN2–producing cell line). β-me, β-mercaptoethanol.

1. MTAASMPVR VAYVVLLAG CERPAMQACS GPCRCDPSEA PRCPAGLVLG 1DGСССС САККЕГЕСТР RDPDPMHHG LCDFGSFAPMK KTVCTA КD
2. APC11PGTIV YRGFSFQSS CKYCTCLCLGA VSCMLCSD VRLPSFFSCF PRVKGDKC CEWVCDEDK QDVVDFALA AYRELDTTG DP dietary CL
3. VQTEGSGAC ITOCMGMCIT VNDNASQG CR ERSSLCHMV PCEADDLENI KGHKCTI Р Т К СПКЕ КЕ SСТСМГТР АРКСГВДТГО РСТРОШИТ
4. LIPVFRCPDF GEMKKNKMF1 КТСАЧВМСР GСДНТФГSL YРМНГЕМА*
5. 15 40 20 60 30
6. Fig. S2
and Cys\textsuperscript{373}-Cys\textsuperscript{307} (Fig. S2g), in congruence with previous data on CCN2 from analysis of the secretome of endothelial cells (33). These disulfide bridges were most likely intraprotein bridges as they were found both in the monomeric and in the dimeric d3-4-CCN2.

As contaminating TGFβ activities in preparations of purified recombinant CCN2 have been observed,\textsuperscript{5} we tested all the isolated CCN2 entities for activation of SMAD transcriptional

\textsuperscript{5} L. F. Lau, personal communication.
activity using a SMAD reporter assay. In our experience, TGFβ activity (i.e. induction of the SMAD reporter) coeluted with CCN2 entities containing domains III-IV from ion-exchange chromatography columns, hydrophobic-interaction chromatography columns, and heparin-affinity chromatography columns. Thus, a subsequent size-exclusion chromatography step was necessary to remove contaminating TGFβ activity from the CCN2 entities. As shown in Fig. S2c, neither of the CCN2 preparations subjected to size-exclusion chromatography displayed detectable SMAD reporter activities except for d3-4-CCN2-M (10 μg/ml), which contained minute amounts of SMAD-stimulating activity.

**Release of the C-terminal domains III-IV of CCN2 is necessary for signaling activity**

Once the various CCN2 entities had been purified and characterized, we proceeded to investigate their relative potencies and efficacies in various signaling assays, starting with the Nano-iAKT biosensor assay. As demonstrated in the real-time recording shown in Fig. 2b, this assay allowed for detection of AKT activity within the first minutes of stimulation with d3-4-CCN2-D. Another noteworthy observation was that the time course of AKT activity following stimulation with d3-4-CCN2-D was similar to that of EGF, the positive control. However, d3-4-CCN2-D elicited more prolonged activation of AKT compared with the transient stimulation of AKT activity in the presence of EGF. As many groups have routinely used the commercially available *Escherichia coli*-derived C-terminal domain IV fragment of CCN2 (d4-CCN2), reported to confer partial CCN2 activity (15, 17), we also included this fragment of CCN2 in the Nano-iAKT assay. Only fragments containing domains III and IV or domain IV of CCN2, d4-CCN2, CT-CCN2, d3-4-CCN2-M, and particularly d3-4-CCN2-D, were able to stimulate AKT activity (Fig. 2c). These results confirmed our initial findings that dimer-enriched fractions of CT-CCN2, purified from the cell line producing FL-CCN2, is the most active form of CCN2. The assay also demonstrated that the CCN2 preparations containing both domains III and IV were substantially more potent and possibly also more effective than d4-CCN2. Thus, domain III appears to be required for full biological activity of CCN2. Strikingly, FL-CCN2-M and FL-CCN2-D, as well as NT-CCN2, completely lacked capacity to stimulate AKT. The concentration-effect relationships of the various CCN2 entities recorded with the AKT biosensor assay were confirmed by LuminesTM bead-based immunoassays of phospho-AKT (Ser473) levels (Fig. 2d). As phosphorylation and activation of ERK1/2 are also a reported CCN2 activity (35, 36), we assayed the concentration-effect relationships of the different CCN2 entities both by biosensor-recorded ERK activity (Fig. 2e) and by determination of phospho-ERK1/2 (Thr202/Tyr204 and Thr185/Tyr187) levels in Rat2 fibroblasts (Fig. 2f). In congruence with the assays of AKT activity, d3-4-CCN2-D was substantially more potent than d3-4-CCN2-M in stimulating ERK1/2 activities. The higher potency of d3-4-CCN2-D was also demonstrated for stimulation of Rac1 activity (Fig. 2g), another rapid signaling activity elicited by CCN2 (18, 37) (see Fig. S3i for real-time activity data). Notably, also the time course of d3-4-CCN2-D stimulated Rac1, and its sensitivity to the EGF receptor inhibitor gefitinib differed from that of EGF-stimulated Rac1 (Fig. S3, i and j). As all of the above assays were performed in Rat2 fibroblasts, we proceeded to investigate whether d3-4-CCN2 might activate the same signaling pathways in another cell line also reported to respond to CCN2. Hence, MCF-7 mammary carcinoma cells (38, 39) were stimulated with d3-4-CCN2-D, confirming that d3-4-CCN2 also stimulates AKT, ERK, and Rac1 activities in these cells (Fig. 2, h–j). Biosensors for p70 S6 kinase (S6K) and p90 ribosomal S6 kinase (RSK) kinase activities, i.e. downstream kinases previously shown to be phosphorylated in tissues overexpressing CCN2 (9), again demonstrated the same order of potencies for d3-4-CCN2-D, d3-4-CCN2-M, and CCN2-CT as those observed for stimulation of AKT and ERK activities (Fig. 2, k and l). Consistent with the assays of AKT and ERK activities, neither FL-CCN2 nor NT-CCN2 was able to stimulate S6K or RSK kinase activities (Fig. 2, k and l). Although the concentration-effect relationships of CT-CCN2– and d3-4-CCN2-M–stimulated AKT and ERK activities displayed some variations of efficacy and potency among the bioassays and immunoassays, a consistent finding was that only CCN2 entities containing domains III and IV or domain IV were capable of eliciting cell signaling activity. Furthermore, the dimeric d3-4-CCN2 (d3-4-CCN2-D) was ~20-fold more potent than the monomeric form (e.g. EC50 for d3-4-CCN2-D–stimulated ERK activities (biosensor), 9.5 × 10−9 M (95% CI, 7.4 × 10−9–1.2 × 10−8), versus EC50 for CT-CCN2-stimulated ERK, 2.2 × 10−7 M (95% CI, 3.9 × 10−8–1.3 × 10−6)). Altogether, the signaling assays consistently confirmed our hypothesis that proteolytic processing of FL-CCN2 is a necessary activation step for biological activity of CCN2.

**C-terminal fragments of CCN2 recapitulate the cell physiologic effects of CCN2**

Next we performed cell physiological assays to investigate whether the differences of efficacy and potency among the various CCN2 entities observed in the signaling assays were also reflected in the cell physiologic actions previously reported for CCN2. CCN2 has been shown to stimulate a number of complex cell physiologic actions in various cells, including cell migration, adhesion, proliferation, and differentiation (4). As these assays are of a prolonged duration that may be sufficient for substantial proteolytic processing of exogenously added CCN2 to occur, we investigated to what extent recombinant FL-CCN2 added to the cell culture medium of Rat2 fibroblasts would be subjected to proteolytic cleavage within the relevant time frame. As shown in Fig. 3a, FL-CCN2 incubated with Rat2 fibroblasts for 24 h underwent proteolytic cleavage and generated a C-terminal fragment of ~18 kDa that was detected with a CCN2 antibody directed toward an epitope in the hinge region (aa 170–207) of CCN2. Thus, the cell physiologic assays may not provide conclusive evidence as to what extent full-length entities of CCN2 (FL-CCN2) lack biological activity; however, they may provide important information as to what extent the CCN2 fragments found to be bioactive signaling molecules also replicate the broad spectrum of cell physiologic actions previously reported for CCN2. First, the often reported capacity of CCN2 to stimulate cell migration was investigated.
CTGF, a matricellular preproprotein

In congruence with previous reports (for a review, see Rachfal and Brigstock (4)), we found that FL-CCN2 to some degree stimulated cell migration of Rat2 fibroblasts. However, CCN2-CT and, to an even greater extent, d3-4-CCN2-D displayed substantially higher efficacy than the full-length CCN2 variants (FL-CCN2-M and FL-CCN2-D) in stimulating cell migration (Fig. 3b and Fig. S4a). We subsequently proceeded to investigate the capacity of CCN2 to increase assembly of focal adhesion complexes (37), a critical step in cell adhesion (40). Again, consistent with the signaling assays, d3-4-CCN2-D was the more effective CCN2 entity in stimulating assembly of focal adhesion complexes. However, neither of the full-length CCN2 entities elicited a significant increase of focal adhesion complexes (Fig. 3c and Fig. S5).

As CCN2 has been reported to stimulate either cell proliferation (31) or senescence (41) depending on cell type, Rat2 cells, an immortalized fibroblast cell line that has previously been shown to respond with cell proliferation upon stimulation with CCN2, was used (31). Thus, in subsequent experiments, we investigated the capacity of the various CCN2 entities to stim-
ulate proliferation of Rat2 fibroblasts. As shown by the concentration-effect curves in Fig. 3d, d3-4-CCN2-D displayed remarkably higher potency and efficacy than any of the other CCN2 entities in stimulating cell proliferation. On the contrary, the full-length CCN2 entities FL-CCN2-M and FL-CCN2-D displayed no or minimal activity below 270 nM (10 μg/ml FL-CCN2-M), consistent with the cell signaling assays. The higher potency of d3-4-CCN2-D was also reflected in DNA synthesis assayed by incorporation of bromodeoxyuridine (BrdU) (Fig. S4, b and c), validating the observation that proteolytic processing of CCN2 and generation of a homodimer of domains III and IV are required for CCN2 to exert its full mitogenic potential.

In subsequent experiments, we investigated to what extent d3-4-CCN2-D could also evoke the reported CCN2-induced osteoclast differentiation (42) and epithelial-to-mesenchymal transition (EMT) (43). Once more, d3-4-CCN2-D recapitulated the previously reported effects of CCN2, first by inducing osteoclast differentiation of RAW264.7 cells (Fig. 3e) and next by inducing the EMT transcription factor SNAIL and stimulating the formation of mammospheres in cultures of MCF-7 cells (Fig. 5, f and g). As the increased levels of SNAIL were not accompanied by a concomitant decrease in E-cadherin, the induction of SNAIL by d3-4-CCN2-D in these experiments most likely reflected partial EMT or induction of EMT in a small proportion of cells only.

**Proteolytic cleavage of the CCN2 hinge releases the C-terminal fragment and is sufficient for activation**

Having firmly established that the homodimer of the C-terminal fragment of CCN2 consisting of domains III and IV is the fully active CCN2 signaling molecule, we turned our focus to the proteolytic processing and activation of FL-CCN2. Although several proteases potentially could cleave CCN2 (28, 44–49), we chose to focus on the matrix metalloproteinases (MMPs), which have previously been shown to cleave CCN2 in the unstructured hinge region between domains II and III (28, 47–49). Incubation of FL-CCN2 with recombinant catalytic domains of various MMP isoforms revealed that FL-CCN2 was indeed susceptible to cleavage by all of the investigated MMPs except MMP11 as shown in the Western blot in Fig. 4a. Thus, we subsequently investigated to what extent the presence of batimatstat, a broad spectrum MMP inhibitor, in the cell culture medium of the CHO cell line producing FL-CCN2 could inhibit endopeptidase cleavage of secreted FL-CCN2 and consequently reduce the generation of smaller fragments of CCN2. As shown in Fig. 4b, inhibition of MMPs did decrease proteolytic fragmentation of secreted FL-CCN2, indicating that MMPs were at least partially responsible for the processing of FL-CCN2 secreted from the CHO cell line. Furthermore, the recombinant MMPs that cleaved FL-CCN2 all generated similar immunoreactive fragments migrating between 15 and 20 kDa as shown in the Western blot probed with an anti-CCN2 antibody recognizing epitopes in the hinge region of CCN2 (Fig. 4a). This cleavage pattern was highly suggestive of the MMPs cleaving FL-CCN2 close to the cleavage site in the hinge region, generating the biologically active CT-CCN2 fragment isolated from the FL-CCN2–producing CHO cell line. The cleavage site of the latter was mapped by Edman sequencing to be the amide bond between Ala180 and Ala181 in the hinge region of CCN2 (177PALAAYRLE185) (Fig. 4c). Incubation of the biosensor with recombinant catalytic domains of various MMPs demonstrated that MMP-7, -8, -12, and -13 cleaved the 177PALAAYRLE185 peptide sequence (Fig. 4d) and thus potentially represent proteases that may generate bioactive CCN2. To address the latter issue, we investigated to what extent any of the above MMP isoforms would generate bioactive CCN2 upon cleavage of FL-CCN2. Recombinant MMP8 was chosen for this purpose for the following reasons. MMP8 could be completely inhibited by batimatstat (Fig. 4a), it could cleave CCN2 within the 177PALAAYRLE185 region and thus generate a C-terminal fragment of CCN2 similar to the bioactive CT-CCN2 (Fig. 4d), and it did not display protease activity that interfered with the Nano-iAKT biosensor per se. As shown in Fig. 4e, incubation of FL-CCN2-M with MMP8 conferred a robust gain of AKT kinase activity that was sensitive to batimatstat, providing proof of principle that cleavage of FL-CCN2 is both necessary and sufficient for generation of bioactive CCN2 as schematically illustrated in Fig. 4f.

**Generation of a dimeric d3-4-CCN2 fusion protein with full agonist activity**

To enable simplified production and purification of dimeric d3-4-CCN2, the fully active form of CCN2, we created fusion proteins of domains III and IV of CCN2 (d3-4-CCN2) and an Fc receptor–silenced version of the Fc fragment of IgG4 (50, 51). Several fusion proteins were engineered with different orientations of d3-4-CCN2 relative to the Fc fragments and varying linkers between the two entities as steric factors are known to be crucial for the efficacy and potency of Fc fusion proteins (51–53). This strategy utilized the disulfide-linked dimerization of the Fc fragment to dictate dimerization of the fusion protein. Different orientations of the Fc fragment relative to d3-4-CCN2, as well as different linkers providing varying degrees of flexibility, generated fusion proteins with various potencies and efficacies in stimulating AKT and ERK activities (Fig. 5, a–c). As shown in Fig. 5, b and c, the fusion protein in which a short linker is connecting IgG4 Fc to the C-terminal end of d3-4-CCN2 displayed agonist properties with efficacy and potency similar to those of homodimeric d3-4-CCN2-D in stimulating AKT and ERK activities, e.g. EC_{50} for d3-4-CCN2-D–stimulated ERK activities (biosensor), 9.5 × 10^{-9} M (95% CI, 7.4 × 10^{-9}–1.2 × 10^{-8}), and for d3-4-CCN2–SL–Fc, 1.3 × 10^{-8} M (95% CI, 1.1 × 10^{-8}–1.4 × 10^{-8}). These findings verified the importance of the homodimeric structure for maximal potency of the CCN2 fragment containing domains III-IV of CCN2.

CTGF, a matricellular preproprotein
CTGF, a matricellular preproprotein

Figure 4. MMP-catalyzed cleavage and activation of CCN2. a, FL-CCN2-M (1 μg of protein) was incubated for 2 h with 0.1 μg of the indicated MMPs in the presence or absence of the broad spectrum MMP inhibitor batimastat (1 μM) and immunoblotted against CCN2 (epitope aa 238–348). A representative blot of two independent experiments is shown. b, FL-CCN2–producing CHO cells were maintained with or without batimastat (1 μM) for 4 days, and conditioned medium was subsequently subjected to Western blot (WB) analysis of CCN2 immunoreactivity (epitope aa 170–207). The panel shows a representative Western blot and histogram of densitometric analysis plotted as density of immunoreactivity migrating at 18 kDa relative to the density of the immunoreactive band around 37 kDa presented as mean ± S.E. (n = 3). c, schematic of a firefly luciferase–based protease biosensor. Upon cleavage of the inserted 177PALAAYRLE185 peptide fragment of the CCN2 hinge, the structural constraints on complementation of activity by the C-terminal and N-terminal parts of firefly luciferase are relieved, and luciferase activity is generated. d, the 177PALAAYRLE185 protease sensor was stably expressed with a signal peptide directing secretion from CHO cells (CHO FreeStyle). Conditioned medium from this CHO cell line was incubated with various MMPs (2.5 ng/μl) as indicated, and luciferase activity was recorded continuously (n = 4 independent experiments). e, FL-CCN2-M was incubated with MMP8 for 120 min, and batimastat was added either from the start of incubation or at the end of the incubation period. The cleavage reactions were subsequently added to the cell culture wells of Rat2 cells stably transfected with the AKT-activity biosensor, and AKT activities were recorded (n = 3 independent experiments). The amount of CCN2 in each well corresponded to 24 μg/ml FL-CCN2-M. f, schematic model of CTGF activation (depicted as monomeric for simplicity). Full-length CTGF requires activation by proteolytic cleavage of the hinge region to release the biologically active C-terminal fragment comprising domains III and IV. Statistical significance was calculated by one-way ANOVA with Dunnett’s post hoc test in d and e two-tailed t test in b and e. *, p < 0.05; **, p < 0.01; ****, p < 0.0001. All error bars represent S.E. (b, d, and e). Uncropped immunoblots are shown in Fig. S7.

C-terminal domains (III-IV) of CCN1 and CCN3 elicit cell signaling activity

Having delineated the structure-activity relationships of CCN2, we subsequently investigated to what extent domains III and IV of other CCN family proteins would also be sufficient for activity and to what extent cleavage of the full-length proteins would be required for agonist activity. CHO cells were genetically engineered to secrete full-length CCN1 (also known as Cyr61) or CCN3 (also known as Nov) as well as the respective C-terminal fragments of CCN1 and CCN3 (containing domains III and IV). The recombinant proteins were subsequently purified from the cell culture medium. As opposed to full-length CCN2, full-length CCN1 displayed significant agonist efficacy as assessed by rapid stimulation of AKT and ERK activities in Rat2 cells (Fig. 5, f and g). However, in congruence with the structure-activity properties of CCN2, the C-terminal fragment of CCN1 containing domains III and IV displayed somewhat higher efficacy and potency than those of full-length CCN1 in stimulating AKT and particularly ERK activities, indicating that proteolytic activation may also be important in regulation of CCN1 activities (Fig. 5, f and g). Indeed, monomeric d3-4-CCN1 displayed similar potency and efficacy as monomeric d3-4-CCN2 in stimulating AKT and ERK activities (e.g. EC50 for d3-4-CCN2–stimulated ERK activities (biosensor), 1.2 × 10−7 M (95% CI, 9.3 × 10−8–1.4 × 10−6), and for d3-4-CCN1-M, 2.5 × 10−8 M (95% CI, 2.0 × 10−8–3.2 × 10−8)). Furthermore, the migration of an immunoreactive band sensitive to β-mercaptoethanol suggested that a putative dimer of d3-4-CCN1 might exist; however, the amounts were too small to enable purification of this entity (Fig. 5e).

Full-length CCN3 was rapidly cleaved following secretion from the CHO cells. Thus, it was not possible to purify full-
length CCN3 from the cell culture medium of CCN3-transfected CHO cells. However, d3-4-CCN3 displayed similar efficacy and potency as d3-4-CCN1 in stimulating AKT and ERK kinase activities as shown in Fig. 5, i–k (e.g. EC_{50} for d3-4-CCN1-M–stimulated ERK activities (biosensor), 2.5 × 10^{-8} \text{ M} (95\% \text{ CI}, 2.0 × 10^{-8} – 3.2 × 10^{-8}) and for d3-4-CCN3-M, 1.8 × 10^{-8} \text{ M} (95\% \text{ CI}, 1.5 × 10^{-8} – 2.2 × 10^{-8}). Thus, d3-4-CCN3 is also sufficient for agonist activity of CCN3.

Subsequently, we investigated to what extent the recombinant C-terminal fragments of CCN1 (d3-4-CCN1) and CCN3 (d3-4-CCN3) were able to stimulate mammosphere formation of MCF-7 mammary carcinoma cells, a more complex biological function. As shown in Fig. 5, h and l, both d3-4-CCN1-M and d3-4-CCN3-M stimulated mammosphere formation of MCF-7 cells, although d3-4-CCN1-M appeared to be substantially more effective than d3-4-CCN3-M.

**Discussion**

Still more than three decades after the discovery of CCN2 (54), a uniform protein structure that defines biologically active CCN2 has not been resolved until now. This report uncovers the novel finding that matricellular CCN2 is synthesized and secreted as a preproprotein that requires proteolytic processing to attain the capacity to elicit cell signaling responses. Furthermore, a homodimer of the active fragment, i.e. the C-terminal fragment comprising domains III and IV of CCN2, was shown to constitute biologically fully active CCN2. Forced generation of a homodimer of the active CCN2 fragment could be obtained by recombinant engineering of the active CCN2 fragment as fusion proteins with the Fc fragment of immunologically defective IgG4. One of these fusion proteins displayed similar efficacy and potency as the dimeric C-terminal fragment of CCN2. This new knowledge on how CCN2 acts as an autocrine/para-
CTGF, a matricellular preproprotein

crine factor may open new avenues of research on the role of CCN2 in disease mechanisms.

The finding that CCN2 is synthesized and secreted as a preproprotein that is autoinhibited by its two N-terminal domains and requires proteolytic processing and homodimerization to become fully biologically active is a mechanism shared by several other autocrine/paracrine factors, e.g. the TGFβ superfamily. This mechanism allows the activity of CCN2 to be controlled by an additional checkpoint, i.e. by specific proteases active under distinct developmental stages or under specific disease mechanisms. Interestingly, in this respect, Overall and co-workers (48) recently reported that cleavage of the hinge region of CCN family members by MMPs was more pervasive than previously appreciated.

Even though the C-terminal cystine knot domain of CCN proteins was postulated to engage in dimer formation 25 years ago (32), this is the first report on the unequivocal existence and isolation of a dimer of a CCN protein. Notably, we provide evidence that a homodimer of full-length CCN2 is not only generated in recombinant cell lines expressing full-length CCN2 but also exists in vivo in a tissue known to express low levels of CCN2 under physiologic conditions, i.e. myocardial tissue. Interestingly, in congruence with our data that CCN2 undergoes proteolytic activation, the domain III-IV fragment of CCN2 could also be demonstrated in the granulation tissue and differentiating scar tissue forming after ischemic necrosis of myocardial tissue, a process in which CCN2 is particularly involved. According to these findings, prepro-CCN2 may first form a homodimer of full-length CCN2 from which the mature homodimeric domain III-IV fragment of CCN2 is generated by proteolytic cleavage. However, we were not able to determine the levels of the homodimeric domain III-IV fragment of CCN2 in myocardial tissue as we currently do not have the means to quantitatively separate the dimeric form of domains III-IV of CCN2 from both the monomeric form of domains III-IV of CCN2 and monomeric full-length CCN2 in tissue extracts. Another hurdle is that the anti-CCN2 antibodies have different avidities toward nonreduced versus reduced forms of CCN2, thus making quantitative analysis of CCN2 fragments even more uncertain.

Although we did not identify any interprotein disulfide bridges responsible for homodimerization of CCN2, the pLink-SS software is limited in that not all disulfide-containing peptides may be identified, especially complex peptides containing both intrapeptide and interpeptide bridges (33, 34). Thus, it is still possible that any of the remaining cysteines of domains III and IV of CCN2 not demonstrated to be involved in disulfide bridge formation may engage in interprotein disulfide bridges and homodimerization of CCN2. Furthermore, another mechanism of homodimerization of CCN2 may be noncovalent metallo cysteine bridge formation as demonstrated for human growth hormone (55).

In addition to conferring autoinhibition of prepro-CCN2, an important function of the N-terminal prodomains may well be to help stabilize domains III and IV of CCN2 in a conformation that would promote dimerization and secretion. Such a stabilizing function of the N-terminal prodomains would be analogous to that reported from resolution of the structure of pro-activin A (56) and from early studies that showed that expression, dimerization, and secretion of activin A and TGFβ required the presence of their prodomains (57). However, the d3-4-CCN2 fragment was undoubtedly able to dimerize and to be secreted in the absence of the N-terminal prodomains because the homodimer of d3-4-CCN2 was readily detectable in the cell culture medium from CHO cells synthesizing and secreting the d3-4-CCN2 fragment. How the prodomain affects the efficacy of dimerization and secretion of endogenously expressed full-length CCN2 remains to be resolved.

The N-terminal propeptide may also contribute to compartmentation and localization of CCN2 close to membrane-bound proteases (46, 49), facilitating activation by proteolytic cleavage of the hinge and release of the active C-terminal fragment. In this regard, it is interesting to note that domain II of CCN1 (the von Willebrand type C repeat (vWC) homology domain) has been shown to interact with transmembrane integrin αβ3 (58), an integrin complex that has also been shown to be important for CCN2 functions (59). Also supporting a critical function of the N-terminal domains in regulation of CCN2 activities are the reports of a monoclonal anti-CCN2 antibody targeting domain II (FG-3019) for inhibition of CCN2 actions in animal disease models of pancreatic ductal adenocarcinoma (10), muscle dystrophy (60), and radiation-induced pulmonary fibrosis (61) and in early-phase clinical trials in patients suffering from idiopathic pulmonary fibrosis (62).

Domain II (vWC domain) of CCN2 has also been shown to bind other proteins in the extracellular matrix, i.e. proteoglycans (aggrecan) (63) and BMP-2 and -4 (64). With regard to the latter, based on X-ray crystallographic studies of the vWC domains of proteins thought to bind BMP-2, such as CV-2, collagen IIa, and CCN3, binding of the vWC domain of CCN proteins and BMP-2 has recently been questioned (65). Other domains of CCN2 may also bind proteins in the extracellular matrix. For example, domain III of CCN2 (TSP1) has been shown to bind and modulate the function of VEGF165 (28, 47). Interestingly, MMP-mediated cleavage of CCN2 released VEGF165 from the complex with CCN2 and caused reactivation of the angiogenic activity of VEGF165 (28, 47). In the context of our current report, a tantalizing consequence of MMP-mediated disruption of the CCN2–VEGF165 complex would be concerted activation of both CCN2 and VEGF165.

Despite extensive investigations, we were not able to demonstrate any cell signaling activity responses stimulated by the N-terminal domains I and II–containing fragment of CCN2. However, in early studies, Grotendorst and Duncan (66) reported that the N-terminal fragment of CCN2 stimulated myofibroblast differentiation and collagen synthesis in the presence of insulin-like growth factor, whereas the corresponding C-terminal fragment could not. These findings were later questioned in studies by Heng et al. (13) and Yang et al. (67), which concluded that the C-terminal fragments of CCN2 could stimulate myofibroblast differentiation and collagen synthesis. Thus, these findings await further clarification.

In this report, we demonstrate that the major cleavage site in the hinge region of CCN2 generating mature CT-CCN2 was Ala180 → Ala181. Interestingly, this site has also been shown to be susceptible to cleavage by ADAM28 (a disintegrin and met-
alloproteinase family endopeptidase), thus providing another example of a protease with the potential capacity to activate CCN2 (47). MMP sites in the hinge region of CCN2 have also been identified distal to the Ala$^{180}$ ↓ Ala$^{181}$ cleavage site (48).

To what extent cleavages of CCN2 at these sites all generate a fully active CCN2 agonist may have to be investigated for each specific fragment. However, the d3-4-CCN2–Fc fusion protein in which the C-terminal fragment of CCN2 was made to commence at Ala$^{197}$ displayed full agonist activity similar to d3-4-CCN2-D. As shown in this study, Cys$^{199}$ forms a disulfide bridge with Cys$^{228}$. Thus, cleavage of CCN2 distal to Cys$^{199}$ may perturb tertiary structure imposed by the disulfide bridge, conceivably reducing or abolishing the agonist activity of CCN2. In this respect, the 10-kDa C-terminal fragment of CCN2 comprising domain IV only, isolated by Briggstock and co-workers (14, 15), was shown to be capable of eliciting rapid cell signaling activity (18–20), although with greatly reduced efficacy and potency compared with the C-terminal fragments comprising both domains III and IV as demonstrated in this study.

For studies of cell biologic and physiologic functions, a fully active version of CCN2 that does not require prior activation would be a great advantage. Even from the dedicated d3-4-CCN2–producing cell line, the majority of the generated product was monomeric d3-4-CCN2. In contrast, the d3-4-CCN2–Fc fusion protein, which displayed agonist potency and efficacy similar to that of homodimeric d3-4-CCN2, alleviated this problem as Fc fusion proteins effectively form homodimers and provide opportunities for simple, quantitative affinity purification of the fusion protein. Furthermore, the d3-4-CCN2 fusion protein lacking immune effector function may also be attractive for investigation of in vivo functions of CCN2 as the Fc fragment confers prolonged half-life in the circulation (68).

Biologically active Fc fusion proteins have also been reported for full-length CCN1 and CCN6 (69, 70). Although the designs of the fusion proteins in these reports were not optimized for activity, they demonstrate the potential for the application of a similar strategy for efficient production and purification of other bioactive CCN proteins as well.

An imminent question was to what extent other members of the CCN family are secreted as preproproteins that require proteolytic processing to generate the mature biologically active signaling molecules. As shown in this study, the C-terminal fragments of CCN1 and CCN3 comprising domains III and IV were clearly sufficient for activation of rapid cell signaling as well as for eliciting cell physiologic responses. Interestingly, the N-terminal domains of CCN1 also appeared to confer some degree of autoinhibition of full-length CCN1. However, full-length CCN1 did not display the strict lack of signaling activity as demonstrated for CCN2 and consequently may not be considered a preproprotein. Relative to CCN2, CCN1 has a very elongated hinge region (63 aa for CCN1 versus 30 aa for CCN2). Thus, the long hinge region of CCN1 could provide greater steric flexibility of the C-terminal domains III and IV, obviating the need for proteolytic cleavage to release the bioactive fragment. The pronounced susceptibility of CCN3 to endopeptidase cleavage, hampering production and purification of recombinant full-length CCN3, is consistent with previous reports (71, 72). Thus, we were not able to obtain full-length CCN3 sufficient for analysis of its agonist activity. However, indirect evidence from genetically engineered mice suggests that CCN3 may also be secreted as a preproprotein. First, mice with genetically targeted deletion of domain II (vWC) of CCN3 produce a truncated variant of CCN3 that immunologically appears to correspond to domains III and IV and displays a markedly different phenotype than both WT mice (73) and mice with complete deletion of CCN3 (74, 75). Furthermore, an N-terminally truncated variant of CCN3 has also been reported to confer transforming activity and support anchorage-independent growth of fibroblasts in vitro, whereas full-length CCN3 was without effect (76). These observations could be explained by proteolytic processing being necessary for activation of full-length CCN3. In this respect it is also interesting to note that the hinge region of CCN3 is very short, i.e. of similar length as that of CCN2. Future studies will be needed to further decipher functional differences between the CCN proteins due to differences in their hinge regions, for instance by generation of recombinant chimeras of a CCN protein and hinge regions from different CCN isoforms.

The implications of CCN2 requiring proteolytic activation to initiate signaling are very important for the interpretation of the many reports on the functions of CCN2 in vivo that have been published over the years. For instance, in the recently reported mice with conditional overexpression of full-length CCN2 in the kidney, no phenotype was present in the absence of a concomitant insult, whereas in the setting of ureteral obstruction, fibrosis was enhanced (20). This observation fits perfectly with the notion that in healthy adult kidney overexpression of full-length CCN2 engenders a limited phenotype, whereas in the presence of insults and concomitant release of endopeptidase activities, the effects of CCN2 overexpression become fully apparent.

Our novel finding that CCN2 is secreted as an inactive preproprotein implies that interaction with a receptor eliciting the observed cell signaling responses is not possible prior to proteolytic activation. A mechanistic explanation for this could be that the receptor-binding site of CCN2 domains III and IV is concealed by domains I and II as schematically depicted in Fig. 4f. Although no complete structure of any of the full-length CCN proteins has yet been reported, this would be in alignment with the structure proposed for CCN3 by Holbourn et al. (77) based on small-angle X-ray scattering. According to this structure, the individual domains of CCN proteins form extended molecules (77); such a structure would allow for the concealment of epitopes essential for receptor recognition and binding by the C-terminal domains III and IV.

This model also implies that the current attempts at targeting CCN2 with an antibody targeting domain II (10, 60, 62) rely on the blockage of activation of the preproprotein, e.g. through interfering with localization of CCN2 to membrane-bound proteases (46, 48, 49) or clearance of all body CCN2 (78). Whether inhibition of activation of CCN2 will be sufficient in disease states where soluble proteases are also often present remains to be seen. Ultimately, the structure of bioactive CCN2 and the mechanism of activation revealed here may facilitate studies on the role of CCN2 and other CCN proteins in pathophysiologic mechanisms of disease, the identification of recep-
CTGF, a matricellular preproprotein

tors mediating the signaling responses of CCN2 and other CCN proteins, and the development of drugs targeting and inhibiting the active part of CCN2 or other CCN proteins.

Experimental procedures

Plasmids

All plasmid constructs were generated by a combination of classical restriction endonuclease–based cloning, Gateway recombination cloning technology, and Gibson assembly. Plasmid maps of all destination vectors and inserts are shown in Fig. S6. All inserts were codon-optimized for human (biosensors) or hamster (recombinant CCN2 entities) expression and synthesized either as “DNA strings” or “gene synthesis” by Thermo Fisher Scientific (Waltham, MA), converted to entry vectors, and recombined with the destination vectors, as indicated in Fig. S6, to yield expression vectors. All constructs based on DNA strings were DNA sequence–verified (GATC Biotech, Constance, Germany), whereas constructs generated by gene synthesis were DNA sequence–verified by the manufacturer.

Protein production

FL-CCN2 was produced from DG44 CHO cells (a dihydrofolate reductase-deficient CHO cell line adapted for suspension culture) stably transfected with an expression vector encoding human CCN2 cDNA (GenBank™ accession number BC087839) and dihydrofolate reductase, allowing selection and amplification of CCN2 expression with methotrexate (subcloning and generation of cell line was performed by Fusion Antibodies Ltd. (Belfast, Ireland)). FL-CCN2 and fragments of CCN2 generated following secretion were all purified from the cell culture medium. For production of FL-CCN2, both monomer and dimer, 1 × 10⁶/ml FL-CCN2–producing cells were seeded in shaker flasks in CD OptiCHO medium (Thermo Fisher Scientific (Gibco)). Following culture for 3 days (with rotatory shaking at 145 rpm), the cells were sedimented by centrifugation, and the cell culture medium was harvested. As prolonged culture (>3 days) increased the amounts of CCN2 entities of lower molecular mass, 1 × 10⁶/ml CCN2–FL–producing cells were cultivated for 5 days (a time point when cell viability was still >95%) before harvest of cell culture medium for production and purification of CCN2-NT and CCN2-CT. For experiments with batimastat, parallel flasks of the FL-CCN2–His–Halo–SUMO, a pool of stably selected DHFR−/− DG44 suspension CHO cells was generated by methotrexate amplification, and 1 × 10⁶/ml cells were seeded in CD OptiCHO and cultivated for 4 days before harvest of the cell culture medium.

For production of FL-CCN1 and d3-4-CCN1, pools of stably selected DHFR−/− DG44 suspension CHO cells were generated by methotrexate amplification. For production of FL-CCN1, 1 × 10⁶/ml FL-CCN1–producing cells were cultivated in shaker flasks in CD OptiCHO medium (Thermo Fisher Scientific (Gibco)) for 4 days before harvest. For production of d3-4-CCN1, 1 × 10⁶/ml d3-4-CCN1–producing cells were maintained in shaker flasks (145 rpm) in ActiPro medium (GE Healthcare). After 3 days of culture, daily feeding with 4% (v/v) Cell Boost 7A and 0.4% (v/v) Cell Boost 7B (both from GE Healthcare) was implemented until harvest of the cell culture medium at day 6. All CHO suspension cells were cultured at 37 °C at 8% CO₂ unless noted otherwise.

For production of d3-4-CCN3, the ExpiCHO Max Titer protocol was utilized according to the manufacturer’s procedure (Thermo Fisher Scientific). Briefly, 100 ml of ExpiCHO culture was transiently transfected with the d3-4-CCN3–encoding expression vector (for details, see Fig. S6d) before switching to 32 °C and an atmosphere with 5% CO₂ after 20 h. Supplements were added at days 1 and 5 as described in the manufacturer’s protocol. The culture was harvested on day 7. Expression of FL-CCN3 in the ExpiCHO system did not yield any full-length product due to protease processing. All d3-4-CCN2–Fc fusions were produced utilizing the ExpiCHO manufacturer’s Max Titer protocol as described above for d3-4-CCN3.

All harvesting of cell culture media was done while cell viability was >95%. At harvest, the media was clarified by centrifugation at 4750 × g for 20 min at 4 °C. Phenylmethylsulfonyl fluoride, EDTA, and MES buffer, pH 6.0, were added to all harvested cell culture media to give final concentrations of 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, and 50 mM MES. The only exception was for purification of the Fc fragment (proteolytically cleaved from d3-4-CCN2–FL–Fc during cultivation) for which MES was replaced with Tris-HCl, pH 7.4, at a final concentration of 25 mM.

Protein purification

The purification procedures for all CCN2 entities (except the FL-CCN2–His–Halo–SUMO fusion protein) used for activity studies are depicted in Figs. S1 and S2. Fractions eluted from cationic ion-exchange chromatography columns were diluted in binding buffer to lower conductivity of the sample before heparin-Sepharose chromatography. FL-CCN1-M and d3-4-CCN1-M were purified using the same strategy as depicted in Fig. S2a for FL-CCN2-M and CT-CCN2, respectively. d3-4-CCN2–Fc fusion proteins and d3-4-CCN3-M were purified using the same strategy as for the untagged d3-4-CCN2 entities depicted in Fig. S2b. For the FL-CCN2–His–Halo–SUMO fusion protein, only a cationic ion-exchange chromatography step was used. For purification of the Fc fragment only, cell culture medium from the d3-4-CCN2–FL–Fc–expressing culture was subjected to protein A–Sepharose chromatography, which captured both the complete product (d3-4-CCN2–FL–Fc) and the Fc fragment with d3-4-CCN2 cleaved off. The Fc fragment was then separated from the d3-4–FL–Fc fusion protein by subsequent size-exclusion chromatography. All protein concentrations were determined with the micro-BCA method (Thermo Fisher Scientific). The chromatography media and
columns (all from GE Healthcare) and buffers (all chemicals from Sigma-Aldrich) used are listed in Table S1.

Cell lines

MCF-7 human mammary adenocarcinoma cells (ATCC HTB-22), Rat2 fibroblasts (ATCC CRL-1764), and murine RAW264.7 macrophage cells (ATCC TIB-71) were obtained from the American Type Culture Collection (LGC Standards, Germany). 293A, FreeStyle CHO-S, and CHO DG44 DHFR<sup>−/−</sup> were obtained from Thermo Fisher Scientific. MCF-7, Rat2, RAW264.7, and 293A were maintained in DMEM with high glucose (Gibco) supplemented with 10% fetal bovine serum and 50 μg/ml Gensumycin (Sanofi). FreeStyle CHO-S was maintained in FreeStyle CHO expression medium (Gibco) supplemented with 1× Glutamax (Gibco). CHO DG44 DHFR<sup>−/−</sup> was maintained in CD DG44 medium (Gibco) supplemented with 1× Glutamax (Gibco) and 0.18% Pluronic F-68 (Gibco) prior to transfection and either CD OptiCHO, BalanCD CHO Growth A, or ActiPro supplemented with puromycin or methotrexate as indicated above.

Antibodies

Anti-GAPDH (sc20357) and anti-CCN2 (L20 (sc14939)/E5 (sc365970)) antibodies were from Santa Cruz Biotechnology. Another anti-CCN2 IgG (ab6992) was from Abcam. Anti-SNAIL (C15D3), anti-E-cadherin (24E10) and anti-focal adhesion kinase (catalog number 3285) antibodies were from Cell Signaling Technology. Anti-vinculin IgG (catalog number V9264) was from Sigma-Aldrich. Secondary antibodies were from Santa Cruz Biotechnology (goat anti-mouse (sc2005) and donkey anti-goat (sc2056)) and GE Healthcare (donkey anti-rabbit (NA934V)).

Because the commercially available antibodies directed against domain III or IV of CCN2 were poor at detecting nonreduced CCN2, antiserum against CT-CCN2 was generated by immunization of rabbits performed by Eurogentec (Belgium). IgG from the antiserum was captured by protein A-Sepharose chromatography (GE Healthcare), and anti-CCN2 IgG was affinity-purified on a column packed with HaloLink Sepharose beads (Promega) coupled with FL-CCN2–His–Halo–SUMO and subsequently concentrated by ultrafiltration (Vivaspin 10,000 molecular weight cutoff, GE Healthcare). Chromatography media and columns were from GE Healthcare, and all other chemical were analytical grade from Sigma-Aldrich (listed in Table S1).

Western blot analysis and staining of SDS-polyacrylamide gels with Coomassie G-250

For experiments with MCF-7 cells, protein was extracted in a boiling lysis buffer composed of 1% SDS and 10 mM Tris-HCl, pH 8.8; sonicated; and centrifuged. Protein concentrations in the resulting supernatants were measured with a micro-BCA assay and adjusted accordingly. The MCF-7 cell extracts were separated on 8 or 12% polyacrylamide gels, whereas CCN2 protein preparations were separated on 4–15% TGX gradient gels (Bio-Rad). For Western blot analysis, proteins were transferred to PVDF membranes using the Trans-Blot Turbo semidytry blotting system (Bio-Rad), and the membranes were blocked in 5% (w/v) nonfat dry milk dissolved in Tris-buffered saline with Tween 20 (20 mM Tris–HCl, pH 7.4, 140 mM NaCl, 2.5 mM KCl, and 0.1% Tween 20; all chemicals were analytical grade from Sigma-Aldrich) for 30–60 min before probing with primary antibody overnight at 4 °C. Secondary antibody incubation was performed at room temperature for 30–60 min before development of SuperSignal<sup>™</sup> West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and analysis of chemiluminescence using the ChemiDoc imaging system (Bio-Rad). For Coomassie staining, gels were washed 3 × 5 min in water before staining with BioSafe G-250 Coomassie Stain (Bio-Rad) and destained with water. Coomassie-stained gels were imaged with the ChemiDoc imaging system.

MS analysis: Protein identification and Edman sequencing

FL-CCN2-D and NT-CCN2 were separated by SDS-PAGE, stained with Coomassie G-250, excised from the gel, and contract analyzed by nano-HPLC-ESI-MS/MS (Proteome Factory AG, Berlin, Germany). For Edman sequencing of CT-CCN2, SDS-PAGE–separated protein was transferred to a PVDF membrane with the Trans-Blot Turbo system, exchanging the standard manufacturer’s blotting buffer with a buffer composed of 50 mM sodium borate, pH 9.0, in 20% methanol. To eliminate N-terminal blockage of the protein, the membrane was washed with deionized water and then incubated for 30 min at room temperature in 1% polyvinylpyrrolidone-40 in 0.1 M acetic acid. Subsequently, the membranes were washed three times (30 min each wash) in deionized water and then incubated overnight in PGAP buffer (manufacturer-supplied; Takara Biosciences) containing 50 milliliters of Pfu pyroglutamate aminopeptidase at 45 °C. Finally, the membranes were washed three times (15 min each wash) in deionized water and air-dried before analysis by Proteome Factory AG.

Identification of disulfide bridges of CCN2

In an attempt to identify the cysteines responsible for dimerization, d3-4-CCN2-M and d3-4-CCN2-D were subjected to LC-MS/MS analysis following the method described previously (33, 34). Briefly, after precipitation with 25% TCA on ice, the proteins were resuspended in 8 M urea and 100 mM Tris, pH 6.5, in the presence of 2 mM N-ethylmaleimide for Lys-C digestion. Then the samples were diluted 4-fold with 100 mM Tris-HCl, pH 6.5, containing 2 mM N-ethylmaleimide for further digestion with trypsin alone or trypsin and Glu-C. Peptide-N-glycosidase F (112 New England Biolabs units/6 μg of proteins) was added to remove glycans 2 h before the digestion was stopped. The resulting peptides were analyzed using an EASY-nLC 1000 system (Thermo Fisher Scientific) interfaced with a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific). A 60-min reverse-phase gradient was used to separate peptides. The top 15 most intense precursor ions from each full scan (resolution, 60,000) were isolated for high-energy collisional dissociation MS2 (resolution, 15,000; normalized collision energy, 27) with a dynamic exclusion time of 30 s. The MS data were analyzed using pLink-SS, a software tool for identification of disulfide bridges as described in detail previously by Dong and co-workers (33, 34). This enables identification of disulfide bridges.
bridges but not differentiation of interprotein or intraprotein linkages.

**Experimentally induced myocardial infarction in mice**

All animal experiments were approved by the national board for animal research, permit number 6288, and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication number 85-23, revised 2010). Fourteen- to 16-week-old female C57BL/6J BomTac mice (Janvier Labs), with ad libitum access to food and water, were subjected to sham operation or myocardial infarction by ligation of the left anterior descending coronary artery (or not) as described previously by Gao et al. (79). Animals were euthanized 7 days after induction of myocardial infarction, the heart was excised, and granulation tissue from the infarcted left ventricle and nonischemic myocardial tissue were sampled.

**2D electrophoresis and immunoblot analyses**

For identification of CCN2 entities in granulation tissue from the infarct zone of mice subjected to myocardial infarction as well as in normal myocardial tissue from sham-operated mice (n = 4 animals/group), the tissue was crushed in liquid nitrogen and subsequently solubilized in buffer containing 1% SDS, 10 mM Tris-HCl, pH 8.8, 2 mM Na3VO4, 10 mM NaF, 4 mM β-glycerophosphate, and 4 mM pyrophosphate. Extracts were then subjected or not to deglycosylation, utilizing Protein Deglycosylation Mix II (New England Biolabs). Extracts were then subjected initially to first dimension electrophoresis on immobilized pH gradient strips (pI range, 7–10; Bio-Rad). Following isoelectric focusing, second dimension electrophoresis was performed on 12% acrylamide gels before semidry blotting (as described above) and detection with anti-CCN2 antibody. A similar protocol was used for investigation of glycosylation of fractions collected from FPLC.

**AKT, ERK, S6K, RSK, and Rac1 biosensor assays**

Plasmids encoding for the various biosensors were generated as described under "Plasmids" and in Fig. S6 and introduced into Rat2 or MCF-7 cells by electroporation (Neon transfection system, Thermo Fisher Scientific) using manufacturer-developed parameters for NIH3T3 or MCF-7, respectively. For generation of stable cell lines, electroporated Rat2 cells were maintained in 5 μg/ml puromycin and MCF-7 cells were maintained in 0.25 μg/ml puromycin until the appearance of isolated colonies, which were expanded and tested for signal intensity. The cell clones with the highest expression levels and preserved cell proliferation were subsequently maintained in 2.5 (Rat2 cells) or 0.1 μg/ml (MCF-7 cells) puromycin. For assays, 10,000 cells/well were seeded in white-walled 96-well cell culture plates, maintained in a CO2 incubator overnight, and subsequently changed to CO2-Independent™ medium (Gibco) without serum. After 5–6 h of serum starvation, 10% (v/v) NanoGlo Live Cell reagent (Promega) was added, and relative light unit measurements were started in a PolarStar Omega plate reader (BMG Labtech, Germany). After 15 min of stabilization, the measurements were paused, and protein preparations were added with a multichannel pipette before continuing the measurements for 30 min. The values from all wells were background-normalized to their individual prestimulatory values (recorded during the last 3 min before stimulation) and then to the vehicle control values. The area under the curve (AUC) for the 30 min after stimulation was calculated for each individual well using GraphPad Prism 6. This conversion to a single value from continuous recordings was performed to enable unbiased comparison of the potencies of different protein preparations. For the concentration-effect curves, protein concentrations were converted to molar units based on calculated molecular weights (subtracting the signal peptide) for unprocessed entities and MS-mapped sequences for the processed CCN2 entities. To enable comparison of results from multiple experiments, the AUC values were expressed as the percentage of the response of the positive control, 0.5 μg/ml EGF (R&D Systems, catalog number 236-EG), which was included in all experiments. For the experiments with activity testing after MMP8 digestion of FL-CCN2-M, the AUC from the cutting reactions with batimastat added 2 h after MMP8 were related directly to the reactions with batimastat added before incubation with MMP8. All inhibitors used for the validation of the biosensors (API-2, AKTi1/2, U0126, SL327, rapamycin, and BI-D1870) were from Tocris (UK).

**TGFβ/SMAD reporter assay**

A shuttle vector, compatible with the RAPAd adenoviral expression system (Cell Biolabs), with four SMAD-binding response elements (4SBE) controlling the expression of firefly luciferase was generated as illustrated in Fig. S6 and used to produce adenovirus by cotransfecting 293A cells together with the RAPAd viral backbone vector. The virus was purified by CaptoCore700 HiTrap column chromatography (GE Healthcare) and titered with the Adeno-X Rapid Titer kit from Clontech (Takara Bio). Rat2 cells were seeded at a density of 10,000 cells/well, transduced with adenovirus encoding the 4SBE reporter at a multiplicity of infection of 1000, and incubated overnight before serum starvation. After 16–20 h of serum starvation, the cells were stimulated for 24 h after which the medium was decanted, and 100 μl of ONE-Glo substrate/lysis reagent (Promega), diluted 1:4 in H2O, was added to each well. The plates were then incubated for 10 min before the lysate was transferred to black-walled plates, and luciferase activity was determined by recording luminescence with the PolarStar Omega plate reader.

**Immunoassay of phosphoproteins**

Rat2 cells were seeded at a density of 350,000/well in 6-well plates and serum-starved for 16–20 h before stimulation with the various CCN2 entities for 20 min. The cells were subsequently lysed in BioPlex lysis buffer (Bio-Rad), corrected for protein content (micro-BCA assay), and analyzed with Luminex bead–based immunoassays (BioPlex immunoassays, Bio-Rad) of phospho-AKT (Ser473) and phospho-ERK1/2 (Thr185/Tyr187 and Thr202/Tyr204) according to the manufacturer’s instructions. Briefly, the phosphoproteins of interest were captured with antibody-coupled beads and labeled with a second antibody coupled to a fluorophore, enabling analysis with the BioPlex 200 instrument (Bio-Rad).
Cell migration assay
To test the effect of various CCN2 entities on cell migration, the transwell migration assay (Thermo Fisher Scientific) (also known as a modified Boyden chamber assay) was used. Cells were seeded on top of a 5-μm-pore-diameter PVDF membrane containing transwells in serum-free medium. CCN2 test fractions were added to the lower chamber of the transwells. Following a 16-h incubation, Cell-ROX dye (Invitrogen, catalog number C10422) was added to the lower chamber to stain migrated cells. Following a 10-min incubation, the top chamber was scraped to remove unmigrated cells. The membranes were then removed from the chamber, immobilized on glass slides, and used for imaging on a Zeiss Axio Observer Z.1 imaging system. Images were analyzed in Adobe Photoshop CC by an investigator blinded to the experimental groups. Results of the migration assays are expressed as the percentage of seeded cells migrated to the lower chamber.

Focal adhesion complex formation
Rat2 fibroblasts were seeded on an eight-chamber glass slide and following overnight serum starvation were stimulated with various CCN2 entities for 24 h. The cells were subsequently fixed with 4% paraformaldehyde in PBS and immunostained for focal adhesion kinase (Cell Signaling Technology, catalog number 3285) and vinculin (Sigma, catalog number V9264) before counterstaining with DAPI. The entirety of cell volume was acquired as Z-stacks (of thickness 0.5 μm) with a Zeiss Axio Observer Z.1 imaging system. Images were analyzed in ImageJ (National Institutes of Health, Bethesda, MD) by an investigator blinded to the experimental groups. Individual cell boundaries were defined as visible from the localization of complexes or based on a distance of 10 μm from the nuclear boundary (DAPI staining). Results are expressed as the number of focal adhesion complexes (spots with overlapping signals for focal adhesion kinase and vinculin) per cell. Data are mean ± S.E. of three independent experiments per group/condition (a total of 250 cells were analyzed per experimental condition).

Cell proliferation assays
Rat2 cells were seeded in 96-well plates at a density of 3000 cells/well and allowed to settle overnight. Cells were then serum-starved for 16–20 h before stimulation with the various protein preparations. After 48 h, the cells were analyzed with the CellTiter-Glo assay system (Promega) according to the manufacturer’s instructions. Results are expressed as the percentage of vehicle control values. For BrdU analysis of DNA synthesis (Roche Applied Science, chemiluminescent ELISA BrdU kit), the cells were labeled with BrdU for 2 h after either 48- or 72-h stimulation with various CCN2 entities. BrdU incorporation was subsequently determined according to the manufacturer’s instructions. Both the CellTiter-Glo and BrdU assays were recorded with a PerkinElmer Life Sciences Victor X5 plate reader.

Mammosphere assay
Single-cell suspensions of MCF-7 cells were generated by trypsinization of adherent MCF-7 cultures followed by trituration through a 25-gauge needle five times and filtering through a 40-μm cell filter prior to seeding at density of 200 cells/well in 96-well ultralow-adhesion plates (Sarstedt) in DMEM/F-12 supplemented with B27 without vitamin A (both from Gibco). Cells were then incubated with or without indicated protein preparations for 7 or 10 days, stained with thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Sigma-Aldrich). Spheres were semiautomatically quantified (40-μm diameter cutoff) with the Oxford Optronix Gelcount™ system.

Tartrate-resistant acid phosphatase (TRAP) assay
RAW264.7 cells were seeded at a density of 10,000 cells/well in DMEM supplemented with 0.5% fetal calf serum and 1 μg/ml d3-4-CCN2-D for 7 days before staining with the TRAP kit from Clontech (Takara Bio). TRAP-positive cells (stained cells) were visualized by light microscopy with a digital camera (Zeiss Axiovert A1 connected to AxiosCam ERc5s), and images were acquired and analyzed with Adobe Photoshop 3.0 and quantified as positive cells/field of view with 20× magnification. For each experiment, two wells were utilized for each condition, and three images were taken per well at the areas of maximal cell density.

MMP 30F-PALA-AYRLE biosensor assay
A protease sensor for the 177PALAAYRLE185 part of CCN2 was generated by inserting the 177PALAAYRLE185 sequence into the protease site of a protease biosensor based on a circularly permuted Photuris pennsylvania luciferase (80) (provided as 30F by Promega). The signal peptide of human albumin was appended to the N terminus, and the resulting protease biosensor, 30F-PALA-AYRLE, was codon-optimized for Chinese hamster and expressed in FreeStyle CHO S cells. After selection of stably transfected cells with 10 μg/ml puromycin, the cells were seeded in fresh cell culture medium at a density of 1 × 106/ml and cultured for 4 days. The cell culture medium was subsequently harvested and stored at −70 °C until use. For assaying MMP activity, 95 μl of freshly thawed cell culture medium (containing the secreted 30F-PALA-AYRLE biosensor) admixed with 2 μl of cAMP reagent (Promega) was distributed in black-walled 96-well plates, and luminescence was recorded with the PolarStar Omega plate reader. Active domains of recombinant MMPs (Enzo Life Sciences) were added (0.25 μg/well) after 5 min, and the signals were monitored for an additional 40 min. To enable statistical comparisons, the continuous recordings were converted to a single value by calculating the AUC for the last 15 min of the assay, when all the MMP reactions had reached plateaus, with GraphPad Prism 6.

MMP cleavage of recombinant CCN2
FL-CCN2-M (final concentration of 1.0 μg/10 μl) was incubated with 1× MMP buffer and recombinant MMPs (final concentration of 80 ng/10 μl) with 1 μM batimastat added either before MMP addition or 2 h after MMP addition. The MMP buffer consisted of 50 mM HEPES, pH 7.0, 10 mM CaCl2, 0.05% Brij 35, and 150 mM NaCl; for MMP3, the HEPES was exchanged with 50 mM MES, pH 6.0, according to the manufa-
CTGF, a matricellular preproprotein

cuter’s instructions. For the experiments with subsequent activity testing, the final concentration of FL-CCN2-M was 24 μg/ml (i.e. 12 μg/ml each part if FL-CCN2-M was cut right in the middle), and Brij 35 was omitted from the MMP buffer to avoid cell lysis.

Statistical analysis

The number of independent experiments and which statisti-
cal methods were used for comparisons is described in the respective figure legends. Error bars in the figures indicate S.E. All statistical comparisons, except for the analysis of the MS data, were performed with GraphPad Prism 6. For comparison of two experimental groups, unpaired Student’s two-tailed t test was used. For comparison of several experimental groups, one-way ANOVA was used followed by Dunnett’s post hoc test when the experimental groups were only compared with the control group (protease biosensor experiments) to avoid com-
parison with an unrepresentative intergroup mean, whereas Šidák’s post hoc test was used when experimental groups were compared with both vehicle control and FL-CCN2-M and for kinase biosensor validation experiments in which more than one pharmacological inhibitor was utilized. Statistical signifi-
cance (p < 0.05) for performed tests is indicated in the figures.

Data availability

All source data used to generate graphs in this report are available from the corresponding author upon request.

Author contributions—O. J. K. and H. A. conceptualization; O. J. K., A. K. G., J.-H. W., V. T. M., and E. M. V. H. data curation; O. J. K., A. K. G., J.-H. W., and E. M. V. H. formal analysis; O. J. K., M.-Q. D., and H. A. supervision; O. J. K., A. K. G., J.-H. W., V. T. M., E. M. V. H., and M.-Q. D. investigation; O. J. K., A. K. G., J.-H. W., V. T. M., and M.-Q. D. visualization; O. J. K., A. K. G., J.-H. W., V. T. M., E. M. V. H., M.-Q. D., and H. A. methodology; O. J. K. and H. A. writing-original draft; O. J. K. and H. A. project administra-
tion; O. J. K., A. K. G., J.-H. W., V. T. M., E. M. V. H., M.-Q. D., and H. A. writing-review and editing; H. A. resources; H. A. funding acquisition.

Acknowledgment—We thank Dr. Lester F. Lau, University of Illinois at Chicago, for advice and for critically reading the manuscript.

References

1. Jun, J.-I., and Lau, L. F. (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat. Rev. Drug Discov. 10, 945–963 CrossRef Medline

2. Schafer, S., Viswanathan, S., Widiyaj, A. A., Lim, W. W., Moreno-Moral, A., DeLaugher, D. M., Ng, B., Patone, G., Chew, K., Khin, E., Tan, J., Chothani, S. P., Ye, L., Rackham, O. J. L., Ko, N. S. J., et al. (2017) IL-11 is a crucial determinant of cardiovascular fibrosis. Nature 552, 110–115 CrossRef Medline

3. Nishida, Y., Yoshioka, M., and St-Amand, J. (2005) The top 10 most abundan
t transcripts are sufficient to characterize the organs functional spec-
cificity: evidences from the cortex, hypothalamus and pituitary gland. Gene 344, 133–141 CrossRef Medline

4. Rachfal, A. W., and Brigstock, D. R. (2005) Structural and functional prop-
eties of CCN proteins. Vitam. Horm. 70, 69–103 CrossRef Medline

5. Leask, A., and Abraham, D. J. (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J. Cell Sci. 119, 4803–4810 CrossRef Medline

6. Murphy-Ullrich, J. E., and Sage, E. H. (2014) Revisiting the matricellular concept. Matrix Biol. 37, 1–14 CrossRef Medline

7. Lau, L. F. (2016) Cell surface receptors for CCN proteins. J. Cell Commun. Signal. 10, 121–127 CrossRef Medline

8. Tong, Z., Chen, R., Alt, D. S., Kemper, S., Perbal, B., and Brigstock, D. R. (2009) Susceptibility to liver fibrosis in mice expressing a connective tissue growth factor transgene in hepatocytes. Hepatology 50, 939–947 CrossRef Medline

9. Ahmed, M. S., Gravning, J., Martinov, V. N., von Lueder, T. G., Edvardsen, T., Czibik, G., Moe, I. T., Vinge, L. E., Øie, E., Valen, G., and Attamahad, H. (2011) Mechanisms of novel cardioprotective functions of CCN2/CTGF in myocardial ischemia-reperfusion injury. Am. J. Physical Heart Circ. Physiol. 300, H1291–H1302 CrossRef Medline

10. Neesse, A., Frese, K. K., Bapiro, T. E., Nakagawa, T., Sternlicht, M. D., Seeley, T. W., Pilarsky, C., Jodrell, D. I., Spong, S. M., and Tuveson, D. A. (2013) CTGF antagonism with mAb FG-3019 enhances chemotherapy response without increasing drug delivery in murine ductal pancreas cancer. Proc. Natl. Acad. Sci. U.S.A. 110, 12325–12330 CrossRef Medline

11. Ball, D. K., Surveyor, G. A., Diehl, J. R., Steffen, C. L., Urumcu, M., Mirando, M. A., and Brigstock, D. R. (1998) Characterization of 16- to 20-
kilodalton (kDa) connective tissue growth factors (CTGFs) and demon-
stration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. Biol. Reprod. 59, 828–835 CrossRef Medline

12. Robinson, P. M., Smith, T. S., Patel, D., Dave, M., Lewin, A. S., Pi, L., Scott, E. W., Tuli, S. S., and Schultz, G. S. (2012) Proteolytic processing of con-
nective tissue growth factor in normal occular tissues and during corneal wound healing. Invest. Ophthalmol. Vis. Sci. 53, 8093–8103 CrossRef Medline

13. Heng, E. C., Huang, Y., Black, S. A., Jr., and Trackman, P. C. (2006) CCN2, connective tissue growth factor, stimulates collagen deposition by gingival fibroblasts via module 3 and α6β1 integrins. J. Cell. Biochem. 98, 409–420 CrossRef Medline

14. Ball, D. K., Moussad, E. E., Rageh, M. A., Kemper, S. A., and Brigstock, D. R. (2003) Establishment of a recombinant expression system for connective tissue growth factor (CTGF) that models CTGF processing in utero. Reproduction 125, 271–284 CrossRef Medline

15. Ball, D. K., Rachfal, A. W., Kemper, S. A., and Brigstock, D. R. (2003) The heparin-binding 10 kDa fragment of connective tissue growth factor (CTGF) containing module 4 alone stimulates cell adhesion. J. Endocrinol. 176, R1–7 CrossRef Medline

16. Steffen, C. L., Ball-Mirth, D. K., Harding, P. A., Bhattacharyya, N., Pillai, S., and Brigstock, D. R. (1998) Characterization of cell-associated and soluble forms of connective tissue growth factor (CTGF) produced by fibroblast cells in vitro. Growth Factors 15, 199–213 CrossRef Medline

17. Brigstock, D. R., Steffen, C. L., Kim, G. Y., Vegurta, R. K., Diehl, J. R., and Harding, P. A. (1997) Purification and characterization of novel heparin binding growth factors in uterine sero
cytes fluids. Identification as hepa-
rin-regulated M, 10,000 forms of connective tissue growth factor. J. Biol. Chem. 272, 20275–20282 CrossRef Medline

18. Lin, C. H., Yu, M. C., Tung, W. H., Chen, T. Y., Yu, C. C., Weng, C. M., Tsai, Y. J., Bai, K. J., Hong, C. Y., Chien, M. H., and Chen, B. C. (2013) Connective tissue growth factor induces collagen I expression in human lung fibroblasts through the Rac1/MLK3/NIK/AP-1 pathway. Biochem. Biophys. Acta 1833, 2823–2833 CrossRef Medline

19. Moe, I. T., Pham, T. A., Hagelin, E. M., Ahmed, M. S., and Attramadal, H. (2013) CCN2 exerts direct cytoprotective actions in adult cardiac myo-
cytes by activation of the PI3-kinase/Akt/GSK-3β signaling pathway. J. Cell Commun. Signal. 7, 31–47 CrossRef Medline

20. Johnson, B. G., Ren, S., Karaca, G., Gomez, I. G., Fligny, C., Smith, B., Ergun, A., Locke, G., Gao, B., Hayes, S., MacDonnell, S., and Duffield, J. S. (2013) Connective tissue growth factor domain 4 amplifies fibrotic kidney disease through activation of LDL receptor-related protein 6. J. Am. Soc. Nephrol. 24, 1769–1772 CrossRef Medline

21. Mokalled, M. H., Patra, C., Dickson, A. L., Endo, T., Stainer, D. Y., and Posp, K. D. (2016) Injury-induced ctfge directs gilad bridging and spinal cord regeneration in zebrafish. Science 354, 630–634 CrossRef Medline

22. Robertson, I. B., and Rifkin, D. B. (2013) Unchaining the beast; insights from structural and evolutionary studies on TGFβ secretion, sequestra-

17968 J. Biol. Chem. (2018) 293(46) 17953–17970

ASBMB
38. Chien, W., O’Kelly, J., Lu, D., Leiter, A., Sohn, J., Yin, D., Karlan, B.,
and connective tissue growth factor (CTGF/CCN2) in breast cancer cells is asso-
ciated with increased migration and angiogenesis. Int. J. Oncol. 38,
1741–1747 CrossRef Medline
39. Hishikawa, K., Oemar, B. S., Tanner, F. C., Nakaki, T., Lüschner, T. F., and
Fujii, T. (1999) Connective tissue growth factor induces apoptosis in hu-
man breast cancer cell line MCF-7. J. Biol. Chem. 274, 37461–37466
CrossRef Medline
40. Tomasek, J. J., Gabbiani, G., Hinz, B., Chapronnier, C., and Brown, R. A.
(2002) Myofibroblasts and mechano-regulation of connective tissue re-
moulding. Nat. Rev. Mol. Cell Biol. 3, 349–363 CrossRef Medline
41. Jun, J. I., and Lau, L. F. (2017) CCN2 induces cellular senescence in fibro-
blasts. J. Cell Commun. Signal. 11, 15–23 CrossRef Medline
42. Nishida, T., Emura, K., Kubota, S., Lyons, K. M., and Takigawa, M. (2011)
CCN family 2/connective tissue growth factor (CCN2/CTGF) promotes
osteostastogenesiss via induction of and interaction with dendritic cell-
specific transmembrane protein (DC-STEMAP). J. Bone Miner. Res. 26,
351–363 CrossRef Medline
43. Zhu, X., Zhong, J., Zhao, Z., Shen, J., Wang, J., Liu, J., Cui, K., Chang, J.,
Zhao, H., and Wong, S. (2015) Epithelial derived CTGF promotes breast
 tumor progression via inducing EMT and collagen I fibers deposition.
Oncotarget 6, 25320–25338 CrossRef Medline
44. Pi, L., Jorgensen, M., Oh, S. H., Protopapadakis, Y., Gjymishka, A., Brown,
A., Robinson, P., Liu, C., Scott, E. W., Schultz, G. S., and Petersen, B. E.
(2015) A disintegrin and metalloproteinase with thrombospondin type 1
motif 7: a new protease for connective tissue growth factor in hepatic
progenitor/ovarian cell niche. Am. J. Pathol. 185, 1552–1563 CrossRef Medline
45. Guillon-Munos, A., Oikonomopoulou, K., Michel, N., Smith, C. R., Petit-
Courty, A., Canepa, S., Reverdiah, P., Heuzé-Vourc’h, N., Diamandis, E. P.,
and Courty, Y. (2011) Kallikrein-related peptide 12 hydrolyzes matricel-
lar proteins of the CCN family and modifies interactions of CCN1 and
CCN5 with growth factors. J. Biol. Chem. 286, 25505–25518 CrossRef Medline
46. Mochizuki, S., Tanaka, R., Shimoda, M., Onuma, J., Fujii, Y., Jinno, H., and
Okada, Y. (2010) Connective tissue growth factor is a substrate of
ADAM28. Biochem. Biophys. Res. Commun. 402, 651–657 CrossRef Medline
47. Dean, R. A., Butler, G. S., Hamma-Kourbali, Y., Delbó, J., Briggstock, D. R.,
Courty, J., and Overall, C. M. (2007) Identification of candidate angiogenic
inhibitors processed by matrix metalloproteinase 2 (MMP-2) in cell-based
proteomic screens: disruption of vascular endothelial growth factor (VEGF/heparin
affin regulatory peptide (pleiotrophin) and VEGF/Connective
tissue growth factor angiogenic inhibitor complexes by MMP-2
proteolysis. Mol. Cell. Biol. 27, 8454–8465 CrossRef Medline
48. Butler, G. S., Connor, A. R., Soumi, N. E., Eckhard, U., Morrison, C. J.,
Noël, A., and Overall, C. M. (2017) Degradomic and yeast 2-hybrid inac-
active catalytic domain substrate trapping identifies new membrane-type 1
matrix metalloproteinase (MMP14) substrates: CCN3 (Nov) and CCN5
(WISP2). Matrix Biol. 59, 23–38 CrossRef Medline
49. Tam, E. M., Morrison, C. J., Wu, Y. I., Stack, M. S., and Overall, C. M.
(2004) Membrane protease proteomics: isoitate-coded affinity tag MS
identification of undescribed MT1-matrix metalloproteinase substrates.
Proc. Natl. Acad. Sci. U.S.A. 101, 6917–6922 CrossRef Medline
50. Canfield, S. M., and Morrison, S. L. (1991) The binding affinity of human
IgG for its high affinity Fc receptor is determined by multiple amino acids
in the CH2 domain and is modulated by the hinge region. J. Exp. Med.
173, 1483–1491 CrossRef Medline
51. Glaesner, W., VICK, A. M., MIllican, R., Ellis, B., Tschang, S. H., Tian,
Y., Bokvist, K., Brenner, M., Koester, A., Porksen, N., Etgen, G., and Bomul, T.
(2010) Engineering and characterization of the long-acting glucagon-like
peptide-1 analogue LY2189265, an Fc fusion protein. Diabetes Metab.
Res. Rev. 26, 287–296 CrossRef Medline
52. Hecht, R., Li, Y. S., Sun, J., Belouski, E., Hall, M., Hager, T., Yie, J., Wang,
W., Winters, D., Smith, S., Spahr, C., Tam, L. T., Shen, Z., Stanislaus, S.,
Chinookoswong, N., et al. (2012) Rationale-based engineering of a potent
long-acting FGF21 analog for the treatment of type 2 diabetes. PLoS One
7, e49345 CrossRef Medline
53. Weng, Y., Ishino, T., Sievers, A., Talukdar, S., Chabot, J. R., Tam, A., Duan,
W., Kerns, K., Sousa, E., He, T., Logan, A., Lee, D., Li, Z., Zhou, Y., Ber-
CTGF, a matricellular preproprotein

Nardó, B., et al. (2018) Glyco-engineered long acting FGF21 variant with optimal pharmaceutical and pharmacokinetic properties to enable weekly to twice monthly subcutaneous dosing. Sci. Rep. 8, 4241 CrossRef Medline

Almendral, I. M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. 8, 2140–2148 CrossRef Medline

Cunningham, B. C., Mulikin, K., Mullen, J. E., and Wells, J. A. (1999) Fisp12/mouse connective tissue growth factor regulates fibroblast proliferation and myofibroblast differentiation. FASEB J. 13, 729–738 CrossRef Medline

Yang, Z., Sun, Z., Liu, H., Ren, Y., Shao, D., Zhang, W., Lin, J., Wolfram, J., Wang, F., and Nie, S. (2015) Connective tissue growth factor stimulates the proliferation, migration and differentiation of lung fibroblasts during paracutaneous injury. J. Biol. Chem. 290, 1091–1097 CrossRef Medline

Roopenian, D. C., and Akilesh, S. (2007) FcRn: the neonatal Fe receptor comes of age. Nat. Rev. Immunol. 7, 715–725 CrossRef Medline

Schütze, N., Kunzi-Rapp, K., Wagemanns, R., Nöth, U., Jatzke, S., and Jakob, F. (2005) Expression, purification, and functional testing of recombinant CYR61/CCN1. Protein Expr. Purif. 42, 219–225 CrossRef Medline

Schütze, N., Schenk, R., Fiedler, J., Mattes, T., Jakob, F., and Brenner, R. E. (2007) CYR61/CCN1 and WISP3/CCN6 are chemoattractant ligands for human multipotent mesenchymal stroma cells. BMC Cell Biol. 8, 45 CrossRef Medline

Su, B. Y., Cai, W. Q., Zhang, C. G., Martinez, V., Lombet, A., and Perbal, B. (2001) The expression of ccn3 (Nov) RNA and protein in the rat central nervous system is developmentally regulated. Mol. Pathol. 54, 184–191 CrossRef Medline

Kysurkchiev, S., Yeger, H., Bleau, A. M., and Perbal, B. (2004) Potential cellular conformations of the CCN3 (NOV) protein. Cell. Commun. Signal. 2, 9 CrossRef Medline

Heath, E., Tahir, D., Andermarcher, E., Schofield, P., Fleming, S., and Boulter, C. A. (2008) Abnormal skeletal and cardiac development, cardiomyopathy, muscle atrophy and cataracts in mice with a targeted disruption of the Nov (CCN3) gene. BMC Dev. Biol. 8, 18 CrossRef Medline

Matsushita, Y., Sakamoto, K., Tamamura, Y., Shibata, Y., Minamizato, T., Kihara, T., Ito, M., Katsube, K., Hiraoka, S., Koseki, H., Harada, K., and Yamaguchi, A. (2013) CCN3 protein participates in bone regeneration as an inhibitory factor. J. Biol. Chem. 288, 19973–19985 CrossRef Medline

Canalis, E., Smerdel-Ramoya, A., Durant, D., Economides, A. N., Beamer, W. G., and Zanotti, S. (2010) Nephroblastoma overexpressed (Nov) inactivation sensitizes osteoblasts to bone morphogenetic protein-2, but Nov is dispensable for skeletal homeostasis. Endocrinology 151, 221–233 CrossRef Medline

Holbourn, K. P., Malfois, M., and Acharya, K. R. (2011) First structural glimpse of CCN3 and CCN5 multifunctional signaling regulators elucidated by small angle x-ray scattering. J. Biol. Chem. 286, 22243–22249 CrossRef Medline

Brener, M. C., Krzyzanski, W., Chou, J. Z., Signore, P. E., Fung, C. K., Guzman, D., Li, D., Zhang, W., Olsen, D. R., Nguyen, V. T., Koo, C. W., Sternlicht, M. D., and Lipson, K. E. (2006) FG-3019, a human monoclonal antibody recognizing connective tissue growth factor, is subject to target-mediated drug disposition. Pharm. Res. 23, 1833–1849 CrossRef Medline

Gao, E., Lei, Y. H., Shang, X., Huang, Z. M., Zuo, L., Boucher, M., Fan, Q., Chuprun, J. K., Ma, X. L., and Koch, W. J. (2010) A novel and efficient model of coronary artery ligation and myocardial infarction in the mouse. Circ. Res. 107, 1445–1453 CrossRef Medline

Galbán, S., Jó, E. H., Bowman, B. M., Stevenson, J., Sebott, K. A., Sharkey, L. M., Laferty, M., Hoff, B. A., Butler, B. L., Wignall, S. S., Binkowski, B. F., Otto, P., Zimmerman, K., Vidugiris, G., Encell, L. P., et al. (2013) Imaging proteolytic activity in live cells and animal models. PLoS One 8, e66248 CrossRef Medline