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CTGF/CCN2 activates canonical Wnt signalling in mesangial cells through LRP6: Implications for the pathogenesis of diabetic nephropathy

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ABSTRACT

We describe the activation of Wnt signalling in mesangial cells by CCN2. CCN2 stimulates phosphorylation of LRP6 and GSK-3β resulting in accumulation and nuclear localisation of β-catenin, TCF/LEF activity and expression of Wnt targets. This is coincident with decreased phosphorylation of β-catenin on Ser 33/37 and increased phosphorylation on Tyr142. DKK-1 and LRP6 siRNA reversed CCN2's effects. Microarray analyses of diabetic patients identified differentially expressed Wnt components. β-Catenin is increased in type 1 diabetic and UUO mice and in *in vitro* models of hyperglycaemia and hypertension. These findings suggest that Wnt/CCN2 signalling plays a role in the pathogenesis of diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN) is a debilitating progressive disease arising from long term complications of diabetes [1]. The hallmark of DN is glomerulosclerosis due to accumulation of extracellular matrix (ECM) [2]. This ultimately causes mesangial expansion and damage to the glomerular basement membrane, leading to proteinuria [3]. Mediators of mesangial dysfunction include oxidative stress [4], glucose [3], TGFβ [5], advanced glycation end products (AGEs) [6] and glomerular hypertension [7]. A number of recent reports have highlighted a role for Wnt signalling in the pathogenesis of DN [8–10]. Importantly, LEF-1, the nuclear transcription factor regulated by Wnt, promotes epithelial to mesenchymal transition (EMT) when its activity is triggered by β-catenin [11]. Mesangial cells cultured in high glucose were shown to be resistant to Akt-dependent apoptosis in the presence of a Wnt ligand and β-catenin nuclear localisation [8]. It is in this context, that Wnt pathways are increasingly seen as viable targets for intervention in the treatment of renal fibrosis. Indeed, the renoprotective effects of many current therapies are thought to be mediated, in part, via modulation of Wnt signalling [12].

Evidence suggests a complex relationship between connective tissue growth factor/CCN family protein 2 (CTGF/CCN2) and the Wnt pathway [13–16]. It was demonstrated in *Xenopus laevis* embryos that CCN2 modulated Wnt signalling by binding to LRP5/6 [15]. Subsequently a number of studies have demonstrated CCN2's interaction with LRP6, while Wnt ligands increase CCN protein expression. A relationship that contributes to increased fibrosis may exist between the Wnts and TGFβ/smad signalling pathways [17–19].

Here we show that CCN2 activates canonical Wnt signalling in human mesangial cells; CCN2 causes nuclear accumulation of β-catenin and TCF/LEF transcriptional activity. We also demonstrate that CCN2 requires the LRP6 receptor to activate Wnt signalling; treatment with DKK-1, the endogenous LRP6 receptor antagonist, and LRP6 knockdown ameliorate CCN2 induced responses. Our studies in animal models of diabetes and in patients suffering from long term complications of diabetic nephropathy highlight significant alterations in Wnt related gene expression, in particular increased expression of β-catenin. The observation that CCN2 can interact with Wnt leads us to propose that the activation of Wnt pathways by CCN2 has pathophysiological significance during the initiation and progression of DN.

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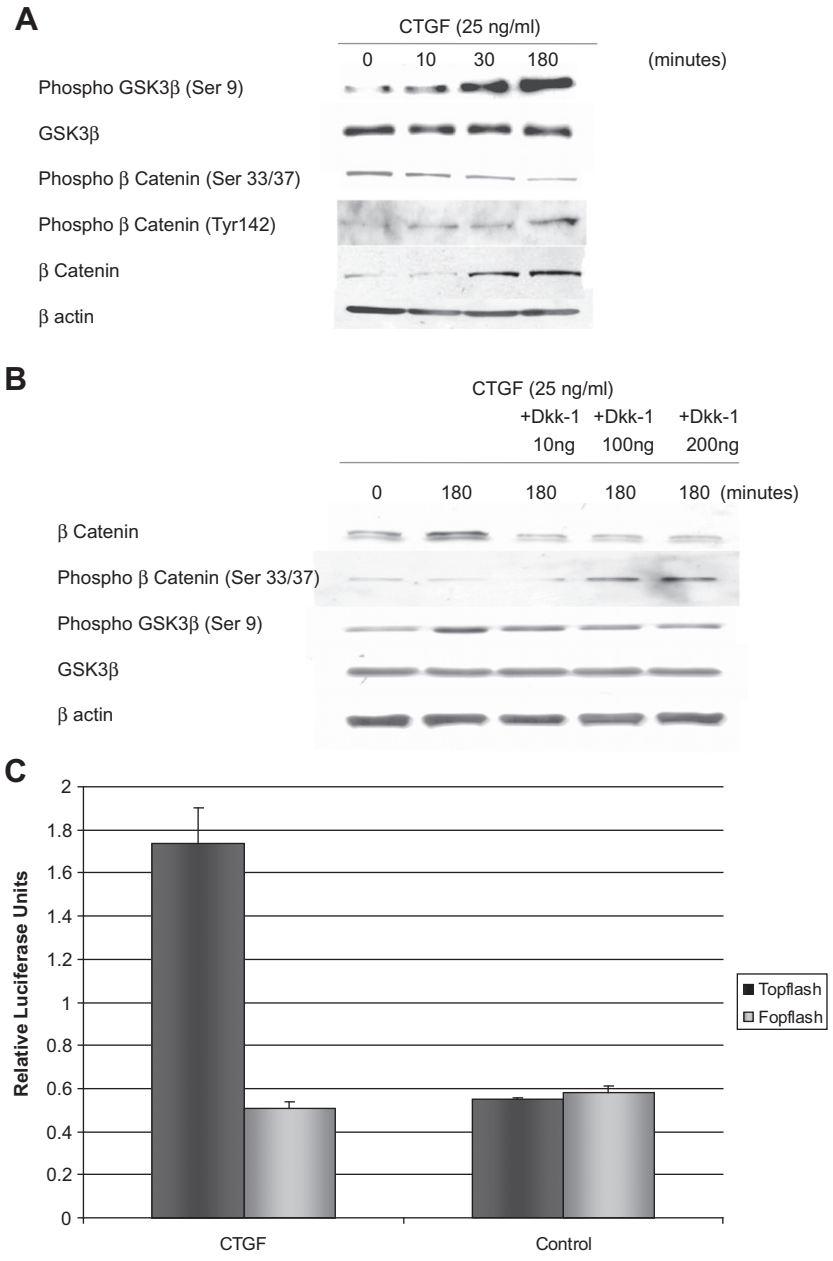

Fig. 1. CCN2 activates canonical Wnt signalling in human mesangial cells. Primary human mesangial cells were grown to 90% confluency and serum starved for 24 h prior to stimulation with recombinant human CCN2 (25 ng/ml). (A, B) Whole cell lysates were analysed by Western blot using the specific antibodies indicated (C). Cells were co-transfected with the reporter Topflash or the mutated TCF/LEF reporter Fopflash and 2 μg of wildtype CCN2. Promoter reporter activity was measured using a dual luciferase kit (Promega). All results are representative of at least three individual experiments.

2. Materials and methods

2.1. Animals

Procedures were licensed by the Irish Department of Health and Children and approved by the UCD Animal Research Ethics Committee. Seven- to ten-week-old male C57Bl/6J mice were divided into two groups: A, treated with streptozotocin (STZ) dissolved in 100 mmol/l citrate buffer, pH 4.5; or B, citrate buffer alone, following AMDCC protocols (http://www.amdcc.org). Diabetes was confirmed by two consecutive daily measurements of fasting blood glucose >15 mmol/l 2 weeks after injection. For unilateral ureteral obstruction (UUO), male C57Bl/6J mice aged 10–12 weeks were placed into two groups, UUO and sham-operated. The UUO group was anaesthetized, received midline laparotomy and identification with ligation of the left ureter. The sham group had the left ureter identified, manipulated but not ligated. On day 10, mice were harvested and UUO was confirmed by dilation of the renal pelvis. Organs were perfused with heparinised saline and the left kidney excised. The renal capsule was removed and the kidney fixed in 10% formalin.

2.2. Tissue culture

Primary human mesangial cells (Lonza) were cultured in MCDB-131, HeLa cells in MEM, both supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. Cells were maintained at 5% CO_2, 37 °C and starved for 24 h prior to stimulation. CCN2, N-half and C-half mutants were expressed and purified from zCCN2 and CCN2-mutant baculovirus-infected insect cells

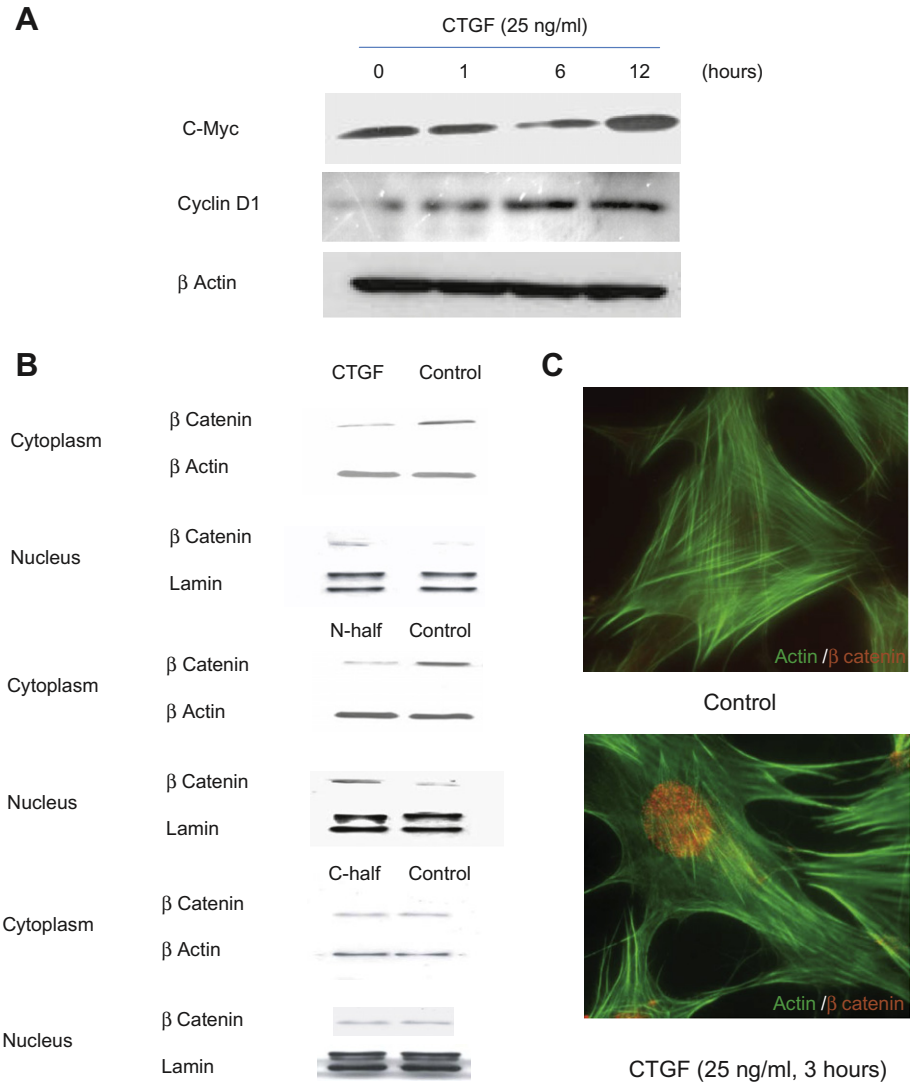

Fig. 2. CCN2 stimulates nuclear accumulation of β-catenin and increased expression of β-catenin-transcriptionally regulated targets. Cells were grown to 90% confluency and serum starved for 24 h prior to stimulation with recombinant human CCN2 (25 ng/ml) and/or deletion mutants (N-half, C-half). (A, B) Whole cell lysates were analysed by Western blot using the specific antibodies indicated. (C) Cells were fixed using standard techniques and probed using specific antibodies to β-catenin. F-actin was visualised with Alexa488 conjugated phallodin. Results are representative of at least three individual experiments.

[20]. Cells were exposed to high glucose (30 mM) for up to 48 h. Osmotic control media was made iso-osmolar with the addition of mannitol (30 mM). Cells were cultured on BioFlex six well laminin-coated culture plates (Dunn Labortechnik GmbH) and subjected to repeated stretch/relaxation cycles by mechanical deformation (60 cycles/min, 8% uniaxial elongation) for up to 48 h.

2.3. RNAi Interference

Cells were transfected with 2 μM LRP6 siRNA or scrambled siRNA, (Dharmacon ON-TARGET/Non-targeting Pool. *D-001810-10-20*), and 6 μl of Fugene HD™ (Roche). Knockdown of LRP6 was confirmed by Western blot. When cells reached ~70% confluency, a migration assay was performed.

2.4. Electrophoresis and Western blotting

Total protein was separated by SDS–PAGE, transferred to nitrocellulose and probed with antibodies to phospho GSK-3β (Ser 9) (1:1000, Cell Signaling Technologies), phospho β-catenin (Ser 33/45) (1:500, Abcam), β-catenin (1:1000, Cell Signaling Technologies), phospho LRP6 (Ser 19) (1:1000, Cell Signaling Technologies), total LRP6 (1:1000, Cell Signaling Technologies), Cyclin D (1:1000), c-myc (1:1000, Cell Signaling Technologies), β-actin (1:20,000, Sigma) and Lamin (1:8000, Cell Signaling Technologies). Proteins were visualised with HRP conjugated secondary antibodies using luminol (Santa Cruz).

2.5. Luciferase assay

Cells were co-transfected with the reporter Topflash or the mutated TCF/LEF reporter Fopflash and 2 μg of wildtype CCN2 plasmid using Fugene HD™. After 24 h, promoter reporter activity was measured using a dual luciferase kit (Promega).

2.6. Immunocytochemistry

Cells were fixed using standard techniques and probed using specific antibodies to β-catenin (1:100, Millipore). F-Actin was visualised with Alexa488 conjugated phallodin. β-Catenin was detected with Alexa594 conjugated anti mouse antibodies (1:1000, Molecular Probes) and images captured using a Zeiss Axioscope equipped with an Axiocam and Axiovision 4.1.

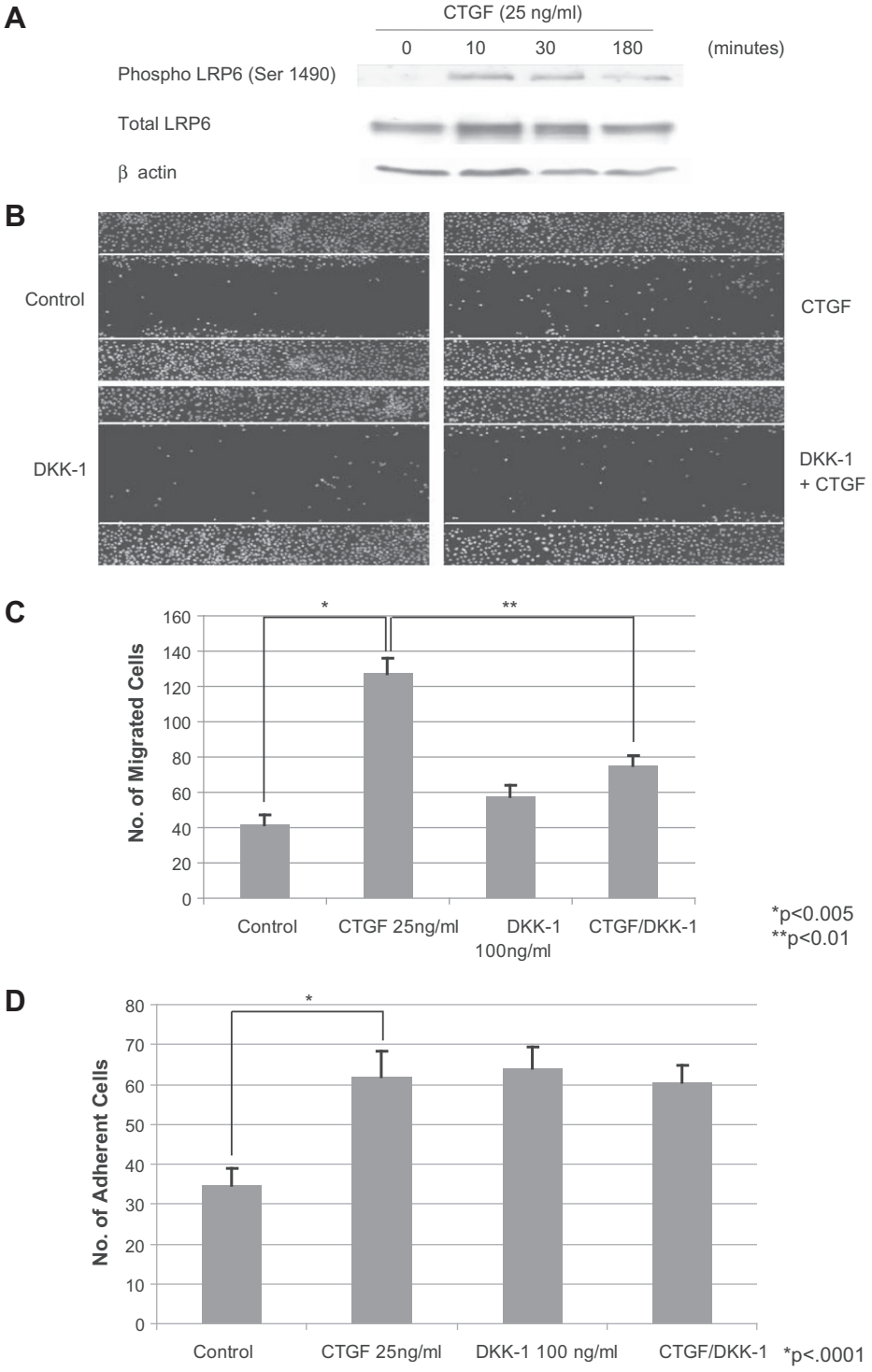

Fig. 3. CCN2 activation of canonical Wnt signalling requires the Wnt co-receptor LRP6. (A) Cells were grown to 90% confluency and serum starved for 24 h prior to stimulation with recombinant human CCN2 (25 ng/ml). Whole cell lysates were analysed by Western blot using antibodies specific for LRP6 and phospho LRP6 (Ser 1490). (B, C) Cells were allowed to reach 70% confluency and a scratch wound applied. Cells were pre-treated with 100 ng/ml of DKK-1 for 1 h then stimulated with 25 ng/ml of CCN2 for 24 h. (D) A 96 well plate was coated with CCN2 (25 ng/ml) and/or DKK-1 (100 ng/ml). Cells were seeded for 3 h and fixed with 3.7% paraformaldehyde. Nuclei were stained with Hoescht 33258 and visualised using a Zeiss Axioscope with Axiovision 4. Results are representative of at least three individual experiments.

2.7. Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded kidney sections. Briefly, post-mortem, mouse kidneys were fixed in situ using 4% (wt/vol) paraformaldehyde, and 3-μm sections stained with anti-β-catenin (Millipore) and visualised with alkaline phosphatase.

2.8. Cell migration

Cells were allowed to reach 70% confluency and a scratch wound applied. Cells were pre-treated with DKK-1 for 1 h then stimulated with CCN2 for 24 h. Images were captured using a Zeiss Axioscope.

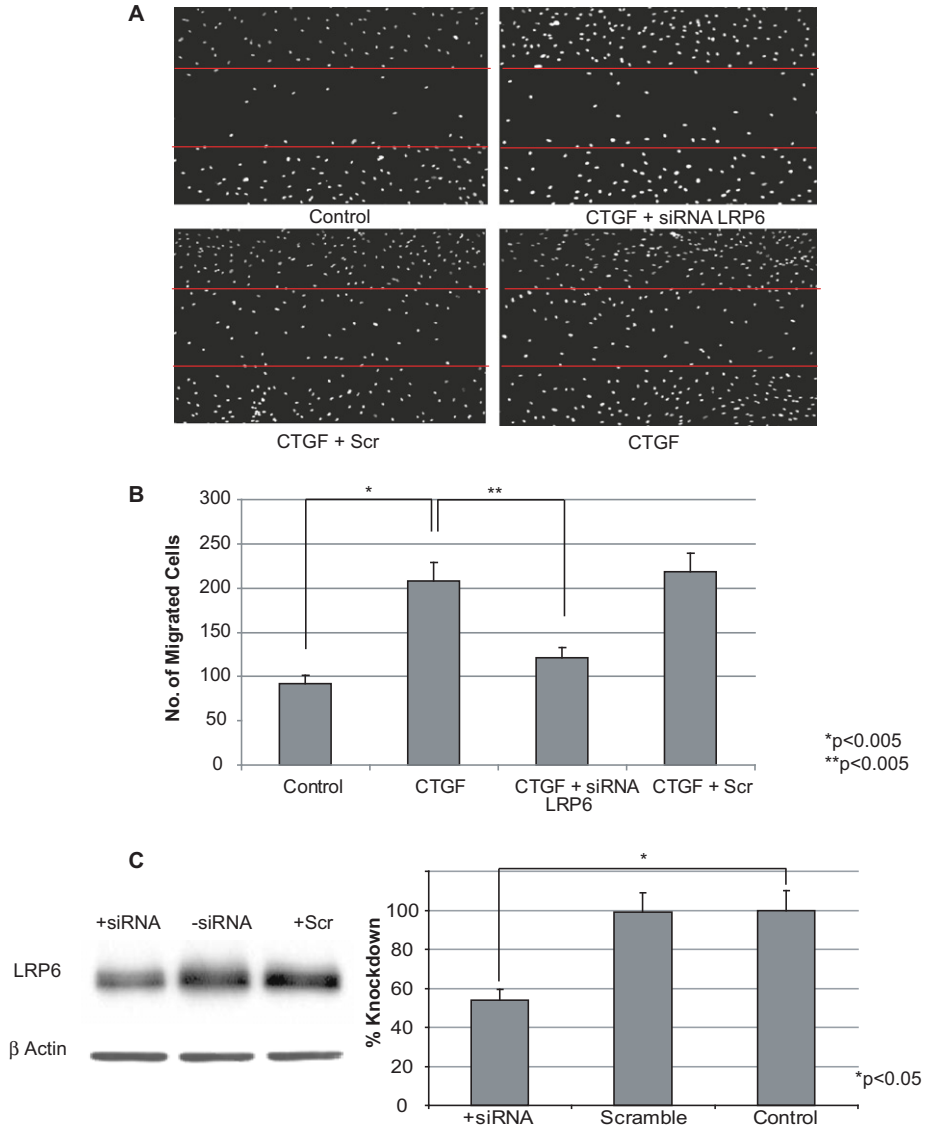

Fig. 4. siRNA knockdown of LRP6 inhibits CCN2 mediated cell migration (A, B). Cells were transfected with LRP6 siRNA or scrambled siRNA, once cells had reached 70–80% confluency a migration/wound assay was carried out as previously (C). Knockdown of LRP6 was confirmed by Western blot.

2.9. Cell adhesion

A 96 well plate was coated with CCN2 (25 ng/ml) and/or DKK-1 (100 ng/ml). Cells were seeded for 3 h and fixed with 3.7% paraformaldehyde. Nuclei were stained with Hoescht 33258 and visualised using a Zeiss Axioscope.

2.10. Microdissection and RNA isolation

Cortical tissue segments were microdissected under and total RNA was isolated using a silica-gel based isolation protocol (Qiagen) and diluted in 30 μl RNase-free water. RNA quality and quantity was assessed on a Bioanalyzer (Agilent Technologies).

2.11. Microarray analysis

Three hundred to eight hundred nanograms of RNA from living donor ($n = 3$), cadaveric donor ($n = 4$), DN ($n = 13$), and MCD ($n = 4$) was reverse-transcribed using SuperScript II (Invitrogen) and T7-$(dT)_{24}$. Second-strand synthesis was performed at 16 °C for 2 h, using DNA Polymerase I, DNA ligase, RNase H (Roche, 1 U/μl) and 1X second-strand buffer. Double-stranded cDNA was blunt-ended using T4 DNA polymerase, purified by phenol/chloroform extraction and transcribed for 16 h at 37 °C in the presence of biotin labeled-ribonucleotides, using the BioArray HighYield RNA transcript labeling kit (Enzo Laboratories). The biotin-labeled cRNA was purified using RNeasy mini-column followed by a quality check using the Bioanalyzer. The fragmentation, hybridization, staining and imaging was performed according to the Affymetrix guidelines.

2.12. Bioinformatic and data analysis

Image files were obtained through Affymetrix GeneChip software (MAS5). Subsequently, robust multichip analysis (RMA) was performed using RMA express. For each in vivo sample, an average RMA value was computed for duplicate microarrays and to ensure the average was statistically representative a *t*-test and *P* value were generated. Only those genes with a *P* value of $\leqslant 0.01$ were included in subsequent analyses. Thereafter, expression data were compared to control and signal log ratio used to generate a heat map using Heat Map Builder®.

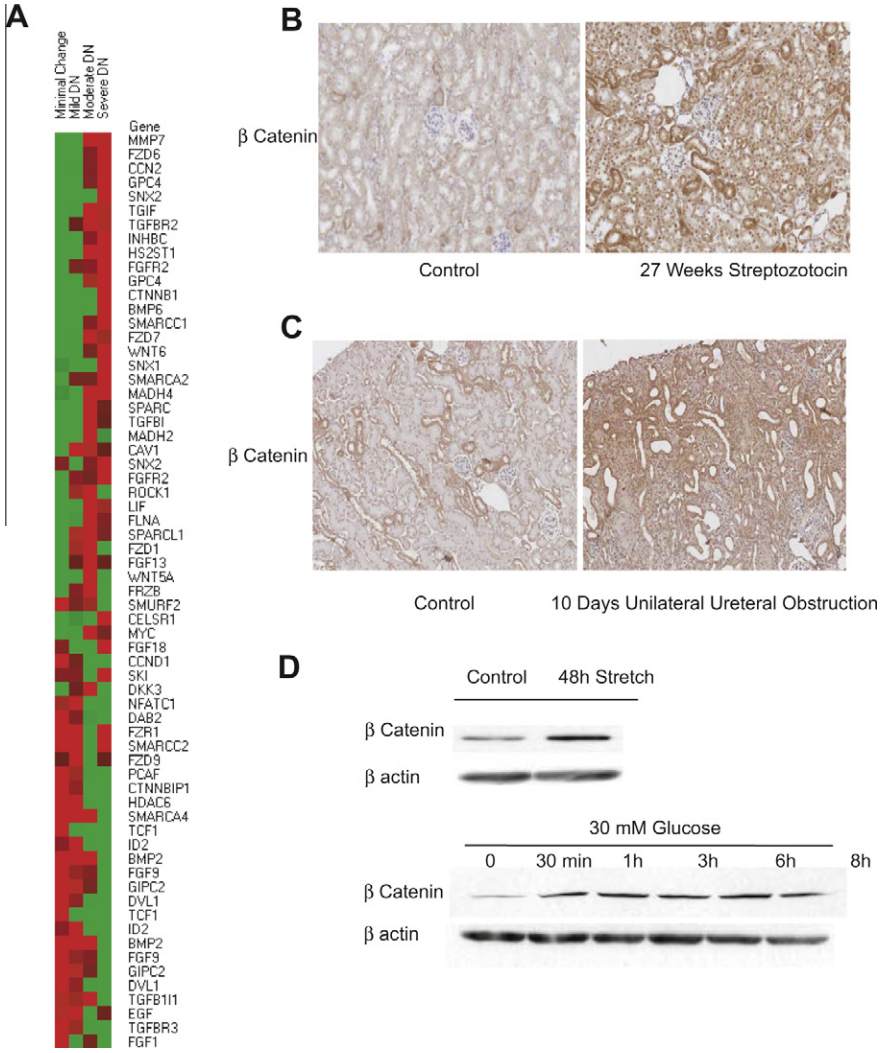

Fig. 5. Wnt pathway and target genes are differentially expressed in renal biopsies (A), animal models of renal disease (B, C) and in vitro cell models of hypertension and hyperglycaemia (D). (A) Microarray analysis of patient samples collected through the European Renal cDNA Biopsy Bank identifies differential expression of several key components of Wnt signalling. (B, C) Immunohistochemistry was performed on paraffin embedded kidney sections from diabetic and UUO mice. (D) Western blots were performed on whole cell lysates from mesangial cells subjected to cyclic mechanical strain for 48 h (upper panel) or cultured in 30 mM glucose for up to 8 h (lower panels).

3. Results and discussion

The nature of its structural organisation has led to the emerging view that CCN2 functions as a matricellular regulator. Supporting this hypothesis, CCN2 modulates a variety of cell signalling pathways and receptors including LRP5/6 [21] TrKA [22], β1 integrins [23] and β3 integrins [24]. Our studies elaborate a role for CCN2 as an activator of the canonical Wnt pathway in human mesangial cells. Treatment of mesangial cells with CCN2 induced phosphorylation of GSK3β on the inhibitory residue serine 9 (Fig. 1A) and increased levels of β-catenin, consistent with stabilisation associated with activation of canonical Wnt pathways. This was characterised by decreased phosphorylation of β-catenin on Ser 33/37 and an increase on Tyr142, markers for degradation and nuclear accumulation, respectively (Fig. 1A). Pretreatment of cells with the Wnt signalling antagonist DKK-1 blocked CCN2 mediated accumulation of β-catenin, resulting in its phosphorylation (Ser 33/37) and degradation (Fig. 1B). Similarly, DKK-1 antagonised CCN-2 phosphorylation of GSK3β. Use of a TCF/LEF luciferase construct (Topflash) confirmed that CCN2 stimulated β-catenin dependent promoter activation (Fig. 1C).

We next determined that expression of β-catenin dependent gene products were increased by CCN2; levels of c-myc and cyclin D-1 were increased after stimulation with CCN2 (Fig. 2A). CCN2 induced nuclear translocation of β-catenin was established by subcellular fractionation and confirmed by immunocytochemistry (Fig. 2B and C). Mutant proteins consisting of either the N-terminal half molecule or the C-terminal half of CCN2 were expressed and purified and the ability to induce cytoplasmic to nuclear translocation of β-catenin was found to reside within the N-terminal half only (Fig. 2B, lower panels).

Expression studies determined that mesangial cells expressed the Wnt co-receptor LRP6 (data not shown). Treatment with CCN2 stimulated the phosphorylation (and subsequent internalisation) of LRP6 on Ser 1490 suggesting that CCN2 can function as an LRP6 agonist (Fig. 3A). Pre-treatment with DKK-1/Dickkopf, blocked CCN2 induced cell migration, but had no effect on CCN2 mediated adhesion (Fig. 3B–D). Furthermore, siRNA knockdown of LRP6 blocked CCN2s' pro-migratory effects. (Fig. 4A–C). Previous studies in Xenopus embryos have shown that CCN2 interacts directly with the LRP6 co-receptor [15] and that over expression of CCN2 results in nuclear accumulation of β-catenin and a concurrent increase in TCF/LEF transcription [25]. Our results here describe a functional relationship between CCN2 and the Wnt co-receptor LRP6 (Fig. 3), resulting in accumulation of β-catenin and promotion of the expression of downstream target genes, with clear pathogenic significance

for the development of diabetic nephropathy. We propose that in human mesangial cells CCN2 modulates Wnt signalling via LRP6 through phosphorylation of its serine 1490 site resulting in nuclear accumulation of β-catenin and TCF/LEF activity.

The pathophysiological significance of Wnt in the progression of nephropathy remains obscure, although recent studies have shown that Wnt target genes are increased following obstructive injury [8,9,26]. Human renal biopsies were collected in a multicenter study, the European Renal cDNA Biopsy Bank (ERCB) and stratified by the reference pathologist according to histological diagnosis. Microarray analyses of these biopsies identified a significant number of differentially expressed genes that are members or direct targets of the Wnt signalling pathway including, frizzled 6, 7 and 9, dickkopf, dishevelled-1, β-catenin, TCF and myc, indicating that Wnt activity is increased in nephropathy (Fig. 5A). These studies suggest a correlation between increased fibrosis of the kidney, loss of kidney function, proteinuria and increased Wnt signalling activity. Microarray analysis was independently confirmed and validated by Schmid et al. [27,28]. Their analyses are available for academic use at www.nephromine.org. Preliminary studies suggest that Wnt-5a and Wnt6 promote cell polarisation with marked rearrangement of focal adhesion, tubulin and actin cytoskeletal networks in mesangial cells. These findings illustrate a complex and conserved nexus between the ligands of the Wnt planar polarity pathway and the secondary transduction mechanism of the traditional Wnt canonical signalling pathway that is capable of remodelling the cytoskeleton of the mesangium, with clear implications for the maintenance of actin mediated contractility during the progression of DN.

Increased β-catenin was observed by immunohistochemistry in kidneys from STZ-induced type 1 diabetic mice (Fig. 5B) at 27 weeks of diabetes, as well as mice that had undergone UUO at 10 days (Fig. 5B), in vivo models for hyperglycaemia and glomerular hypertension mediated damage to the kidney respectively. Staining of β-catenin was particularly evident in the tubular epithelium in diabetic kidneys (Fig. 5B). Previous studies have suggested that acute exposure of cells to elevated glucose can activate Wnt signalling [29]. Increased β-catenin was also observed in mesangial cells cultured in 30 mM glucose or subjected to cyclic mechanical strain (Fig. 5D), in vitro models of hyperglycemia and hypertension, respectively. Increased expression of CCN2 during the progression of DN, likely leads to activation of Wnt signalling and subsequent initiation of TCF/LEF transcription; Wnt target genes are increased in the kidney following obstructive injury [26], while inhibition of Wnt signalling by DKK-1 leads to decreased expression of Wnt target genes such as C-myc, fibronectin and twist, and improved renal function following UUO and hypertensive injury [30]. Pharmacological intervention has shown that modulation of Wnt signalling via GSK 3β regulation is a potential therapeutic target in treatment of organ hypertrophy [11] and constitutes an emerging target in renal disease. CCN2 had previously been shown to interact with the Wnt co-receptor, LRP6 [15]. Although Wnt signalling is crucially important during renal development, evidence suggests that it may be reactivated in the setting of renal disease. Consequently, it was suggested that CCN2 can interact with Wnt signals in the setting of diabetic nephropathy, thereby expanding its detrimental repertoire [31]. We propose CCN2 antagonism as a potential therapeutic intervention is at the centre of multiple pathways, including Wnt, that modify the pathogenesis and progression of diabetic nephropathy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.004.

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