



REVIEW ARTICLE

Fell–Muir lecture: connective tissue growth factor (CCN2) – a pernicious and pleiotropic player in the development of kidney fibrosis

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SUMMARY

Connective tissue growth factor (CTGF, CCN2) is a member of the CCN family of matricellular proteins. It interacts with many other proteins, including plasma membrane proteins, modulating cell function. It is expressed at low levels in normal adult kidney cells but is increased in kidney diseases, playing important roles in inflammation and in the development of glomerular and interstitial fibrosis in chronic disease. This review reports the evidence for its expression in human and animal models of chronic kidney disease and summarizes data showing that anti-CTGF therapy can successfully attenuate fibrotic changes in several such models, suggesting that therapies targeting CTGF and events downstream of it in renal cells may be useful for the treatment of human kidney fibrosis. Connective tissue growth factor stimulates the development of fibrosis in the kidney in many ways including activating cells to increase extracellular matrix synthesis, inducing cell cycle arrest and hypertrophy, and prolonging survival of activated cells. The relationship between CTGF and the pro-fibrotic factor TGF β is examined and mechanisms by which CTGF promotes signalling by the latter are discussed. No specific cellular receptors for CTGF have been discovered but it interacts with and activates several plasma membrane proteins including low-density lipoprotein receptor-related protein (LRP)-1, LRP-6, tropomyosin-related kinase A, integrins and heparan sulphate proteoglycans. Intracellular signalling and downstream events triggered by such interactions are reviewed. Finally, the relationships between CTGF and several anti-fibrotic factors, such as bone morphogenetic factor-4 (BMP4), BMP7, hepatocyte growth factor, CCN3 and Oncostatin M, are discussed. These may determine whether injured tissue heals or progresses to fibrosis.

Keywords

CCN2, chronic kidney disease, connective tissue growth factor, connective tissue growth factor-receptors, fibrosis, TGF β

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Introduction

Chronic kidney disease (CKD) can develop in widely divergent disorders such as diabetes, auto-immune diseases and drug toxicity but it invariably leads to a single outcome, renal fibrosis. This has devastating effects on kidney function, and frequently long-term dialysis and renal transplantation are required to maintain the life of those affected by it. About 13% of the US population have CKD, a figure

that is probably typical for western societies and that may be even higher in some other populations.

The kidney has a complex structure. In the adult human kidney, afferent arterioles carry blood into approximately 600,000 glomerular capillary tufts, across whose basement membranes it is filtered. Each glomerulus is connected to a tubular system through which the filtrate passes and in which it is modified, before entering collecting ducts as urine for transport to the ureter for passage to the bladder.

The tubules are surrounded by a usually sparse interstitium which is permeated by blood capillaries and other small blood vessels. The many different cellular components of these structures are susceptible to injury, after which either repair occurs or progression to a state where cells lay down excessive amounts of extracellular matrix, forming fibrous scar tissue that either replaces normal tissue or impedes its function. In the glomeruli, mesangial cells become activated to a myofibroblast-like state and secrete abundant collagen and fibronectin, obstructing glomerular capillaries and filtration (Figure 1a). In the renal interstitium, excessive extracellular matrix is produced by a variety of cells including activated fibroblasts, pericytes and tubular epithelial cells, accompanied by tubular atrophy and peritubular capillary loss (Figure 1b). Many cellular and molecular processes that lead to this state have been identified (Liu 2011; López-Hernandez & López-Nova 2012). This review will focus on the role of connective tissue growth factor, a

secreted 36–38 kDa protein that modulates inflammatory and fibrotic processes and that plays a key role in kidney fibrosis.

Ethical approval

Ethical approval was obtained for investigations in the authors laboratory using human tissue and animal experiments were carried out under the authority of a UK Home Office Licence.

CTGF is a CCN protein

Connective tissue growth factor (CTGF or CCN2) is a member of the CCN family of secreted matricellular proteins (Figure 2). The name CCN is derived from the first letter of three members of the family (underlined). The other family members are cysteine-rich angiogenic inducer 61

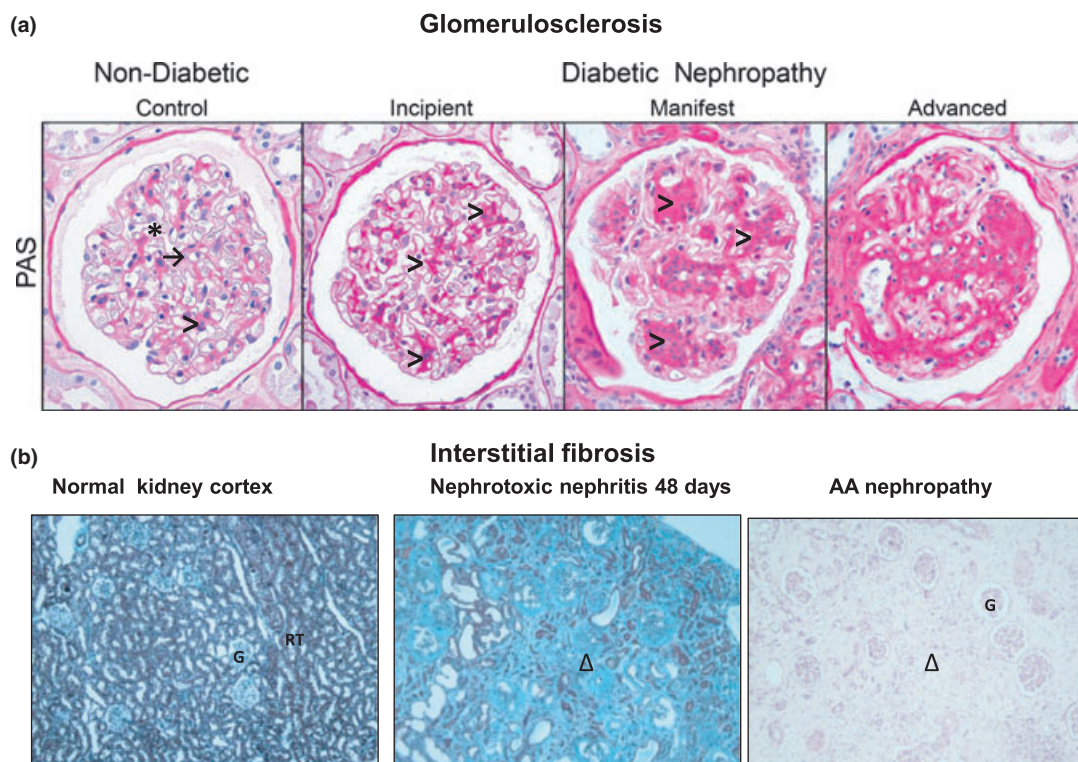


Figure 1 Normal and fibrotic kidney structure. (a) Glomerulosclerosis (glomerular fibrosis). The four frames show cross-sections of typical glomeruli from normal human kidney and from patients with incipient, manifest and advanced stages of diabetic nephropathy stained with periodic acid Schiff reagent (PAS; Wahab *et al.* 2005a, reproduced with permission from Diabetologia). Blood is filtered across the glomerular capillary basement membranes (→) into the urinary space (*) from where the filtrate drains into the tubular system. The capillary tuft is supported by the mesangium composed of mesangial cells and extracellular matrix (>). With incipient disease, there is diffuse expansion of mesangial matrix (>), strong pink staining), but otherwise normal cellularity and vasculature. At later stages (manifest and advanced), further mesangial expansion leads to increasing diffuse and nodular sclerosis with encroachment on and occlusion of capillary lumina and progressive loss of cells. $\times 400$. (b) Renal interstitial fibrosis (pictures courtesy of Prof Terry Cook). Normal WKY rat kidney cortex, like human cortex, has an abundance of renal tubules (RT) with scattered glomeruli (G). Interstitial matrix between neighbouring tubules is barely detected. Forty-eight days after induction of nephrotoxic nephritis, the tubules have been lost, or have distorted morphology, and are replaced by abundant interstitial extracellular matrix. This fibrotic tissue is stained light blue with Masson's trichrome (Δ). Remnant glomeruli are also fibrotic. Human aristolochic acid nephropathy (AA nephropathy, syn Chinese herbal nephropathy) results in profound tubular loss and massive interstitial fibrosis (Δ) but glomeruli (G) show only mild ischaemic changes. Stain, heamatoxylin and eosin.

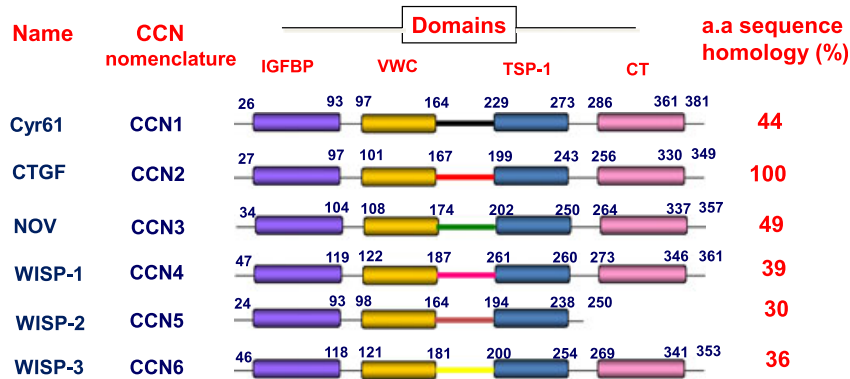


Figure 2 Domain structure and amino acid sequence homology of the CCN proteins. IGFBP, insulin-like growth factor binding protein domain; VWC, von Willebrand factor C domain; TSP-1, thrombospondin type 1 repeat domain; CT, C-terminal domain with cysteine knot motif; CTGF, connective tissue growth factor. Numbers refer to the amino acid (a.a.) sequence for each CCN protein. The per cent a.a. homology shown is with reference to CTGF/CCN2.

(CYR 61 or CCN1), nephroblastoma overexpressed gene (NOV or CCN3) and the Wnt-inducible signalling pathway proteins 1–3 (WISP 1–3 or CCN 4–6). The genes for CCN proteins encode a modular structure comprising a signal peptide followed by four domains, each named for its homology with other proteins. CCN5 lacks the C-terminal domain. Each domain is able to bind multiple ligands. In the case of CTGF, these include IGF-1, IGF-2 (IGF-BP domain); $\alpha 5\beta 3$ integrin, TGF β and bone morphogenetic factor-4 (BMP4; von Willebrand factor C domain); LRP-1, VEGF (TSP1 domain); and Wnt, heparan sulphate proteoglycan, integrins, LRP-5, LRP-6 (CT domain). With this large number of interactions, it is not surprising that CCN proteins, which are generally expressed at low levels in healthy adult tissue but increased in many disease settings, are able to affect many different biological functions. Alternative splicing of CCN mRNAs (Perbal 2009) and post-translational proteolytic cleavages of CCN proteins (De Winter *et al.* 2009) may result in yet further complexity in the biological roles of these molecules. These include effects on cell adhesion and migration, cell proliferation, survival and apoptosis, cellular transdifferentiation and extracellular matrix synthesis and turnover. Proteolytic processing of CTGF complexed with other proteins may also generate biological responses, for example, angiogenesis, when VEGF is released from an angiogenic-inhibitory complex with CTGF by matrix metalloproteinase 2 (MMP2) (Dean *et al.* 2007). Moreover, CCN proteins have been implicated as having key functions in pathologies ranging from wound healing to fibrosis to cancer and are potential therapeutic targets in these disorders (Jun & Lau 2011).

Human CTGF and the mouse homologue, FISP12, were discovered independently in 1991 (Bradham *et al.* 1991; Ryseck *et al.* 1991). It was soon recognized that CTGF was induced by TGF β 1 in a sequential manner in wound healing and that it may be involved in tissue repair (Igarashi *et al.* 1993). However, it was found that there was a strong correlation between skin sclerosis and CTGF expression in

dermal fibroblasts of patients with systemic sclerosis, implying that CTGF may also be involved in fibrosis (Igarashi *et al.* 1995). This was confirmed after injecting CTGF and TGF β together into the skin of neonatal mice which, in contrast to either cytokine alone, induced persistent fibrosis (Mori *et al.* 1999). Subsequently, CTGF has been implicated in fibrosis in a variety of tissues and organs (Shi-Wen *et al.* 2008).

Connective tissue growth factor and inflammation

Chronic inflammation usually precedes and accompanies the development of fibrosis in organs. Tissue injury from infectious, immune, mechanical, chemical or other agent is followed by the acute release of inflammatory mediators, recruitment and activation of inflammatory cells (macrophages, leucocytes and T-lymphocytes), removal of damaged cells and pathogens, activation of fibroblasts to myofibroblasts and finally repair (Wynn 2007). Activation of inflammatory cells releases many factors including profibrotic cytokines such as TGF β . With repeated or prolonged injury, myofibroblast activation persists, leading to fibrosis rather than repair. Activation of the transcription factor NF- κ B, a key marker of inflammatory activity, occurs in tubular epithelial cells and in the glomerulus in human kidney diseases and in animal models, particularly when proteinuria is present (Mezzano *et al.* 2001, 2004). It seems likely that CTGF, which is expressed in tubular and other renal cells in kidney disease (Ito *et al.* 1998; Nonaka *et al.* 2008), promotes cellular infiltration by activating NF- κ B (Sanchez-Lopez *et al.* 2009). Experimentally, 24 h after injecting normal mice i.p. with CTGF, inflammatory cells infiltrate the renal interstitium, with marked increases in CD68⁺ macrophages and CD3⁺ T-lymphocytes. NF- κ B was activated in the kidney, and the cellular infiltration was attenuated by prior treatment with an NF- κ B inhibitor. Expression of MCP-1 and

RANTES, chemokines that promote inflammation, was increased in both tubular epithelial cells and infiltrating interstitial cells and was also dependent on NF- κ B activation (Sanchez-Lopez *et al.* 2009). Connective tissue growth factor itself is chemotactic for monocytes *in vitro* (Cicha *et al.* 2005). These data indicate that CTGF provokes an inflammatory response in the kidney which is likely to contribute to the initiation of fibrosis in kidney disease.

Connective tissue growth factor and kidney fibrosis

Exploring potential mechanisms involved in fibrosis in diabetic nephropathy, we and others used suppression subtraction hybridization to reveal changes in gene expression in mesangial cells exposed to high glucose conditions (Mason *et al.* 1997; Murphy *et al.* 1999). Both studies showed that CTGF expression was upregulated in these conditions. Moreover, an earlier report implicating CTGF in the development of skin fibrosis (Igarashi *et al.* 1995) raised questions as to whether it is also involved in the development of glomerulosclerosis in diabetic nephropathy. A subsequent study confirmed that both mRNA and protein levels of glomerular CTGF increase progressively with advancing glomerulosclerosis in this disease (Wahab *et al.* 2005a). Moreover, a study on renal biopsies from patients with CKD of various origins indicated that those with glomerulosclerosis or interstitial fibrosis showed increased CTGF expression in cells at those sites, suggesting a role for CTGF in renal fibrosis generally (Ito *et al.* 1998). Subsequently, Suzuki *et al.* (2003) reported that CTGF expression was higher in glomerular mesangial and epithelial cells in diseases characterized by mesangial matrix expansion (diabetic nephropathy and IgA nephropathy) than it was in disorders associated with changes in the glomerular basement membrane (membranous nephropathy and minimal change nephrotic syndrome). Another study reported that CTGF was strongly expressed in cellular and fibrocellular crescents in human crescentic glomerulonephritis and proposed that it was involved in extracellular matrix production by parietal epithelial cells and fibrosis (Kanemoto *et al.* 2004). Animal models of kidney disease also showed changes in CTGF expression. For example, CTGF expression was markedly increased in interstitial fibroblasts during the development of tubular damage and interstitial fibrosis in the rat remnant kidney model (Frazier *et al.* 2000) and CTGF was expressed *de novo* in dilated proximal tubules of a rat model of diabetic nephropathy (Wang *et al.* 2001a,b). In a non-human primate (*Papio hamadryas*) model of type 1 diabetes, tubular CTGF protein scores after 5 years of disease predicted the degree of albuminuria at 10 years, suggesting CTGF as a contributory factor in the development of incipient nephropathy and an early marker of kidney disease (Thomson *et al.* 2008).

Whilst observational studies in man and animals are highly suggestive of a link between CTGF expression and the development of fibrosis in the kidney, they do not prove it. In con-

trast, interventional studies firmly establish this. Experimentally induced unilateral ureteric obstruction (UUO) in rodents leads to rapid development of renal interstitial fibrosis and is used as a model of CKD (Klahr & Morrissey 2002). Seven days after performing UUO, the number of myofibroblasts in the interstitium is markedly increased, accompanied by increased expression of type 1 collagen, fibronectin, EDA-fibronectin (a splice variant associated with fibrosis), CTGF and the development of fibrous tissue incorporating these molecules. Administration of CTGF antisense oligonucleotide (ASO) via the renal vein markedly attenuated these molecular events and the development of fibrosis in UUO (Yokoi *et al.* 2004). Interestingly, TGF β levels that were much increased in UUO were not affected by the CTGF-ASO treatment. Another model of renal fibrosis, sub-total nephrectomy (5/6) in TGF β -transgenic mice, leads to the development of glomerulosclerosis and interstitial fibrosis over the following 56 days (Okada *et al.* 2002). Intravenous administration of CTGF-ASO from days 29 to 56 after surgery blocked the expression of CTGF in proximal tubular epithelial cells and attenuated the development of interstitial fibrosis as shown by decreased peritubular collagen 1 deposition, decreased numbers of interstitial fibroblasts and reduced levels of α 1COL1 and FN-III mRNA in the remnant kidney. This occurred despite the continued expression of TGF β (Okada *et al.* 2005). Connective tissue growth factor expression in the glomerulus and glomerulosclerosis was unaffected in the CTGF-ASO-treated mice, the authors attributing this to their earlier observation that intravenously injected oligonucleotides are not absorbed by glomerular cells. However, in another study, mice with streptozotocin-induced type 1 diabetes and mutant *db/db* mice with type 2 diabetes were given CTGF-ASO subcutaneously for 16 weeks. The mesangial matrix expanded in the type 1 mice, accompanied by an increase in kidney cortex mRNA for CTGF, collagen 1 (α 1), fibronectin and TGF β . The increase in glomerular size was attenuated, and the mRNA levels returned to those of controls in the ASO-treated animals. CTGF-ASO treatment had similar effects in the *db/db* mice, and improvements in kidney function tests (urinary protein loss, serum creatinine) were also noted (Guha *et al.* 2007). Collectively, these studies provide convincing evidence of a key role for CTGF in the development of both glomerulosclerosis and interstitial fibrosis following kidney injury. The experiments in mice provide proof of principle that anti-CTGF therapies are successful in attenuating the development of renal fibrosis. Moreover, a phase 1 trial of the effects of an anti-CTGF antibody in human diabetics with microalbuminuria (indicative of early renal changes) showed not only that the antibody was safe to use but also that it reduced microalbuminuria (Adler *et al.* 2010).

Investigation of mice in which CTGF expression has been genetically modified also supports the notion that CTGF is a key factor in the development of renal fibrosis. CTGF was overexpressed in fibroblasts in mice transgenic for the murine homologue, *fisp12*, under the control of the minimal promoter from the *Col1a2* gene and a fibroblast-specific

6 kb upstream enhancer (Sonnlyai *et al.* 2010). Mice that were homozygous for the transgene developed fibrosis in many tissues, including focal glomerulosclerosis and interstitial fibrosis in the kidney.

The promoter of the human *nephrin* gene was used to drive overexpression of murine CTGF specifically in podocytes. These transgenic mice had normal kidney histology but 12 weeks after induction of diabetes with streptozotocin they showed glomerular hypertrophy, mesangial expansion and proteinuria compared to diabetic non-transgenic mice. Glomerular CTGF mRNA levels were markedly increased in the diabetic transgenic mice compared to diabetic non-transgenic controls, but mRNAs for matrix encoding genes were not significantly increased. However, whilst expression of matrix metalloproteinase 2 (MMP) was increased in control diabetic mice, it was lowered in the diabetic transgenics. This difference in MMP2 expression was accompanied by a decline in MMP2 activity in the diabetic transgenics, so the mesangial matrix expansion associated with increased CTGF expression was likely due to decreased matrix degradation rather than to increased matrix protein expression. Interestingly although CTGF protein was increased in podocytes in diabetic transgenics, as expected, it was also increased in the mesangium. Thus, the podocyte transgene-CTGF likely had a paracrine inductive effect on mesangial cell CTGF expression (Yokoi *et al.* 2008).

Complementing these experiments, another group induced diabetes in CTGF^{+/-} and CTGF^{+/+} mice and later subjected them to UUO to accelerate pathological changes in the kidney (Nguyen *et al.* 2008). After 17 weeks, renal cortex, plasma and urinary levels of CTGF were all increased in the CTGF^{+/+} mice, whilst cortex and plasma levels in CTGF^{+/-} mice were similar to those in non-diabetic controls. Moreover, gelatinase activity (due to MMP2 and MMP9) was decreased in CTGF^{+/+} mice, but was maintained at control levels in the CTGF^{+/-} mice, inferring again that higher CTGF levels decrease these metalloproteinases, which could slow matrix degradation.

It is noteworthy that the expression of CTGF in the renal cortex of transgenic mice does not always enhance the development of fibrosis in disease models. In contrast to homozygotes (see above), heterozygote *Col1a2-CTGF* mice have normal kidney structure, even though they have higher levels of cortical CTGF mRNA (Fragiadaki *et al.* 2011). When aristolochic acid (AA) nephropathy was induced in heterozygote *Col1a2-CTGF* mice, CTGF mRNA expression in the kidney cortex was markedly increased and there was a modest increase in CTGF protein compared to AA-treated wild-type litter mates. However, they only developed the same degree of interstitial fibrosis as that found in the AA-treated controls (Fragiadaki *et al.* 2011). This finding is similar to the observation that enhanced CTGF expression in cardiomyocytes did not evoke cardiac fibrosis in unchallenged transgenic mice, or increased cardiac damage when they were subjected to ischaemia-reperfusion (Panek *et al.* 2009). Thus, the relationship between CTGF and the development of fibrosis in a tissue appears to depend on context

and may differ depending on factors such as the level of CTGF expressed, the cell types exposed to the factor, the receptors for CTGF expressed by them, the levels of the profibrotic factor TGFβ and the activity of its signalling pathway.

The relationship between CTGF and TGFβ in fibrosis

TGFβ has long been recognized as a key factor in the development of renal inflammation and fibrosis (Floege & Johnson 1993). The discovery that TGFβ stimulated expression of CTGF (Igarashi *et al.* 1993) raised questions about the relationship between the two factors in driving fibrosis. These have still not been completely resolved. Connective tissue growth factor binds to TGFβ [Kd 30 nM] promoting interaction with its receptor and increasing downstream signalling (Abreu *et al.* 2002). However, the observation that fibrosis could be ameliorated in some animal models by treatment with CTGF-ASO whilst TGFβ levels were unaffected (Okada *et al.* 2005) led to the idea that CTGF acted 'downstream' of TGFβ. Nevertheless, experimental data suggest that the two factors act in a cooperative rather than in a sequential manner to promote fibrosis. For example, repeated daily subcutaneous injections of TGFβ1, TGFβ2, TGFβ3 or CTGF into the skin of neonatal mice failed to induce fibrosis, but persistent fibrosis developed at the site when TGFβ and CTGF were injected together, or when TGFβ was injected for 3 days, followed by CTGF for 4 days (Mori *et al.* 1999). Using a similar protocol in transgenic mice harbouring a reporter gene for the *COL1A2* gene promoter, the same group reported that injections of TGFβ3 or CTGF for 1 week failed to activate the promoter above control levels, but the sequential procedure stimulated a 12-fold increase in activity (Chujo *et al.* 2005). In another report, when CTGF and TGFβ2 were injected intraperitoneally together, neonatal mice developed severe fibrosis of the peritoneal membranes. However, injection of either TGFβ2 alone, or CTGF alone, did not induce fibrosis (Wang *et al.* 2011).

In vitro experiments have not yet fully explained the molecular basis of the cooperative effects of the two cytokines in driving fibrosis *in vivo*. In the TGFβ canonical signalling pathway, Smad2 and Smad3 are phosphorylated following TGFβ binding to its receptor (ALK5) and then translocate to the nucleus in a complex with Smad4, where they bind to the promoters of TGFβ-responsive genes, stimulating their transcription (Miyazawa *et al.* 2002). For example, the *COL1A2* promoter contains a classical Smad-binding element (SBE) for Smad3 (Chen *et al.* 2000), whilst the *COL1A1* promoter contains a CC(GG)-rich element to which Smad2 binds in association with Sp1 (Sysa *et al.* 2009). Smad2-deficient mice are embryonic lethal, whereas Smad3-deficient mice are viable and are protected from developing fibrosis in several different models (Flanders 2004). Mori *et al.* (2008) investigated whether responses to TGFβ differed in CTGF^{-/-} and CTGF^{+/+} murine embryonic fibroblasts (MEF). Although there were differences in cell proliferation rate and fewer α-smooth muscle actin positive

cells formed in response to TGF β in CTGF^{-/-} cells, activation of the TGF β canonical signalling pathway was unaffected when compared to CTGF^{+/+} cells. This was evidenced by normal Smad2 phosphorylation, Smad2/3 and Smad4 translocation to the nucleus, Smad3-dependent transcriptional activity, and synthesis of type 1 collagen and fibronectin in the null cells. However, in contrast to these results, in human mesangial cells, Smad2 and Smad3 phosphorylation was stimulated more when treated with TGF β plus exogenous rCTGF than it was with TGF β alone. Moreover, CTGF enhanced TGF β -induced transcriptional activity of the SBE4-luc reporter gene in these cells, whilst treatment with an antisense CTGF-ASO abrogated much of the transcriptional response to TGF β (Wahab *et al.* 2005b). The CTGF-ASO also markedly reduced TGF β -stimulated transcription of TGF β -responsive genes such as those for collagen III, PAI-1 and the cyclin-dependent kinase inhibitor, p15^{INK} in mesangial cells. One factor that is likely to be relevant to the different results of these two studies is that mesangial cells exhibit basal expression of CTGF whilst the MEF line studied by Mori *et al.* (2008) does not (Wahab *et al.* 2005b). Liu *et al.* (2011) investigated TGF β responses in adult dermal fibroblasts derived from wild-type mice and from mice in which CTGF expression in fibroblasts/smooth muscle cells was conditionally deleted. TGF β induced the same level of expression of α -smooth muscle cell actin and Col1a1 mRNA in both cell types. They proposed that in cells in which CTGF is normally basally expressed, CTGF mediates induction of TGF β -inducible mRNAs, whilst in cells lacking constitutive CTGF expression, responses to TGF β are unchanged. Interestingly, *in vivo*, wild-type mice were susceptible to bleomycin-induced skin fibrosis, whereas mice in which fibroblast/smooth muscle cell CTGF expression was deleted were not susceptible. The authors concluded that lack of a fibrotic response to bleomycin in CTGF-knockout mice may relate to a failure to recruit myofibroblasts (derived from pericytes) to the site, possibly because cell migration requires CTGF/integrin interactions.

In another recent study, CTGF was depleted in foreskin fibroblasts with an adenoviral siRNA. These cells were unable to upregulate type 1 collagen mRNAs and protein in response to subsequent TGF β treatment (Nakerakanti *et al.* 2011). Connective tissue growth factor depletion had no effect on Smad3 phosphorylation in response to TGF β , but Smad1 phosphorylation was abolished and ERK1/2 phosphorylation much reduced. Smad1 was first reported as being activated by a BMP receptor (ALK1) in endothelial cells but subsequent studies showed that it is also activated by TGF β in other cell types, including fibroblasts and epithelial cells, either via ALK1 or ALK5 (Miyazawa *et al.* 2002; Trojanowska 2009). Smad1 binds directly to the CTGF promoter activating it and increasing CTGF. It is proposed that secreted CTGF then binds to $\alpha_v\beta_3$ integrin activating the ERK1/2 MAP kinase pathway in a Src-dependent manner, this pathway being required for TGF β -induced activation of Smad1 (Trojanowska 2009; Nakerakanti *et al.* 2011). Thus, the pathway has the potential for persistent

cell activation. Importantly, Smad1 and p-Smad1 levels are elevated in systemic sclerosis (SSc) skin biopsies and siRNA depletion of Smad1 in SSc fibroblasts normalized their production of CTGF and collagen 1, indicating that the Smad1 pathway is involved in this fibrotic disease (Pannu *et al.* 2008). Collectively these reports highlight the importance of taking into account the possible involvement of the Smad1 pathway, as well as the Smad2/3 pathway, in future studies of the role of TGF β and CTGF in fibrotic diseases.

It is possible that the concentrations of TGF β and CTGF may affect the outcome of some experiments. Abreu *et al.* (2002) treated foetal mink lung cell cultures with TGF β and CTGF. Whilst 6 nM CTGF strongly potentiated the phosphorylation of Smad2 in cells stimulated with 10 pM TGF β , it had no effect with 50 pM TGF β , suggesting the concentration ratio between the two factors is important in cooperative effects. Moreover, in cells in which TGF β concentration was constant, even 3 nM CTGF stimulated 3TP-Lux activity, whilst maximum activity was achieved with 15 nM CTGF and 35 nM had no further effect.

There are also potential mechanisms for attenuating TGF β responses by driving down CTGF expression. Both TGF β 1 and 2 upregulated the expression of sphingosine kinase-1 (SK-1) in an immortalized podocyte cell line. CTGF expression was increased in TGF β -treated cells, but even more when they were transfected with an siRNA targeting SK-1. Conversely, CTGF expression decreased when SK-1 was overexpressed in TGF β -treated cells and further up-regulated when such cells were exposed to an SK-1 pharmacological inhibitor (Ren *et al.* 2009). Collectively these results indicate that SK-1 exerts a braking effect on TGF β -induced CTGF expression. Glomerular SK-1 expression was noted in human diabetic nephropathy biopsies but was barely detectable in controls, suggesting that the brake is applied in pathological situations where CTGF is upregulated. Similarly, glomerular CTGF was increased and albuminuria was worse in diabetic SK-1^{-/-} mice than in SK-1^{+/+} mice (Ren *et al.* 2009). Further mechanisms that decrease CTGF actions, but which involve opposition by other cytokines, are described later.

Pro-fibrotic effects of CTGF on renal cells in vitro

Connective tissue growth factor has effects on many cell types in the kidney. Selected examples of CTGF-induced effects are discussed to illustrate the diversity of its potential roles in kidney fibrosis.

Stimulation of extracellular matrix deposition

In the glomerulus, mesangial cells lay down an extracellular matrix around themselves, providing support for the glomerular tuft and also contract in response to vasoconstrictors, helping to regulate blood flow through the glomerular capillaries. Glomerulosclerosis in diabetic nephropathy is due to the expansion of the mesangial extracellular matrix. The expression of both TGF β and CTGF is increased in DN

(Wahab *et al.* 2005a). Connective tissue growth factor stimulates the expression of fibronectin in mesangial cells *in vitro*, as does TGF β . The stimulatory effect of the latter is mediated through CTGF (Wahab *et al.* 2001a,b). Both factors also increase the incorporation of the newly synthesized fibronectin into an insoluble extracellular matrix around the cells. This process is dependent on an increase in expression of cell surface $\alpha 5\beta 1$ integrin that provides anchoring points for the formation of the extracellular fibronectin matrix (Weston *et al.* 2003). This TGF β -driven effect was attenuated by blocking CTGF expression with antisense oligonucleotides, demonstrating a key role for the factor in matrix formation. The deposition of an insoluble fibronectin matrix in the mesangium provides a scaffold to which other matrix proteins such as collagens can attach to form a mature fibrotic tissue.

Induction of mesangial cell cycle arrest and hypertrophy

Mesangial cell hypertrophy occurs early in diabetic nephropathy and is due to the cells undergoing cell cycle arrest. The arrest, which occurs in the G1 phase of the cycle, was attributed to TGF β , itself induced by high glucose conditions (Wolf & Ziyadeh 1999). Our subsequent study showed that CTGF induces mesangial cells to enter the G1 phase from their quiescent G0 state and to express cyclin D1 and the cyclin-dependent kinase inhibitors (CDKIs), p15^{INK4}, p21^{Cip1} and p27^{Kip1} which are known to bind and inactivate cyclinD/CDK4/6 and the cyclin E/CDK2 kinase complexes. This in turn leads to the maintenance of pRB protein in its hypophosphorylated state, thereby preventing cell cycle progression. TGF β induces the same events but these are CTGF-dependent because they were attenuated by prior treatment with a CTGF-ASO (Wahab *et al.* 2002).

Prolonging survival of activated cells

It was reported that CTGF induces apoptosis in mesangial cells (Hishikawa *et al.* 2001), but we found no evidence of this. Indeed we reported that CTGF induces rapid synthesis of MAPK phosphatase-1 (MKP-1), an enzyme that dephosphorylates p38MAP kinase, inactivating it (Wahab *et al.* 2007). Inactive p38MAP kinase is unable to phosphorylate the anti-apoptotic protein Bcl-2, protecting it from degradation and allowing its dimerization with Bax, protecting the cell from apoptosis triggered by formation of Bax-Bax homodimers. siRNA depletion of MKP-1 in mesangial cells allowed p38 phosphorylation and under these conditions CTGF did promote apoptosis. In summary, arrest in G1 is likely to be important in the development of fibrosis because the cells are highly active in protein synthesis during this phase of the cycle, including extracellular matrix proteins. Continued production of matrix, as in fibrosis, depends on persistently activated cells, in contrast to wound healing and recovery from injury that requires apoptosis of previously active cells. Connective tissue growth factor is clearly implicated in cycle arrest and the accompanying

elevated protein synthesis and in cell survival in diabetic glomerulosclerosis.

Transition of quiescent renal cells to a myofibroblastic phenotype

Connective tissue growth factor is able to stimulate transition of differentiated cells such as tubular epithelial cells, endothelial cells and interstitial fibroblasts to an activated myofibroblast cell phenotype *in vitro*. In this transition, proteins that are characteristically expressed by the differentiated cell are lost (e.g. E-Cadherin in epithelial cells), whereas proteins that are characteristically expressed by myofibroblasts, such as fibrillar collagens and α -smooth muscle actin, are expressed *de novo* (Fragiadaki & Mason 2011). Myofibroblasts occupy the intertubular spaces in renal interstitial fibrosis, generating the abundant collagenous extracellular matrix which is characteristic of this condition. They also express CTGF which is considered to be a marker of myofibroblasts and fibroproliferative disease (Leask *et al.* 2009). The transition of tubular epithelial cells to myofibroblasts (EMT) was thought to provide an important source of interstitial cells in *in vivo* models of renal interstitial fibrosis (Iwano *et al.* 2002). However, more recent cell lineage studies indicate that pericytes that support peritubular capillary walls are the major source of renal interstitial myofibroblasts in fibrosis disease models (Humphreys *et al.* 2010). Under stimulus of disease factors, pericytes detach from the capillary wall, migrate into the interstitium and undergo transition to myofibroblasts (Schimpf & Duffield 2011). Liu *et al.* (2010) reported that about 85% of myofibroblasts are derived from CTGF-expressing pericytes in a bleomycin-induced model of skin fibrosis. Connective tissue growth factor stimulates the migration of retinal pericytes *in vitro* (Pi *et al.* 2011). We observed that CTGF can induce expression of Snail1 (Fragiadaki *et al.* 2012), a transcriptional regulator which is expressed in pericytes isolated from UUO kidney (Lin *et al.* 2008). Snail1 initiates the expression of fibronectin in epithelial cells and fibroblasts in association with NF- κ B p65 subunit and PARP-1 (Stanisavljevic *et al.* 2011) and overexpression of Snail1 in transgenic mice induces interstitial fibrosis in the kidney (Boutet *et al.* 2006). Collectively these results strongly suggest that CTGF may be a key factor in promoting pericyte migration and transition to interstitial myofibroblasts in renal fibrosis. However, the debate concerning the role of tubular cell EMT as a source of myofibroblasts in renal fibrosis continues. We have reviewed the evidence for and against this recently (Fragiadaki & Mason 2011).

Recruitment of inflammatory cells

As described earlier, intraperitoneal injection of CTGF in mice activated NF- κ B, induced an inflammatory cell infiltrate in the interstitium and stimulated the expression of cytokines in the kidneys. To define this pathway Sanchez-Lopez *et al.* (2009) investigated the effect of CTGF on murine

proximal tubular epithelial cells (MCT cells) and on HK2 cells. NF- κ B was rapidly activated in both cell types, as were the MAP kinases, ERK1/2, p38 kinase and JNK. NF- κ B activation was dependent on the activation of all three kinases as it was blocked by specific kinase inhibitors. Connective tissue growth factor also upregulated expression of MCP-1, ICAM-1 and IL-6 in MCT and HK2 cells in an NF- κ B-dependent manner. These results support the view that CTGF is likely to play an important role in the recruitment of inflammatory cells to the tubulointerstitial compartment and in the induction of chemokines, cytokines and extracellular matrix (ECM) proteins. We observed that CTGF also activates NF- κ B in human mesangial cells, so this pathway is likely to be important in glomerular inflammation and subsequent sclerosis (Wahab and Mason, unpublished results).

Cellular receptors for CTGF and downstream signalling

Exposure of cells to CTGF activates numerous signalling molecules. For example, in mesangial cells ERK1/2, JNK, PKB, CamKII, PKC α and PKC δ are activated, suggesting that CTGF functions via one or more signalling receptors (Wahab *et al.* 2005c). Several CTGF receptors have been reported but none are unique for the cytokine. They include low-density lipoprotein receptor-related protein (LRP-1; Segarini *et al.* 2001), low-density lipoprotein receptor-related protein-6 (LRP-6; Mercurio *et al.* 2003), tropomyosin-related kinase A (TrkA; Wahab *et al.* 2005c), a 280 kDa chondrocyte receptor (Nishida *et al.* 1998) and several adhesion receptors (Jun & Lau 2011) such as β 3 integrin (Crean *et al.* 2002), α V β 3 integrin (Gao & Brigstock 2004), α 5 β 1 integrin (Gao & Brigstock 2005) and α M β 2 integrin (Schober *et al.* 2002). The TGF β type III receptor is also reported to bind CTGF (O'Donovan *et al.* 2012). Heparan sulphate proteoglycans function as co-receptors with integrin receptors (Gao & Brigstock 2004) and LRP-1 (Gao & Brigstock 2003; Jun & Lau 2011). To date, only some of these receptors have been reported in renal cells.

Low-density lipoprotein receptor-related protein-1

Low-density lipoprotein (LDL) receptor-related protein-1, a member of the LDL-receptor family of proteins can bind and endocytose many different proteins such as ECM proteins, growth factors and antigens, in addition to LDL. LRP-1 was proposed as a master regulator of the plasma membrane proteome, adapting it to the prevailing microenvironment by binding other plasma membrane proteins via bridging proteins, followed by endocytosis and lysosomal degradation. Binding of ligands, including extracellular matrix proteins, may also trigger phosphorylation of the cytoplasmic tail of LRP-1, promoting its interaction with intracellular adaptor proteins and downstream cell signalling (Gonias *et al.* 2004).

Low-density lipoprotein receptor-related protein-1 was isolated as a CTGF-binding protein from the bone marrow

stem cell line, BMS2. Radiolabelled CTGF was displaced from BMS2 cells by known ligands of LRP-1 such as the receptor-associated protein (RAP), and another cell line, deficient in LRP-1, was unable to bind CTGF, demonstrating the receptor–ligand relationship. Connective tissue growth factor endocytosis was blocked by RAP, indicating that cellular uptake was mediated by LRP-1 (Segarini *et al.* 2001). Renal fibrosis follows activation of cells to myofibroblasts in the interstitium. CTGF alone had no effect on normal rat kidney fibroblast cells (NRK-49F) but augmented the effect of TGF β in stimulating fibronectin synthesis and *de novo* expression of α SMA, evidence of activation to myofibroblasts (Yang *et al.* 2004). Connective tissue growth factor, but not TGF β , induced phosphorylation of LRP-1 in the NRK-49F cells. RAP inhibited this activation and reduced the synergistic action of CTGF on TGF β -induced α SMA expression. The synergistic action of CTGF was dependent on its activation of the ERK1/2 signalling pathway, although whether this was downstream of LRP-1 phosphorylation or not was not investigated (Yang *et al.* 2004). Others showed subsequently that tissue-type plasminogen activator (tPA) also promoted TGF β -stimulated transition of NRK-49F cells to myofibroblasts. This was dependant on tPA-induced phosphorylation of LRP-1 which led to recruitment of β 1 integrin to form a complex with LRP1, activating integrin-linked kinase (ILK; Hu *et al.* 2007). This kinase can activate several downstream pathways including phosphorylation of Akt. Hu *et al.* also showed that the tPA/LRP-1 pathway is active *in vivo* in the UUO model of renal fibrosis. Moreover *in vivo* inhibition of either ERK1/2 or Akt activation decreased myofibroblast markers in the UUO model, indicating a key role for both signalling pathways in myofibroblast proliferation in interstitial fibrosis (Rodriguez-Pena *et al.* 2008). Collectively these results indicate an important role for LRP-1 activation by different ligands, including CTGF, in triggering signalling involved in renal fibrosis.

Low-density lipoprotein receptor-related protein-6

Connective tissue growth factor-induced phosphorylation of glycogen synthase kinase 3 β (GSK3 β) and increased levels of β -catenin in mesangial cells, which could be blocked by DKK-1, a Wnt signalling pathway antagonist, observations consistent with activation of the canonical Wnt signalling pathway by CTGF (Rooney *et al.* 2011). The authors showed that mesangial cells expressed LRP-6, shown previously to be a Wnt co-receptor that interacts with CTGF (Mercurio *et al.* 2004). Connective tissue growth factor induced serine-phosphorylation of LRP-6 followed by nuclear accumulation of β -catenin and transcriptional activity as measured by a TCF/LEF-luciferase reporter. Rooney *et al.* (2011) identified several upregulated Wnt target genes in human nephropathy biopsies, in UUO, and in streptozotocin-induced diabetes in animal models. However, further research is needed to establish the precise role of the CTGF-activated LRP-6 pathway in mesangial cells. Chronic dysregulated Wnt signalling has been implicated in renal fibrosis,

but Wnt pathways are also involved in healing processes following acute renal injury (Nelson *et al.* 2011).

Tropomyosin-related kinase A

Tropomyosin-related kinase A is a member of the neurotrophin receptor family (TrkA, TrkB and TrkC). Each forms a transmembrane homodimeric receptor tyrosine kinase on binding its ligand. Neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4)] bind to one or more of these receptors, triggering phosphorylation in the cytosolic domain, binding of adaptor proteins and activation of downstream signalling pathways. Tropomyosin-related kinase A is the cognate receptor for NGF. All neurotrophins also bind to a common low-affinity receptor p75^{NTR} that can associate with the Trk receptors, modifying their neurotrophin-binding selectivity (Allen & Dawbarn 2006). Other proteins have been reported to associate directly with Trk receptors, for example, the GPI-linked membrane protein Ephrin A5 to TrkB. In addition, Trk receptors can be transactivated by the Src-related kinase Fyn following the activation of some G-protein coupled receptors such as the A2a adenosine receptor and LRP-1 (Schechterson & Bothwell 2010).

Connective tissue growth factor binds and activates TrkA

We found that CTGF bound to the TrkA/p75^{NTR} receptor in human mesangial cells, inducing phosphorylation at Y490 in the juxtamembrane region of TrkA (Wahab *et al.* 2005c). This is the docking site for the adaptor protein Shc that initiates signalling through the Ras-ERK1/2 and PI3 kinase/Akt (PKB) pathways in other cell types (Chao *et al.* 2006). We also reported that CTGF induced phosphorylation of Y674/675, key sites in TrkA activation for regulating its catalytic activity (Cunningham *et al.* 1997). The TrkA inhibitor K252a blocked CTGF-induced phosphorylation of ERK 1/2, Akt, JNK (Wahab *et al.* 2005c). In a subsequent investigation of the involvement of TrkA in diabetic nephropathy, we showed that high glucose conditions induce CTGF in mesangial cells which activates TrkA and downstream phosphorylation of ERK1/2. These steps were blocked by either siRNA knock-down of either CTGF or TrkA, confirming the specific involvement and importance of CTGF–TrkA-initiated signalling in the response to high glucose (Fragiadaki *et al.* 2012).

Connective tissue growth factor activation of TrkA induces TIEG-1, potentiating TGFβ signalling

Connective tissue growth factor induces TGFβ-inducible early-response gene-1 (TIEG-1) in mesangial cells, an action that was inhibited by K252a and thus likely mediated via TrkA activation (Wahab *et al.* 2005b). This transcriptional repressor binds to the promoter of Smad7, a negative regulator of the TGFβ/Smad signalling pathway, inhibiting its expression and so potentiating signalling (Johnsen *et al.* 2002). Connective tissue growth factor enhanced TGFβ-

induced phosphorylation of Smad2 and 3, their nuclear translocation, increased activity of the SBE4-Luc reporter gene and the transcription of several TGFβ responsive genes in mesangial cells. Antisense oligonucleotide knock-down of TIEG-1 in mesangial cells showed that this CTGF potentiation of TGFβ-stimulated effects was indeed due to induction of TIEG-1 and downregulation of Smad7 expression. Thus, we proposed that under pathological conditions where CTGF expression is elevated the induction of TIEG-1 is a key step in promoting continued activation of the TGFβ signalling pathway (Figure 3).

Tropomyosin-related kinase A expression is induced by hypoxia, a common feature of CKD

Although expressed on mesangial, tubular epithelial and podocyte cells in culture, TrkA expression in normal renal cells *in vivo* is very low. In contrast, it is expressed on both glomerular cells and tubular epithelial cells in diabetic nephropathy (Fragiadaki *et al.* 2012) and in other CKDs associated with glomerulosclerosis and interstitial fibrosis (Bonofiglio *et al.* 2007). We also showed TrkA activation in DN kidney biopsies. It seems likely that TrkA expression is induced in CKD by hypoxia which is commonly present. It has four canonical hypoxia responsive elements in its promoter and hypoxia inducible factor 1α (HIF1α), a transcription factor that binds these elements, accumulates in proximal tubular epithelial cells exposed to either dimethyl-oxaloylglycine (DMOG) or desferrioxamine (DFO), agents which are commonly used to mimic hypoxic changes in cells. Moreover, *in vivo*, TrkA is expressed *de novo* in renal tubular epithelial cells in the hypoxia/reperfusion model of kidney injury (Fragiadaki *et al.* 2012). It is noteworthy that CTGF is also induced by hypoxia (Higgins *et al.* 2004).

CTGF–TrkA signalling may require co-receptors

Mason (2009) speculated that CTGF–TrkA/p75 signalling may require ligation of other cell surface proteins such as integrins and heparan sulphate proteoglycans following clustering in the plasma membrane. Recently, Edwards *et al.* (2011) reported that CTGF binds to a β1 integrin/TrkA plasma membrane complex in glioma tumour-initiating or tumour stem cells, activating NF-κB. In another setting, CTGF induced expression of the chemokines fractalkine, MCP-1 and RANTES in mesangial cells via an ERK1/2- and PI3-K/PKB/NF-κB-dependent pathway. It was proposed that this was initiated by CTGF interaction with TrkA and a heparan sulphate proteoglycan co-receptor as it was inhibited by K252a or heparin (Wu *et al.* 2008).

Connective tissue growth factor and TrkA are endocytosed and may participate in intracellular signalling

Connective tissue growth factor is internalized from the mesangial cell surface in endosomes and accumulates in a

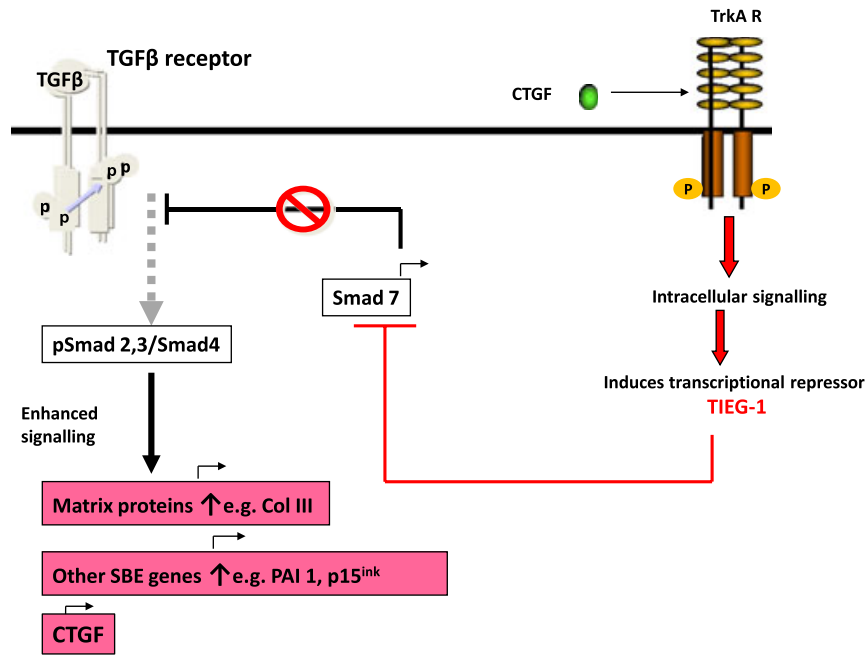


Figure 3 Connective tissue growth factor (CTGF) stimulates the TGF β -Smad signalling pathway. Connective tissue growth factor activation of the tropomyosin-related kinase A (TrkA) receptor induces TIEG-1, a transcriptional repressor of Smad7. Decreased Smad7, a natural repressor of TGF β signalling, promotes increased phospho-Smad2/3, promoting transcription of Smad-responsive genes. TIEG-1, TGF β -inducible early-response gene 1; SBE, Smad binding element.

juxtannuclear organelle. Some CTGF then translocates to the cytosolic compartment where it is phosphorylated by protein kinase C before translocating to the nucleus (Wahab *et al.* 2001b). In unstimulated cells, TrkA is distributed in a punctate pattern over the whole cell and also in a ring-like structure around the nucleus. No p-TrkA is present. Following CTGF stimulation juxtannuclear TrkA becomes more prominent and punctate p-TrkA staining appears in the nucleus within 20 min (Fragiadaki and Mason unpublished results). Taken together these observations suggest that, as well as initiating signalling pathways at the plasma membrane, CTGF/TrkA may participate in complex intracellular signalling mechanisms, first in signalling endosomes (Miaczynska *et al.* 2004) and then possibly within the nucleus itself. The internalization of NGF/TrkA to form signalling endosomes is well established (Grimes *et al.* 1996). Treatment of isolated nuclei with CTGF markedly increased their RNA pool size, but no specific mRNAs encoding collagens, fibronectin, etc., were detected, leading us to conclude that the increased transcription may be attributed to ribosomal RNA (Wahab *et al.* 2001b).

Integrin adhesion receptors

All CCN family members interact with integrins. At least eight different integrins are involved, with Lau and colleagues proposing that CCN proteins mediate their diverse biological actions on many different cell types primarily through interactions with these cell adhesion receptors (Chen & Lau 2009; Jun & Lau 2011). Integrins are

expressed at the sites of focal adhesions and are a family of heterodimeric membrane-spanning proteins that interact with a variety of proteins extracellularly. This initiates conformational changes that promote their assembly with protein complexes on their cytoplasmic domains. These both form bridges to the cytoskeleton of the cell and activate associated signalling kinases such as ILK and focal adhesion kinase (FAK). Integrins participate in both outside-in and inside-out signalling and are involved in many biological functions ranging from cell adhesion to sensing the extracellular environment and mediating cell responses to it. Ligand binding may promote clustering and cross-talk between integrin complexes and receptor tyrosine kinases (Campbell & Humphries 2011; McDonald *et al.* 2011; Millard *et al.* 2011).

Crean *et al.* (2002) reported that CTGF mediates signalling in mesangial cells at least in part through a β 3-integrin-mediated process. Treatment with anti- β 3 antibodies inhibited CTGF-induced activation of ERK1/2 (p42/44) and Akt (PKB) and blocked CTGF-stimulated fibronectin expression. Furthermore, CTGF-induced mesangial cell migration that was dependent on ERK1/2 and Akt signalling was blocked by antibodies against either α 1, α 2, β 3 or α v β 3 integrins. We observed that K252a, a TrkA inhibitor, attenuated CTGF-induced ERK1/2 and Akt phosphorylation (Wahab *et al.* 2005c) and that siRNA depletion of either CTGF or TrkA blocked high glucose-induced ERK1/2 activation (Fragiadaki *et al.* 2012) in mesangial cells. Collectively these results suggest that CTGF activation of these signalling pathways in mesangial cells may involve crosstalk or interaction between integrins and the TrkA receptor, as discussed

earlier. Heparan sulphate proteoglycans may be a further component of such a complex (Gao & Brigstock 2004).

As well as stimulating intracellular signalling, CTGF induces dissolution of focal adhesions in mesangial cells and actin filament disassembly, promoting cell migration *in vitro* and, perhaps more importantly, preventing a contractile response to vasoconstrictors. Hypocontractility is thought to occur in diabetic nephropathy. These changes involve dephosphorylation of FAK and paxillin, an intracellular protein interacting with both the integrin cytoplasmic domain and FAK (Crean *et al.* 2004). Src recruitment and activation follow exposure to CTGF, and the dephosphorylations appear to depend on increased activity of the Src-dependent tyrosine phosphatase, SHP-2. The detailed mechanism underlying this action of CTGF remains to be elucidated.

TGF β receptor III

O'Donovan *et al.* (2012) proposed that CTGF switches TGF β 1 signalling from Smad-dependent to Smad-independent signalling, mediated at least in part by CTGF interaction with TGF β receptor III (TGF β RIII). They reached this conclusion after finding that (i) CTGF co-immunoprecipitated with a V5-TGF β RIII fusion protein expressed in mesangial cells; (ii) treating TGF β -stimulated mesangial cells with CTGF reduced pSmad2 and pSmad3 levels, whilst simultaneously inducing a positive shift toward non-canonical ERK1/2 MAPK signalling; (iii) CTGF also reduced TGF β -transcriptional responses in HeLa cells transfected with the 3TP-Lux reporter gene; (iv) TGF β RIII knock-down with a shRNA reversed these signalling changes; (v) TGF β 1 binding to TGF β 1-receptor in HK2 cells was reduced in the presence of CTGF. These findings are, however, difficult to reconcile with previous reports. For example, CTGF treatment increased p-Smad2, p-Smad3 and transcriptional activity measured by the SBE4-Luc reporter gene and transcription of TGF β -responsive genes in TGF β -stimulated mesangial cells (Wahab *et al.* 2005c). In this study, p-Smad levels were measured in nuclear extracts since they modulate transcription in the nucleus, rather than in the whole cell lysates from which nuclear debris had been removed, used by O'Donovan *et al.* (2012). Secondly, Abreu *et al.* (2002) found that CTGF promoted TGF β binding to its receptor, as discussed earlier. Thirdly, strong evidence to date indicates that CTGF promotes TGF β -Smad signalling *in vivo* to induce fibrosis in models such as the UO model (Yokoi *et al.* 2004) and the subtotal nephrectomy model (Okada *et al.* 2002), also discussed earlier. Thus, the findings by O'Donovan *et al.* (2012) and the role of the CTGF/TGF β RIII interaction require further clarification.

Interactions between CTGF and other cytokines may impact on renal fibrosis

Bone morphogenetic factor-4

Bone morphogenetic factor-4 (BMP4) is a member of the TGF β superfamily. Connective tissue growth factor binds

directly to BMP4 [Kd 5 nM] with a higher affinity than it does to TGF β [Kd 30 nM]. Interaction of BMP4 with its receptor and downstream signalling via Smad1 phosphorylation were decreased in the presence of CTGF (Abreu *et al.* 2002), and this was attributed to direct binding between CTGF and BMP4. However, *in vivo*, although glomerular CTGF is increased in murine and human DN (Riser *et al.* 2000; Wahab *et al.* 2005a) diabetic mice with mesangial matrix expansion have increased glomerular BMP4 expression and p-Smad1 levels (Tominaga *et al.* 2011). Indeed, overexpression of BMP4 in transgenic mice leads to marked glomerulosclerosis and albuminuria, changes akin to those in diabetic nephropathy. Diabetic BMP4^{+/-} mice have reduced glomerulosclerosis compared to BMP4^{+/+} mice, confirming a role for BMP4 in this disease (Tominaga *et al.* 2011). The interaction of CTGF with BMP4 in DN has yet to be explored but it seems unlikely that CTGF significantly suppresses the actions of BMP4, as might be predicted from the findings of Abreu *et al.* (2002).

Bone morphogenetic factor-7

Bone morphogenetic factor-7 is another member of the TGF β superfamily. It also binds to CTGF (Kd 14 nM; Nguyen *et al.* 2008). Following reports that administration of BMP7 reduced glomerulosclerosis in several different animal models of renal fibrosis Wang and Hirschberg (2003) showed that this factor antagonizes TGF β -induced fibrogenic responses in mesangial cells, including reducing fibronectin and type IV collagen accumulation, CTGF expression, PAI-1 promoter activity and MMP2 gelatinase activity. Bone morphogenetic factor-7 is expressed normally in tubular epithelial cells but this is lost during STZ-induced diabetes, as is expression of the BMP type II receptor and the ALK2 type I receptor. Gremlin, a BMP antagonist, increased. This and *in vitro* studies gave rise to the proposal that loss of BMP7 activity is profibrogenic (Wang *et al.* 2001b). Subsequently, Nguyen *et al.* (2008) found that nephropathy was attenuated in diabetic CTGF^{+/-} mice in which glomerular CTGF levels are reduced compared to CTGF^{+/+} mice. Whilst BMP7 signalling was reduced in diabetic CTGF^{+/-} mice as evidenced by decreased p-Smad1/5 levels and expression of the downstream target Id1, this was relatively preserved in the CTGF^{+/+} mice. Moreover, when CTGF was injected into non-diabetic mice, renal p-Smad 1/5 levels fell. *In vitro* studies on renal cells gave similar results (Nguyen *et al.* 2008). Thus, the higher levels of CTGF in DN, and probably in other CKDs, correlate with decreased BMP7-signalling and disease advancement.

Hepatocyte growth factor

Hepatocyte growth factor (HGF) is expressed widely in tissues, including in interstitial and endothelial cells of the kidney. Proximal tubular epithelial cells (PTECs) express the c-Met receptor for HGF. Mice that are transgenic for TGF β 1 under the control of the albumin promoter express

TGF β in the liver and have increased levels of plasma TGF β compared to controls. When these transgenic mice were subjected to 5/6 nephrectomy, CTGF levels in tubular and glomerular epithelial cells in the remnant kidney increased markedly compared to controls, probably as a result of the higher plasma TGF β levels, and the development of interstitial fibrosis was enhanced. Treatment with HGF significantly reduced the CTGF expression and attenuated the expression of collagen and development of fibrosis (Inoue *et al.* 2003). We have summarized previously the likely pathway, involving HGF induction of SnoN and TGIF (Wahab & Mason 2006). *In vitro* experiments with co-cultures of PTECs and a tubulointerstitial fibroblast cell line showed that HGF attenuated TGF β -stimulated CTGF expression in the epithelial cells and reduced collagen expression in the fibroblasts which was dependent, at least in part, on CTGF secreted by the PTEC. Thus, HGF is able to attenuate TGF β -stimulated fibrogenic responses by down-regulating CTGF (Inoue *et al.* 2003).

CCN3

Riser *et al.* (2009) discovered an intriguing relationship between CTGF (CCN2) and Nov (CCN3). CCN3 is expressed in unstimulated mesangial cells, whilst CTGF expression is negligible. Following stimulation with TGF β , CCN3 expression declined and CTGF levels increased, followed by increase in collagen1 transcription and translation. However, addition of either conditioned medium enriched in CCN3 or purified recombinant CCN3 was able to block CTGF and collagen 1 expression in TGF β -stimulated cells, suggesting a reciprocal or 'ying-yang' relationship between the two CCN proteins. The mechanism of CCN3 inhibition of CTGF expression is unknown but is not due to downregulation of Smad3 signalling. The authors hypothesize that CCN3 is an endogenous negative regulator of ECM and fibrosis. Thus, in normal wound healing, CCN3 may be expressed at the stage of resolution, switching off CTGF and extracellular matrix synthesis, whilst in fibrosis CCN3 levels remain low and CTGF continues to stimulate ECM production.

Oncostatin M

Oncostatin M (OSM) belongs to the IL-6 family of cytokines. We proposed that it may play a role in renal interstitial inflammation, reporting that it was expressed by activated peripheral blood mononuclear cells, that media conditioned by these cells induced the expression of the OSM-specific receptor β -subunit in human PTECs, and that OSM activated the Jak/Stat signalling pathway in these cells, which subsequently underwent EMT *in vitro* (Nightingale *et al.* 2004). Pollack *et al.* (2007) later speculated that OSM may be able to act as either a pro-fibrosis cytokine or, conversely, as a pro-healing cytokine, depending on the prevailing cellular microenvironment or nature of the cellular injury. They went on to report that OSM attenuated TGF β -

induced expression of CTGF and several other matricellular proteins in human PTEC. This effect was at least partly dependent on OSM-receptor-mediated Stat1/Stat3 signalling and independent of the Smad2/3 pathway (Sarkozi *et al.* 2011). Investigations into the relationship between OSM and CTGF *in vivo* would be of interest, especially whether OSM is able to suppress matricellular protein expression by PTECs in renal inflammation.

In summary, following tissue damage, the local concentrations of various cytokines acting on CTGF are likely to contribute importantly to whether injury is followed by healing and resolution, or by fibrosis.

Other factors that may impact on CTGF expression and renal fibrosis

MicroRNAs

MicroRNAs (miRNAs) are short non-coding RNAs which inhibit the expression of target genes. When dicer, an enzyme required to generate miRNAs, was selectively deleted in murine podocytes the mice developed proteinuria and glomerular lesions including glomerulosclerosis, demonstrating the importance of miRNAs in maintaining normal kidney structure and function (Shi *et al.* 2008). MicroRNAs are clearly involved in regulating TGF β expression and fibrosis in the kidney (Patel & Noureddine 2012). Information about whether miRNAs are involved in regulating CTGF expression in the kidney is lacking. However, two miRNAs (miR-133 and miR-30c) that interact with the 3' untranslated region of the CTGF transcript and are negative regulators of CTGF expression have been implicated in the pathophysiology of rodent and human left ventricular hypertrophy (LVH)/cardiac fibrosis (Duisters *et al.* 2009). Both miRNAs are down-regulated in LVH with concomitant increases in CTGF. Collectively these results suggest there may be an important role for miRNA regulation of CTGF expression in the development of kidney fibrosis.

Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) occurs in the CTGF promoter at G-945C. The GG genotype occurred more frequently in patients with systemic sclerosis than in controls in a large UK study (Fonseca *et al.* 2007). Moreover, the authors observed that the C allele has a high affinity for Sp3, a transcriptional regulator, and is associated with reduced transcriptional activity, repressing CTGF expression. However, a further large study on subjects of wide European origin did not find any significant association of this genetic variant with systemic sclerosis (Rueda *et al.* 2009). To date, there is little information concerning the possible effects of CTGF SNPs and susceptibility to renal fibrosis, although a study on the G-945C polymorphism in patients with diabetes with and without evidence of nephropathy did not find any correlation between the polymorphism and renal disease or the plasma CTGF level

(Dendooven *et al.* 2011). Further studies on CTGF SNPs and the onset and progression of renal fibrosis in different kidney diseases are required to reveal whether such SNPs are important or not.

Concluding comments

A large body of evidence implicates CTGF in the development of renal fibrosis. This comes from both *in vivo* and *in vitro* experiments, from the investigation of naturally occurring diseases in which kidney fibrosis develops, from animal models and from experiments with renal cells. Connective tissue growth factor is involved in the early stages of inflammation before overt fibrosis and at later stages when it is well established. Connective tissue growth factor is a pleiotropic player in these processes, its actions ranging from receptor activation inducing cell signalling and *de novo* gene transcription, to participating in a delicate 'yin-yang' balance with other cytokines and growth factors, steering injured tissues towards fibrosis if CTGF predominates or healing if it does not. A recurrent but not exclusive theme is the role of CTGF acting in a cooperative manner with TGF β to promote fibrotic responses. Overall its effects in adult tissues are pernicious. The evidence points to CTGF, its receptors, and the signalling pathways initiated by their activation, providing a range of targets for new therapeutic interventions in fibrotic kidney diseases. Current therapies for fibrotic diseases have very limited success. Reaching a complete understanding of mechanisms that control the yin-yang balance between CTGF and other factors, including miRNAs, in determining fibrosis or healing, the ways in which TGF β and CTGF work together or not, and the relative importance of various receptors for CTGF and how they work cooperatively together, or independently, are likely to provide key challenges in developing successful new therapies to counter the development of renal fibrosis.

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Conflict of interest

None.

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