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A perspective on anti-CCN2 therapy for chronic kidney disease

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FULL REVIEW

ABSTRACT

Kidney fibrosis is the common end point of chronic kidney disease independent of aetiology. Currently, no effective therapy exists to reduce kidney fibrosis. CCN2 appears to be an interesting candidate for anti-fibrotic drug targeting, because it holds a central position in the development of kidney fibrosis and interacts with a variety of factors that are involved in the fibrotic response, including transforming growth factor (TGF) β and Bone morphogenetic proteins. Although CCN2 modifies many pathways, it does not appear to have a membrane receptor of its own. Numerous experimental and clinical studies lowering CCN2 bioavailability have shown promising results with minimal adverse side effects. This review aims to provide an overview of the current state of CCN2 research with a focus on anti-fibrotic therapy.

Keywords: CCN2, CKD, fibrosis, intervention, kidney

INTRODUCTION

The common and important feature of chronic kidney disease (CKD), independent of aetiology, is development of fibrosis. Fibrotic processes are characterized by an increase in the number of myofibroblasts and a change in extracellular matrix (ECM) composition, quality and quantity. Accumulation of ECM (e.g. collagen, fibronectin and proteoglycans), increase in non-degradable collagen cross-linking and a decrease in ECM degradation are a hallmark of fibrosis and thought to be mostly mediated by myofibroblasts.

Myofibroblasts are activated fibroblasts with a specialized contractile phenotype. The exact origin of the interstitial myofibroblasts remains unclear, but evidence suggests that they might originate from resident fibroblasts, pericytes, circulating fibrocytes or through endothelial- or epithelial-to-mesenchymal transition [1–3].

Currently, although many different approaches with varying results have been taken, no clinical therapy exists that targets the progression of fibrosis effectively.

A promising potential target might be the matricellular protein CCN2 (also known as CTGF, the second member of the Cyr61, CTGF, Nov family). A wide and still growing variety of pro-fibrotic properties has been attributed to CCN2, and the anti-fibrotic efficacy of CCN2 inhibition observed in many pre-clinical models is now being studied in clinical trials. This review aims to describe the physiological role of CCN2, its main pro-fibrotic properties and the current scientific evidence regarding CCN2 inhibition as an effective method for hampering the development of kidney fibrosis.

CCN2 FUNCTIONS IN NORMAL PHYSIOLOGY

CCN2 is a matricellular matrix molecule consisting of four distinct, conserved domains. Domain 1 is an insulin-like growth factor (IGF)-binding protein domain, Domain 2 a von Willebrandt factor type C repeat, Domain 3 a thrombospondin type 1 repeat and Domain 4 a cysteine knot [4]. Located between Domains 2 and 3 is a linker region susceptible to proteolytic cleavage.

Genetic deletion revealed that CCN2 is required for many different developmental processes, such as normal chondrogenesis and vertebral disc development, [5–8]. Skeletal deformities are a hallmark of CCN2 knockout mice, and skeletal overexpression of CCN2 results in osteopoenia [5, 9]. Thoracic skeletal deformities and pulmonary malformation contribute to early postnatal death from respiratory failure in these CCN2 knockout mice [10, 11]. During development, CCN2 is also involved in angiogenesis, pulmonary development, vascular basal membrane formation and normal pericyte–endothelium interaction in the skin, and it has been proposed that reduced CCN2 expression is responsible for the lower collagen content found in the ageing skin [12]. Paradoxically, in adult mice, local overexpression of CCN2 is associated with similar phenomena, such as 'loss' of pericyte–endothelial interaction as a hallmark of interstitial fibrosis in the kidney, whereas local overexpression of CCN2 in the lung leads to decreased alveolarization and angiogenesis in mice [13].

In the kidney, CCN2 is predominantly expressed in the podocytes during development. During adulthood, CCN2 is weakly expressed in podocytes, parietal epithelial cells and interstitial fibroblasts [14]. CCN2 seems not to be required for normal glomerular development, since CCN2 full knockout mice show no gross morphological differences in glomerular morphology at E16.5. Furthermore, no abnormalities were observed in podocytes, pericyte/mesangial or endothelial cell localization and number in neonatally lethal CCN2 knockout mice (Figure 1). It cannot be excluded that CCN2 might play a role in kidney development at postnatal stages, but thus far, 90% CCN2 reduction for 10 days did not lead to structural changes or albuminuria in healthy mice (Figure 2).

CCN2 AND KIDNEY FIBROSIS

Murphy et al. [15] identified CCN2 as one of the genes that are most markedly up-regulated glomerular mesangial cells exposed to high glucose. Furthermore, stimulation of mesangial cells with CCN2 resulted in increased matrix deposition. CCN2 overexpression in tubulointerstitial fibroblasts was also associated with increased ECM synthesis [16], and subsequently, numerous in vitro experiments in different renal cell types have suggested a central role for CCN2 in the development of kidney fibrosis [17].

Up-regulation of kidney CCN2 has been observed in human and experimental kidney fibrosis of various aetiologies, including diabetic nephropathy, hypertensive nephrosclerosis, crescentic glomerulonephritis and chronic allograft nephropathy [18, 19]. CCN2 protein levels are also elevated in plasma and urine of patients with various kidney diseases, including diabetic nephropathy and chronic allograft nephropathy [20–22]. Moreover, plasma CCN2 was found to be an independent predictor of end-stage renal disease and mortality in patients with type 1 diabetes mellitus [23]. Numerous factors have been identified that lead to up-regulation of CCN2 during fibrogenic processes in the kidney, including a variety of growth factors, lysophosphatidic acid, glucose, reactive oxygen species, mechanical stress and hypoxia [24–27]. The most important CCN2-inducing growth factors involved in kidney fibrosis are transforming growth factor (TGF) β, IGF-1, Wnt, angiotensin II, tumour necrosis factor-α and platelet-derived growth factor [28, 29]. In the kidney and other organ systems, CCN2 overexpression is now widely appreciated as a marker of fibrotic activity.
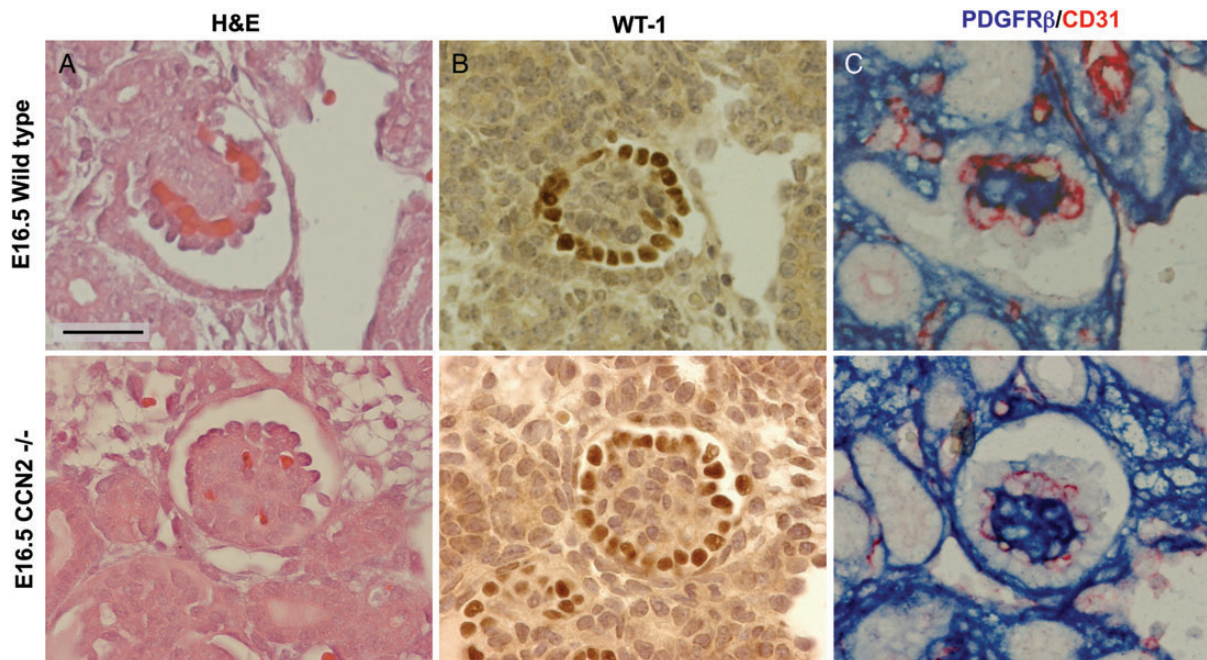
FIGURE 1: Constitutional CCN2 deletion does not lead to morphological changes in the developing glomerulus. Shown are representative images of the same glomerulus stained with respectively hematoxylin & eosin (A), the podocyte marker Wilms' tumor 1 (WT-1) (B) and PDGFRβ/CD31 (C). Sections are from embryonic mouse kidneys at E16.5. (Bar represents 25 μm.)
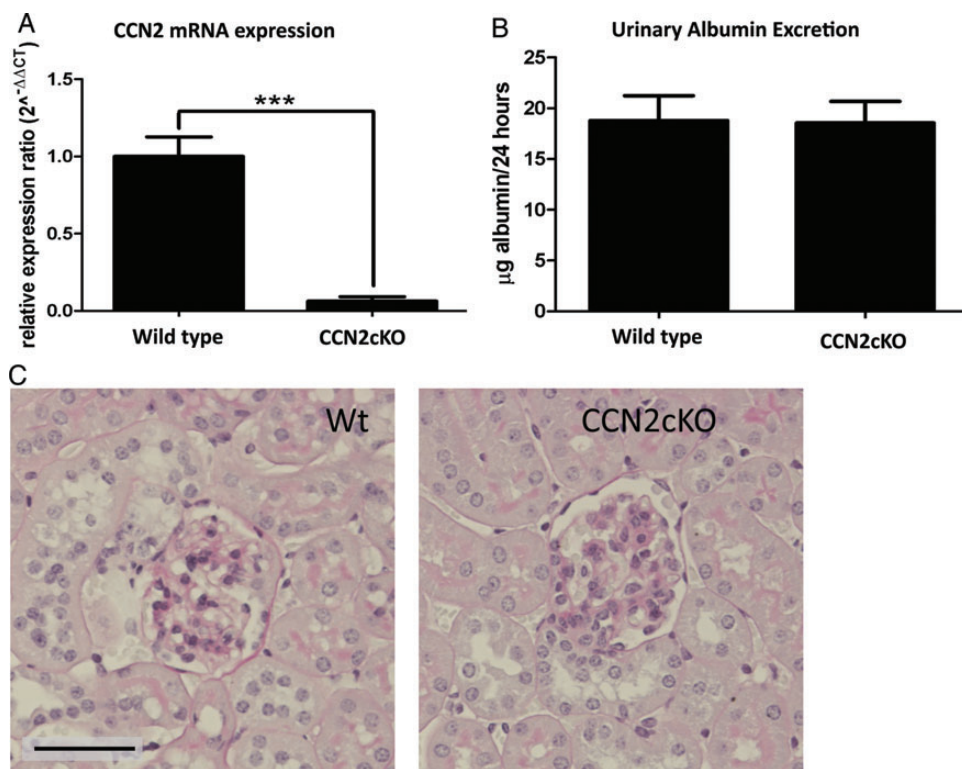
FIGURE 2: Decreased CCN2 expression does not lead to alterations in urinary albumin excretion or kidney morphology in conditional full knockout mice. (**A**) CCN2 mRNA expression in transgenic ER-ROSA26/CCN2 flox mice injected with either corn oil vehicle solution (wild type) or tamoxifen (CCN2cKO) (***$P < 0.001$, wild-type $n = 3$, CCN2cKO $n = 5$). (**B**) Twenty-four-hour urinary albumin excretion in wild-type versus CCN2cKO mice (wild-type $n = 5$, CCN2cKO $n = 5$). (**C**) PAS stained sections showing glomerular and tubular morphology. Bar represents 50 μm (All bars represent SEM).

CCN2 INTERACTION WITH FACTORS INVOLVED IN KIDNEY FIBROSIS

Direct binding and activation of cell surface receptors

CCN2 can directly activate cell surface receptors LRP1 and LRP6, TrkA and epidermal growth factor receptor (EGFR), mainly through binding of the possibly not very specific 'sticky' C-terminal cysteine knot (Domain 4) [30–32]. Significant efforts over 20 years have failed to identify a receptor exclusively or even predominantly functioning as a transmitter of CCN2 signalling, and it seems plausible that such a receptor might not exist. As a matricellular protein consisting of four distinct domains, CCN2 can simultaneously interact with multiple different extracellular proteins including growth factors, cell surface molecules such as integrins, and ECM proteins such as fibronectin and proteoglycans (Figure 3). This suggests that CCN2 might primarily function as a modulator and spatial coordinator of signalling activities in multiple distinct pathways.

Modulation of pro-fibrotic pathways

Of the many fibrosis-inducing factors identified, TGFβ1, two are commonly regarded as the most potent, and key players in myofibroblast activation, differentiation and proliferation, as well as in initiating ECM component transcription in the kidney.

Increased levels of circulating TGFβ1 are sufficient to induce proteinuria and robust kidney fibrosis within 5 weeks [33]. After the initial discovery that the combination of CCN2 with TGFβ aggravates fibrosis, it was shown that CCN2 could bind directly to TGFβ hereby enhancing the binding of TGFβ to its receptor [34]. Consistently, intradermal injection of TGFβ alone induces a fibrotic response, but addition of CCN2 is needed for sustained and progressive fibrosis [35, 36]. Also, fibroblasts deficient of CCN2 could not be activated and did not express typical pro-fibrotic factors upon TGFβ stimulation [37, 38]. CCN2 increases TGFβ-induced Smad2/3 and extracellular-signal regulated kinase (ERK) phosphorylation (Figure 3B). This has been attributed to the disruption of negative feedback as a result of suppression of the inhibitory Smad7 due to CCN2 signalling through TrkA [39].

Very recently, it was discovered that CCN2 can also directly bind and activate the EGFR leading to ERK phosphorylation and an influx of inflammatory cells in the kidney. Consistent with previous observations with other ligands, cross-talk was observed between EGFR and TrkA activation by CCN2 [30]. Other important pro-fibrotic pathways enhanced by CCN2 include IGF-1 and Wnt. CCN2 binds to IGF-1 with low affinity and enhances the pro-fibrotic effects of IGF-1 (Figure 3A)
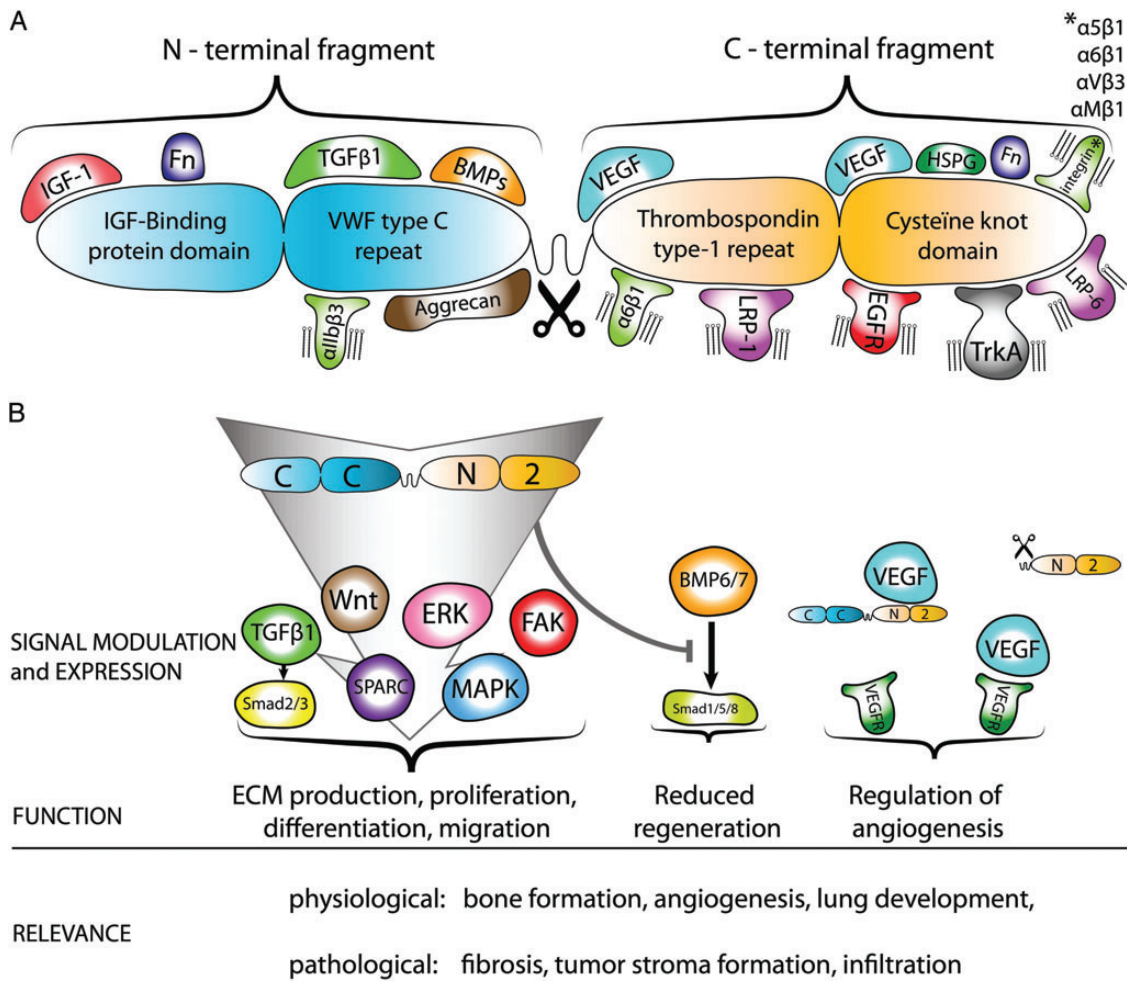
FIGURE 3: Schematic overview of factors interacting tumor stroma with CCN2 (**A**) and downstream regulatory effects (**B**).

[34, 40]. In cultured renal fibroblasts, collagen type 1 expression was only enhanced upon co-stimulation with both IGF-1 and CCN2, and IGF-1-induced expression of collagen type 3 was synergistically enhanced by CCN2 [40]. CCN2 activates Wnt signalling in mesangial cells and kidney pericytes, where its pro-fibrotic effect largely relies on binding to the low-density lipoprotein receptor-related protein-6 receptor [31, 41] (Figure 3B).

Modulation of anti-fibrotic pathways

CCN2 not only enhances a multitude of pro-fibrotic signals but also hampers the reno-protective and regenerative effects of bone morphogenetic proteins [42] (Figure3B). As CCN2 directly binds Bone morphogenetic proteins, the anti-fibrotic effect of anti-CCN2 therapy might not only involve reduction of the activity of pro-fibrotic stimuli but also involve preservation of BMP signalling [34, 43]. Another factor that can protect against kidney fibrosis is vascular endothelial growth factor (VEGF) [44]. Binding to Domain 3 in full-length CCN2 inhibits VEGF activity, but interestingly, upon proteolytic cleavage of CCN2 in the hinge region between Domains 2 and 3, VEGF is released in its active form (Figure 2B) [45]. Thus, reno-protective VEGF activity might depend on CCN2 degradation by proteases in the kidney microenvironment.

CCN2 INHIBITION IN FIBROSIS MODELS

Possible efficacy of anti-CCN2 therapy has been explored by a number of different approaches, including genetic deletion, RNA interference and (neutralizing) antibodies. The first published report on an *in vivo* interventional CCN2 reduction was RNAi-based CCN2 down-regulation in a model of liver fibrosis induced by carbon tetrachloride (CCl4) [46]. This revealed reduction of *col1a2* mRNA expression. Subsequently, CCN2 inhibition was also shown to reduce liver fibrosis score [47].

As for lung fibrosis, in a bleomycin-induced mouse model, fibrosis was attenuated by a single-chain anti-CCN2 antibody [48]. Furthermore, CCN2 RNAi prevented the proliferation of vascular smooth muscle cells in a rat model of pulmonary vascular remodelling [48, 49]. Moreover, although CCN2 expression by fibroblasts was not required for normal skin development, CCN2 inhibition did effectively reduce bleomycin-induced skin fibrosis [50]. CCN2 RNAi treatment also proved effective in a rabbit model of hypertrophic scar formation of the skin, while it appeared not to compromise wound closure [51].

CCN2 INHIBITION IN KIDNEY FIBROSIS

Evidence for efficacy of CCN2 reduction during the development of experimental kidney fibrosis was first established by Yokoi *et al.* In a 7-day unilateral ureteral obstruction model an ~50% reduction of CCN2 was achieved by injection of antisense nucleotides. This was associated with a lower fibrosis score and reduced collagen, fibronectin and α-smooth muscle actin expression. However, proliferation of tubular epithelium was not altered in the mice treated with CCN2 antisense nucleotides, suggesting that a reduction in CCN2 might not affect regenerative capacity [52].

Another study showed that in TGFβ transgenic overexpressing mice, a significant reduction in kidney fibrosis was obtained by intravenous administration of CCN2 antisense oligonucleotides [12]. In a rat model of renal allograft nephropathy, a CCN2 reduction of only 24% by RNAi appeared sufficient to prevent up-regulation of fibrotic markers [53].

A protective effect of CCN2 reduction in diabetic kidney disease has been observed by several groups. A 50% reduction of CCN2 by RNAi diminished glomerulosclerosis in mouse models of type 1 (streptozotocin; STZ) and type 2 diabetes (db/db) [54]. Concordantly, upon induction of diabetes by STZ injection, hemizygous CCN2 mice (CCN2+/−) with 50% lower CCN2 expression than their wild-type littermates developed less glomerular damage and proteinuria, which was associated with preserved activity of renoprotective BMP7 signalling activity in mice with reduced CCN2 expression [42]. Consistently, nephrin-1 promoter-driven specific overexpression of CCN2 in podocytes worsened diabetic glomerulosclerosis [55].

In several models including peritoneal sclerosis and 14-day unilateral ureteral obstruction, it was shown that a fully human monoclonal antibody against CCN2 (FG-3019) significantly inhibited collagen deposition [56]. In a small Phase 1 study in diabetic patients with microalbuminuria, FG-3019 lowered microalbuminuria without any adverse effects [57]. Interestingly, FG-3019 treatment as well as genetic hemizygous CCN2 deletion also slowed down functional and structural deterioration of mdx muscle dystrophy, dramatically improving fibrosis score, exercise performance, electrophysiology and also efficacy of stem cell therapy [58]. In addition, this same antibody, FG-3019, has also been shown to hamper tumour growth in mouse models of pancreatic cancer and metastatic melanoma [59, 60].

Although anti-CCN2 therapy thus appears promising, its effectiveness might be more limited in severe and CKD. For example, 50% reduction of CCN2 expression by hemizygous deletion did not improve outcome in three models of severe kidney disease, i.e. 14-day UUO, high-dose aristolochic acid nephropathy and long-term diabetic nephropathy. Specifically, renal function decline, morphological damage and the up-regulation of collagens and αSMA were not different in wild-type and hemizygous CCN2 mice [61]. Of note, in all these models, the remaining CCN2 level was still above the baseline in wild-type control mice. Interestingly and in concordance with a possible threshold phenomenon, further increase of already elevated CCN2 levels by fibroblast-specific overexpression of CCN2 did not aggravate the severe nephrotoxicity and fibrosis induced by aristolochic acid [62].

Thus, in severe CKD, CCN2 level might have to be reduced more profoundly than was achieved by hemizygous deletion. Alternatively, CCN2 might no longer be rate limiting in the context of massive up-regulation of a multitude of other pro-fibrotic factors. Although a constitutional full knockout mouse is not viable, studies in conditional CCN2 knockout mice or more profound suppression of CCN2 levels with antibodies or iRNAs are warranted to clarify this.

Safety and efficacy of anti-CCN2 therapy

Both in long-term preclinical and clinical safety studies, as well as in completed and ongoing pre-clinical efficacy studies and clinical trials with anti-CCN2 therapy, no serious adverse effects have been observed to date.

ClinicalTrials.gov lists several ongoing trials regarding efficacy and safety of FG-3019 anti-CCN2 antibody therapy outside the renal field, including idiopathic pulmonary fibrosis, liver fibrosis associated with hepatitis B infection and locally advanced or metastatic pancreatic cancer. It will be interesting to see for which diseases anti-CCN2 therapy might be effective. Another important question is whether restrictions will apply in terms of disease severity, and/or tolerable levels of residual free CCN2, for anti-CCN2 therapy to be clinically effective. Currently, no active trials of CCN2 inhibition in renal disease are listed, but in a small already completed study, a reduction of urinary albumin excretion was noted upon treatment with of FG-3019 in microalbuminuric patients with diabetes [57].

Another unresolved issue is that different members of the CCN family have overlapping as well as antagonistic properties, and are subject to parallel as well as compensatory regulation. This might at least in part explain the dissimilar net result of CCN2 reduction observed in different conditions. For example, like CCN2, CCN1 is also up-regulated in kidney disease and the biological functions of CCN1 and CCN2 partially overlap [63]. However, CCN3 might directly bind CCN2 and serve as an endogenous inhibitor of CCN2 in renal disease [64]. In this respect, it is noteworthy that we observed a compensatory increase of CCN1, but not of CCN3 or CCN5, in CCN2 suppressed mice with AA-nephropathy (Falke *et al.* unpublished).

UNRESOLVED ISSUES FOR CLINICAL TRANSLATION

Controversies

CCN2 was originally identified as a gene that is up-regulated in atherosclerotic aorta, but it still remains to be established whether it serves a predominantly protective, or a detrimental role in cardiovascular disease conditions [65]. For example, CCN2 overexpression is positively associated with plaque stability in atherosclerotic carotid arteries [66]. The role of CCN2 in myocardial response to injury is also controversial. CCN2 is up-regulated in the infarct zone after myocardial infarction, and many studies have shown that myocardial fibrosis and heart

failure are associated with overexpression of CCN2 in the heart [67]. Panek *et al.* [68] showed that transgenic cardiomyocyte-specific CCN2 overexpression caused age-related heart failure with compensatory myocardial hypertrophy, whereas Gravning *et al.* [69] reported that transgenic cardiomyocyte-specific CCN2 overexpression in mice *prevented* hypertrophy. Strikingly, both studies show a protective effect of CCN2 overexpression in myocardial infarction and pressure overload. Moreover, serum CN2 levels after a myocardial infarction were positively correlated with patient survival [69]. The beneficial effects of CCN2 overexpression in cardiomyocytes might relate to direct protection against hypoxia by activation of PI3 K/Akt/GSK3-β and stabilization of HIF1α, as has been observed also in breast cancer cells [70, 71]. However, HIF1α stabilization in the kidney has also been associated with an increase in epithelial-to-mesenchymal transition and fibrosis [72]. Under which conditions the potential beneficial CCN2 effects on HIF1α stabilization during ischemic disease might outweigh the HIF1α-dependent and -independent effects in the development of fibrosis remain to be established.

PERSPECTIVE

Available evidence suggests that anti-CCN2 therapy is safe and might be an effective approach to reducing progressive CKD, but clinical evidence of efficacy in severe and strongly progressive disorders is still pending.

Since CCN2 is eliminated from plasma primarily by glomerular filtration, plasma CCN2 is a potential uraemic toxin [73]. Of note, conventional haemodialysis does not reduce plasma CCN2, but haemodiafiltration does effectively eliminate CCN2 from the circulation and can thus be regarded as an alternative or additional mode of anti-CCN2 therapy.

Given the broad involvement of CCN2 in numerous pathways driving the progression of kidney disease, CCN2 inhibition might have significant potential beyond the application as a stand-alone therapy. Also in combination with other therapies, CCN2 inhibition might be of value by enhancing the efficacy and possibly allowing for dose reduction of individual pathway inhibitors.

CONFLICT OF INTEREST STATEMENT

The data presented in this review article have not been published elsewhere.

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Transforming growth factor-β and the progression of renal disease

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ABSTRACT

Transforming growth factor-β (TGF-β) is a profibrotic cytokine found in chronic renal diseases, which initiates and modulates a variety of pathophysiological processes. It is synthesized by many renal cell types and exerts its biological functions through a variety of signalling pathways, including the Smad and MAPK pathways. In renal diseases, TGF-β is upregulated and induces renal cells to produce extracellular matrix proteins leading to glomerulosclerosis as well as tubulointerstitial fibrosis. Different types of renal cells undergo different pathophysiological changes induced by TGF-β, leading to apoptosis, hypertrophy and abnormalities of podocyte foot processes, which ultimately result in renal dysfunction. In this review, we describe the effects of TGF-β on different renal cell types and the means by which TGF-β participates in the pathomechanisms of glomerular and tubulointerstitial diseases.

Keywords: diabetic nephropathy, EMT, fibroblasts, progression of renal diseases, renal fibrosis

TRANSFORMING GROWTH FACTOR BETA AND ITS PATHWAYS

Transforming growth factor-beta (TGF-β) is a multifunctional regulator that modulates cell proliferation, differentiation, apoptosis, adhesion and migration of various cell types and induces the production of extracellular matrix proteins (ECM) [1]. Most cell types, including immature haematopoietic cells, activated T and B cells, macrophages, neutrophils and dendritic cells, produce TGF-β and/or are sensitive to its effects [1]. The TGF-β superfamily, characterized by 6 conserved cysteine residues, is encoded by 42 open reading frames in humans and consists of >30 related members in mammals, including 3 TGF-βs, 4 activins and over 20 bone morphogenetic proteins (BMPs) [1–3]. Although the diverse TGF-β ligands elicit very different cellular responses, they all share a set of common sequence and structural features [2]. The three mammalian isoforms of the TGF-β subfamily (TGF-β1, TGF-β2, TGF-β3) share 70–82% amino acid homology and have qualitatively similar activities in different systems [4]. The active form of a TGF-β cytokine is a dimer stabilized by hydrophobic interactions, which are further strengthened by an intersubunit disulphide bridge in most cases [2]. TGF-β initiates intracellular signalling by binding to receptor complexes that contain two distantly related transmembrane serine/threonine kinases called receptors type I and type II (TβR-I and TβR-II) (Figure 1) [5]. Both of these receptors have an N-glycosylated extracellular domain that is rich in cysteine residues, one transmembrane domain and an intracellular serine/threonine kinase domain [1]. The type II receptor kinase is a constitutively active kinase, whereas the type I receptor kinase needs to be activated by the type II receptor kinase [1]. On most cell

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