CCN3 (NOV) Is a Negative Regulator of CCN2 (CTGF) and a Novel Endogenous Inhibitor of the Fibrotic Pathway in an *in Vitro* Model of Renal Disease

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Fibrosis is a major cause of end-stage renal disease, and although initiation factors have been elucidated, uncertainty concerning the downstream pathways has hampered the development of anti-fibrotic therapies. CCN2 (CTGF) functions downstream of transforming growth factor (TGF)- β , driving increased extracellular matrix (ECM) accumulation and fibrosis. We examined the possibility that CCN3 (NOV), another CCN family member with reported biological activities that differ from CCN2, might act as an endogenous negative regulator of ECM and fibrosis. We show that cultured rat mesangial cells express CCN3 mRNA and protein, and that TGF- β treatment reduced CCN3 expression levels while increasing CCN2 and collagen type I activities. Conversely, either the addition of CCN3 or CCN3 overexpression produced a marked down-regulation of CCN2 followed by virtual blockade of both collagen type I transcription and its accumulation. This finding occurred in both growth-arrested and CCN3-transfected cells under normal growth conditions after TGF- β treatment. These effects were not attributable to altered cellular proliferation as determined by cell cycle analysis, nor were they attributable to interference of Smad signaling as shown by analysis of phosphorylated Smad3 lev-

els. In conclusion, both CCN2 and CCN3 appear to act in a yin/yang manner to regulate ECM metabolism. CCN3, acting downstream of TGF- β to block CCN2 and the up-regulation of ECM, may therefore serve to naturally limit fibrosis *in vivo* and provide opportunities for novel, endogenous-based therapeutic treatments. (Am J Pathol 2009, 174:1725–1734; DOI: 10.2353/ajpath.2009.080241)

Renal fibrosis is a common endpoint of chronic injury in the kidney, is central in diabetic renal disease, and thus is critical to the current increase in end-stage renal disease. Although angiotensin II inhibitor therapy is being used to successfully slow progression in many patients, there remains a need for more effective treatments capable of blocking or reversing progression. Recognition of multiple initiating factors in renal fibrosis has led to a search for novel, and common, downstream targets.

A molecule recently identified as playing a critical role in fibrosis development is CCN2, originally termed connective tissue growth factor (CTGF). It is a member of a now recognized CCN family of six cysteine-rich proteins demonstrating similarities in their multimodular structure, but differences in function.^{1,2} Three of the four constitutive modules present in all members show partial identity with insulin-like growth factor binding proteins, von Willebrand factor, and thrombospondin 1. The C-terminal module contains a cysteine knot motif that is important in the heterodimerization of several growth factors and matrix proteins.^{3,4}

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CCN2 is produced by kidney mesangial cells (MCs), a cell type critical in the formation of glomerulosclerosis.⁵ We and others have shown that up-regulation of CCN2 in MCs occurs in response to factors known to be responsible for driving fibrosis in renal disease, including a high glucose environment, hypertensive force, and elevated transforming growth factor (TGF)- β ,⁵ the latter being a well-established profibrotic cytokine in fibrosis.^{6,7} A number of factors, including mechanical strain, appear to stimulate CCN2 expression without the up-regulation of TGF- β , supporting the idea that CCN2 may provide a more downstream and essential target for regulation of matrix metabolism in fibrosis.^{5,8} This idea is supported by recent data in several animal models of renal fibrosis, including that induced by diabetes, whereby CCN2 inhibition by antisense oligonucleotides (AS-ODN) is able to block progression.^{9–11}

The biological process of fibrosis initiation is not unlike normal wound healing. However in fibrosis, perhaps because of the chronic insult, there is an inability to terminate the cytokine up-regulation that drives the remodeling of the extracellular matrix (ECM). We hypothesized that an endogenous regulatory molecule(s) exists that may be active in shutting down this process in normal wound healing, ie, after new tissue and matrix are generated. If identified, such a factor might be used therapeutically to prevent or reverse the progression of fibrosis. Toward this end, we suspected that one possibility for negative regulation of CCN2 might be CCN3 [formerly known as nephroblastoma overexpressed gene (NOV)]. CCN3 has not been previously studied as an element of the fibrotic pathway, or as a CCN2 regulatory molecule. Whereas CCN2 has often been associated with proliferative disease, CCN3 has anti-proliferative effects in a number of cell systems, and likely in disease.^{12,13} We also noted that cell types expressing high levels of CCN2 generally expressed low levels of CCN3 (Li CL, Perbal B, and Riser B, unpublished observations). This study was therefore conducted to test the hypothesis.

Materials and Methods

Reagents

RPMT from Invitrogen (Grand Island, NY) and fetal bovine serum (FBS) from Gemini (Woodland, CA) were used for growth medium. TGF- β 1 was from R&D Systems (Minneapolis, MN). Purified rat collagen type I (COLI) was from Upstate Biotechnology (Lake Placid, NY) and polyclonal anti-rat COLI from Chemicon International (Temecula, CA). The production of full-length recombinant human CCN2 protein in a baculovirus expression system has been described previously.⁵ Recombinant human and mouse CCN3 (rhCCN3 and rmCCN3) were from R&D Systems and were generated from a DNA sequence encoding a mature CCN3 protein expressed in a mouse myeloma cell line. Anti-CCN3 antibodies included a rabbit polyclonal produced by us¹⁴ and monoclonals from R&D Systems.

Cell Culture

The MCs used were from a cloned line (16KC₂) derived from Fischer rat glomeruli as previously described.¹⁵ MCs were long term cultured in RPMI 1640 medium containing antibiotics and 5 mmol/L glucose, plus 10% FBS. For many experiments, cells were seeded in normal growth medium in 24-well tissue culture plates, and grown for 3 days. On the day 4, cells were washed with RPMI containing 1% FBS (RPMI-1), then incubated in fresh RPMI-1. Twenty-four hours later, the cells were washed and exposed to RPMI-1 with added cytokines, as indicated in the figures. After 48 hours, all cells were again washed and incubated in RPMI-1 plus cytokines for 1 or 2 additional days before harvest. In all cases, heparin (50 μ g/ml) was added to all wells before harvest to induce the release of cell- or matrix-bound CCN2, as previously described.⁵ For determination of cellular proliferation and standardization of enzyme-linked immunosorbent assay (ELISA) data, results were expressed per cell, based on the amount of DNA determined using the CyQuant cell proliferation assay kit (catalog no. C-7026; Molecular Probes, Eugene, OR). By running samples against a standard curve generated using increasing numbers of MCs, the cell number in each test well could be determined.

Generation of a Stable Cell Line Overexpressing Human CCN3

To generate a stable cell line expressing hCCN3, MCs were seeded into culture plates and grown for 24 hours. The transfecting mixture containing 20 μ g of CCN3 expression vector PCB6+¹⁶ was incubated with 15 μ l of transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, CA), in 1 ml of serum-free RPMI for 30 minutes at room temperature. This preparation was then added to cells with fresh medium and incubated. The medium was replaced the next day and, after two additional days, cells were replated with 1 mg/ml of G418 antibiotic, to select for stable antibiotic-resistant colonies. These G418-resistant cell lines were then analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using human-specific CCN3 primers to assess expression of hCCN3.

Transfection of the *col1* Promoter Construct and Promoter Analysis by Luciferase Measurement

Cells were seeded on 24-well plates and transfected 18 hours later with COL1a2 constructs kindly provided by Dr. William Schnaper, Northwestern University, Evanston, IL.¹⁷ Renilla-luciferase pRL-SV40 was used as a control to normalize for transfection efficiency. Transfection was performed with the Invitrogen reagent Lipofectamine 2000. Briefly, 0.8 μ g of collagen promoter constructs and 0.01 μ g of pRL-SV40 control constructs were mixed with 1 μ l of Lipofectamine 2000 in 100 μ l of serum-free me-

Table 1. Sequences of Primers Used in PCR Amplification

Gene	Strand	PCR primer sequence
hCCN3	Sense	5'-ATGCAGAGTGTGCAGAGCAC-3'
	Anti-sense	5'-TTACATTTTCCCTCTGGTAGTC TTCA-3'
rCCN3	Sense	5'-TCTGTGGGATCTGCAGTGAC-3'
	Anti-sense	5'-ATTGTTCTGAGGGCAGTTGG-3'
rCCN2	Sense	5'-AAGGGTCTCTTCTGCGACTT-3'
	Anti-sense	5'-ATTTGCAACTGCTTTGGAAG-3'
rCOL1	Sense	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Anti-sense	5'-AAGGTGCTGGGTAGGGAAGT-3'
r18s rRNA	Sense	5'-GACCATAAACGATGCCGACT-3'
	Anti-sense	5'-AGACAAATCGCTCCACCAAC-3'

dium. The mixtures were incubated for 30 minutes at room temperature and added to the cells with 1 ml of fresh medium. After 18 hours the medium was replaced with one containing 2% FBS, and cells were incubated for an additional 18 hours. Either TGF- β (2 ng/ml) or control vehicle was added to the cells. In some experiments, the transfected cells were pretreated for 4 hours with 0 to 300 ng/ml CCN3 before adding TGF- β . Then, 24 hours later, the cells were washed with PBS and extracts were prepared using 150 μ l of reporter lysis buffer (Promega Inc., Madison, WI). Luciferase activity of the promoter construct and the internal control were measured by adding 15 μ l of extract with 50 μ l of luciferase substrate and 50 μ l of stop-and-go reagent. The luciferase activity determined in the assay was normalized using the transfection efficiency. The experiments were performed with triplicate samples.

RNA Extraction and RT-PCR

RNA extraction was performed using Trizol reagent under methods provided by Invitrogen.¹⁸ Synthesis of cDNA was performed using random hexamers and Moloney murine leukemia virus reverse transcriptase at 42°C for 1 hour starting with 5 μ g of total RNA. Two μ l of cDNA was then used for PCR. The sequences of primers used in PCR amplification are shown in Table 1. PCR analyses were done using a thermocycler (Applied Biosystems, Foster, CA). Electrophoresis of the amplification products was in 1 to 2% agarose gels. Bands were visualized by ethidium bromide staining, and intensities determined by densitometer scanning with subsequent analysis using the NIH Image program (National Institutes of Health, Bethesda, MD).

ELISA

An ELISA was used to quantify levels of cytokines and collagen. An indirect ELISA was used for CCN2 protein measurements for the conditioned media, as we have previously described.⁵ For CCN3, a direct ELISA was used for tissue-culture samples from rat cells, and an indirect ELISA was used for rat cells transfected to express hCCN3. In brief, for the direct ELISA, the samples and recombinant standards (diluted in the same medium as the samples) were incubated at room temperature to

allow binding to the 96-well plate. The unbound sites were then blocked with 1% bovine serum albumin (BSA) + 0.05% Tween 20. After washing, a primary antibody was added (MAB1640, R&D Systems) or K19-immunized rabbit serum.¹⁴ For the indirect ELISA, the plate was first coated with MAB1640, then blocked, then incubated with sample and standard before giving K19-immunized rabbit serum. After further washing, horseradish peroxidase-conjugated secondary antibody (anti-rabbit 111-035-003 or anti-mouse 115-035-003; Jackson ImmunoResearch, West Grove, PA) was added, followed by more washing and horseradish peroxidase substrate (Enhanced K- β Blue TMB substrate, 308175; Neogen Corp., Lexington, KY). The color intensity was allowed to develop before being read at 650 nm using a microplate reader (Thermo Max; Molecular Devices Corp., Sunnydale, CA).

The measurement of COL1 was done by direct ELISA, as previously described¹⁹ and similar to the method described here for CCN3, except that incubations were performed at 4°C to minimize aggregation of the molecule, and the samples and standards were incubated in the plate overnight. The blocking solution and secondary antibody dilution buffer contained 5% nonfat dry milk + 0.05% Tween 20. The BSA blocking solution above was used to dilute the primary antibody (AB755P, Chemicon). The substrate color was developed at room temperature, and read as above.

Western Blotting

Biological samples were prepared by mixing 1 vol of a sample with 1 vol of the loading buffer with 10% 2-mercaptoethanol and 2% sodium dodecyl sulfate. Twenty μ l of each prepared sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 15% Tris-HCl gradient gel (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) + 0.1% Tween 20 for 1 hour at room temperature and then incubated with monoclonal anti-CCN3 antibody (1:500 dilution, R&D Systems). For phospho-Smad3 activity a phospho-Smad3-specific rabbit monoclonal antibody (Cell Signaling, Danvers, MA) was used. A horseradish peroxidase-conjugated secondary antibody (1:15,000 dilution; Amersham, Piscataway, NJ) and horseradish substrates (Pierce, Rockford, IL) were used to label the bands, which were enhanced with the chemiluminescence system (Pierce) and were developed using Amersham X-ray film.

Cell Cycle Analysis

A single cell suspension was prepared from the cell cultures and fixed in ice-cold 70% ethanol for 2 hours. Cells were then washed with PBS and incubated in PBS containing 0.1% Triton X-100, 0.2 mg/ml RNase A, and 5 μ g/ml propidium iodide for 30 minutes at the room temperature. Fluorescence-activated cell sorting analysis was conducted using a Becton Dickinson (Mountain

View, CA) LSRII system using PC-based FACSDiva acquisition and analysis software with ModFit program for determining cell cycle.

Immunohistochemistry

MCs grown on chamber slides were fixed in methanol. Immunoperoxidase labeling was performed at ambient temperature in a humidified chamber. Endogenous peroxidase was first blocked in 0.3% hydrogen peroxide/methanol. Cells were then rinsed in TBS and nonspecific binding was blocked in 1% BSA/TBS. Primary antibodies were anti-CCN3 (K19, 1:250 dilution in 1% BSA/TBS), anti-CCN2 (polyclonal, affinity-purified rabbit antibody recognizing the COOH-terminal portion of CCN2), and anti-rat polyclonal COL1 (all described above) were applied, then cells washed 5× in TBS/0.025% Brij35. Secondary antibody conjugate (1:10 in BSA/TBS; horseradish peroxidase polymer conjugate, broad spectrum; Zymed Laboratories, San Francisco, CA) was applied, and then washed in TBS/Brij and 1 in TBS only. Stable diaminobenzidine (Invitrogen, Burlington, Canada) was applied while monitoring color development. Cells were counterstained in hematoxylin and dehydrated before mounting.

Statistics

The procedure MIXED (SAS software; SAS Institute, Cary, NC) was used to perform specific analyses. Differences of least squares means estimates were performed for the pairwise comparisons of groups. *P* values less than 0.05 were considered statistically significant. For analyses in which there were only two groups, a Student's *t*-test was performed and *P* values less than 0.05 were considered statistically significant. Analysis of variance was used with a Tukey test when appropriate.

Results

Exogenous CCN3 Treatment Attenuates TGF- β -Stimulated CCN2 Expression and Blocks COL1 Production

To first examine the potential effect of CCN3 on CCN2 and the resulting fibrosis, we used a previously reported *in vitro* model.²⁰ In this model cultured MCs are grown to near confluence, then the medium is replaced for 24 hours with one containing a low concentration (2%) of FBS to place the cells in a near growth-arrested state. Cells are then exposed for 24 to 96 hours to medium containing TGF- β 1 (2 ng/ml), or control medium only. This TGF- β treatment results in an increase in CCN2 mRNA and protein, and is followed some hours later by a concomitant increase in the baseline level of type I collagen (COL1) mRNA and in turn increased secreted COL1.^{20,21} TGF- β stimulation of collagen synthesis can be blocked by CCN2-specific antisense oligonucleotides (ODNs), demonstrating a critical role for CCN2 in this pathway *in*

vitro^{22,23} similar to that shown *in vivo* in models of renal fibrosis.^{9–11} To test the effects of CCN3 in the above *in vitro* model, we used two different preparations. The first was a conditioned medium from the NCI-H295R human cell line. This cell expresses biologically active CCN3 at high levels, as we have described.²⁴ The second preparation used was a purified recombinant mouse CCN3 (rmCCN3). Although recombinant proteins can lack the biological activity of the native molecule, this CCN3 has been shown to induce cellular adhesion of Balbc/3T3 cells, thus confirming the capability for, at least this biological function.²⁵

After a 96-hour total treatment period, there was little or no measurable constitutive CCN2 present, but a strong stimulation of CCN2 production followed the exposure to TGF- β , as expected (Figure 1A). Under these TGF- β -treated conditions, the addition of conditioned medium-CCN3 at increasing concentrations produced a clear dose-dependent reduction in CCN2 levels that began at the lowest concentration tested (0.5 ng/ml). The effect reached an approximate 50% inhibition at the highest CCN3 concentration (50 ng/ml) used (Figure 1A). A similar level of CCN2 blockade was observed when 5 ng/ml of the purified rmCCN3 was used. As the dose of this purified molecule was increased to 50 ng/ml, total blockade of CCN2 was achieved, and remained unchanged as the dose was further increased (500 ng/ml rmCCN3). A similar, but less potent, CCN3 dose-inhibitory response was seen after a 24-hour exposure to the same preparations (data not shown). Baseline secretion of COL1 protein was also increased in response to the same TGF- β exposure (Figure 1B). As was observed for CCN2, the conditioned medium-CCN3 produced a dose-dependent inhibition of collagen production. In this case, there was a total blockade of collagen at the highest concentration used (50 ng/ml). The purified rmCCN3 produced a similar inhibition, but this time required 500 ng/ml for a complete blockade (Figure 1B). A similar, but less potent, collagen-inhibitory dose response to CCN3 was seen after 24 hours of exposure, reaching an approximate 40% and 80% reduction with the conditioned medium-CCN3 and rmCCN3, respectively, at the highest concentrations tested (data not shown).

As a second method for examining the effects of CCN3 on collagen regulation, the human *col1* promoter linked to luciferase was expressed in this MC line using a transient transfection method. The cells were then exposed to either 1 or 100 ng/ml of rmCCN3 24 hours after transfection, and the luciferase activity was measured at three subsequent periods. Results showed little collagen gene activation at 24 hours after transfection and little or no effect of CCN3 at the same period (Figure 1C). However, *col1* activation increased greatly at 48 hours after transfection, and there was a dose-dependent inhibition of this strong promoter activity in response to the added CCN3, reaching ~50% inhibition at 100 ng/ml of rmCCN3. By 72 hours after transfection, as the cells became confluent, the level of collagen activation began to fall on its own (Figure 1C, control). Nevertheless, exogenous CCN3 treatment further reduced, in a dose-dependent manner, the *col1* promoter activity.

Figure 1. Exogenous CCN3 treatment down-regulates MC CCN2 and COL1 protein and COL1 promoter activity. Secretion of CCN2 (**A**) or COL1 (**B**) per cell in response to conditioned medium (cond)-CCN3 or purified rmCCN3 as quantified by ELISA. **P* < 0.05 versus TGF-β; ***P* < 0.05 versus control. Box shows the range, the dot the average, and the line the median of values. The amount of CCN3 in the conditioned medium from NCI-H295R cells was predetermined by ELISA. **C:** Activation of *COL1* promoter as measured by luciferase activity, in response to rhCCN3 at the times indicated after transfection. Each bar represents an individual treatment under the conditions indicated. The experiment was repeated with very similar results. **A:** The low baseline CCN2 secretion was markedly increased by TGF-β exposure and both unpCCN3 (cond. NCI) and rmNOV blocked CCN2 production in a dose-dependent manner. **B:** A similar effect was seen on COL1 levels. **C:** *COL1* promoter activity was the strongest 48 hours after transfection and was greatly inhibited by CCN3.

Last, immunohistochemical staining was used to examine the localization of CCN2 and COL1 in MCs, and the response to CCN3 treatment. Results showed that both CCN2 and COL1 protein in control unstimulated MCs were present and localized primarily to discrete peripheral areas, ie, near the cell membrane (Figure 2). After 48 hours of TGF-β exposure there was a loss of this discrete localization, and a new and more intense staining was now apparent and diffusely distributed throughout the cytoplasm, with the greatest concentration appearing paranuclear in distribution. Our previous results (Figure 1, A–C) support the idea that this represents new synthesis of both molecules. A 1-hour pretreatment of the cells with CCN3 virtually blocked this TGF-β-induced effect on both CCN2 and COL1 (Figure 2), thus supporting the previous results (Figure 1, A and B).

CCN3 Is Expressed in MCs and Is Down-Regulated by TGF-β

We reasoned that because exogenous CCN3 was able to block the stimulation of CCN2 and collagen by TGF-β, then if these MCs are capable of producing and secreting the molecule, paracrine activity might be important in shutting down the wound healing and/or fibrotic processes. In contrast, to up-regulate ECM metabolism there might be a requirement to decrease CCN3 activity. Indeed, PCR analysis demonstrated CCN3 expression in our unstimulated MCs in culture (Figure 3A). Whereas, exposure to TGF-β resulted in an increase in both CCN2 and COL1 mRNA expression as expected (Figure 3A), there was a clear reverse- or down-regulation of CCN3 expression (reduced band intensity) in response to

Figure 2. The inhibitory effects of CCN3 on TGF-β stimulation of CCN2 and COL1 localization in MCs are illustrated using immunocytochemistry. Immunocytochemical staining of CCN2 and COL1 was performed on cultured rat MCs, in the absence or presence of exogenously administered cytokines. Representative fields show that treatment with TGF-β alone (5 ng/ml) caused localization of CCN2 to change from a primarily peripheral localization (**A**) to a more homogenous pattern (**B**) throughout the cytoplasm. **C:** Treatment with both TGF-β (5 ng/ml) and rmCCN3 (500 ng/ml) prevented this change from occurring. Similarly, TGF-β alone (5 ng/ml) caused localization of COL1 to change from a primarily peripheral localization (**D**) to a more homogenous cytoplasmic distribution with concentration in the perinuclear area (**E**). **F:** As with CCN2, addition of both TGF-β (5 ng/ml) and rmCCN3 (500 ng/ml) prevented this change from occurring for collagen I.

Figure 3. Expression and negative regulation of CCN3 in MCs by TGF- β . **A:** RT-PCR results for the indicated mRNA species run from the same set of three MC cultures in the presence or absence of TGF- β 1 (2 ng/ml). Molecular weight markers are shown in the left lane. **B:** CCN3 protein secretion per cell in control and TGF- β -treated cultures after 96 hours. Shown are average $\pm$ SEM. * $P < 0.05$ versus control. Untreated MCs in culture gave low levels of CCN2 and COL1 mRNA and protein and high levels of CCN3 mRNA and protein. Exposure to TGF- β increased CCN2 and COL1 activity while simultaneously decreasing CCN3 activity. The experiment was repeated with similar results.

TGF- β , as the constitutive expression was markedly diminished (Figure 3A). Quantification by ELISA demonstrated that the low CCN2 and COL1 mRNA levels in non-TGF- β -stimulated cultures were associated with marked CCN3 protein production (Figure 3B). TGF- β exposure, while increasing CCN2 and COL1 transcript levels (Figure 3A), simultaneously and strongly down-regulated the level of secreted CCN3 protein (Figure 3B). Under the same conditions described above, immunostaining of untreated control MCs demonstrated a strong localization of diffuse, homogenously distributed CCN3 throughout the cytoplasm and nucleus, as well as areas of dense focal accumulations near the cell membrane (Figure 4C). After the exposure to TGF- β , CCN3 was greatly reduced in the areas of focal deposit at the cell membrane and in the nucleus, with little diffuse staining now present in the cytoplasm (Figure 4D). As observed previously, CCN2 in control and in TGF- β -exposed cultures exhibited a nearly opposite expression pattern (Figure 4, A and B).

Targeted Overexpression of the Human CCN3 Gene Down-Regulates CCN2 and Collagen I Activity in Rat MCs

Exposure of rat MCs to either an unpurified, native, or purified rhCCN3 protein resulted in reduced CCN2 and

Figure 4. The converse effects of TGF- β on CCN2 and CCN3 localization in MCs are illustrated using immunocytochemistry. Immunocytochemical staining of CCN2 or CCN3 was performed on cultured rat MCs, in the absence and presence of TGF- β (5 ng/ml). Representative fields show that CCN2 accumulates as dense deposits in the peripheral regions of the cells without TGF- β stimulation (A), but appears to redistribute throughout the cytoplasm in response to TGF- β (B). Conversely, CCN3 is homogenously distributed throughout the cytoplasm in the absence of TGF- β (C), but accumulates as dense deposits in the cell periphery when treated with TGF- β (D).

COL1 secretion, whereas the unstimulated cells produced low constitutive CCN3. Therefore, we sought to determine the effect of a selective up-regulation of cellular CCN3 expression. Accordingly, MCs were transfected with the human CCN3 gene *in vitro*, and a stable transfected cell line was generated with high expression of hCCN3 (Figure 5A). Because the primers used were selective for human CCN3, no mRNA signal was observed in the control (empty vector) transfected cells, but clear expression occurred in the hCCN3-transfected cells (Fig-

Figure 5. Targeted expression of hCCN3 reduces CCN2 and blocks COL1 activity in rat MCs. **A:** Results of RT-PCR on mRNA levels from rat MCs transfected with the human CCN3 gene versus control (empty vector) transfected cells. Results are from the same MC lines analyzed by ELISA for CCN3 (B), CCN2 (C), or COL1 (D) proteins produced. Human CCN3 transfection resulted in a high level of human CCN3 mRNA expression and secreted protein. This transfection of CCN3 significantly decreased CCN2 secretion and blocked COL1 production. Cell counts at the time of these measurements showed no significant affect of transfection on cell replication (not shown).

Figure 6. Comparison of exogenous versus endogenous CCN3 on COL1 promoter activity. Control, empty vector, transiently transfected MCs were exposed to mCCN3 at the indicated concentrations. hCCN3-transfected MCs received no added CCN3. COL1 activation was measured by luciferase production. * $P < 0.05$ compared with no added CCN3 (control-transfected). In the absence of TGF- β stimulation there was little activation of the COL1 promoter and no effect of CCN3 except a slight reduction in cells transfected with CCN3 (white bars). With TGF- β treatment (2 ng/ml, black bars) the COL1 promoter was strongly activated and was markedly reduced by either increased endogenous or exogenous CCN3.

ure 5A). This selective, high expression of hCCN3 resulted in a marked decrease in the level of *Col1* transcripts, as compared with the control transfected cells as determined in a semiquantitative RT-PCR assay (Figure 5A). Quantitative ELISA analysis of cell culture supernatants from these stably transfected rat MCs demonstrated that the marked, increased expression of the human CCN3 gene (Figure 5A) led to a high production and secretion of human CCN3 protein (Figure 5B). This marked level of hCCN3 activity produced a greater than 50% reduction in CCN2 secretion (Figure 5C) and a near total blockade of the COL1 produced (Figure 5D).

Because separate experiments indicated that either exogenously added CCN3 or targeted high expression of CCN3 were both able to reduce collagen activity, we compared the two in a single side-by-side experiment at equal cell numbers. The strong stimulation of the *Col1* promoter by TGF- β , was partially blocked by 30 ng/ml of added CCN3, and was further blocked as the concentration was increased to 300 ng/ml (Figure 6). Transfection with high expression of CCN3 reduced COL1 promoter activity equal to, or greater than, the reduction observed with the highest concentration of exogenously added CCN3. Only in cells overexpressing CCN3 was there an observed effect on constitutive promoter activity, but this did not reach statistical significance (Figure 6).

The Effects of CCN3 on CCN2 and COL1 Are Not Mediated Through Alteration in Cell Growth

One possibility for the observed effects of CCN3 on CCN2 and COL1 was through an alteration of cell growth. In the previous experiments, cell numbers were determined by measurement of total DNA at the time of assay and showed no significant effect of any treatment on replication (not shown). However, to address this further,

Figure 7. Transfection and overexpression of CCN3 does not affect cell cycling under the conditions tested. The number of cells for a given cell-cycle phase (labeled) is plotted in rat MCs that have either been transfected with an empty vector (A, control) or the gene for CCN3 (B, transfected) in representative experiments. Flow cytometric analysis of cellular DNA was conducted after 2 days of incubation with media containing 10% serum. Both plots are similar, indicating that transfection with the CCN3 gene causes little or no change in cell cycling. This is confirmed in C, in which the percentage of total cells is plotted for each cycle from three separate experiments. The profiles for control and CCN3-transfected cells at each cell-cycle phase are not significantly different, as determined by a group *t*-test.

a cell-cycle analysis was conducted using fluorescent-assisted cell sorting. In the first experiments, control, empty vector, and CCN3-transfected MCs were dispersed and grown under identical conditions in 10% FBS for 2 days before analysis. The two cell lines produced very similar plots (Figure 7, A and B), and a quantitative analysis of the percentage of total cells in each phase confirmed that there was no significant difference (Figure 7C). When cells were then cultured under the same low-serum conditions described in the experiments above, the cycle profile showed that cells were synchronized in predominately one phase, and were not affected by exposure to either TGF- β , CCN3, or a combination of both cytokines (Figure 8, A–E).

The Effect of CCN3 on Smad Signaling

Finally, we examined the possibility that the observed effects of CCN3 were mediated through a blockade of the effect of TGF- β . To test this, we examined phosphorylated Smad3 (pSmad3) signaling after TGF- β stimulation in both the presence and absence of elevated endogenous or exogenous CCN3. As expected, in control-transfected MCs, pSmad3 activity was low or absent, as evidenced by the absence of a pSmad3 band, but was strongly up-regulated immediately after TGF- β (Figure 9A). In the CCN3-transfected cells, expressing high levels of CCN3, there was little, or no, effect on this TGF- β stimulation of pSmad3 (Figure 9A). Quantitation of bands from three experiments confirmed that there was no significant effect of CCN3 on pSmad3 (control, 7372 ± 467

Figure 8. Treatment of MCs with exogenous CCN3 does not alter cell cycling. The number of cells for a given cell-cycle phase (labeled) is plotted in cultured rat MCs from representative experiments under varying conditions as follows: control untreated cells (**A**), cells treated exogenously with TGF-β1 (3 ng/ml) (**B**), cells given CCN3 (300 ng/ml) (**C**), cells exposed to both CCN3 (300 ng/ml) and TGF-β (3 ng/ml) (**D**). Cells were first cultured in a medium containing 10% serum for 2 days, then replaced with fresh medium containing 2% serum for 1 day, before adding the exogenous cytokines indicated above. Flow cytometric analysis of cellular DNA was performed after 2 additional days of culture with the cytokines. All of the plots are similar, indicating that treatment with TGF-β and/or CCN3 did not affect the cell cycle in these cells, under the experimental conditions. This is confirmed in **E**, in which the percentage of total cells at each cell-cycle phase is quantified from three separate experiments. The profiles for the G₀/G₁ and S phases are unchanged by the various treatments, as determined by analysis of variance, using a Tukey posthoc analysis.

density units versus transfected 5627 ± 1457 ; $P = 0.09$). Similarly, when normal MCs were exposed for 2 days to a high level of added CCN3 (300 ng/ml), there was no marked effect on pSmad3 activity (Figure 9B).

Discussion

We suspected that an endogenous molecule might exist that acts to down-regulate ECM metabolism in the late stages of normal wound closure or healing. However, in chronic injury in which the wound healing response often progresses to a fibrotic or scarring situation, this negative regulatory molecule or process might either not be activated, or is activated, but insufficient to overcome the chronic insult. Abundant data now points to CCN2 as a very downstream effector molecule driving increased ECM turnover that is necessary for both normal wound healing and also the production of a fibrotic lesion. Because CCN2 is often associated with proliferative diseases and CCN3 with anti-proliferation but not previously associated with fibrosis, we speculated that CCN3 might play a regulatory role in CCN2 activity.

In this study, we showed for the first time that when cells (here MCs) were exposed to exogenous CCN3 protein, there was indeed a dose-dependent attenuation, or

Figure 9. Phosphorylation of pSmad3 in cultured rat MCs is not altered by CCN3. Both CCN3 transfected (**A**) and exogenously treated (**B**) cells are shown. Cells were first cultured in a medium containing 10% FBS for 2 days, after which the medium was exchanged to one containing 2% serum for 1 day. Transfected cells were incubated for another 2 days before analysis. For the exogenously treated cells, recombinant CCN3 (300 ng/ml) was added to the plates indicated above, and both treated and untreated plates were incubated for an additional 2 days. Before harvesting the cells, the plates indicated above were treated with TGF-β (3 ng/ml), and all plates were incubated for another hour. Nuclear extracts were then prepared for immunoblotting. Results in **A** are duplicate samples from two different sets of cultures, and **B** is a single sample from combined cultures. The experiments were both repeated with very similar results. The data indicate that TGF-β increases phosphorylation of pSmad3 in rat MCs under the experimental conditions used, and that CCN3 does not affect its phosphorylation.

blockade of the TGF-β stimulated increase in CCN2 transcript and cell-associated and secreted protein levels. This has the potential then to be critically important, because TGF-β is a potent profibrotic element in the disease occurring in a variety of organ systems, including the kidney. The relevance of this CCN3 response was shown by the finding of a linked, and marked, reduction in the activation of the collagen promoter with a near blockade of the TGF-β-stimulated synthesis, secretion, and redistribution of intracellular COL1. This CCN2 and collagen inhibitory response was seen after exposure to either a CCN3-enriched conditioned medium, or a purified, recombinant hCCN3. In addition, the ability of the CCN3 protein to down-regulate CCN2 and ECM activity was confirmed by our additional studies in which the CCN3 gene was overexpressed in a stably transfected MC line. This constitutive overexpression of the single gene with subsequent production of endogenous CCN3 resulted in a potent reduction, and a near blockade, of TGF-β-stimulated CCN2 and collagen activity, respectively. As was the case with exogenous CCN3 treatment, this was shown to occur at both the mRNA and protein levels. The influence of CCN3 on CCN2 and collagen was reduced when cells were not stimulated by added TGF-β, but was nevertheless present as shown in transfected cells (see Figure 3).

The relevance of our findings above was shown in two further sets of experiments. First, it was demonstrated that these MCs, under normal culture conditions, produce and secrete CCN3. Immunostaining demonstrated CCN3 was present in a diffuse distribution throughout the cytoplasm and nucleus, with additional focal areas of dense deposit at the cell membrane. Under conditions in which there is a requirement, or action, to increase CCN2

and therefore ECM (here by TGF- β stimulation), we showed that there is simultaneous and marked reduction in cellular mRNA and secreted CCN3 protein, with a simultaneous loss of the diffuse and focal cytoplasmic accumulation. This then supports the idea that endogenous CCN3 activity plays a role in ECM metabolism in MCs, and that CCN2 and CCN3 work in opposition to regulate collagen promoter activation and the secretion of its protein. The finding of a negative effect of TGF- β on CCN3 expression is supported by previous findings with cultured murine osteoblasts and adrenal cortical cells.^{26,27} However, these studies did not examine the effect of CCN3 on ECM metabolism. Although we and others have reported CCN3 mRNA present in multiple organs, including the kidney, very little is known about cell-specific localization, regulation, and normal function of CCN3 in any organ, including that of the kidney.^{16,28} We have previously shown that during normal nephrogenesis, CCN3 protein is tightly associated with normal differentiation of glomerular podocytes and is expressed in Wilms' tumors.¹⁶ A number of reports demonstrate a link between cancer cell growth and CCN3 expression, most showing an inverse relationship.^{12,29,30} Additionally, a recent report has shown a role for CCN3 as a regulator of human hematopoietic stem or progenitor cell growth.³¹

Exactly how CCN3 acts to down-regulate CCN2 and collagen activity was not identified in our studies. Our dosing and fixed dose studies indicated a close relationship between CCN3-mediated lowering of CCN2 levels and the reduction in COL1, suggesting that this effect was mediated by a direct effect on CCN2 activity. However, in some cases, a less than complete reduction in CCN2 was associated with a near total blockade of COL1. This might be interpreted as at least a partial effect on COL1 independent of CCN2. Alternatively, because CCN2 changes typically precede those of COL1, then the time selected for collection of samples could explain such results. Interestingly, in a very recent report, CCN3 expression was shown to be inversely associated with glomerular cell proliferation and was negatively regulated by platelet-derived growth factor (PDGF)-BB.³² In proliferative diseases of the kidney, PDGF-BB is thought to play a stimulatory role in cell growth. This same study showed that glomerular cell proliferation is negatively correlated with CCN3 expression in necrotizing glomerulonephritis.³² The relationship between CCN3 expression and ECM accumulation or fibrosis was not studied, nor was the interaction with CCN2 determined. In comparison, we used an *in vitro* model of renal fibrosis that seeks to mimic the primarily nonproliferating conditions found in chronic kidney disease, eg, that associated with diabetes,³³ using low FBS concentrations during the experimental portion of culture. Because there is data to support a role for PDGF in fibrosis development in a number of models of diabetic renal fibrosis, and in patients with diabetic nephropathy,^{34–36} this molecule is likely to also contribute to the regulation of CCN3, and thus ECM production and fibrosis, albeit to a lower level than in highly proliferative disease. This remains to be tested.

We conducted a series of experiments to determine whether a CCN3-mediated suppression of cell replication

Figure 10. Hypothesis for the regulation, by CCN3, of CCN2 and collagen in fibrosis. **Solid arrows** show the current paradigm for up-regulation of ECM accumulation and fibrosis by TGF- β and CCN2. **Dashed arrows** show the pathway identified in the present work. **Red arrows** depict the effect (positive or negative) of the molecule on the further downstream elements. Currently unknown are factors that may induce endogenous CCN3 in renal disease or fibrosis.

might be responsible for the observed effect on CCN2 and COL1. Our analysis of total DNA in cultures at the time of harvest indicated that this was not the case. In a follow-up cell-cycle analysis, we demonstrated that under the conditions of low FBS used, cells were synchronized in a growth-arrested state and growth was not affected by treatment with TGF- β or CCN3. Even under conditions of 10% FCS, cells transfected to overexpress CCN3 had no effect on cell cycle or their response to TGF- β . This indicated that the observed effects of CCN3 were not the result of an alteration in cell growth.

Last, we investigated whether the observed effects of CCN3 might occur via direct blockade of TGF- β stimulation. To test this, we measured pSmad3 activity as an indicator of TGF- β signaling that is required for both CCN2 and COL1 up-regulation.³⁷ We showed that pSmad3 was strongly up-regulated after TGF- β exposure, but neither CCN3-transfection nor exposure to exogenous CCN3 significantly suppressed this response. This supports the idea that the observed effects of CCN3 on CCN2 and COL1 are not mediated through blocking TGF- β signaling.

In summary, the data support our novel hypothesis that in renal injury, including chronic kidney disease and diabetic nephropathy, a rapid response is the up-regulation of TGF- β , which in turn stimulates CCN2 and down-regulates CCN3 (Figure 10). This combination is responsible for the increased metabolism and remodeling of COL1 and other ECM proteins. With acute injury we speculate that as the wound is repaired, CCN3 is up-regulated by a currently unknown mechanism, and has the effect of shutting down CCN2 and subsequently the remodeling of ECM. With chronic injury, for example in diabetic renal fibrosis, CCN3 may be increased in later stage disease, but alone is insufficient (or not in sufficient quantity) to shut down the action of CCN2. Overall, these results suggest a novel target for therapeutic intervention in renal fibrosis, and likely that affecting a variety of other organs. Further, *in vivo* studies will now be necessary to confirm this hypothesis.

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