CCN3 is a novel endogenous PDGF-regulated inhibitor of glomerular cell proliferation

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CCN proteins affect cell proliferation, migration, attachment, and differentiation. We identified CCN3 as a suppressed gene following platelet-derived growth factor (PDGF)-BB or -DD stimulation in a cDNA-array analysis of mesangial cells. *In vitro* growth-arrested mesangial cells overexpressed and secreted CCN3, whereas the addition of the recombinant protein inhibited cell growth. Induction of mesangial cell proliferation by PDGF-BB or the specific PDGF β-receptor ligand PDGF-DD led to downregulation of CCN3 mRNA, confirming the array study. Specific PDGF α-receptor ligands had no effect. CCN3 protein was found in arterial smooth muscle cells, the medullary interstitium, and occasional podocytes in the healthy rat kidney. Glomerular CCN3 was low prior to mesangial proliferation but increased as glomerular cell proliferation subsided during mesangioproliferative glomerulonephritis (GN). Inhibition of PDGF-B in mesangioproliferative disease led to overexpression of glomerular CCN3 mRNA. CCN3 localized mostly to podocytes in human glomeruli, but this expression varied widely in different human glomerulonephritides. Glomerular cell proliferation negatively correlated with CCN3 expression in necrotizing GN. Our study identifies CCN3 as an endogenous inhibitor of mesangial cell growth and a modulator of PDGF-induced mitogenesis.

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Increased mesangial cell proliferation and matrix expansion characterize IgA nephropathy, most types of lupus nephritis, diabetic nephropathy, and other proliferative glomerular diseases. Among the most potent mitogens for mesangial cells are the platelet-derived growth factor (PDGF) isoforms PDGF-BB and -DD.[1–6] Both are overexpressed in glomeruli in various diseases. Inhibition of PDGF-BB or -DD in mesangioproliferative nephritis can prevent renal failure, glomerulosclerosis, and tubulointerstitial fibrosis.[1,4–6] Whereas the role of PDGF in glomerular cell proliferation is thus well established, nothing is known about modulators of PDGF bioactivity and the feedback mechanisms that might limit its actions.

In the course of a systematic search for genes that are downregulated by PDGF-BB or -DD in mesangial cells, we discovered *CCN3* as one of the most prominently suppressed genes. *CCN3*, also known as nephroblastoma overexpressed gene (*NOV*), belongs to the CCN protein family (Cyr61, CTGF, nephroblastoma overexpressed gene), which currently consists of six members: cystein-rich 61 (Cyr61/CCN1), connective tissue growth factor (CTGF, CCN2), CCN3, and Wnt-1-induced secreted proteins-1, -2, and -3 (CCN4, 5, 6). All CCN proteins are involved in the regulation of cell proliferation, migration, attachment, and differentiation.[7]

Little is known about CCN3 and the kidney. In the developing human kidney, the mesonephric and paramesonephric ducts as well as the stromal and other tubular cells expressed *CCN3* mRNA.[7,8] Very high CCN3 protein levels were observed in podocytes of meso- and metanephric glomeruli, whereas in the adult kidney, CCN3 is expressed at a low level only.[8]

Given the potent downregulation of *CCN3* by PDGFs (see above) and the overexpression of the PDGF-B and -D chain in a large number of glomerular diseases, we asked whether CCN3 represents a downstream target and modulator of PDGF activity in the glomerulus.

RESULTS

A cDNA array analysis identifies CCN3 as a potently suppressed gene in PDGF-stimulated HMC

Using the Affymetrix human genome chip U133A, which detects more than 18 400 transcripts derived from approximately 14 500 human genes, we found that the gene

most potently suppressed in PDGF-BB-stimulated human mesangial cells (HMC) was *CCN3* (reduction 61±9% in comparison to cells exposed to medium only; $n=3$). Similarly, PDGF-DD stimulation led to a 48±7% downregulation of *CCN3* mRNA. Downregulation of *CCN3* in both situations was verified by reverse transcriptase (RT)-PCR (data not shown).

Table 1 | Expression pattern of CCN3 in rat and human kidney

Location	Rat	Human
Glomerulus		
Endothelial cells	–	–
Mesangial cells	*De novo* in disease	–
Podocytes	++	++
Parietal epithelial cells	–	–
Circulating leukocytes, platelets	?	?
Arteries		
Smooth muscle cells	++	ND
Endothelial cells	–	ND
Tubules	++	ND
Interstitium		
Interstitial cells	++	ND
Macrophages	?	ND

ND, not determined; ++, positive staining; –, absent staining; ?, not specified.

Growth arrest stimulates CCN3 mRNA and protein expression in HMC and rat mesangial cells

We next asked whether CCN3 expression in HMC is affected by their proliferative status. Growth arrest of HMC was induced by incubation in medium complete with trace elements (MCDB) medium for 72 h (Figure 1a). MCDB medium is a serum-free medium, which permits HMC culture in the absence of growth factors or fetal calf serum (FCS).[9] The growth arrest was reversible since the cells could be re-stimulated by PDGF-BB to achieve similar DNA synthesis rates as cells kept in medium with FCS at all time points (Figure 1a). Growth-arrested HMC exhibited a time-dependent increase of *CCN3* mRNA levels relative to HMC growing in medium with FCS (Figure 1b). Full-length CCN3 protein could be detected in supernatants of growth-arrested, but not of growing, HMC (Figure 1c). Potential truncated

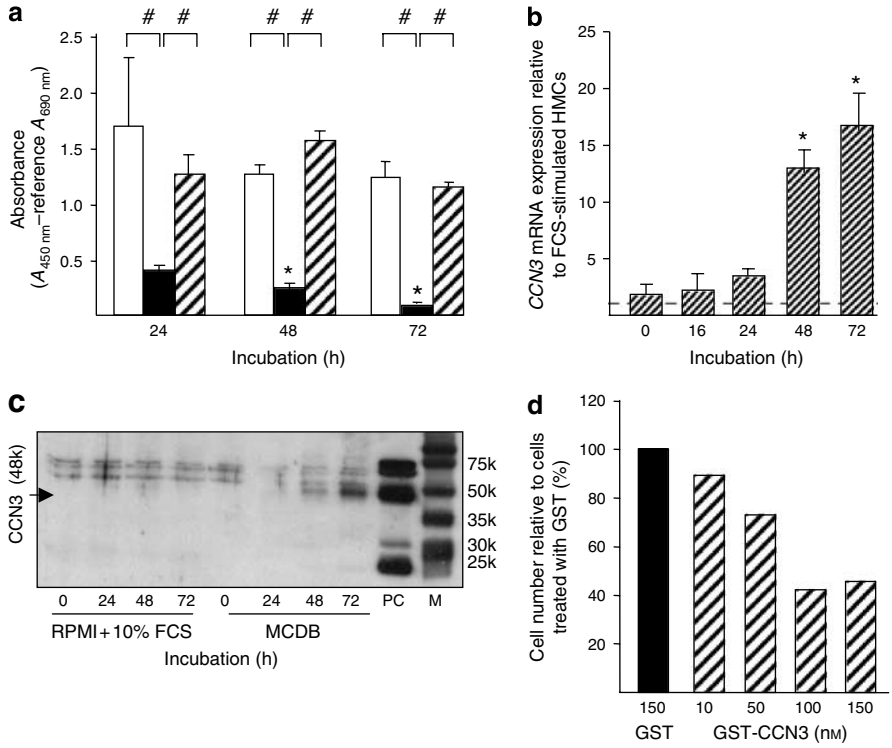

Figure 1 | CCN3 is involved in growth arrest of mesangial cells. (**a**) Proliferation of HMC cultivated in MCDB (filled bars) or RPMI media with 10% FCS (open bars). To demonstrate the reversibility of the growth arrest, the cells were stimulated with PDGF-BB (50 ng ml^{-1}) for 24 h after incubation with MCBD media (hatched bars). Cell proliferation was assessed by seeding HMC in 96-well plates (Nunc, Wiesbaden, Germany), growing them to subconfluence and then incubating them in the above media. Data are means±s.d. of four independent experiments. *$P<0.05$ versus cells treated with MCDB media for 24 h, #$P<0.05$. (**b**) *CCN3* mRNA, as detected by real-time RT-PCR and normalized to *glyceraldehyde-3-phosphate dehydrogenase* mRNA, following growth arrest in HMC. For this, HMC were switched from RPMI plus 10% FCS at 0 h to MCDB medium. Data are means±s.d. of three independent experiments. *$P<0.05$ versus 0 h. (**c**) Expression of CCN3 protein by western blotting in proliferating HMC (RPMI + 10% FCS) or following transfer into growth-arrest medium (MCDB). PC, positive control (supernatant from adrenocortical cell cultures, which are known to secrete CCN3); M, molecular weight markers. One representative experiment out of two is shown. (**d**) Proliferating HMC can be growth inhibited by adding recombinant CCN3 protein. A total of 10 000 cells were seeded on day 0 and cultivated in RPMI + 10% FCS. At day 1, GST-CCN3 (10, 50, 100, and 150 nM) or GST alone (150 nM) was added and the cells were incubated for a further 3 days. Cell number was counted in duplicate using a Malassez hemocytometer. One representative experiment out of two is shown.

CCN3 protein isoforms, which have been described to induce proliferation in other cell types,[10–12] were not observed. Full-length or truncated CCN3 was not detectable in cell lysates at any time point (data not shown), indicating that the CCN3 protein was completely secreted.

Similar to the above observations in HMC, *CCN3* mRNA overexpression was also noted in rat mesangial cells after cultivation in MCDB medium for 16–48 h, but not in RPMI medium containing 10% FCS (data not shown).

To assess the functional role of extracellular CCN3 in HMC, we incubated HMC, proliferating in RPMI medium with 10% FCS, with either recombinant glutathion-*S*-transferase (GST)-tagged CCN3 or GST alone. The GST-tag did not influence the activity of recombinant CCN3 as described previously.[13] After 3 days, numbers of HMC dose-dependently decreased following incubation with GST-CCN3 in comparison to cells growing in the presence of GST control protein (Figure 1d).

Ligands of the PDGF β-receptor, but not of the α-receptor, downregulate CCN3 mRNA expression

PDGF-BB and the specific β-receptor ligand PDGF-DD are potent mitogens for mesangial cells, whereas ligands of the PDGF α-receptor, that is PDGF-AA and -CC, usually elicit lower-level and fewer biological effects in mesangial cells.[5,14,15] In agreement with these observations, stimulation of HMC with PDGF-BB or -DD resulted in a significant downregulation of *CCN3* mRNA expression after 24 h, whereas incubation with PDGF-AA or -CC did not influence the *CCN3* mRNA expression (Figure 2).

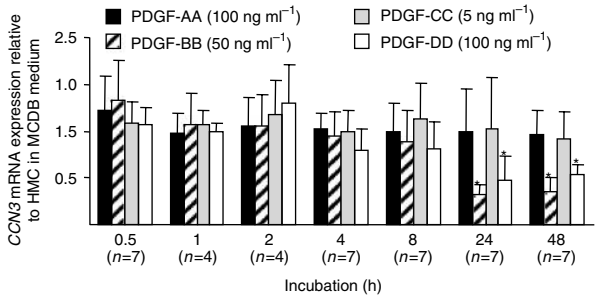

Figure 2 | Incubation with PDGF-BB or -DD, but not PDGF-AA or -CC, results in a downregulation of *CCN3* mRNA. PDGF-BB and -DD, but not PDGF-AA and -CC, induce a downregulation of *CCN3* mRNA as determined by real-time RT-PCR and normalized to *glyceraldehyde-3-phosphate dehydrogenase* mRNA. Prior to the experiment, HMC had been cultured for 72 h in MCDB medium to maximally upregulate *CCN3* expression. Data are means ± s.d. *$P < 0.05$ versus 0.5 h.

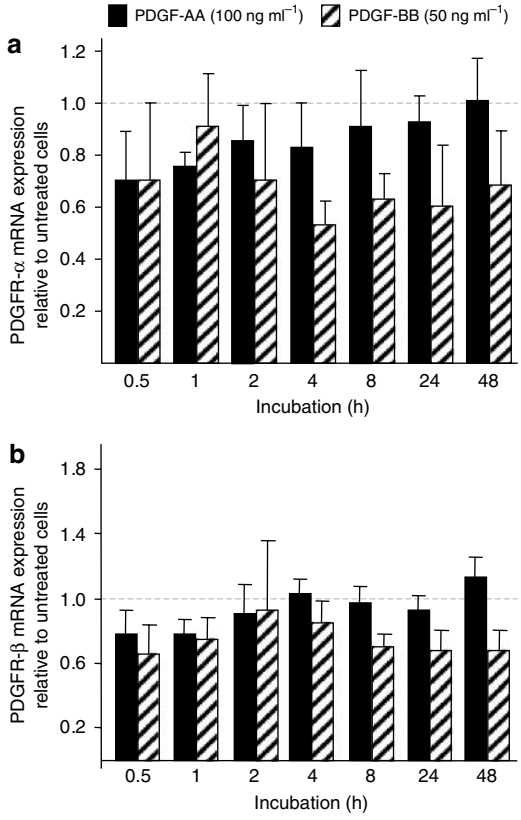

Figure 3 | Incubation with PDGF-BB or -AA does not affect the expression the *PDGF receptor* α- and *β-chain* mRNA in HMC. Real-time RT-PCR-based expression data of *PDGF receptor* (**a**) *α- or* (**b**) *β-chain* normalized to *glyceraldehyde-3-phosphate dehydrogenase* mRNA. Data are means ± s.d. of four independent experiments.

Expression of the *PDGF receptor* α- and *β-chains* is not altered in HMC stimulated with PDGF-AA or -BB

As HMC express both PDGF-receptors,[16] we asked whether a loop exists whereby PDGFs altered CCN3 release, which in turn modifies PDGF receptors and thus PDGF-responsiveness. However, neither the stimulation of HMC with PDGF-AA nor with -BB resulted in a significant change of *PDGF* α- and *β-receptor* mRNA (Figure 3).

Expression of CCN3 in normal rat kidney and during experimental mesangioproliferative glomerulonephritis

In normal adult rat kidney, CCN3 was expressed in arterial smooth muscle cells and in the medullary interstitium (Figure 4a–c) (Table 1). In addition, collecting duct cells were also positive as demonstrated in serial sections using antibodies for CCN3 and aquaporin-2 (Figure 4g and h). About 20% of the rats exhibited CCN3 expression in a podocyte-specific pattern (Figures 4e, 5a and b). No convincing CCN3 positivity was detected in the mesangium or glomerular endothelium (Figure 4d). Constitutive expression of *CCN3* in glomeruli was confirmed by RT-PCR (Figure 6a).

Anti-Thy 1.1 mesangioproliferative glomerulonephritis (GN) in rats is characterized by early mesangiolysis, a phase of overshooting mesangial cell proliferation and matrix accumulation and subsequent resolution of disease over the next 28 days.[17] In nephritic rats, we detected CCN3 protein within the expanded mesangium in addition to the constitutive podocytic CCN3 expression at day 9 after disease induction (Figures 4f, 5c and d) (Table 1). Glomerular *CCN3* mRNA expression decreased during the early phase of mesangial damage and subsequently was overexpressed starting on day 4 after disease induction (Figure 6a). The

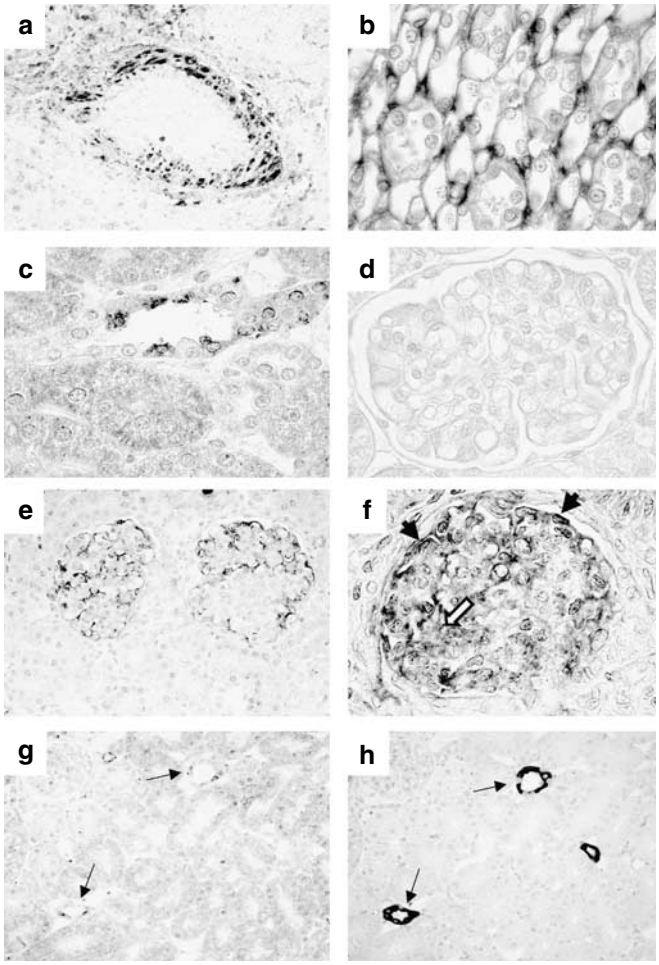

Figure 4 | CCN3 protein expression in kidneys of healthy and nephritic rats. In the healthy rat kidney, (**a**) CCN3 is expressed in arterial smooth muscle cells, (**b**) in the medullary interstitium, (**c**) and in some cells of the collecting duct. (**d**) CCN3 staining is absent in the mesangium. (**e**) About 20% of the normal rats expressed CCN3 in a podocyte pattern. (**f**) On day 9 after induction of mesangioproliferative anti-Thy 1.1 nephritis, an apparent *de novo* expression of the CCN3 protein in the mesangium (open arrow) in addition to the constitutive podocytic expression (filled arrows) was observed. Serial sections labeled for (**g**) CCN3 and (**h**) aquaporin-2 identifies the CCN3-positive tubular segments as collecting ducts. Original magnifications: $\times$ 200 in A, E, G, H; $\times$ 600 in B, C, D, F.

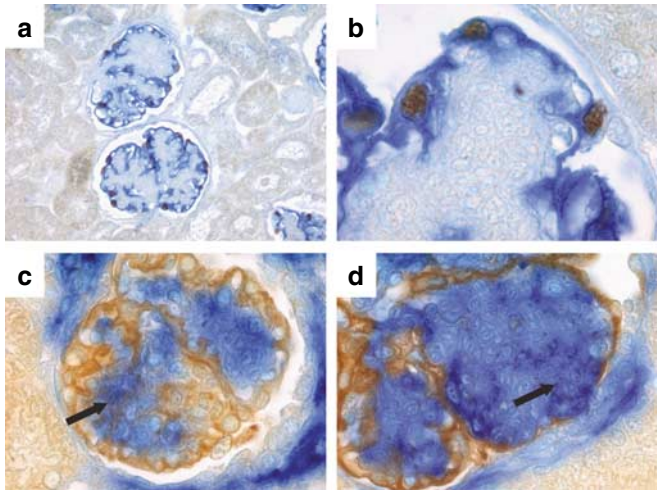

Figure 5 | Podocytic and mesangial CCN3 protein expression in kidneys of healthy and nephritic rats. The podocytic CCN3 protein expression in the healthy rat kidney was confirmed by double immunostaining for CCN3 (blue color) and nuclear WT-1 expression (brown) (**a**, original magnification $\times$ 200; **b**, original magnification $\times$ 1000). In the nephritic rat at day 9 after induction of anti-Thy 1.1 nephritis, the activated mesangium exhibits *de novo* expression of α-smooth muscle actin (blue). In addition to the constitutive podocytic CCN3 expression (brown), CCN3 protein could be detected in a (**c**) diffuse or (**d**) spotty pattern within the activated mesangium as indicated by the arrows (original magnification $\times$ 600).

10.4-fold peak induction of *CCN3* mRNA expression in glomeruli occurred about 2 days after the peak of glomerular cell proliferation (Figure 6a), which in this phase of the disease is almost exclusively due to mesangial cell proliferation.[17] Western blot analysis of glomerular protein extracts confirmed the overexpression of full-length 48 kDa CCN3 protein on days 7 and 9 after disease induction (Figure 6b). A second CCN3 isoform (75 kDa) was also overexpressed (Figure 6b).

Antagonism of PDGF-B restores CCN3 mRNA expression in experimental mesangioproliferative GN

Antagonism of PDGF-B using antagonistic aptamers from day 3 until day 6 after anti-Thy 1.1 nephritis induction results in a significant reduction of mesangial cell proliferation.[1] In glomeruli isolated from nephritic rats, which had been treated with PDGF-B aptamers, *CCN3* mRNA expression on day 7 was significantly higher as compared to nephritic animals receiving inactive control compound (PEG40) (Figure 6c).

Decreased CCN3 protein expression correlates with reduced glomerular cell proliferation in human necrotizing GN

In normal human kidney, a predominantly glomerular staining with a podocytic expression pattern of CCN3 could be observed in almost all glomeruli (Figure 7a). Outside of glomeruli, nonspecific background staining precluded an unequivocal detection of positive cells (Table 1). To assess disease-specific alterations, we measured the CCN3-positive glomerular area by morphometry in a large number of human biopsies. Whereas the positively stained area was relatively constant within a particular biopsy, a wide variability was observed within disease entities and between different diseases, so that no specific pattern of altered CCN3 expression was detected (Figure 7a and b).

To specifically assess the relationship between glomerular CCN3 expression and cell proliferation, we evaluated the glomerular immunostaining for CCN3, the proliferation-associated protein mib/Ki-67 and p27, a negative regulator of cell proliferation. Whereas glomerular cell proliferation in most disease entities was very low and no clear relationship with CCN3 evolved, in necrotizing GN, we observed a significant negative correlation between CCN3 protein

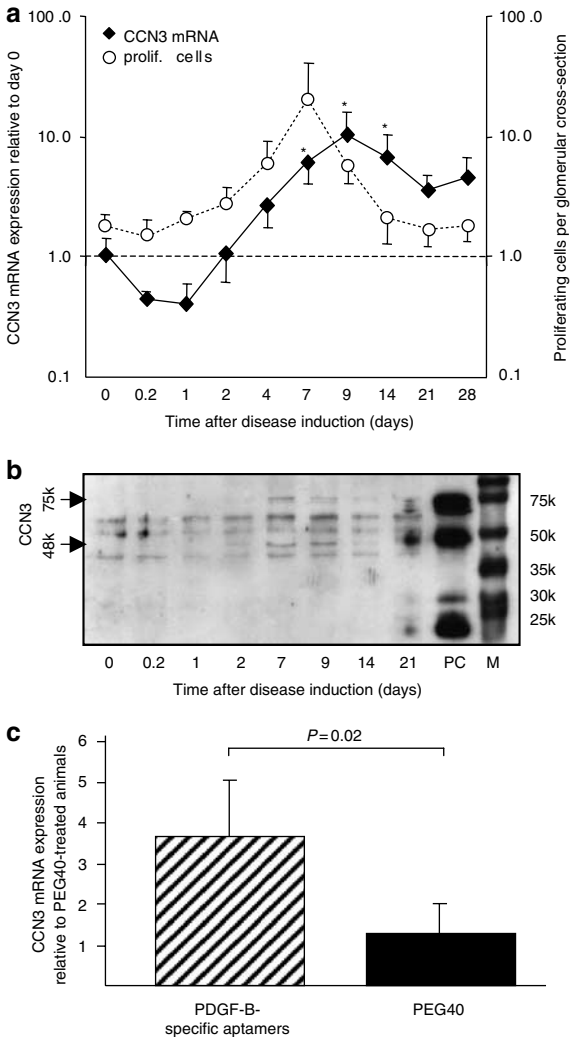

Figure 6 | Glomerular expression of CCN3 in the course of anti-Thy1.1 mesangioproliferative nephritis. Rats were killed at 4 h and at days 1, 2, 4, 7, 9, 14, 21, and 28 after disease induction ($n = 9$ each). (**a**) RNA was isolated from the glomeruli and the expression of *CCN3* mRNA was measured by real-time RT-PCR. The figure shows the transcript expression (means ± s.d.) in relation to the expression in non-nephritic rats. A parallel evaluation of proliferating cells per glomerular cross-section shows that its peak antedates the peak of *CCN3* mRNA. Data are depicted on a half logarithmic scale. *$P < 0.05$ versus non-nephritic rats. (**b**) The expression of the CCN3 protein was detected by western blot analysis ($n = 4$ each, PC, positive control, M, molecular weight markers). (**c**) Glomerular *CCN3* mRNA expression in nephritic rats treated with PDGF-B specific aptamers or PEG40 from day 3 to 6 after disease induction. Rats were killed at day 7 ($n = 4$ each).

expression and numbers of MIB-positive cells and a positive correlation between CCN3 and numbers of p27-positive cells (Figure 7a and c).

DISCUSSION

In this study, we identify CCN3 in the glomerulus as a potent endogenous growth inhibitor of mesangial cells as evidenced by (1) increased *CCN3* mRNA expression in growth-arrested human as well as rat MC in comparison to proliferating cells, (2) the detection of extracellular full-length CCN3 protein in mesangial cell supernatants and glomerular lysates, and (3) reduced cell proliferation of HMC following the addition of recombinant CCN3 protein.

The role of CCN3 in regulating cell proliferation has been described variably. Overexpression of *CCN3* in chicken embryonic fibroblasts, glioblastoma or Ewing's sarcoma cell lines inhibits their growth *in vitro* and *in vivo*.[7,13,18] In glioma and choriocarcinoma cell lines, CCN3 mediates connexin 43-induced growth inhibition,[19,20] and in chronic myeloid leukemia, the constitutively active BCR-ABL protein kinase downregulated CCN3 expression. Transfection or incubation of BCR-ABL$^+$ cells with CCN3 inhibited leukemia cell proliferation.[21] However, in other situations, CCN3 was described to promote growth or to amplify the activity of FGF2 and PDGF.[22–24] In part, these variable functions of CCN3 may relate to its complex biology given that for normal cell proliferation the balance of full-length and truncated CCN3 proteins is critical and the biological activity may be context dependent.[7] Further post-translational modifications or the subcellular localization might be important for the biological functions.[10–12] For example, Liu *et al.*[24] described an induction of cell proliferation by an approximately 40 kDa CCN3 protein, purified from supernatants of transfected 293 cells, whereas our primary mesangial cells secreted a 48 kDa CCN3 protein. In another study, Lafont *et al.*[22] described an induction of proliferation by recombinant CCN3 in the C2C12 myoblast cell line. However, in contrast to the experimental conditions employed in our study, Lafont *et al.* coated culture plates with recombinant CCN3, which resulted in increased adhesion of the cells and might thereby have affected cell proliferation in a different manner.

The second major finding of this study was that *CCN3* in mesangial cells and glomeruli is regulated by PDGF-BB and -DD, that is the two known ligands of the PDGF β-receptor chain. Consistent with this, the treatment of nephritic rats with PDGF-B-specific aptamers resulted in both a pronounced reduction of mesangial cell proliferation[1] and higher expression of *CCN3* mRNA as compared to control animals. *In vitro*, the decrease in mesangial cell *CCN3* production in response to PDGF-BB (Figure 2a) directly mirrored PDGF-BB and -DD-induced mesangial cell proliferation, which is maximal after 24 h.[16] In contrast, selective ligands of the PDGF α-receptor, that is PDGF-AA and -CC, did not affect *CCN3* mRNA expression, indicating that the downregulation of *CCN3* is mediated by the PDGF β-receptor chain. These data are in accordance with the finding that PDGF-BB and -DD are potent mitogens for mesangial cells *in vitro* and *in vivo*, whereas PDGF-AA usually elicits lower-level and fewer biological effects in mesangial cells despite their expression of the PDGF α-receptor.[14,15] We also excluded, at least at the mRNA level, that the interaction between PDGFs and CCN3 involves modulation of the mesangial cell PDGF receptor expression. This was of importance given that in glioblastoma cells, the

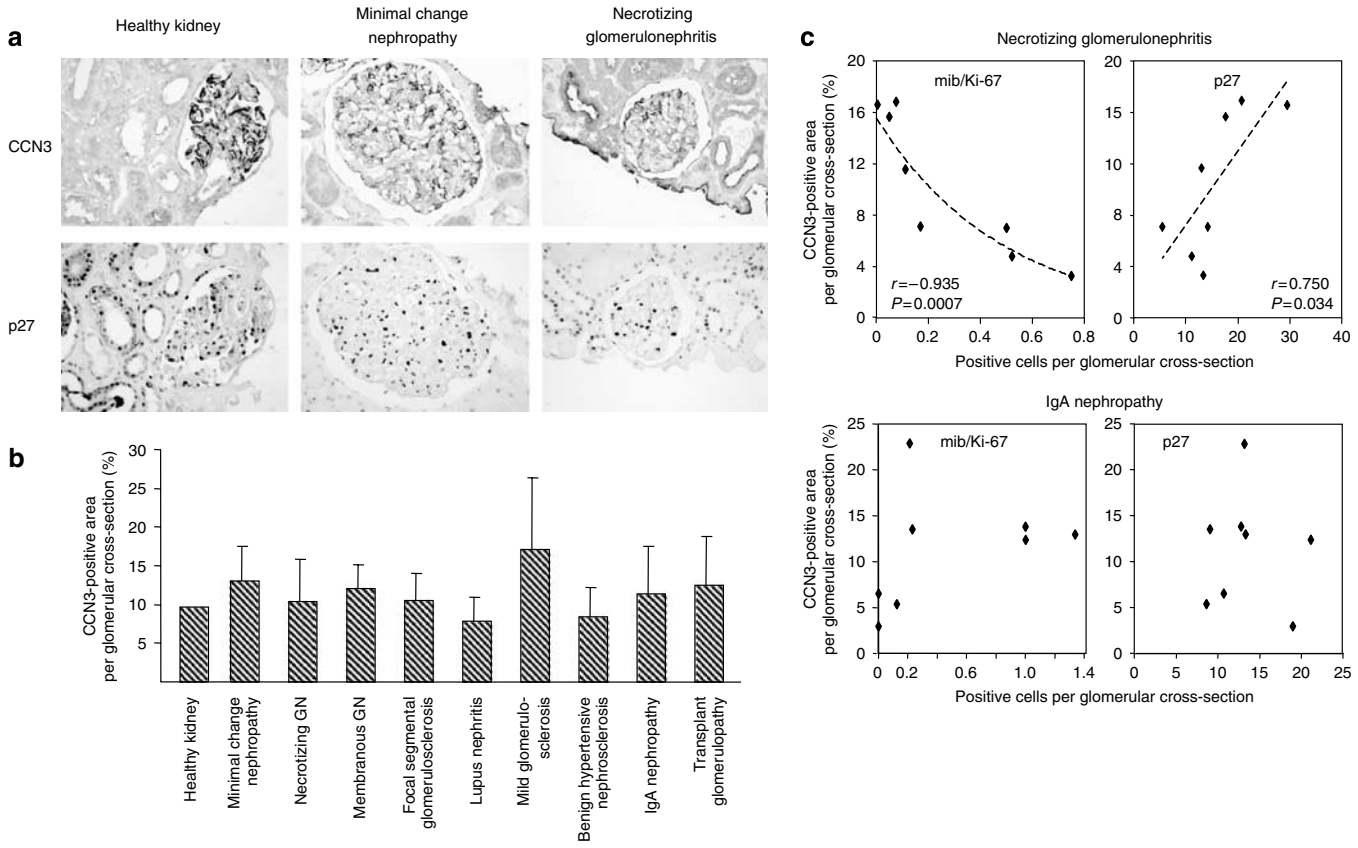

Figure 7 | CCN3 protein expression in diseased human kidney. (**a**) In human glomeruli, CCN3 is expressed in a podocyte-specific pattern. CCN3 glomerular staining pattern of healthy kidney as well as in biopsies of patients with minimal change nephropathy and necrotizing GN are shown. In serial sections, p27-positive glomerular cells were demonstrated in the same glomeruli. (**b**) CCN3 protein immunostaining in glomeruli of renal biopsies from patients with various renal diseases. The percentage of positively stained area in each glomeruli was measured by computer-based morphometry. Data are means ± s.d. of eight biopsies each. (**c**) Renal biopsies from patients with necrotizing GN and IgA nephropathy were stained for CCN3, mib/Ki-67, or p27. The relative glomerular area staining positively for CCN3, as determined by computer-based morphometry, was correlated to the number of MIB/Ki-67 or p27-positive cells per glomerular cross-section ($n = 8$). In necrotizing GN, but not in IgA, nephropathy glomerular cell proliferation correlated negatively with CCN3 expression. Statistics were performed using the two-tailed Pearson's test.

expression of the PDGF receptor α-chain is upregulated by CCN3.[25]

Third, we provide the first data on the CCN3 expression pattern in rat and human kidney. We found that renal cells expressing CCN3 in normal kidney largely overlap with those producing PDGF-B, -C, and -D.[5,26,27] Although the physiologic function of both PDGF- and CCN3-expression in some locations, such as the collecting duct, is completely unknown, we have recently provided first evidence that the production of these growth factors in the podocyte may serve as an important regulatory mechanism of intraglomerular cell proliferation. Thus, overexpression of PDGF-D in murine podocytes resulted in extensive proliferative changes within the glomerular tuft.[28] It is therefore conceivable that in normal glomeruli, a finely tuned balance between pro- and anti-proliferative factors, such as the PDGF-B and -D/CCN-3 system produced by podocytes, governs the physiological glomerular cell turnover. At least in rat glomeruli, we only detected the full-length CCN3 protein (48 kDa), which inhibits cell proliferation, and a 75 kDa isoform, which represents a dimer, that is still resistant to denaturing conditions used for western blot.[7] Potential shorter isoforms, which appear to be involved in malignant transformation and growth induction,[12] were not detected.

The fourth major finding of the study is that in experimental mesangioproliferative nephritis, an apparent *de novo* expression of CCN3 in the mesangium can be detected. In normal rat glomeruli, CCN3 protein is largely confined to podocytes. During the peak proliferative phase of anti-Thy1.1 nephritis, glomerular *CCN3* mRNA expression increased and the protein also appeared in the activated mesangium. Thus, it appears that, as in the case of other growth factors, such as vascular endothelial growth factor, CCN3 is normally acting as a paracrine mediator but that there may be an autocrine component in disease. The maximal glomerular *CCN3* mRNA and protein expression occurred at about day 9 after disease induction. This time point is remarkable, as it followed the peak intraglomerular cell proliferation by 2 days and the peak *PDGF-B* mRNA

expression in the glomeruli by 5 days.[5] The time course thereby is consistent with our hypothesis that CCN3 may be a (patho-)physiological regulator, which limits mesangial cell proliferation *in vivo* and which may contribute to the restitution of normal glomerular morphology. In the anti-Thy 1.1 nephritis model, the latter usually occurs in the course of 4 weeks.[17]

Two other members of the CCN-family have also been invoked in the pathogenesis of mesangioproliferative anti-Thy 1.1. CCN1 (Cyr61) is upregulated in nephritic glomeruli between days 3 and 7 after disease induction. However, in contrast to CCN3, CCN1 remains exclusively expressed by podocytes and inhibits PDGF-induced mesangial cell migration rather than proliferation.[29] A second member, CCN2 (CTGF), is also strongly upregulated in extracapillary and mesangial proliferative lesions and in areas of periglomerular fibrosis. In anti-Thy 1.1 nephritis, *CCN2* mRNA overexpression was already detected at day 1 after disease induction and maximum expression was reached by day 7.[30] In contrast to CCN3, which may be involved in glomerular healing (see above), CCN2 acts as a downstream mediator of TGF-β and appears to be involved in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis.[19,30]

In the final part of this study, we assessed the CCN3 expression pattern in a large collection of human renal biopsies with a variety of glomerular pathologies. Although no disease-specific pattern evolved, we noted in the glomerular disease with the most intense inflammatory and proliferative activity, namely necrotizing GN, that the CCN3 expression correlated negatively with glomerular cell proliferation and positively with the number of p27-positive nuclei. As p27 is a cell cycle inhibitor, levels are high in quiescent mesangial cells *in vitro* as well as *in vivo*[31–33] and reduced upon induction of mesangial cell proliferation *in vitro* and during anti-Thy1.1 nephritis.[31,32] Our observations in human necrotizing GN are therefore consistent with the assumed role of CCN3, whereby high levels are antiproliferative and low levels permit a proliferative response in glomeruli.

Up to now, the participation of other members of the CCN family in the pathogenesis of human renal disease is largely unknown. Some data are available, which invoke CCN2 (CTGF) in the development of renal fibrosis. *CCN2* mRNA expression is increased in diabetic nephropathy, IgA nephropathy, chronic transplant rejection, crescentic GN, focal and segmental glomerulosclerosis, lupus nephritis, and membranoproliferative GN.[19,34,35]

In conclusion, we identify CCN3 as the first endogenous inhibitor of mesangial cell growth and as a negative feedback regulator of the important mitogens PDGF-BB and -DD. We also add CCN3 to a growing list of podocyte-derived growth factors, which may convert the proliferative activity within the glomerular tuft. A better understanding of such regulatory systems may yield novel therapeutic approaches to glomerular diseases.

MATERIALS AND METHODS

Mesangial cell culture

Primary HMC were obtained from Biowithaker (Verviers, Belgium) and were cultivated as described.[36] Rat mesangial cells were established and maintained as described previously.[37,38]

Affymetrix gene chip array

Growth-arrested HMC were stimulated with PDGF-BB (10 ng ml^{-1}; Sigma-Aldrich, Deisenhofen, Germany), PDGF-DD (100 ng ml^{-1}; a kind gift of W LaRochelle, Curagen, Branford, CT, USA), or with RPMI 1640 alone as described.[16] After cultivation for 24 h, the cells were lysed and RNA was isolated, reverse transcribed into double-stranded cDNA and subsequently transcribed into biotinylated cRNA probes. The probes were finally hybridized to an Affymetrix human Genome U133A microarray as described.[16]

Mesangial cell culture experiments

Subconfluent HMC were incubated for 0, 16, 24, 48, or 72 h in MCDB-media (Sigma-Aldrich) or supplemented RPMI 1640 medium.[36] After 72 h in MCDB medium, PDGF-AA (100 ng ml^{-1}; Sigma-Aldrich), PDGF-BB (50 ng ml^{-1}), PDGF-CC (5 ng ml^{-1}; a kind gift of U Eriksson, Ludwig Institut for Cancer Research, Stockholm, Sweden), or PDGF-DD (100 ng ml^{-1}) in MCDB with 100 μg ml^{-1} of streptomycin and 100 U ml^{-1} penicillin were added and the cells were incubated for 0.5, 1, 2, 4, 8, 24, or 48 h.

Cell proliferation was assessed as described.[16]

For the incubation of HMC with recombinant CCN3 protein, 10 000 cells were seeded in 500 μl of complete RPMI medium with 10% FCS in a 24-well plate at day 0. At day 1, recombinant CCN3 protein, tagged with GST or GST alone, produced as described,[13] was added to a final concentration of 10, 50, 100, and 150 nM GST-CCN3 or 150 nM GST. Cells were counted using a Malassez hemocytometer at day 4 ($n = 4$).

Rat model of mesangioproliferative GN

All animal experiments were approved by the local review boards. Anti-Thy 1.1 mesangial proliferative GN was induced in male Wistar rats (Charles River, Sulzfeld, Germany) weighing 180 g, by injection of 1 mg kg^{-1} anti-Thy 1.1 monoclonal antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England).

To study the kinetics of CCN3 expression during anti-Thy 1.1 nephritis, 81 rats received anti-Thy 1.1 monoclonal antibody and were killed at time points 4 h, day 1, 2, 4, 7, 9, 14, 21, and 28 after monoclonal antibody injection ($n = 9$ each). Following killing, renal tissue as obtained and glomeruli were isolated by differential sieving.[39]

To study the effects of PDGF-B antagonism *in vivo*, anti-Thy1.1 mesangioproliferative GN was induced in male Wistar rats as described above. Rats received twice-daily intravenous injections of 0.33 mg PDGF-B-specific aptamers ($n = 5$) or control/PEG40 ($n = 4$), starting at day 3 after the induction of anti-Thy1.1 GN as described previously.[1,4] Renal biopsies were obtained at day 7.

Real-time quantitative RT-PCR

Total RNA was isolated from HMC or rat mesangial cells using the Invisorb Spin Cell-RNA Mini Kit (Invitek, Berlin, Germany) or from isolated glomeruli using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA purity determination, cDNA synthesis, and RT-PCR were performed as described.[16] Primer sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase cDNA amplification was used as an internal standard.

Table 2 | Primers for real time RT-PCR

Gene	Forward primer	Reverse primer	Probe
(a) Primers for detection with SybrGreen (qPCR Core Kit for SYBR Green I)			
CCN3 (human)	TGAGGTGCCTGGAGAGTGCT	AGGGTAAGGCCTCCCAGTGA	
GAPDH (human)	ÀGCCACATCGCTCAGACACC	GCGCCCAATACGACCAAA	
(b) Primers for detection with Taqman probe (qPCR Core Kit)			
CCN3 (rat)	CTACAGAGTGGAGCGCGTGTT	GGAAGATTCCTGTTGGTGACCC	AAGAGCTGTGGAATGGGCTTGTCCAC
GAPDH (rat)	ACAAGATGGTGAAGGTCGGTG	AGAAGGCAGCCCTGGTAACC	CGGATTTGGCCGTATCGGACGC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-PCR.

Western blot analysis

Supernatants of growing or growth-arrested cells were centrifugated for 5 min at 10 000 *g*. The cells were lysed as described.[16] The proteins from supernatant and cell lysates were concentrated using heparin sepharose. The heparin sepharose was washed four times with phosphate-buffered saline containing protease inhibitors, dissolved in phosphate-buffered saline/protease inhibitor and incubated with 500 µg protein over night at 4°C. The complexes were washed with phosphate-buffered saline/protease inhibitor and the proteins were eluated with 100 µl Laemmli buffer without bromophenol blue (10 min 95°C). A 30 µl probe was loaded in each lane and western blot analysis was performed as described,[40] using a polyclonal antibody against CCN3 (K19M), which recognizes a C-terminal 19-aminoacid peptide of human CCN3.[11] As a positive control, a supernatant from adrenocortical cell cultures, which are known to secrete CCN3, was used.

Immunohistochemistry in rat and human tissues

Immunohistochemistry was performed in methyl Carnoy's fixed, paraffin-embedded tissue sections as described.[27] CCN3 expression was assessed using the polyclonal rabbit anti-CCN3 antibody K19M.[11] Glomerular cell proliferation was analyzed using a mouse monoclonal antibody to the MIB-1/Ki-67 antigen (clone MIB-1, DAKO, Hamburg, Germany). In addition, we stained for the cell cycle inhibitor p27 (clone 57, BD Biosciences, Erembodegem, Belgium). Negative controls consisted of substitution of the primary antibody with non-immune rabbit IgG or irrelevant monoclonal mouse IgG (DAKO). For the double immunostaining of WT-1 and CCN3, sections were first stained for WT-1 (sc-192, Santa Cruz, CA, USA) using 3,3′diaminobenzidine as a chromogen as described above, followed by detection of CCN3 using the blue alkaline phosphatase substrate kit III (Vector, Grünberg, Germany). In the case of CCN3 and α-smooth muscle double staining, the sections were stained first for CCN3 (3,3′diaminobenzidine staining) followed by the staining for α-smooth muscle actin followed by the blue alkaline phosphatase substrate kit III.

Sections of human biopsies were obtained from healthy kidney and patients with IgA nephropathy, lupus nephritis, necrotizing GN, membranous nephropathy, minimal change nephropathy, benign nephrosclerosis, glomerulosclerosis, focal segmental glomerulosclerosis, and transplant glomerulopathy ($n = 8$ each). Biopsies contained between 1 and 26 glomeruli per section. Formalin-fixed paraffin-embedded human biopsy sections were pretreated with unmasking solution in a microwave and blocked with host serum prior to incubation with the antibodies.

Immunohistochemical staining was analyzed by computer-based morphometry (Soft Imaging system GmbH, Münster, Germany). The relative area staining positively for CCN3 in each glomerulus was calculated. For p27 and MIB staining, positively stained cells per glomerulus were counted. In all analyses, the investigator was unaware of the origin of the slides.

Statistical analysis

All values are expressed as means ± s.d. Statistical significance (defined as $P < 0.05$) was evaluated using analysis of variance and Bonferroni *t*-tests, and the two-tailed Pearson's test, where appropriate.

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