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Connective tissue growth factor antagonizes transforming growth factor-β1/Smad signalling in renal mesangial cells

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The critical involvement of TGF-β1 (transforming growth factor-β1) in DN (diabetic nephropathy) is well established. However, the role of CTGF (connective tissue growth factor) in regulating the complex interplay of TGF-β1 signalling networks is poorly understood. The purpose of the present study was to investigate co-operative signalling between CTGF and TGF-β1 and its physiological significance. CTGF was determined to bind directly to the TβRIII (TGF-β type III receptor) and antagonize TGF-β1-induced Smad phosphorylation and transcriptional responses via its N-terminal half. Furthermore, TGF-β1 binding to its receptor was inhibited by CTGF. A consequent shift towards non-canonical TGF-β1 signalling and expression of a unique profile of differentially regulated genes was observed in CTGF/TGF-β1-treated mesangial cells. Decreased levels of Smad2/3 phosphorylation were evident in STZ (streptozotocin)-induced diabetic mice, concomitant with increased levels of CTGF. Knockdown of TβRIII restored TGF-β1-mediated Smad signalling and cell contractility, suggesting that TβRIII is key for CTGF-mediated regulation of TGF-β1. Comparison of gene expression profiles from CTGF/TGF-β1-treated mesangial cells and human renal biopsy material with histological diagnosis of DN revealed significant correlation among gene clusters. In summary, mesangial cell responses to TGF-β1 are regulated by cross-talk with CTGF, emphasizing the potential utility of targeting CTGF in DN.

Key words: connective tissue growth factor (CTGF), p42/44 MAPK (mitogen-activated protein kinase), signalling cross-talk, Smad signalling, transforming growth factor-β (TGF-β), transforming growth factor-β1 receptor.

INTRODUCTION

CCN proteins (cysteine-rich angiogenic inducer 61, connective tissue growth factor, nephroblastoma overexpressed) comprise a family of homologous matricellular proteins that regulate diverse cell processes [1]. CTGF (connective tissue growth factor)/CCN2 was identified as a pro-fibrotic mediator in both *in vivo* and *in vitro* models of DN (diabetic nephropathy) [2,3], where it modulates matrix accumulation, cell migration and reorganization of the actin cytoskeleton [4,5], paralleling pathogenic alterations to the mesangium during the progression of nephropathy. Structurally, CTGF is characterized by four functional domains [6], reflecting the widely accepted view that CTGF functions as a matricellular protein, modulating and integrating other signalling networks. Previous studies have identified an interaction between CTGF and certain TGF-β (transforming growth factor-β) superfamily ligands which leads to altered cellular function [7,8], suggesting that there is a requirement for co-operation between these and other pro-fibrotic agents in the progression of fibrosis.

The actions of TGF-β in mammalian cells are mediated by two distinct serine/threonine kinase receptors, the TβRI (TGF-β type I receptor) and the TβRII (TGF-β type II receptor) [9]. Recent years have seen an increasing focus on the regulation of ligand receptor interaction by other extracellular molecules and receptors, including the TβRIII (TGF-β type III receptor, also known as betaglycan), which binds to TGF-β with high affinity [10].

Although the Smad canonical pathway accounts for many effects of TGF-β signalling, it does not readily explain how TGF-β signalling generates pleiotropic responses. It has long been accepted that the proteomic composition of a cell influences the cellular response to TGF-β signalling [11]. These 'context-dependent' events control the output response of the canonical Smad2/3-dependent pathway and the activation of Smad2/3-independent pathways that regulate additional TGF-β responses [9], yet remain critically undefined in DN.

In the present paper we demonstrate that CTGF binds to TβRIII and negatively regulates TGF-β1 Smad-dependent signalling and transcriptional activity in human mesangial cells. We show that the VWC (Von Willibrand repeat type C) domain/domain 2 of CTGF mediates the regulation of TGF-β signalling responses. We also demonstrate a subsequent switch towards non-canonical signalling and a consequential unique

Abbreviations used: CCN, cysteine-rich angiogenic inducer 61, connective tissue growth factor, nephroblastoma overexpressed; CTGF, connective tissue growth factor; Δ1 CTGF, CTGF with domain 1 deleted; Δ2 CTGF, CTGF with domain 2/VWC domain deleted; Δ3 CTGF, CTGF with domain 3 deleted; Δ4 CTGF, CTGF with domain 4 deleted; C-CTGF, C-terminal-half CTGF; DMEM, Dulbecco's modified Eagle's medium; DN, diabetic nephropathy; ERK, extracellular-signal-regulated kinase; FDR, false discovery rate; FL CTGF, full-length CTGF; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grb2, growth-factor-receptor-bound protein 2; GSEA, gene-set enrichment analysis; HK2, human kidney proximal tubule; HMC, human mesangial cell; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; KD, kinase dead; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; N-CTGF, N-terminal-half CTGF; rhCTGF, recombinant human CTGF; shRNA, short hairpin RNA; Shc, Src homology and collagen homology; STZ, streptozotocin; TAK1, transforming growth factor-β-activated kinase 1; TGF-β, transforming growth factor-β; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; TβRIII, TGF-β type III receptor; TRAF6, tumour-necrosis-factor-receptor-associated factor 6; VWC, Von Willibrand repeat type C.

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profile of differentially expressed genes in CTGF and TGF-β1 co-treated mesangial cells. A number of these genes were confirmed to be similarly differentially expressed in biopsies from DN patients. Cross-talk between CTGF and TGF-β1 has likely pathophysiological consequences for the progression of glomerulosclerosis, highlighting the importance of targeted therapeutic strategies.

EXPERIMENTAL

Reagents and antibodies

Antibodies against phospho-p44/42 MAPK (mitogen-activated protein kinase; Thr^{202}/Tyr^{204}), total p44/42 MAPK, phospho-Smad2 (Ser^{465}/Ser^{467}), total Smad2, phospho-Smad3 (Ser^{423}/Ser^{425}), total Smad3, phospho-Smad2 ($Ser^{245}/Ser^{250}/Ser^{255}$), phospho-Smad1 ($Ser^{463}/Ser^{465}$)/Smad5 ($Ser^{463}/Ser^{465}$)/Smad8 ($Ser^{426}/Ser^{428}$), total Smad5 and T$\beta$RIII (#2519) were from Cell Signaling Technology. The anti-αV5 antibody was from Invitrogen. The anti-[p-Smad2/3 (Ser^{423}/Ser^{425})] and anti-CTGF (SC-L20) antibodies were from Santa Cruz Biotechnology. Anti-CTGF D2, a fully human IgG1κ monoclonal antibody which recognizes amino acids 142–157 of CTGF [12], and anti-CTGF D1, which recognizes domain 1 of CTGF, were from FibroGen. Anti-rabbit/mouse HRP (horseradish peroxidase)-conjugated secondary antibodies were from Promega. All other reagents were purchased from Sigma–Aldrich unless otherwise stated.

Preparation of recombinant human CTGF, and N-terminal and C-terminal CTGF

RhCTGF (recombinant human CTGF), N-CTGF (N-terminal-half CTGF) and C-CTGF (C-terminal-half CTGF) were expressed and purified from baculovirus-infected insect cells [13]. The purity of rhCTGF, N-CTGF and C-CTGF was assessed by immunoblotting with specific antibodies.

Cell culture

Primary HMCs (human mesangial cells; Clonetics) were maintained in MCDB-131 medium (Gibco); HK2 (human kidney proximal tubule) cells were maintained in DMEM (Dulbecco's modified Eagle's medium)/Hams F12 medium and HeLa cells were maintained in DMEM. MCDB-131 and DMEM media was supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 10 mM L-glutamic acid and 5 mg/ml penicillin/streptomycin, whereas DMEM/Hams F12 medium was supplemented with 10 mM L-glutamic acid, 5 mg/ml penicillin/streptomycin, 1× ITS Liquid Media Supplement, 10 ng/ml EGF (epidermal growth factor), 36 ng/ml hydrocortisone and 3 pg/ml tridothyronine. Cells were maintained at 37 °C in a humidified 95/5 % air/CO_2 atmosphere. Following serum-starvation for 24 h, cells were treated with rhCTGF (25 ng/ml or 0.7 nM), TGF-β1 (10 ng/ml or 0.2 nM) or both together for the indicated times or with rhCTGF, N-CTGF (12.5 ng/ml) or C-CTGF (12.5 ng/ml) for 30 min. MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]/p42/44 MAPK, Src and TβRI activity was inhibited by adding PD98059 (5–10 μM; Merck), PP2 (10 μM; Enzo Life Sciences) and SB431542 (10 μM; Sigma) respectively to the cells for 1 h prior to treatment. The Src family kinase inhibitor PP2 is potent, reversible, ATP-competitive and a selective inhibitor of the Src family of protein tyrosine kinases. Recent findings have also shown that it can have off-target effects on the TβRI [13a].

Viral transduction of HMCs

Cells were seeded at 5×10^4 cells per 60 mm tissue culture dish and transduced with control shRNA (short hairpin RNA; catalogue number sc-42224-V, Santa Cruz Biotechnology) or a pool of three TβRIII shRNAs (multiplicity of infection = 30; catalogue number sc-108080, Santa Cruz Biotechnology) in medium containing polybrene (5 μg/ml). The medium was replaced 24 h after transfection with medium without polybrene. Transduced cells were selected for puromycin (8 μg/ml) resistance, expanded and screened for expression of TβRIII by Western blotting and quantitative PCR.

Preparation of cellular protein extracts and Western blotting

Protein extracts were prepared in lysis buffer consisting of Tris/HCl, pH 7.5 (50 mM), sodium deoxycholate (0.25 %), NaCl (150 mM), EGTA (1 mM), NaF (1 mM), Igepal CA-630 [1 % (v/v)], PMSF (1 mM), protease inhibitor cocktail (1×) and phosphatase inhibitor cocktail. After incubation at 4 °C for 20 min, nuclear and cellular debris were removed by centrifugation at 20000 ***g*** for 20 min at 4 °C. Protein was quantified by Bradford assay (Bio-Rad Laboratories). Samples were resolved by SDS/PAGE, transferred on to nitrocellulose and blocked for 1 h in TBS-T (Tris-buffered saline containing 0.05 % Tween 20) and 5 % (w/v) skimmed milk. Primary antibody incubations were performed overnight at 4 °C and HRP-conjugated secondary antibody incubations were at room temperature (25 °C) for 1 h. Densitometry analysis of band intensity was performed using Scion Image Version 4.0.

TGF-β1 receptor-binding assay

The TGF-β1 receptor-binding assay was carried out as per the manufacturer's instructions (NFTG0, R&D systems). Briefly, HK2 cells were detached using accutase (Sigma), washed in dPBS (Dulbecco's PBS) and resuspended to a final concentration of 4×10^6 cells/ml. Biotinylated TGF-β1 (7.5 nM) in the presence or absence of rhCTGF (10.9 nM) was incubated with the washed cells (1×10^5) for 60 min at 4 °C. Avidin–FITC (10 μl) was added and incubated at 4 °C in the dark for 30 min. Cells were washed with 1× RDF1 buffer (R&D Systems), and resuspended in 1× RDF1 buffer for flow cytometric analysis. Cells with TGF-β1 bound to its receptor are fluorescently stained and can be quantified (percentage positively stained cells) by analysis of fluorescence at 488 nm by flow cytometry.

3TP-lux luciferase assay

Readily transfectable HeLa cells were grown to ~60 % confluence and transiently co-transfected with *Renilla* luciferase (hereafter called *Renilla*) (0.1 μg) and 3TP-lux (0.4 μg) using Fugene 6 (Invitrogen). Cells were treated with rhCTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both together for 24 h or were co-transfected with the mammalian expression vector pDEST40 (Invitrogen) containing either FL CTGF (full-length CTGF), Δ1 CTGF (CTGF with domain 1 deleted), Δ2 CTGF (CTGF with domain 2/VWC domain deleted), Δ3 CTGF (CTGF with domain 3 deleted) or Δ4 CTGF (CTGF with domain 4 deleted), followed by treatment with TGF-β1. Δ1, Δ2 and Δ3 CTGF cDNA were obtained by loop-out mutagenesis of FL CTGF cDNA. Δ4 CTGF was created by introduction of a stop codon after domain 3. 3TP-lux promoter reporter activity was measured using a dual luciferase kit (Promega).

TβRII KD (kinase dead) transfection of HMCs

Mesangial cells were seeded on to 10 cm tissue culture dishes and were allowed to reach 80 % confluency before being transfected with 10 μg of KD TβRII (Addgene plasmid 11762) [14] using Fugene HD (Promega) in serum-free medium. After 24 h, the cells were treated with rhCTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both together for 15 min.

Motility assay

Mesangial cells were seeded at a density of 5×10^4 cells per well of a six-well plate, grown to 90 % confluence and rendered quiescent by starving them from serum for 24 h. A wound was scratched using a sterile pipette tip, and the cells were washed once to remove debris and then re-incubated with medium containing either rhCTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both together for 24 h. Movement was assessed by microscopic examination at 0 and 24 h and multiple fields were photographed with a Nikon TMS microscope equipped with a video camera (JVC).

Adhesion assay

96-well culture plates were left uncoated or were coated with CTGF (25ng/ml), TGF-β1 (10ng/ml), both CTGF and TGF-β1 combined or fibronectin as a positive control for adhesion at 4 °C overnight. Human mesangial cells were then seeded on to plates at 1×10^5 cells per well and allowed to attach for 50 min. The wells were washed three times with PBS, fixed, permeabilized and stained with Hoechst for 1 min. Cells were counted in a field of $\times$40 magnification and the average number of adherent cells per field in eight fields were plotted.

Quantitative RT-PCR

Total DNaseI-treated RNA (2 μg) was reverse transcribed using random hexamers and Superscript II (Invitrogen). RT-PCR was performed on an Applied Biosystems 7900HT fast real-time system using gene-specific primers and SybrGreen. The primers were: TβRIII forward, 5′-CCAAGATGAATGGCACACAC-3′; TβRIII reverse, 5′-GATTTCAGGTCGGGTGAACAG-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward, 5′-CAATGACCCCTTCATTGACC-3′; and GAPDH reverse, 5′-CTAGACGGCAGGTCAGGTC-3′. Gene expression was normalized to GAPDH.

Construction of V5–TβRIII

TβRIII ORFEXPRESS™-Shuttle Clone (GeneCopoeia) was inserted into pcDNA3.1/nV5-DEST (Invitrogen) using a Gateway LR Clonase reaction as described in the manufacturer's instructions (Invitrogen).

Immunoprecipitation assay

Mesangial cells were grown to ~80 % confluence and transiently transfected with V5–TβRIII (10 μg) using Fugene HD (Roche). Cells were scraped into 300 μl of PBS and left untreated or incubated with CTGF (1 μg), or CTGF and TGF-β1 (100 ng) for 1 h. Cells were pelleted and lysed in CelLytic™ M cell lysis reagent containing PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail. After incubation at 4 °C for 20 min, nuclear and cellular debris were removed by centrifugation at 20 000 ***g*** for 20 min at 4 °C. Cell lysates were incubated with anti-V5 agarose (clone V5-10) for 90 min on an orbital shaker. The resin was washed four times with PBS, 2$\times$ SDS sample buffer (20 μl) was added to the beads and samples were resolved by SDS/PAGE (10 % gel).

CTGF/TGF-β1 human genome array

Mesangial cells were starved for 24 h followed by treatment with TGF-β1 (10 ng/ml), CTGF (25 ng/ml) or both together. Total RNA was reverse-transcribed, fragmented and hybridized to an Affymetrix human genome U133 plus 2.0 array (Affymetrix). Data from replicates of three arrays per experimental condition were normalized by GC Robust Multi-array. Average before a linear model was applied, and differentially expressed genes at $P < 0.05$ were identified using a modified Student's *t* test and Benjamini–Hochberg correction for multiple testing.

Human DN biopsy gene array

Renal biopsies were collected in a multicentre study, the ERCB (European Renal cDNA Bank), after informed consent was obtained and local ethics approval. Clinical and histological patient characteristics were described by Schmid et al. [15]. Total RNA was reverse-transcribed, fragmented and hybridized to an Affymetrix human genome array U133. Subsequently, RMA (robust multichip analysis) was performed. Expression data from DN biopsies were compared with the control, and signal log ratios were used to generate a heat map using Hierarchical Clustering Explorer 3.5.

Gene-set enrichment analysis

Gene-set enrichment analysis was performed on a ranked list of probes and two gene sets were derived from the top 50 overexpressed and top 50 underexpressed genes from Nephromine (http://www.nephromine.org). Probes were ranked on the basis of fold change of the combination treatment (TGF-β1 and CTGF) over the control. The analysis was conducted using the open source GSEA (gene-set enrichment analysis) v2.0 software package (http://www.broadinstitute.org/gsea). Significance thresholds were set at a nominal $P < 0.05$ and a false discovery rate <0.25 as recommended by the Broad Institute and the GSEA software developers.

STZ (streptozotocin)-induced diabetes in mice

Procedures were licensed by the Irish Department of Health and approved by the local animal research ethics committee. C57BL/6J mice were treated with STZ dissolved in 100 mM citrate buffer (pH 4.5) or treated with citrate buffer alone (http://www.diacomp.org). Diabetes was confirmed by two consecutive daily measurements of fasting blood glucose >15 mmol/l two weeks after STZ injection. Mice were killed at 18 and 27 weeks of hyperglycaemia. Portions of the renal pole were lysed in 50 mM Tris/HCl (pH 7.4), 1 % (v/v) Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl and 1 mM EDTA, supplemented with 1 mM PMSF, 1$\times$ protease inhibitor cocktail, 1 mM NaF, 40 mM β-glycerophosphate, 2 μM microcystine and 1 mM sodium vanadate. This model of diabetes was verified and published previously [16]. These lysates were then used to examine Smad2/3 phosphorylation by Western blot analysis and CTGF expression by RT-PCR.

RESULTS

CTGF antagonizes TGF-β mediated Smad activity

Evidence for an interaction between CTGF and TGF-β [7] led us to explore signalling networks regulated by CTGF

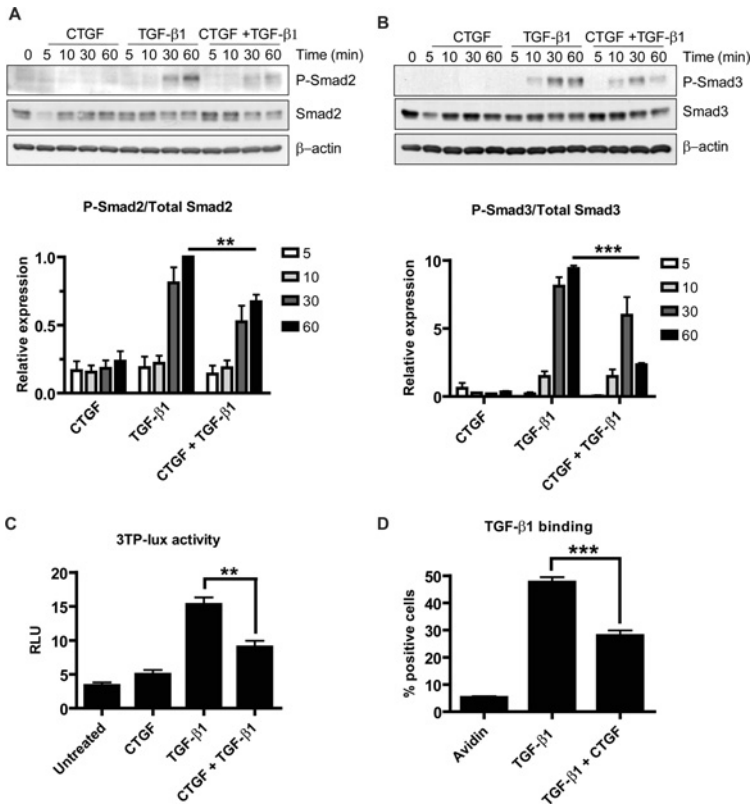


Figure 1 CTGF inhibits TGF-β1-induced Smad2/3 phosphorylation, 3TP-lux promoter activity and TGF-β1 receptor binding

HMCs were serum-starved for 24 h followed by treatment with CTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both CTGF and TGF-β1 together for the times indicated. Smad2 phosphorylation (P-Smad2) (**A**) and Smad3 phosphorylation (P-Smad3) (**B**) was determined by Western blotting. Densitometry was performed using Scion Image and statistical analysis calculated with GraphPad Prism software. (**C**) HeLa cells were transiently co-transfected with *Renilla* and the 3TP-lux promoter reporter plasmid. The cells were treated with CTGF, TGF-β1 or both CTGF and TGF-β1 together 24 h later for a further 24 h. Relative 3TP-lux activity in luciferase units (RLU) was obtained using *Renilla* as an internal control. (**D**) HK2 cells were stained with biotinylated TGF-β1 (7.5 nM) in the absence or presence of CTGF (10.9 nM) followed by fluorescein-conjugated avidin. Cells with biotinylated TGF-β1 bound to its receptor (positively stained cells) emitted fluorescence at 488 nm which was determined by flow cytometric analysis. Results are the percentage of positively stained cells $\pm$ S.E.M. and are representative of three individual experiments. $^{**}P < 0.01$; $^{***}P < 0.005$ (one-way ANOVA).

and TGF-β1. Mesangial cells were treated with CTGF (25ng/ml), TGF-β1 (10 ng/ml) or both together. CTGF had no effect on Smad phosphorylation compared with untreated cells, but antagonized TGF-β1-induced phosphorylation of Smad2 (Figure 1A) and Smad3 (Figure 1B). This effect was not dose dependent (results not shown). CTGF had no effect on TGF-β1-induced Smad1/5/8 phosphorylation (Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410499add.htm).

The antagonistic effect of CTGF on TGF-β1 was also demonstrated using a promoter reporter activity assay. Co-treatment with rhCTGF and rhTGF-β1 decreased TGF-β1-induced 3TP-lux activity (Figure 1C). Furthermore, CTGF decreased binding of TGF-β1 to its receptor by approximately 40 % (Figure 1D) in HK2 renal cells. Mesangial cell expression of TβRII was too low to decipher meaningful binding of TGF-β1. Collectively, these findings indicate that CTGF inhibits TGF-β1 receptor-binding and regulates Smad2/3 phosphorylation and downstream transcriptional activity.

To determine whether these effects can be ascribed to a specific domain, we generated baculovirus constructs encoding N-terminal and C-terminal half deletion mutant proteins (Figure 2A). The N-CTGF and C-CTGF mutant proteins were shown to be pure when run on a gel (Figure 2B). Mesangial cells were then treated with full-length rhCTGF, N-CTGF or C-CTGF in the absence or presence of TGF-β1. Full-length CTGF and N-CTGF inhibited TGF-β1-induced Smad2/3 phosphorylation, whereas C-CTGF had no effect (Figure 2C). This suggests that the N-terminal half of CTGF mediates the inhibition of TGF-β1 activity.

An interaction between CTGF and TGF-β1 has been proposed to occur via the CR/VWC repeat [7]. To test if this domain of CTGF was responsible for its inhibition of TGF-β1 activity, we generated constructs expressing FL CTGF, Δ1 CTGF, Δ2 CTGF, Δ3 CTGF or Δ4 CTGF (Figure 2D). Following transfection of these mutant constructs, CTGF was expressed in the cell lysates (Figure 2E, left-hand panel) and secreted into the medium (Figure 2E, right-hand panel) for all of the constructs. Secretion of CTGF after expression of the constructs was also verified by ELISA (results not shown). Each of these constructs was co-overexpressed with the 3TP-lux promoter reporter and the internal control renilla and then stimulated with TGF-β1. Overexpression of FL CTGF, Δ3 CTGF and Δ4 CTGF decreased TGF-β1-activated 3TP-lux activity (Figure 2F). However, deletion of domain 1 and domain 2 of CTGF prevented this inhibition of TGF-β1-activated 3TP-lux activity (Figure 2F), implicating both domains of the N-terminus of CTGF in the regulation of TGF-β1 activity.

CTGF causes a shift towards non-canonical TGF-β1 cell signalling

Both CTGF [17] and TGF-β1 [18] have been shown previously to activate p42/44 MAPK. We were interested in investigating the effect on p42/44 MAPK as it has been shown to regulate Smads

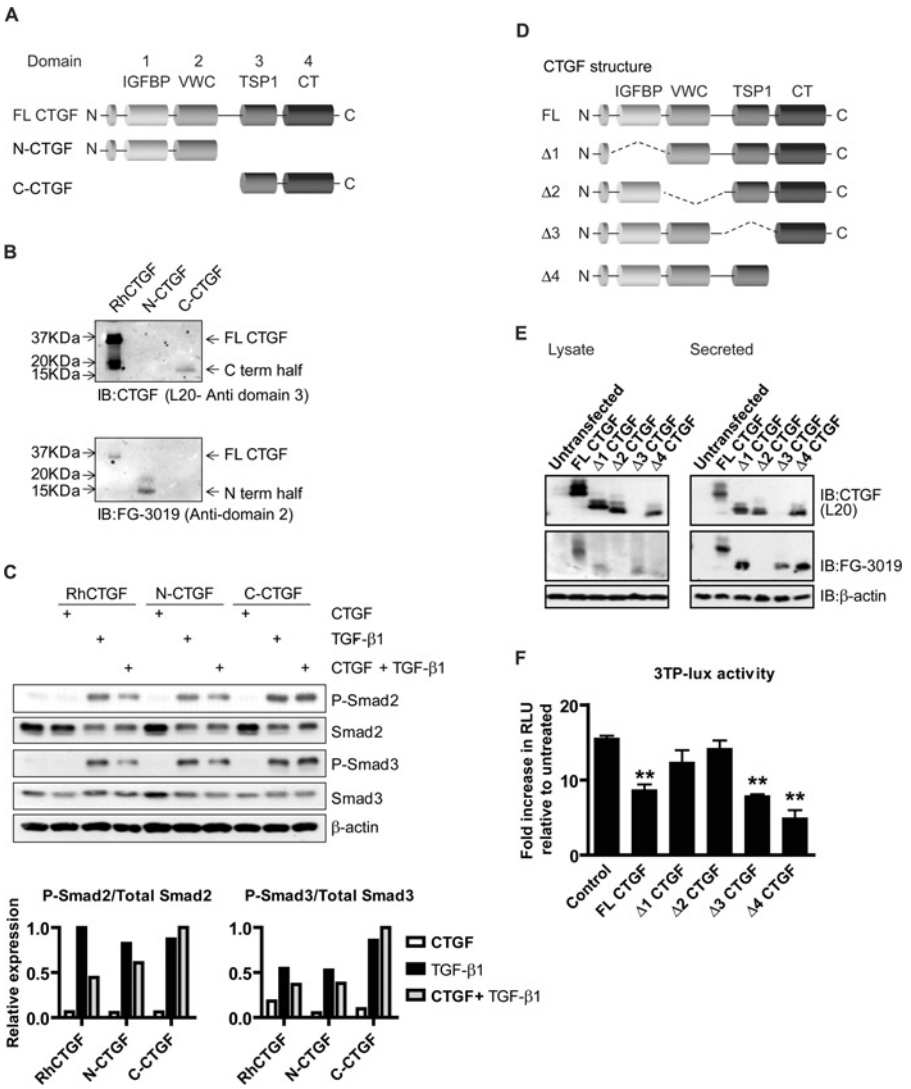


Figure 2 CTGF-mediated abrogation of TGF-β1 activity is mediated by the N-CTGF

(**A**) Illustration of N-CTGF and C-CTGF structure. (**B**) Recombinant FL CTGF and N- and C-CTGF are pure preparations. FL CTGF and N- and C-CTGF fragments of CTGF were resolved by SDS/PAGE (10 % gel) and were immunoblotted (IB) with antibodies directed against domain 3 in the C-terminus (anti-CTGF SC-L20) and domain 2 in the N-terminus (anti-FG-3019) of CTGF. FL CTGF was detected by both antibodies. (**C**) HMCs were serum-starved for 24 h followed by treatment with full-length recombinant (rh) CTGF (25 ng/ml), N-CTGF (12.5 ng/ml) or C-CTGF (12.5 ng/ml) for 1 h. Smad2 and Smad3 phosphorylation levels were analysed by Western blotting. Densitometry was performed using Scion Image software. (**D**) Illustration of full-length (FL) CTGF and Δ1, Δ2, Δ3 and Δ4 CTGF structure. (**E**) Expression of FL, Δ1, Δ2, Δ3 and Δ4 CTGF was tested by transfecting cells with the precursor clones and lysing whole cell lysates 24 h post transfection (left-hand side) or collecting conditioned medium containing 100 μg/ml heparin (right-hand side) at 48 h post-transfection. CTGF expression levels were detected using CTGF (sc-L20) and FG-3019 antibodies. (**F**) HeLa cells were transiently co-transfected with FL, Δ1, Δ2, Δ3 and Δ4 CTGF expression constructs, *Renilla* and the 3TP-lux reporter plasmid. Twenty four hours later, the cells were left untreated or treated with TGF-β1 (10 ng/ml) for a further 24 h. Relative 3TP–lux activity in luciferase units was obtained using *Renilla* as an internal control. Results are graphed as relative fold increase in luciferase units relative to untreated $\pm$ S.E.M. Results are representative of three individual experiments, $^{**}P < 0.01$ using one-way ANOVA. CT, C-terminal; IB, immunoblot; IGFBP, IGF-binding protein; TSP1, thrombospondin-1.

through phosphorylation of the linker region of Smad [19,20] or through cross-talk between the signalling pathways [21]. To first determine whether p42/44 MAPK phosphorylation changed upon co-treatment with CTGF and TGF-β1, mesangial cells were arrested for 24 h followed by treatment with CTGF, TGF-β1 or both together for the times indicated. Both CTGF and TGF-β1 increased phosphorylation of p44/44 MAPK (Figure 3A). Interestingly, however, p42/44 MAPK phosphorylation was markedly increased upon co-stimulation with CTGF and TGF-β1 for up to 30 min (Figure 3A).

We next investigated if this increase was mediating the decrease in Smad phosphorylation observed in the CTGF/TGF-β1-treated cells. We found, however, that there was no change in Smad2 phosphorylation in its linker region when cells were treated with both CTGF and TGF-β1 (Figure 3B). In addition, cells were pretreated with the MEK inhibitor PD98059 for 1 h followed by co-treatment with CTGF and TGF-β1 to determine if reducing p42/44 MAPK phosphorylation could rescue Smad signalling. PD98058 inhibited p42/44 MAPK phosphorylation as expected; however it had no effect on Smad2 phosphorylation (Figure 3C) and Smad3 phosphorylation (Figure 3D) in co-treated cells, suggesting that TGF-β1-mediated Smad2/3 phosphorylation is not regulated by p42/44 MAPK.

We have shown previously that Src is required for CTGF-induced p42/44 MAPK phosphorylation [17] and others have demonstrated that the Src family tyrosine kinase inhibitor PP2

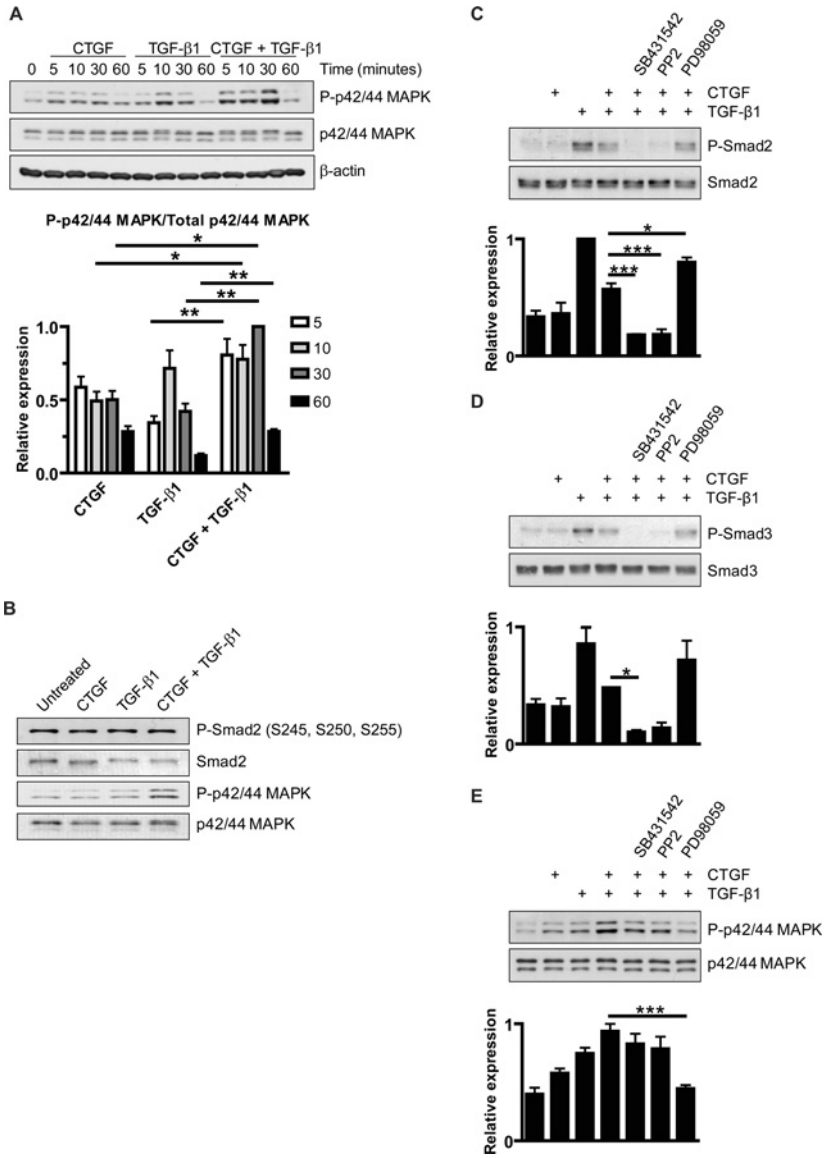


Figure 3 Co-treatment of mesangial cells with CTGF and TGF-β1 induces a shift towards non-canonical signalling

HMCs were serum-starved for 24 h followed by treatment with CTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both CTGF and TGF-β1 together for the indicated times. (**A**) The lysates were analysed by Western blotting using P-p42/44 MAPK and total p42/44 MAPK. β-Actin was used as a loading control. Densitometry was performed using Scion Image and statistical analysis was calculated with GraphPad Prism software. (**B**) Cells were treated with CTGF, TGF-β1 or both CTGF and TGF-β1 together for 30 min. Smad2 linker region [P-Smad2 (Ser^{245}, Ser^{250} and Ser^{255}) and p42/44MAPK phosphorylation was determined by Western blotting. (**C**–**E**) Following serum starvation, cells were pretreated with the TβRI inhibitor SB431542 (10 μM), the Src inhibitor PP2 (10 μM) and the MEK inhibitor PD98059 (20 μM) for 1 h. Cells were then treated with CTGF, TGF-β1 or both together for 30 min. The lysates were analysed by Western blotting using P-Smad2 and total Smad2 (**C**), P-Smad3 and total Smad3 (**D**) and P-p42/44 MAPK and total p42/44 MAPK (**E**). Results are $\pm$ S.E.M. and representative of three individual experiments. Densitometry was performed using Scion Image and statistical analysis was calculated with GraphPad Prism software. $^{*}P < 0.05$;$^{**}P < 0.01$;$^{***}P < 0.005$ (one-way ANOVA).

inhibits TGF-β1-induced p42/44 MAPK phosphorylation [22]. To investigate the cross-talk between Smad2/3 and p42/44 MAPK and determine if Src is playing a role in the regulation of Smad or p42/44 MAPK signalling in mesangial cells, we pretreated the cells with a TβRI inhibitor (SB431542) and PP2 for 1 h followed by co-treatment with CTGF and TGF-β1. Pretreatment with both SB431542 and PP2 completely inhibited Smad2 (Figure 3C) and Smad3 (Figure 3D) phosphorylation and p42/44 MAPK phosphorylation to a lesser degree (Figure 3E), suggesting that Src is involved in regulating Smad signalling in mesangial cells.

As pretreatment with SB431542 only partially attenuated the increased p42/44 MAPK phosphorylation observed upon co-treatment with CTGF and TGF-β1, it would suggest that there are other paths to p42/44 MAPK activation. In order to address this, we transfected mesangial cells with a KD TβRII mutant and looked at the effect on CTGF- and TGF-β1-mediated p42/44 MAPK phosphorylation. Expression of KD TβRII completely inhibited Smad2 and Smad3 phosphorylation as expected compared with the empty vector, but increased basal p42/44 MAPK phosphorylation indicating that p42/44 MAPK phosphorylation is likely independent of the kinase activity of the type II receptor (Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410499add.htm).

Renal cell migration is altered by CTGF and TGF-β1

In vitro [2,17,23] and *in vivo* [24] studies suggest disruption of actin cytoskeletal structures in mesangial cells exposed

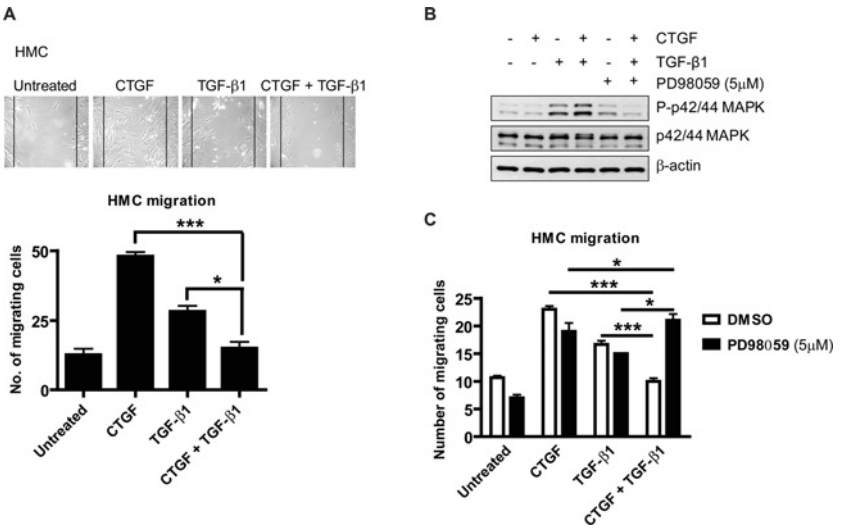


Figure 4 Renal cell migration is decreased upon co-treatment with CTGF and TGF-β1

(**A**) HMCs were serum-starved for 24 h, a wound was scratched, followed by treatment with CTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both CTGF and TGF-β1 together for a further 24 h. For inhibition of MEK, serum-starved cells were pretreated with PD98059 (5μM) for 1 h prior to treatment with CTGF and TGF-β1 as above. Images were captured under phase contrast at $\times$10 magnification using a Nikon TMS inverted microscope (**A**) or were fixed with paraformaldehyde (3.75 %) for 15 min, permeabilized with Triton X-100 (0.1 %) for 10 min, blocked with 5 % goat serum for 1 h and the nuclei were stained with Hoechst for 2 min. Images were captured using a Zeiss Axioscope equipped with an Axiocam and Axiovision 4.1. (**B**) Cell lysates were analysed by Western blotting using P-p42/44 MAPK, total p42/44 MAPK and β-actin as a loading control. (**C**) Migrated cells were counted and plotted. Results are $\pm$ S.E.M. and representative of three individual experiments. $^{*}P < 0.05$; $^{***}P < 0.005$ (one-way ANOVA.)

to high glucose and growth factor stimuli. *In vitro*, actin-mediated contractile responses are frequently modelled using cell migratory responses. Given the observed signalling shift from canonical to non-canonical pathways, we hypothesized that this might manifest as altered contractile/migratory behaviour. Consistent with previous observations, both CTGF and TGF-β1 alone strongly promoted migration, whereas, intriguingly, cell migration was decreased in the co-treated cells (Figure 4A).

A significant body of evidence has implicated MAPK in the regulation of cell migration and contractility [17,25,26]. To assess whether the decrease in cell migration was mediated by p42/44 MAPK, cells were pretreated with PD98059 (5μM) or DMSO. Pretreating with PD98059 at a concentration that was sufficient to inhibit p42/44 MAPK to levels similar to CTGF-induced p42/44 MAPK phosphorylation alone (Figure 4B) resulted in a rescue of cell migration in the co-treated cells (Figure 4C).

In order to address possible regulatory effects of TGF-β1 on CTGF-mediated responses we utilized a well-characterized effect of CTGF, that is, increased cell adhesion. Although CTGF stimulated mesangial cell adhesion, TGF-β1 had no effect, indicating that migratory responses and adhesive responses are distinct events (Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410499add.htm).

CTGF binds TβRIII and antagonizes TGF-β1-mediated Smad phosphorylation and migratory responses

Due to the involvement of TβRIII in the regulation of TGF-β signalling, we hypothesized that TβRIII may regulate CTGF/TGF-β1 signalling. TβRIII-knockdown cells were generated by virally transducing mesangial cells with TβRIII shRNA with 60 % knockdown of TβRIII at the mRNA level (Supplementary Figure S4A at http://www.BiochemJ.org/bj/441/bj4410499add.htm) and 50 % knockdown at a protein level (Supplementary Figure S4B). Inhibition of TGF-β1-induced Smad2 and Smad3 phosphorylation by CTGF was reversed in TβRIII shRNA cells (Figure 5A). Additionally, basal and CTGF- and TGF-β1-induced p42/44 MAPK phosphorylation was decreased when TβRIII expression was knocked down (Figure 5B), suggesting that TβRIII positively regulates p42/44 MAPK. To determine if CTGF associates with TβRIII to elicit its effects on TGF-β1, a V5-tagged TβRIII construct was overexpressed in mesangial cells. Co-immunoprecipitation studies demonstrated a clear physical association between the overexpressed TβRIII and recombinant CTGF (Figure 5C). Furthermore, migratory responses in CTGF/TGF-β1-treated cells were partially restored (50 %) in the TβRIII shRNA-tranduced cells compared with the control shRNA-transduced cells (Figure 5D). The partial restoration may be due to the fact that TβRIII expression is only decreased 50 % at the protein level. Perhaps with 100 % knockdown migration may have been fully restored.

CTGF antagonism of TGF-β1-mediated Smad signalling has clear transcriptional consequences in mesangial cells

Given the altered signalling and phenotypic responses, we hypothesized that a signalling shift would have significant transcriptional consequences. We identified a large number of genes (1654) that were uniquely differentially expressed in the cells treated with both CTGF and TGF-β1 ($P < 0.05$) (Figure 6A). We compared this dataset with that from patients with DN. We generated a heat map from the most over-represented genes that were regulated only in the presence of CTGF and TGF-β1 together and confirmed their expression in the patient biopsies (http://www.nephromine.org) (Figure 6B). Overall, these results suggest that the altered signalling response observed in CTGF/TGF-β1-treated mesangial cells gives rise to the expression of a unique pathologically significant gene-expression profile.

We employed a GSEA [27,28] with the top 50 overexpressed and the top 50 underexpressed genes. The expression data from the combination of TGF-β1 and CTGF treatments were assessed for enrichment of the two gene sets mentioned above. The top 50

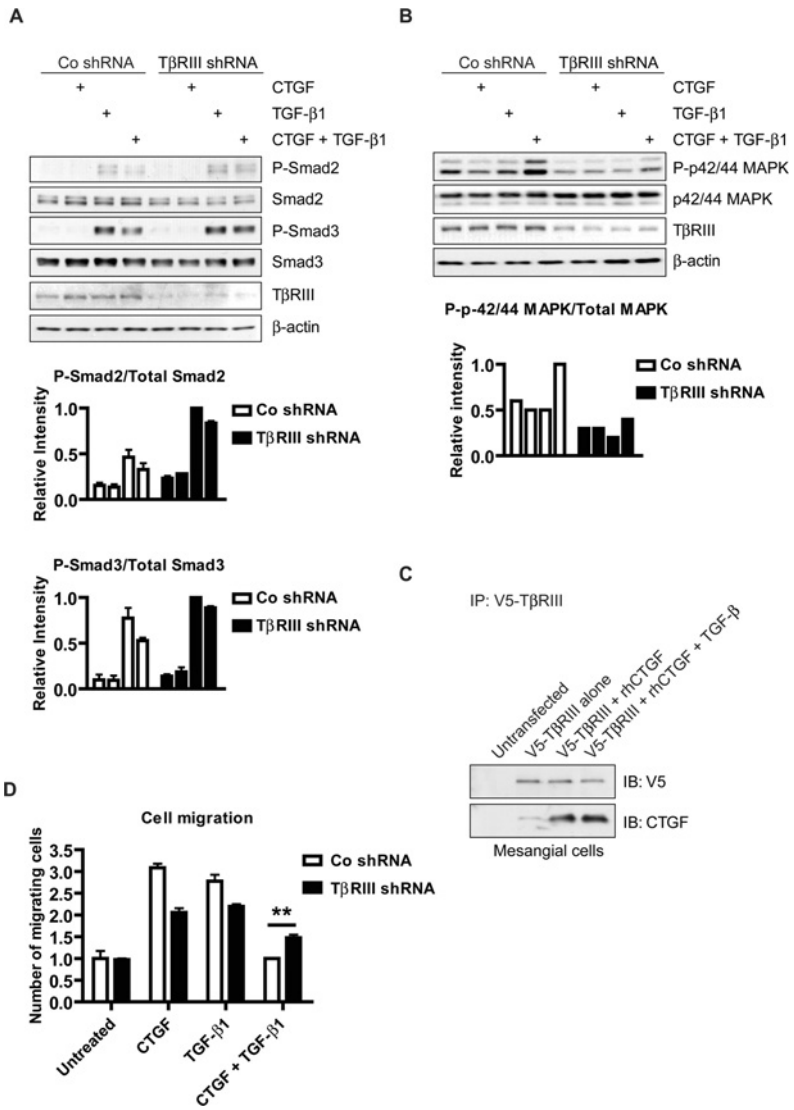


Figure 5 TβRIII binds to CTGF and mediates its inhibition of TGF-β1 canonical cell signalling and cell migration

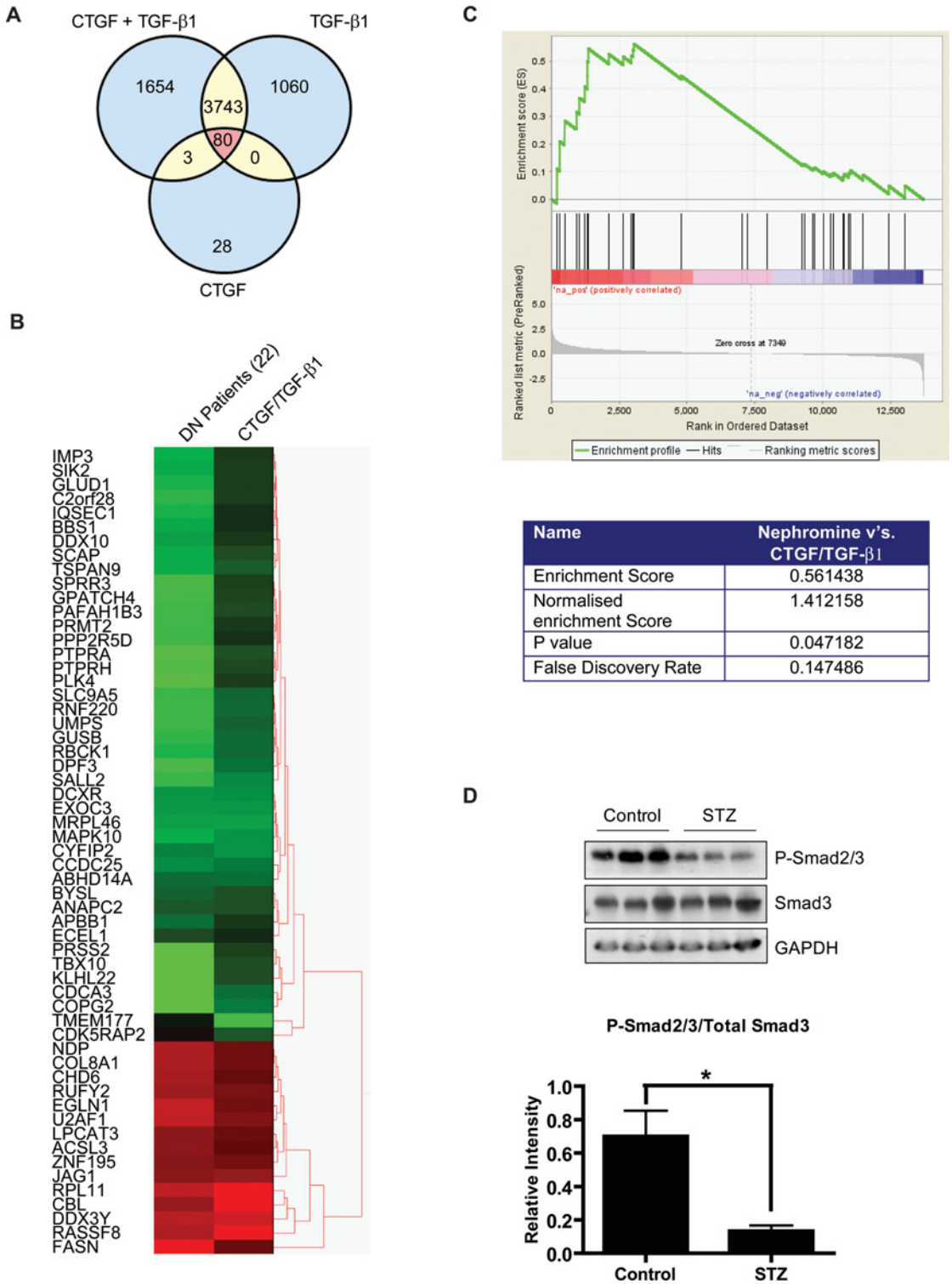
HMCs were virally transduced with either control shRNA or TβRIII shRNA. The control (Co) shRNA and TβRIII shRNA cells were treated with CTGF, TGF-β1 or both together for 1 h. Smad2 and Smad3 phosphorylation (**A**) and p42/44 MAPK phosphorylation (**B**) was determined by Western blotting. Densitometry was performed using Scion Image software. (**C**) Mesangial cells were transfected with V5–TβRIII and treated with CTGF (1 μg in 100 μl PBS) or CTGF (1 μg in 100 μl PBS) and TGF-β1 (100 ng) for 1 h followed by immunoprecipitation (IP) with anti-V5 antibody and immunoblotting (IB) with anti-CTGF and anti-V5 antibodies. (**D**) A wound was scratched in cell monolayers of control shRNA or TβRIII shRNA-transduced cells followed by treatment with CTGF, TGF-β1 or both together. Cells which had migrated into the wound were counted and the counts are represented in a graph. Results are ± S.E.M. and are representative of three individual experiments. $^{**}P < 0.01$ (one-way ANOVA).

overexpressed genes as determined from Nephromine (biopsies from DN patients compared with the controls) were determined to be significantly enriched in a ranked list of probes from the array with a *P*-value of 0.047 and a FDR (false discovery rate) of 0.147 (Figure 6C). Enrichment of this gene set derived from expression in DN patients within our array data from expression of mesangial cells treated with a combination of TGF-β1 and CTGF suggests that the overexpression of these genes by the two treatments are involved in DN.

CTGF is well documented to be elevated in renal fibrosis [29,30] and we found that CTGF mRNA expression is increased in STZ-induced diabetic mice at 27 weeks of diabetes [16,31]. We wanted to determine if there was an effect on Smad2 and Smad3 phosphorylation in the STZ diabetic mouse to support our *in vitro* results. Assessment of diabetes and features of early DN in this STZ model was published previously by our group [16]. Using the same lysates, we found a significant reduction in Smad2/3 phosphorylation at 27 weeks of diabetes with no change in Smad3 levels (Figure 6D). At the earlier time point of 18 weeks of diabetes, CTGF mRNA expression levels (Supplementary Figure S5A at http://www.BiochemJ.org/bj/441/bj4410499add.htm) and Smad2/3 protein phosphorylation levels were unchanged, whereas total Smad3 protein expression levels were increased (Supplementary Figure S5B) implying that under *in vivo* conditions where CTGF is elevated, there is a decrease in Smad2/3 phosphorylation in the STZ diabetic mouse.

DISCUSSION

Elevated expression of CTGF is a pathological hallmark of fibrosis in many disease processes [32–35]; however, transient overexpression of CTGF results in only a minimal fibrotic



Name	Nephromine v's. CTGF/TGF-β1
Enrichment Score	0.561438
Normalised enrichment Score	1.412158
P value	0.047182
False Discovery Rate	0.147486

Figure 6 Co-treatment with CTGF and TGF-β1 results in a unique gene profile

(**A**) Venn diagram generated from Human Genome Affymetrix array results illustrating how there are a large number of genes (1654) that are regulated only when HMCs are treated with CTGF and TGF-β1 together. There are a number of genes common to two (yellow) or all three (pink) experimental conditions or genes that are unique to each of the treatments (blue). (**B**) A heat map was generated comparing microarray gene expression ($\log_2$ values) from DN patient biopsies ($n = 22$) with the top 100 most over-represented genes that are regulated only in the presence of both CTGF and TGF-β1 in mesangial cells. Red represents an increase in expression whereas green represents a decrease in gene expression. (**C**) GSEA of the expression data of mesangial cells treated with TGF-β1 and CTGF. A gene set derived from the top 50 overexpressed genes in DN patients was found to be significantly enriched within the array data. A FDR less than 25% is significant. (**D**) Protein extracts from renal poles of 27-week diabetic mice ($n = 3$) were probed via Western blotting using anti-(P-Smad2/3), anti-(total Smad3) and anti-(GAPDH) antibodies. Densitometry was performed using Scion Image software. Results are $\pm$ S.E.M. and are representative of three individual experiments. *$P < 0.05$ (Student's unpaired t test).

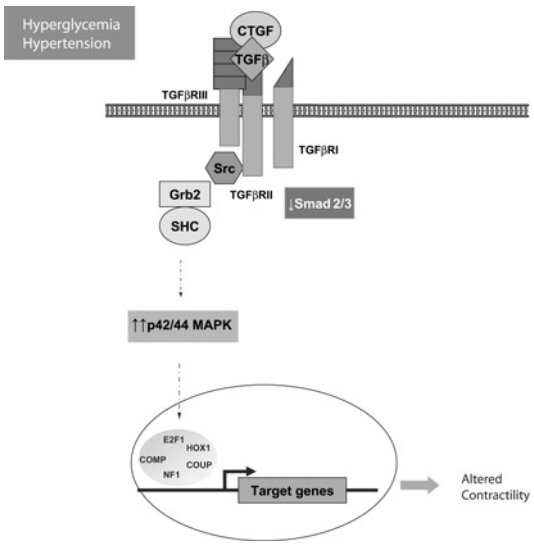


Figure 7 Proposed mechanism by which CTGF and TβRIII regulate TGF-β cell signalling in renal cells

Combinatorial stresses of hyperglycaemia and hypertension in the diabetic milieu result in increased mesangial expression of the matricellular growth regulator CTGF. In this microenvironment, TβRIII regulates the interplay between CTGF and TGF-β1 with consequent altered signalling responses, undergoing a shift from canonical to non-canonical pathways, resulting in increased activation of p42/44 MAPK. This signalling response controls differential gene expression leading to altered mesangial contractility. COMP (cartilage oligomeric matrix protein); COUP, NRF2 (nuclear receptor subfamily 2 group F); HOX1, homoeobox 1; NF1, neurofibromin 1.

response [36]. This has led to the hypothesis that CTGF alone is not pro-fibrotic, but that it creates a permissive environment for other factors to promote fibrosis [37]. In the present study, we provide evidence for a signalling switch in mesangial cells treated with CTGF and TGF-β1, with pathophysiological implications for the development and progression of DN. This involves the negative regulation of TGF-β1-mediated Smad signalling and transcriptional responses by CTGF, mediated by TβRIII, and a switch to Smad-independent pathways accompanied by differential gene expression associated with DN.

CTGF causes a clear inhibition of TGF-β1-dependent Smad2 and Smad3 phosphorylation and reporter gene expression. This observation was supported by a previous study showing that during the chronic stages of fibrosis, where CTGF expression was increased a Smad3-independent mode of TGF-β signalling occurred [38]. This is probably mediated by CTGF's ability to decrease TGF-β1 receptor binding. We show that overexpression of a full-length construct of CTGF inhibits TGF-β1-responsive reporter gene expression, whereas constructs with domain 1 or domain 2/VWC of CTGF deleted did not inhibit TGF-β1 activity, suggesting that both domains may be involved in mediating this inhibition. The observation that the VWC domain of CTGF mediates the antagonism of TGF-β1 responses highlights this sequence as a putative binding domain; the VWC domain of CTGF is structurally similar to the binding domain of known kielin/chordin-like BMP (bone morphogenetic protein) superfamily antagonists [38a].

Whereas TGF-β1-dependent Smad phosphorylation and activity was decreased, phosphorylation of p42/44 MAPK was markedly increased by co-treatment with CTGF and TGF-β1. Although p42/44 MAPK has been shown to negatively regulate Smad phosphorylation, it does not appear to be responsible for its decrease with CTGF/TGF-β1 as evidenced by the observation that the MEK inhibitor PD98059 had no effect. This is in agreement with Chen et al. [39] that inhibition of Ras/MEK/ERK does not reduce phosphorylation of Smads in mesangial cells. Moreover, phosphorylation of the Smad linker region was not increased by co-treatment with CTGF and TGF-β1.

Two key studies published in 2007 best illustrate the dichotomy of p42/44 MAPK activation at the heart of non-canonical TGF-β receptor signalling. It should be noted that TβRII undergoes autophosphorylation on three tyrosine residues: Tyr^{259}, Tyr^{336} and Tyr^{424}, albeit at a much lower level than autophosphorylation on serine and threonine residues. Galliher and Schiemann [40] showed that TβRII can also be phosphorylated by Src, a non-RTK (receptor tyrosine kinase), on Tyr^{284}, which can serve as a docking site for the recruitment of Grb2 (growth-factor-receptor-bound protein 2) and Shc (Src homology and collagen homology), thereby bridging TβRII to MAPK activation. In the same year, a key paper by Lee et al. [41] showed that TβRI can also be tyrosine phosphorylated after TGF-β stimulation; activated TβRI can recruit and directly phosphorylate ShcA on tyrosine and serine residues. The subsequent formation of a ShcA–Grb2–Sos (Son of sevenless) complex is then capable of activating Ras at the plasma membrane, leading to sequential activation of c-Raf, MEK and p42/44 MAPK. It thus seems that there are at least three routes to p42/44 MAPK activation in response to TGF-β; first, via autophosphorylation, secondly via Src activation and thirdly via TβRI/II Shc recruitment. The partial inhibition of the MAPK response by pretreatment with SB431542 suggests multiple paths to p42/44 MAPK activation. The observation that basal p42/44 MAPK phosphorylation is markedly increased in cells overexpressing a KD mutant of the type II receptor supports the hypothesis that p42/44 MAPK activation is independent of receptor dimerization. Indeed, given the fact that CTGF blocks TGF-β receptor binding, it seems likely that inhibition of receptor dimerization is at the core of the modulatory effects observed. Our previous studies identified Src activation in mesangial cells in response to CTGF [17]. The partial inhibition of p42/44 MAPK phosphorylation and the complete inhibition of Smad2/3 phosphorylation by the Src inhibitor PP2 again highlight a role for recruitment and activation of Src kinase in the modulation of TGF-β responses, supporting the findings of Galliher and Schiemann [40]. This does not exclude the possibility that the increased MAPK activity observed when cells are treated with CTGF and TGF-β is a result of concerted additive crosstalk between CTGF-activated Src and TGF-β-activated Src.

It should be noted that alternate pathways to TGF-β-mediated MAPK phosphophorylation have also been identified, including the TRAF6 (tumour-necrosis-factor-receptor-associated factor 6)–TAK1 (transforming growth factor-β-activated kinase 1) p38/JNK (c-Jun N-terminal kinase) pathway [41a]. The interaction between TβRI and TRAF6 is necessary for TGF-β-mediated ubiquitylation of TRAF6 and subsequent activation of the TAK1–p38/JNK pathway. Interestingly, TβRI kinase activity is required for activation of Smad signalling, whereas TRAF6 regulates the activation of TAK1 in a receptor kinase-independent manner, again illustrating the divergence and complexity at the heart of TGF-β receptor mediator signalling, strengthening the hypothesis that multiple distinct signalling pathways are induced by the active TβR complex. Indeed, it is possible that the effects of CTGF on TGF-β signalling are mediated in a similar intrinsic receptor kinase-independent mechanism, the elucidation of which remains a significant research goal.

The ability of TGF-β to promote migratory responses is increasingly accepted to occur through the integration of signals arising not only from Smad2/3, but including Rho family

GTPases, p42/44 MAPK, p38 MAPK and PI3K (phosphoinositide 3-kinase) [42]. In the present paper, we show that whereas CTGF and TGF-β1 alone promote mesangial cell migration, together they negatively regulate migration, mediated in part by p42/44 MAPK as PD98059 reverses the effect. This observation is similar to that of Del Carpio-Cano et al. [43] who found that CTGF overexpression negatively regulates TGF-β1-induced cell aggregation. Conversely, Abreu et al. [7] observed cell aggregation in Mv1Lu cells upon treatment with TGF-β1 and purified CTGF but not with either treatment alone, strengthening the hypothesis that these events are context dependent. Under permissive microenvironments, Smad1/5/8 phosphorylation is involved in TGF-β-mediated cell migration, causing a switch to a pro-migratory phenotype [44]. In mesangial cells, however, TGF-β1-induced Smad1/5/8 phosphorylation is unchanged with CTGF/TGF-β1 treatment, indicating that this switch is distinct.

Significant efforts have been made to identify receptors involved in mediating the effects of CTGF; however, it appears not to rely on a single specific receptor but, rather, binds to other signalling components, modulating their activity. We demonstrated that TβRIII binds CTGF in human mesangial cells. Knockdown of TβRIII results in a restoration of TGF-β-induced Smad signalling and migratory responses, suggesting that the formation of a ternary complex between CTGF, TGF-β1 and the TβRIII may be required for the moderation of signalling responses. In addition, knockdown of TβRIII markedly reduced the level of p42/44 MAPK phosphorylation basally and in response to CTGF and TGF-β1 treatments, indicating that TβRIII has a positive regulatory effect on TGF-β1-induced p42/44 MAPK activation. A recent study has again highlighted the co-operative nature of CTGF and TGF-β1 in the promotion of fibrosis [37]. It seems that CTGF can either enhance TGF-β1 Smad signalling/transcription [7] or not, depending on the cell type [45,46]. Our results suggest a compounding factor in regulating TGF-β1–TβR binding is the expression of TβRIII. Mv1Lu cells have a low level of expression of TβRIII, with a reduced capacity for TGF-β1 binding compared with other cell types [10]. In contrast, glomerular cells have abundant levels of TβRIII and there is evidence that expression is increased in glomerulosclerosis [47]. The presence of soluble TβRIII may alter the presentation of TGF-β1 to the type II receptor, explaining the observed reno-protective effect of soluble TβRIII in the *db*/*db* mouse model [48].

We found a unique profile of differentially expressed genes in CTGF and TGF-β1 co-treated mesangial cells. This finding is supported by Shi-wen et al. [49] who demonstrated that TGF-β was only able to induce one-third of the genes in *Ccn2*$^{-/-}$ MEFs (mouse embryonic fibroblasts) compared with *Ccn2*$^{+/+}$ MEFs. The results demonstrate that the overexpressed genes from Nephromine are significantly over-represented in the TGF-β1/CTGF co-treatment dataset compared with the control with a P-value of <0.05, and a FDR of 0.147, well below the recommended threshold of the Broad Institute. Furthermore, these genes were found to be differentially expressed in a co-ordinate fashion in biopsies from patients with DN compared with the controls, further implying a necessity for co-operation between CTGF and TGF-β1 in DN. In support of our *in vitro* model, we found that Smad2/3 phosphorylation was decreased in STZ diabetic mice where CTGF expression is elevated.

In summary, we propose that CTGF causes a switch in TGF-β1 signalling from Smad-dependent to Smad-independent with attendant changes in gene transcription, mediated at least in part by the VWC domain of CTGF and an interaction with the TGF-β co-receptor TβRIII. These results support the hypothesis that co-operative signalling/cross-talk between CTGF and TGF-β1 are involved in the progression of fibrosis, highlighting the potential for CTGF-directed therapies.

AUTHOR CONTRIBUTION

Helen O'Donovan performed experiments, wrote the paper and reviewed/edited the paper prior to submission; Fionnuala Hickey performed experiments; Derek Brazil performed experiments and reviewed/edited the paper prior to submission; Noelynn Oliver provided materials and reviewed/edited the paper prior to submission; Catherine Godson and Finian Martin reviewed/edited the paper prior to submission; John Crean contributed to the research design and paper preparation and reviewed/edited the paper prior to submission.

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online data

SUPPLEMENTARY ONLINE DATA

Connective tissue growth factor antagonizes transforming growth factor-β1/Smad signalling in renal mesangial cells

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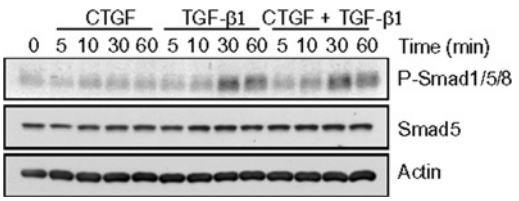

Figure S1 CTGF has no effect on TGF-β-induced Smad1/5/8 phosphorylation

HMCs were serum-starved for 24 h followed by treatment with CTGF (25 ng/ml), TGF-β1 (10 ng/ml) or both CTGF and TGF-β1 together for the times indicated. Smad1/5/8 phosphorylation was determined by Western blotting.

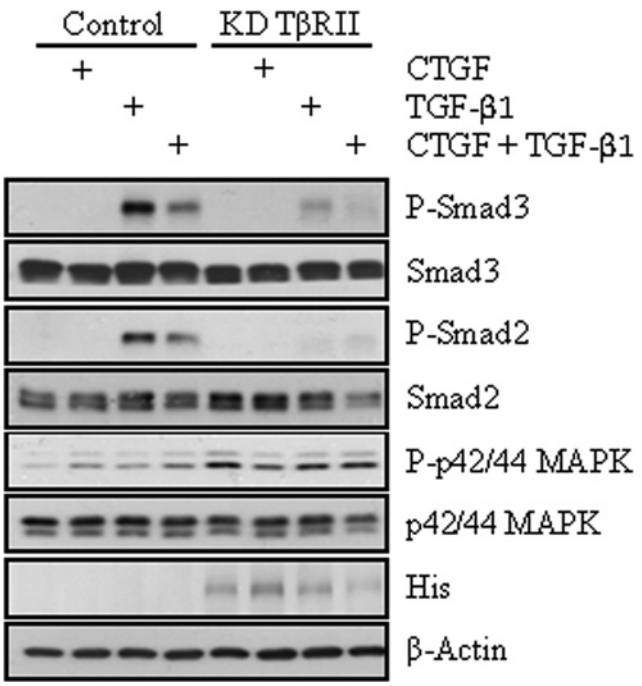

Figure S2 CTGF and TGF-β1-induced P-p44/44 MAPK phosphorylation is independent of TβRII kinase activity

Mesangial cells were transfected with a His-tagged KD TβRII (pCMV5B-TGFβ receptor II K227R) which prevents receptor dimerization. The cells were serum-starved for 24 h followed by stimulation with CTGF (25ng/ml), TGF-β1 (10ng/ml) or both CTGF and TGF-β1 combined for 15 min. Smad2, Smad3 and p42/44 MAPK phosphorylation was determined by Western blotting. Immunoblotting with anti-His antibody was used to confirm expression of KD TβRII.

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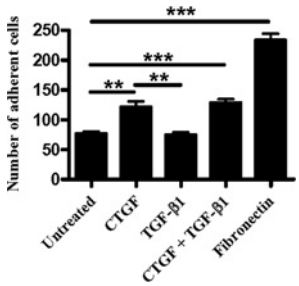

Figure S3 TGF-β1 had no effect on CTGF-mediated adhesion

Culture plates (96 well) were left uncoated or were coated with CTGF (25 ng/ml), TGF-β1 (10 ng/ml), both CTGF and TGF-β1 combined or fibronectin as a positive control for adhesion at 4 °C overnight. HMCs were then seeded on to the plates at 1×10^5 cells per well and allowed to attach for 50 min. The wells were washed three times in PBS, fixed, permeabilized and stained with Hoechst for 1 min. Cells were counted in a field of $\times 40$ magnification and the average number of adherent cells per field in eight fields were plotted. Results are $\pm$ S.E.M. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ (one-way ANOVA).

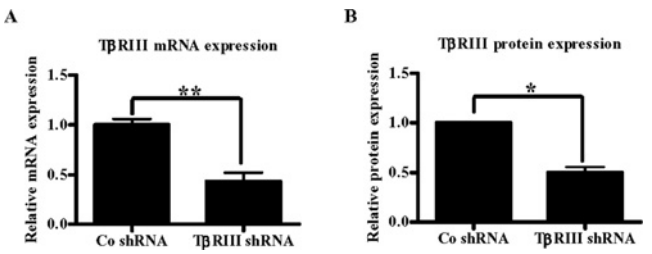

Figure S4 TβRIII mRNA and protein expression

HMCs were virally transduced with either control shRNA or TβRIII shRNA. (**A**) TβRIII mRNA expression was assessed by RT (reverse transcription)–PCR using GAPDH as an internal control. (**B**) Protein expression was assessed using an anti-TβRIII antibody and was normalized to β-actin levels and plotted as relative intensity. Results are $\pm$ S.E.M. $^*P < 0.05$; $^{**}P < 0.01$ (Student's unpaired t test).

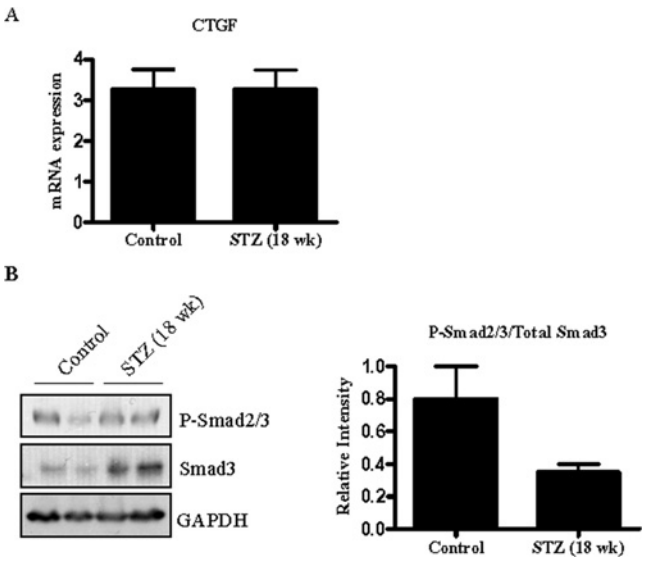

Figure S5 CTGF expression and Smad2/3 phosphorylation is unchanged whereas total Smad3 levels are increased in 18 week STZ-induced diabetic mice

(**A**) Protein extracts from renal poles of 18 week diabetic mice ($n = 2$) were analysed for CTGF expression by RT–PCR using 18S as an internal control. Cell lysates were assessed for Smad2/3 phosphorylation and total Smad3 expression. (**B**) Densitometry was performed using Scion Image software. P-Smad2/3 intensities were normalized to total Smad3 levels and plotted as relative intensity. Results are $\pm$ S.E.M.

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