TGFβ-modulates cell-cell-communication in early epithelial-to-mesenchymal transition

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Abbreviations

AFM	Atomic force microscopy
AJ	Adherens Junction
CKD	Chronic kidney disease
Cx	Connexin
DAPI	4’,6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbeccos modified eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
Elisa	enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERK	extracellular regulated kinase
FCS	Fetal calf serum
FSP1	Fibroblast specific protein
GJIC	Gap junctional intercellular communication
HK2	Human Kidney
MAPK	mitogen activated protein kinases
PBS	phosphate buffered saline
PT	Proximal tubule
siRNA	small interfering RNA
SMADS	small mothers against decapentaplegic
TGF-β	Transforming growth factor beta
TRITC	Tetramethyl Rhodamine Isothiocyanate
ZO	Zona Occludens

ABSTRACT

Aims/hypothesis A key pathology in diabetic nephropathy is tubulointerstitial fibrosis. Characterized by increased deposition of the extracellular matrix, fibrotic scar formation and declining renal function, the prosclerotic cytokine TGF-β1 mediates many of these catastrophic changes. The current study investigates a role for TGF-β1 induced epithelial to mesencyhmal transition (EMT) in alterations in cell-adhesion, cell coupling and cell communication in the human renal proximal tubule.

Methods: Whole-cell and cell-compartment expression of E-cadherin, N-cadherin, Snail, vimentin, β-catenin and connexin-43 (Cx43) was determined in HK2 and human proximal tubule cells (hPTC) +/-TGF-β1, using western blotting and immunocytochemistry, and quantified using densitometry. The contribution of Cx43 in PT cell communication was quantified using small interfering RNA knockdown, whilst dye-transfer assessed gap-junction mediated intercellular communication (GJIC). Functional tethering was assessed by single-cell force spectroscopy +/-TGF-β1 or immunoneutralization of cadherin ligation.

Results: High glucose (25mmol/l) increased the secretion of TGF-β1 from HK2 cells. Analysis confirmed early TGF-β1 induced morphological and phenotypic changes of EMT with altered expression of adhesion and adheren junction proteins. These changes correlated with impaired cell adhesion and decreased tethering between coupled cells. Impaired E-cadherin mediated adhesion induced a loss of Cx43 expression and GJIC, an effect mimicked by neutralizing E-cadherin ligation. Up-regulation of N-cadherin failed to restore adhesion or Cx43 mediated GJIC.

Conclusions: Our study provides compelling evidence that TGF-β1 induced EMT instigates a loss in E-cadherin, cell adhesion and ultimately connexin-mediated cell communication in the proximal tubule under diabetic conditions ahead of overt signs of renal damage.

INTRODUCTION

The crucial pathology underlying progressive chronic kidney disease (CKD) in diabetes is tubulointerstitial fibrosis [1-2]. Central to this process is epithelial-to-mesenchymal transformation (EMT), or the trans-differentiation of tubular epithelial cells into myofibroblasts [3-5]. Overwhelming evidence implicates the beta1 isoform of transforming growth factor (TGF-β1) as the predominant cytokine mediating these phenotypic fibrotic changes [6-7]. In diabetes, production of TGF-β1 in the proximal tubule is stimulated by high glucose [8-9]. TGF-β1 modulates the expression of several epithelial cell recognition and organizational proteins, whilst contributing to the reciprocal loss of tubular epithelial cells and accumulation of interstitial fibroblasts, changes associated with declining excretory function [10-12]. In EMT, the loss of epithelial characteristics, e.g. epithelial (E)-cadherin and the zonula occludens protein ZO-1 coincides with the acquisition of proteins associated with a mesenchymal phenotype, e.g. α-smooth muscle actin, Fibroblast Specific Protein (FSP1) and vimentin, culminating in cytoskeletal remodelling and disruption of the tubular basement membrane [13-14]. Loss of cell-adhesion, associated with reduced E-cadherin expression, represents a pivotal step of those early phenotypic and morphological changes previously observed in response to TGF-β1 induced tubular injury [15]. Cadherins have a central role in forming the multi-protein adherens junction (AJ) that links cell-cell contact to the actin cytoskeleton and various other signalling molecules [16]. The extracellular domain mediates ligation with E-cadherin on adjacent cells [17], whilst the cytoplasmic domain binds to β-catenin linking cadherin to the actin cytoskeleton via α-catenin. Interaction of cadherin with F-actin, via the catenins, not only serves to increase adhesive strength of the junction but also acts as a signalling 'node' for proteins that influence adhesiveness &/or initiate intracellular signalling. Co-localised with E-cadherin and β-catenin at the sites of cell-cell contact [18], connexins (Cx) oligomerise into hexameric hemichannels (connexons) connecting the cytoplasm of adjoining cells and forming gap junctions. Gap-junctions (GJ) allow transfer of

solutes, metabolic precursors and electrical currents [19], and are essential for synchronising activity to ensure appropriate function. Inhibiting cadherin-based cell adhesion inhibits GJ-assembly [20], whilst expression of recombinant cadherins into cells lacking strong coupling, increases Cx-phosphorylation at the AJ [21] and increases cell-to-cell communication [22]. Since intercellular adhesion precedes GJ-formation and inhibiting cadherin-based cell adhesion is known to inhibit GJ-assembly, we hypothesise that glucose-evoked increases in TGF-β will compromise cell-communication and therefore function in the proximal tubule.

In retinal capillaries of diabetic mice, Cx43 expression is reduced and apoptosis increased resulting in a loss of cell-communication and a decline in the number of pericytes and acellular capillaries [23]. This suggests that a loss in Cx-expression may be crucial in the development of vascular lesions observed in diabetic retinopathy. Similar findings from vascular endothelial cells, confirm that high glucose decreases Cx-expression/function and that this is an early trigger for apoptosis [24]. These data highlight the importance of GJ-mediated cell-coupling and suggests that a loss in cell-cell communication may contribute to some microvascular complications of the disease. Glucose decreases GJ-conductance and disrupts cellular homeostasis in a variety of cell systems [25-26] and glucose-dependent down-regulation of Cx43 expression and GJ-communication has been reported in bovine retinal pericytes [27], endothelial [28-29], and epithelial cells [30].Whilst the presence of GJs in the kidney has long been known, specific details regarding their function in the proximal tubule (PT) is sparse. Studies on renal vasculature have confirmed a role for various Cxs on renin secretion and the regulation of blood pressure [31], but minimal data exists on their role in tubular function where expression is also high. In the current study we present novel findings demonstrating a link between high glucose, TGF-β1, impaired cell adhesion and reduced GJ-expression in the proximal tubule. These changes will have profound effects on overall cellular integrity and function and may represent key events orchestrating loss of function in diabetic nephropathy.

METHODS

Tissue culture supplies were purchased from Invitrogen (Paisley, UK). Immobilon P membrane was from Millipore, Watford, UK and ECL; Amersham Biosciences, Buckinghamshire, UK. A Qproteome kit was obtained from Qiagen (Sussex, UK). Antibodies and siRNA were obtained from Santa Cruz (CA, USA), R&D systems (Abingdon, UK) and Affinity Bioreagents (Cambridge, MA, UK). Recombinant human TGF-β1, Fibronectin, Lipofectamine and Lucifer yellow were obtained from Sigma (Poole, UK) as were all other general chemicals. Anti-TGF-β1 ELISA was obtained from R&D systems.

Model cell line: HK2 cells (passages 18-30) were maintained in DMEM/Hams F12 (DMEM/F12) medium, supplemented with 10% fetal calf serum (FCS), glutamine (2mM), and EGF (5ng/ml) and cultured at 37^{o}C in a humidified atmosphere of 5%CO_2**.** Prior to treatment, cells were transferred to DMEM/F12 low glucose (5mM) for 48hr as described previously [32]. Cells were serum starved overnight before applying either TGF-β1 (2-10ng/ml), anti-E-cadherin neutralising antibody (20μg/ml) or anti-N-cad neutralising antibody (10μg/ml) for 48hr. To assess the effect of high glucose, cells were treated with either 5mmol/l (control), 25mmol/l (high) glucose, or 25mmol/l mannitol (osmotic control) for 48hr or 7days.

Human proximal tubular cells (hPTCs): Following patient consent and ethics approval from South Staffordshire Research Ethics Committee (REC application number 08/H1203), cells were obtained from anonymised nephrectomy for renal carcinoma. Renal cortex was longitudinally sectioned, the fibrous capsule removed and 1cm^3 portions cut from the outer region. Pieces were placed into DMEM and further cut into 1mm^3 sections. Each piece was placed into the well of a 24-well plate

which had been previously coated with gelatin for 20min followed by FCS overnight. Sections were cultured in DMEM/Nutrient Ham's F12 1:1 supplemented with 5mg:ml insulin, 5mg:ml transferrin, 5ng:ml sodium selenite, 36ng:ml hydrocortisone, 4pg:ml tri-iodothyronine 10ng:ml epidermal growth factor, 2mM glutamine 10 000U:ml penicillin and 10 000mg:ml streptomycin at 37°C, 5% CO_2. Immunohistochemical staining showed cells to be positive for cytokeratin, human epithelial antigen and alkaline phosphatase, but negative for factor VIII-related antigen and actin.

Quantification of TGF-β1: Total TGF-β1 was measured by specific enzyme-linked immunosorbent assay (ELISA) of cell culture supernatant collected from growth-arrested HK2 cells stimulated under serum-free conditions. Active TGF-β1 is measured directly and latent TGF-β1 can be measured indirectly following acid activation of samples. This assay has <1% cross-reactivity for TGF-β2 and TGF-β3. TGF-β1 concentration was normalized to mg/ml of protein. Data are expressed as picograms of TGF-β1 per milliliter per mg of protein.

Immunoblotting: Cytosolic proteins were prepared and separated by gel electrophoresis and electro-blotting onto Immobilon P membranes as described previously [32]. For determination of protein localisation, proteins were harvested using the Qproteome cell compartment kit. Membranes were probed with specific polyclonal antibodies against anti- E-cadherin (R&D systems), N-Cadherin (Sigma), Snail (R&D systems), vimentin (Affinity Bioreagents), beta-catenin (Santa Cruz), and Cx43 (Santa Cruz) at dilutions of 1:1000, 1:500, 1:500, 1:800, 1:1000, and 1:400 respectively.

Cx43Knockdown: Cells were grown to 40% confluence in six-well plates or on cover-slips treated with 3-aminopropyltriethoxy-silane (APES). Knockdown of Cx43 expression was achieved using small interfering RNA (siRNA). Transfection of siRNAs was carried out using Lipofectamine as

described previously [33]. Cells were harvested and assayed 48, 72 and 96hr after transfection. Negative controls included untransfected cells, lipid alone, two scrambled siRNAs, one of which was fluorscein conjugated. Cx43 knockdown was confirmed by Western blot analysis.

Immunocytochemistry: Cells at 80% confluence were fixed with 4% paraformaldehyde (PFA). Following blocking, the nuclear stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1mM) was added for 3mins. Cells were then incubated with TRITC-conjugated phalloidin (Sigma) diluted at 1:100 in PBS-Triton for 1hr at 25^{o}C or incubated overnight at 4^{o}C with primary antibody (1:100) diluted in PBS-Triton. Candidate proteins were visualized using Alexa488-conjugated secondary antibody (1:400) in PBS-Triton for 1hr at 25^{o}C. Fluorescence was visualized using an Axiovert 200 fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK).

Dye transfer: Lucifer yellow was dissolved in 250µl of fresh LiCl (150mmol/l)/HEPES (10mmol/l; pH 7.2). Individual cells within a cell cluster were injected using an Injectman/Femtojet 5247 delivery system (Eppendorf, Hamburg, Germany). The duration of injection was set at 1sec with an injection pressure of 2psi and a compensation pressure of 0.7psi. Dye transfer between coupled cells was recorded over 4min using Metamorph software (Molecular Devices) and a Cool Snap HQ CCD camera (Roper Scientific).

Single cell force spectroscopy: Atomic force microscope (AFM)-force spectroscopy (CellHesion® module, JKP Instrument Germany) was used to measure cell-cell adhesion and the separation forces required in uncoupling these cells. A single HK2 cell was bound to a cantilever using fibronectin (20mg/ml) and poly-l-lysine (25µg/ml) and subsequently brought into contact with an adherent cell

(in a cluster of coupled cells) using a known force (1nN). The two cells remained in contact for a defined period of time (10sec) whilst bonding formed. The cantilever was then retracted at a constant speed (5μm/sec) and force (nN) versus displacement (deflection of the cantilver) measured using a laser, until the cells were completely separated (pulling length 60-80μm). Each cell-cell recording was repeated in triplicate with a 30sec pause interval between successive measurements. Retraction recordings from multiple cells (approx 50) in separate experiments (n=5) were made and the maximum unbinding force (nN) and the detachment energy (fJoules) calculated.

Analysis: Autoradiographs were quantified by densitometry using TotalLab 2003 (NonLinear Dynamics, Durham, NC USA). Where data was quantified, the non-stimulated, low glucose control condition was normalized to 100% and data from all other experimental conditions compared to this. Statistical analysis of data was performed using a one-way ANOVA test with a Tukey's multiple comparison post-test. Data are expressed as mean±SEM, and 'n' denotes the number of experiments. Probability (P) <0.05 was taken to signify statistical significance.

RESULTS

HK2 and hPTC express adherens junction proteins:

Western blot analysis confirmed expression of (a) E-cadherin, (b) β-catenin, (c) Snail and (d) vimentin in HK2 and hPTC, giving rise to bands at 97kDa, 92kDa, 28kDa and 50kDa respectively (Fig1).

High glucose increases secretion of TGF-β1:

ELISA of supernatant from HK2 cells showed a 68% increase in total TGF-β1 secretion after exposure to high glucose (25mmol/l) for 7days under serum-free conditions (67±2.9pg/ml; Fig2), as compared to 5mM glucose (40±3.9pg/ml; n=3; P<0.01). Differences in TGF-β1 were only detected following acidification of the samples, suggesting that TGF-β1 was produced in its latent form. Incubation with a TGF-β1 neutralising antibody (10μg/ml) blocked the effects of high glucose restoring TGF-β1 secretion to basal (40±8.2pg/ml). Mannitol (25mmol/L) failed to increase TGF-β1 secretion. There was no difference in TGF-β1 secretion in supernatant taken from cells exposed to high glucose for 48hrs (data not shown).

TGF-β1 alters adherens junction protein expression in HK2-cells:

TGF-β1 (48hr; 2-10ng/ml) altered cell morphology from an epithelial-like cobblestone appearance, to an elongated fibroblast-like phenotype (see ESM Fig 1a). These changes were accompanied by re-organisation of the actin cytoskeleton into peripheral stress fibres (see ESM Fig 1b). The cytokine (48hr; 2-10ng/ml) induced a concentration-dependent decrease in whole-cell expression of E-cadherin (Fig3a), attributable to a loss in expression from both the membrane (M) and nuclear (N)

cell compartments (Fig3b). In contrast, TGF-β1 increased expression of the transcriptional repressor Snail throughout the cell (Fig3c and Fig3d). Integral to E-cadherin cell adhesion under basal conditions, β-catenin is primarily localised at the membrane (M) (Fig3f). In the current study, TGF-β1 failed to alter whole cell expression of β-catenin (Fig3e), but redistributed the catenin from the membrane (M) to nuclear (N) and cytoskeletal (CK) fractions (Fig3f). TGF-β1 produced a concentration-dependent increase in whole cell expression of the intermediate filament protein vimentin (Fig3g), with the greatest change being in the cytoskeleton and nucleus (CK and N, Fig3h).

TGF-β1 alters adherens junction protein expression in hPTC:

Human proximal tubule cells (hPTC) were incubated for 48hrs in low glucose (5mmol/l) with increasing concentrations of TGF-β1 (2-10ng/ml). TGF-β1 reduced expression of E-cadherin by 68±3.7%, 51±2.3% and 45±4.6% of control at 2, 4 and 10ng/ml respectively (n=3; $P<0.001$, Fig4a); up-regulated Snail expression to 110±10.4%, 127±7.8% (n=3; $P<0.05$) and 154±8.5% (n=3; $P<0.01$) of control (Fig4b), but failed to alter the expression of β-catenin (Fig4d). The expression of the intermediate filament protein, vimentin, increased to 104±7.5%, 133±9.4% and 155±8.4% of control at 2, 4 and 10ng/ml (n=3; $P<0.01$, Fig4c).

TGF-β1 decreases Cx43 expression:

Following *in vitro* activation of latent TGF-β1, by repeated cycles of freeze-thaw of 7day conditioned medium [34], subsequent 48hr exposure of cells to the activated media significantly decreased Cx43 expression to 72±2.1% compared to conditioned 5mmol/l control medium (n=3; $P<0.05$; Fig5a). Mannitol (25mmol/l) failed to alter Cx43 expression. In Fig5b, TGF-β1 (48hrs) decreased Cx43 expression in HK2 cells to 40±6.1%, 37±7.9% and 30±12.6% of control at 2, 4 and

10ng/ml (n=3; $P<0.001$). These data were comparable to cytokine-induced inhibition of Cx43 in hPTC, where expression fell to 53±4.8%, 53±3.7% and 51±3.5% of control at similar concentrations of TGF-β1 (n=3; $P<0.001$, Fig5c). In the absence (Fig5d) or presence (Fig5e) of the cytokine (10ng/ml) Cx43 is predominantly localised to the cell membrane (M). The reduction in whole cell expression of the protein therefore reflects a loss in expression, rather than redistribution to other cell compartments.

Loss of Cx43 decreases GJIC in proximal tubule cells:

Both HK2 cells and hPTC express Cx43 (Fig6a). Small interfering RNA (siRNA) was used to knockdown Cx43 expression to 30±3.4% of control ($n=4$, $P<0.001$), whilst scrambled siRNA and transfection reagent (TR) alone had no effect (Fig6b). In separate experiments, cells were transfected with fluorescein-tagged Cx43 siRNA to identify transfected cells expressing reduced Cx43 (false red colour in Fig6 panel e). Injection of Lucifer yellow into the transfected cells failed to exhibit dye transfer (green; Fig6 panels g-j) whilst dye moved away from the site of injection in non-transfected cells within the same cell cluster, confirming Cx43-mediated intercellular communication (Fig6 panels m-p).

Loss of E-cadherin ligation replicates TGF-β1 induced changes in Cx43-expression

To determine if the reduction in GJIC induced by TGF-β1 depended on a loss in E-cadherin expression or reflected reduced E-cadherin mediated ligation between adjacent cells, HK2 cells were treated for 48hr with an immuno-neutralising antibody for E-cadherin. The antibody prevents ligation, but has no effect on expression of the protein (antiuvomorulin, 20μg/ml; Sigma). Neutralising E-cadherin ligation altered cell morphology consistent to those changes observed in

response to TGF-β1 (Fig7a). The cytokine and anti-E-cadherin significantly down-regulated whole cell expression of Cx43 to 43±4.0% and 37±7.6% of control (n=3; P<0.001; see Fig7b), an effect mainly attributable to a loss in membrane expression (Fig7be). Co-application of TGF-β1 and anti-E-cadherin did not potentiate the changes in Cx43 expression compared to TGF-β1 alone (data not shown). Although TGF-β1 significantly reduced E-cadherin expression to 32±6.1% of control (n=3; P<0.01), as expected, neutralising ligation failed to alter either expression (112.7±12.2% of control; n=3, see Fig7d), or localisation of E-cadherin (Fig7e). In agreement with data presented in Fig3 and 4, TGF-β1 increased the expression of Snail to 125±13.8% of control (n=3; P<0.05), whilst neutralising E-cadherin ligation had little effect on either whole-cell expression (Fig 7f), or localisation of the protein (Fig7g). Whole cell expression of β-catenin was unchanged by TGF-β1 or loss of E-cadherin ligation, however the latter did re-localise to the cytoskeleton (CK) (Fig7h & i).

TGF-β1 reduces GJIC in proximal tubule cells

HK2 cells were cultured for 48hrs in low (5mmol/l) glucose, high (25mmol/l) glucose, low glucose +/- TGF-β1 (2-10ng/ml) or low glucose +/- anti-E-cadherin antibody (20μg/ml). Individual cells within a cluster were injected with Lucifer yellow (green) and the degree of dye spread assessed 4min after injection (Fig8; data representative of 3 repeats from each of 3 separate experiments). In control cells, dye permeated away from the injected cell into neighbouring cells (Fig8a). This cell-cell coupling was reduced under high (25mM) glucose conditions (Fig8b). TGF-β1 produced a concentration-dependant reduction in the extent of dye-transfer (2-10ng/ml; Fig 8c-e). This loss in GJIC was mimicked in cells treated with anti-E-cadherin (Fig8f).

Loss in E-cadherin increases N-cadherin expression:

N-cadherin expression (135kDa, Fig9a) was predominantly localised to the cell membrane (Fig9b). This expression was significantly up-regulated by TGF-β1 (48hrs) to 176±23%, 280±17% and 325±62% of control at 2, 4 and 10ng/ml in HK2-cells (panel c, n=3; P<0.05), and 237±45%, 240±18% and 305±27.5% of control in hPTC (panel d, n=3; P<0.01). In a separate series of experiments in HK2-cells, TGF-β1 (10ng/ml) up-regulated N-cadherin to 310±66% compared to control (Fig9e, n=3; P<0.05), whilst neutralising E-cadherin ligation only increased expression to 172±47% of control. The neutralising antibody had little effect on Cx43 expression (91±8.4% of control, n=3; see Fig9f), as compared to either TGF-β1 or anti-E-cadherin which significantly decreased Cx43 expression by 54±4.8% and 40±8.9% respectively (n=3; P<0.001).

TGF-β1 reduces adherence between coupled cells of the proximal tubule:

Atomic force microscope (AFM)-force spectroscopy was used to measure cell-to-cell adhesion and the separation forces required to uncouple cells. Prior to attachment, cells were cultured for 48hrs under identical conditions +/-TGF-β1 (10ng/ml). A single HK2 cell (cell-1) was bound to a cantilever and subsequently brought into contact with an adherent cell (cell-2) within a cluster, using a fixed force (Fig10 phase inserts). After 10sec, the cantilever was then retracted (5μm/sec) and force versus displacement measured until the cells were completely separated. Retraction force-displacement curves provide important information regarding the adhesion between two cells, such as the energy required to separate them (the grey area in panel a), and maximum force of detachment (red circle). The former is normally referred to as "detachment energy" (panel c) and the later the "maximum unbinding force" (panel b). The retraction measurements of control (47cells in 5 separate experiments) and TGF-β-treated HK-2 cells (53cells in 5 separate experiments) are shown in the figure 10b & c. The results indicate that TGF-β1 decreases the maximum unbinding force by 20±6% (n=5; P<0.001), whilst the detachment energy was decreased to 53±6% (n=5; P<0.001). Neutralizing

E-cadherin ligation had minimal effect on detachment energy (112% of control; 47cells in 5 separate experiments), but actually increased maximum unbinding force by 32±0.5%.

DISCUSSION

TGFβ1 is important in many tubulointerstitial diseases where disassembly of the adherens junction represents the initial overt change in epithelial organisation, well before any suggested cellular migration associated with EMT [34-35]. The cadherin-catenin complex is crucial in epithelial cell-cell adhesion and facilitates cell-communication via gap-junctions. We confirm that TGF-β1 reduces membrane expression of E-cadherin and show for the first time that the cytokine decreases functional tethering between cells of the proximal tubule. Whilst TGF-β1 fails to alter whole cell expression of β-catenin, accumulation in the nucleus is synonymous to that shown previously and may signify release and subsequent re-localization of β-catenin in response to reduced expression of the cadherin [36]. Our data provide compelling evidence that cell-cell adhesion and Cx43 GJIC are dramatically reduced in the presence of TGF-β1, events likely to represent an early stage in glucose-induced renal damage in the proximal tubule.

To establish a direct link between E-cadherin and Cx43-expression, a neutralising antibody against E-cadherin ligation was used to mimic changes in cell adhesion seen in response to TGF-β1. Negating E-cadherin ligation did not affect expression of the protein, nor did it up-regulate the transcriptional repressor Snail which accompanied the down-regulation of E-cadherin expression in response to TGFβ. Importantly however, loss of ligation reduced Cx43 expression to a comparable degree to that observed in response to the cytokine, suggesting that it is a loss in tethering between cells, rather than a change in protein expression per se, which controls Cx-expression and GJIC. Retraction force-displacement curves confirmed that TGF-β1 reduced the maximum unbinding force required to begin separation of two cells by 20%, whilst halving the detachment energy required to completely separate them. The greater decrease in the detachment energy could be partly explained due to the increase in cell rigidity following TGF-β treatment as demonstrated by re-arrangement of the cytoskeleton into peripheral stress fibres (see electronic supplementary material ESM).

A switch in cadherin isoform from E-cadherin to neural (N)-cadherin is associated with EMT [37]. TGF-β dramatically increased N-cadherin expression in our model. However, this switch was unable to reverse morphological changes or the loss in cell-to-cell adhesion in response to the cytokine. Surprisingly, neutralizing E-cadherin ligation actually increased maximum unbinding force. Counter-intuitive, this observation suggests that the modest increase in N-cadherin, evoked by blocking E-cadherin ligation (Fig9e), can maintain tethering between coupled cells when the cadherin-catenin complex is intact, i.e. when only ligation is impaired. However, TGF-β1 dramatically reduced E-cadherin expression and forced β-catenin to move away from the membrane. In this scenario, and in the absence of a catenin binding partner, up-regulation of N-cadherin is redundant, and the switch is unable to maintain tethering. These data suggest that it is the loss in E-cadherin expression and dissolution of the catenin/cadherin complex which drives the detachment of cells in EMT.

Type 2 EMT is commonly defined as the ability of adult epithelial cells to undergo de-differentiation, traverse the TBM into the interstitium and trans-differentiate into a myofibroblast phenotype capable of synthesising and increasing the deposition of ECM. Whilst these activated myofibroblasts are thought of as key effector cells in the pathogenesis of renal fibrosis, it is clear that they originate from multiple lineages. Accumulating evidence suggests that local interstitial fibroblasts [38], pericytes [39], local mesenchymal stem cells [40] or the injured epithelium itself [10] may contribute to this pool, and there is considerable debate both for and against a role of EMT in renal fibrosis [41]. The established criteria supporting EMT in fibrosis is based upon identifying morphological changes and altered expression of key epithelial/mesenchymal markers. Failure of fibroblasts to fully migrate and traverse the TBM is more commonly known as partial EMT (p-EMT), a phenomenon where cells express epithelial and mesenchymal markers yet lack migratory capacity. The argument against full phenotypic transformation has been fuelled by data from the

Duffield group who suggest that not only is EMT unlikely to occur *in vivo*, but that vascular pericytes are the source of fibrosis generating myofibroblasts [39].

As summarised in a recent review TGF-β1 binds to a trans-membrane TBRII receptor and initiates several intracellular signalling cascades, including the small mothers against decapentaplegic (SMADs) and mitogen activated protein kinases (MAPK), such as extracellular regulated kinase (ERK), p38 and Jun Kinase [42]. Smads are subdivided into three classes; Receptor regulated (R) Smads (Smad1, 2, 3, 5 and 8), the Common (Co) Smads (Smad4) and the Inhibitory (I) Smads (Smad6 and 7) [43]. Following TβRII activation, R-Smads form oligomeric complexes with the common Smad (Co-Smad) prior to translocation into the nucleus and regulation of gene transcription, The majority of TGF-β1 targeted genes regulated in EMT rely on Smad3-dependent transcriptional regulation. Recent studies in cells from the proximal tubule demonstrated angiotensin II-induced tubular EMT was Smad3-dependent [44]. Similarly, (β)1-integrin gene expression, a potential therapeutic target of renal fibrosis is also up-regulated in both unilateral obstruction and in chronic tubulointerstitial fibrosis via Smad3-dependent mechanism [45]. However, despite the predominant involvement of Smad3, a role for Smad2 should not be discounted [46].

Hyperactive Smad signalling observed in certain types of renal disease reflects aberrant levels of both Smad co-repressors and their subsequent regulators [47]. The inhibitory Smads (Smad6 and Smad7) inhibit R-Smad phosphorylation by blocking their access to TβRI, &/or by promoting the degradation of the receptor complexes. Smad7 represents a general antagonist of both TGF-β1 and BMP signalling, with reports showing that induction of Smad7 blocks tubular EMT and the development of fibrotic lesions [48]. The role and regulation of Smad signalling in regulation of gap junction expression and GJIC in the proximal tubule remains to be confirmed; however it is highly likely that these effects are Smad-dependent and subject to regulation via endogenous inhibitors. The potential of exogenous agonist application in reversing these disrupted changes in cell-to-cell

communication represents an area of therapeutic interest and forms the basis of our continuing research.

Reduced cell-adhesion, cell-coupling and cell-to-cell communication will have profound effects on overall integrity and function of the proximal tubule and altered GJIC and renal haemodynamics have recently been reported in a Zucker fatty rat model of type2 diabetes [49]. In the current study we concede that our *in vitro* data provides a minimalistic model of the early events in EMT and we recommend caution in translating these novel findings to the *in vivo* situation where the multifactorial molecular pathology of renal fibrosis may modify responses. However, in spite of this caveat, current data provide a compelling foundation to identify future therapies aimed at maintaining or restoring renal function in diabetes, a supposition supported by recent data from mesangial cells, where glucose-induced hypertrophy has been reversed by Cx43 over-expression [50].

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CONTRIBUTION STATEMENT

All authors contributed to the conception, design and analysis of the data and drafting of the manuscript and have approved the final version for publication.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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FIGURE LEGENDS

Figure 1: ***HK2 and hPTC express adherens junction proteins*****:**

Western blot analyses of HK2 and hPTC cell lysates (5μg protein/lane) using antibodies against human (a) E-cadherin, (b) β-catenin, (c) Snail, and (d) vimentin detected appropriate bands of approximately 97kDa, 92kDa, 28kDa and 50kDa respectively. Controls included antibody pre-absorbed with a 10-fold excess of immunizing peptide (data not shown).

Figure 2: ***High glucose stimulates TGF-β1 secretion in HK2 cells:***

HK2 cells were grown in either low glucose (5mmol/l control), or high glucose (25mmol/l) ±TGFβ-1 neutralising antibody (10μg/ml) for 7days under serum-free conditions. Mannitol (25mmol/l) was used as an osmotic control. The supernatant was removed and subsequently TGF-β1 secretion was quantified by ELISA and expressed as pg/ml of TGF-β1 secreted per mg of protein. Results are representative of 3 separate experiments. Key significances are shown where **P*<0.05, ***P*<0.01 and *** *P*<0.001.

Figure 3: ***TGF-β1 evoked changes in AJ-protein expression in HK2-cells*****:**

To assess the effect of TGF-β1 on expression of key AJ proteins, HK2 cells were grown in low glucose (5mmol/l) alone (control), or together with 2, 4 or 10ng/ml TGFβ-1. Whole cell expression of E-cadherin and its transcriptional co-repressor Snail were determined by immunoblotting. TGF-β1 decreased E-cadherin expression (panel a**)** and reciprocally increased the expression of Snail (panel c). Compartmental localisation of E-cadherin and Snail expression were determined in membrane (M), cytosol (C), nuclear (N) and cytoskeletal (CK) fractions of HK2 cells +/-TGF-β1 (10ng/ml).

The cytokine altered the cellular localization of both proteins, as compared to control (panels b and d respectively). The cytokine did not alter whole cell expression of β-catenin (panel e), but increased vimentin expression in a concentration-dependent manner (panel g). The distribution of both proteins between the different cell compartments was altered in response to TGF-β1 (panel f and h respectively). Representative blots for each protein were re-probed for GAPDH as a loading control.

Figure 4: ***TGF-β1 evoked changes in AJ-protein expression in hPTCs*****:**

hPTCs were grown in low glucose (5mmol/l) +/- 2-10ng/ml TGFβ-1 for 48hrs and whole cell expression of (a) E-cadherin, (b) Snail, (c) Vimentin and (d) β-catenin was determined by immunoblotting. Upper panels show representative blots for each protein and re-probed for GAPDH as a loading control. Lower panels show mean (±SEM) densitometry data, normalised against the non-stimulated low glucose control (100%), from 3 or more separate experiments. Each bar in the histogram corresponds to the associated lane in the representative blot. Key significances are shown, **P*<0.05, * *P*<0.01, *** *P*< 0.001.

Figure 5: ***TGF-β1 down-regulates Cx43 expression in proximal tubule cells*****:**

To assess the effects of glucose on Cx43 expression, HK2 cells were cultured in either low glucose (5mmol/l), high glucose (25mmol/l) or mannitol (25mmol/l) for 7days. Secreted TGF-β1 was activated by repeat freeze-thaw and the conditioned media applied for 48hrs (panel a). In a separate series of experiments, HK2 (panel b) and hPTC (panel c) cells were grown in low glucose (5mmol/l) ±2, 4 or 10ng/ml TGFβ-1. Whole cell Cx43 expression was determined by immunoblotting. Upper panels are representative immunoblots showing changes in protein expression versus the same blots stripped and re-probed for GAPDH as a loading control. Lower panels show mean (±SEM)

densitometry data from 3 or more separate experiments. Data normalised against the unstimulated low glucose control (100%). Each bar in the histogram corresponds to the associated lane in the representative blot and key significances are shown where *** $P< 0.001$. Compartmental localisation of Cx43 expression was determined in membrane (M), cytosol (C), nuclear (N) and cytoskeletal (CK) fractions of control (panel d) and TGF-β1 treated (10ng/ml) HK2 cells (panel e).

Figure 6: ***Knock-down of Cx43 prevents GJIC in HK2 cells:***

Panel a confirms Cx43 expression in 3 separate preparations from both HK2 cells and hPTC. In panel b, Cx43 siRNA significantly reduced CX-expression in HK2 cells. The upper panel is a representative immunoblot showing changes in protein expression for the transfection reagent (TR) alone, Cx43 siRNA and scrambled siRNA versus the same blot stripped and re-probed for GAPDH as a loading control. Blots were quantified by densitometry and data normalised against the non-stimulated low glucose (5mM; 100%). The lower panel shows mean (±SEM) densitometry data from 3 separate experiments. Each bar in the histogram corresponds to the associated lane in the representative blot where *** signifies $P<0.001$. In panel c, red fluorescent protein (RFP) was used to identify cells exhibiting anti-Cx43 transfection within a cluster of non-transfected cells. The red transfected cell (d and f) was injected with Lucifer yellow (g) and dye spread (green) examined between 0min (h) and 4min (i). An overlay (yellow) of red transfection and green dye spread is shown in panel j and with phase (k), 4min after the initial injection of dye. In panel i, the micro-injector is repositioned over a non-transfected cell in the same cluster. Injection of Lucifer yellow (m and n), led to a spreading of dye (green) between 0min (panel o) and 4min (panel p). An overlay (yellow) of red transfection and green dye spread is shown in panel q and a triple overlay with a phase image is shown in panel r.

Figure 7: ***Loss of E-cadherin ligation partially mimics the effects of TGF-β1 on cell morphology and Cx43 expression in HK2 cells*****:**

Phase contrast microscopy demonstrated that TGF-β1 (10ng/ml) and loss of E-cadherin ligation altered gross cell morphology in favour of a fibroblast phenotype (panel a, magnification x20). The effects of the cytokine and anti-E-cadherin on the expression of Cx43 (b), E-cadherin (d), Snail (f) and β-catenin (h) were examined in HK2 whole cell lysates and cell fractions. Upper panels are representative immunoblots showing changes in candidate protein expression versus the same blots stripped and re-probed for GAPDH as a loading control. Blots were quantified by densitometry and data normalised against the unstimulated low glucose (5mM) control (100%). Lower panels show mean (±SEM) densitometry data from 3 or more separate experiments. Each bar in the histogram corresponds to the associated lane in the representative blot. In panels c,d,g and h, compartmental localisation for each protein was determined in membrane (M), cytosol (C), nuclear (N) and cytoskeletal (CK) fractions of HK2 cells +/-48hrs with anti-E-cadherin antibody (20μg/ml). Key significances are shown where *P<0.05, **P<0.01 and *** P<0.001.

Figure 8*: TGF-β1 reduces GJIC in HK2 cells:*

GJIC was determined by the extent of dye (green) spread following micro-injection of Lucifer yellow into a single HK2 cell within a cell cluster. Control cells (panel a) exhibited a rapid (0-to-4min) transfer of dye between neighbouring cells. 48hr exposure to either high glucose (25mmol/) (b) or low glucose (5mmol/l) incubated with TGF-β1 at either 2ng/ml (c), 4ng/ml (d) and 10ng/ml (e) evoked a concentration-dependent decrease in dye spread. Neutralising E-cadherin ligation, using an immuno-neutralising antibody (20μg/ml; panel f), also restricted intercellular communication and prevented dye transfer.

Figure 9: ***TGF-β1 turns on the 'cadherin switch'*****:**

Western blot analyses of HK2 and hPTC cell lysates (5μg protein/lane) using antibodies against human N-cadherin detected bands of approximately 135kDa in 3 different protein preps (panel a). Membrane localization was confirmed by immunocytochemistry where N-cadherin immunoreactivity was visualized by ALEXA488 (green) and nuclear staining for DAPI is blue (panel b). HK2 cells (panel c) and hPTC (Panel d) were grown in low glucose (5mmol/l) alone or ±2, 4 or 10ng/ml TGFβ-1 for 48hrs and whole-cell expression of N-cadherin determined by immunoblotting. A comparison between the effects of TGF-β and anti-Ecad on N-cadherin expression in HK2 cells was assessed in panel e. Finally comparisons between the effects of TGF-β1, anti-Ecad and anti-Ncad on Cx43 expression were determined in HK2 cells (panel f). Upper panels are representative immunoblots showing changes in protein expression or the same blots stripped and re-probed for GAPDH as a loading control. Blots were quantified by densitometry and normalized against the non-stimulated low glucose control (100%). Lower panels show mean (±SEM) densitometry data from 3 or more separate experiments. Each bar in the histogram corresponds to the associated lane in the representative blot. Key significances are shown, **P*<0.05, * *P*<0.01, *** *P*< 0.001.

Figure 10: ***TGF-β reduces cell adhesion*****:**

AFM-force spectroscopy was used to measure the detachment energy (fJoules) and maximum unbinding force (nN) required to uncouple two HK2 cells. In panel a, a single HK2 cell can be seen bound to the cantilever (cell-1, phase insert). This was bought into contact with an adherent cell-2 using a fixed force (1nN) for 10sec, whilst bonding formed. The cantilever was then retracted

(5μm/sec) and force versus displacement measured until the cells separated (approximate pulling length 60-80μm). The energy required to separate the cells (grey area in panel a), and maximum force of detachment (red circle) was measured. The former is known as the "detachment energy" (panel c) and the later is "maximum unbinding force" (panel b). The cytokine (10ng/ml) decreased the maximum unbinding force by 20%, and the work of adhesion decreased by 53% compared to control. Data is expressed as mean ± SEM. of multiple cells from 5 separate experiments, where key significances are shown, *** $P<0.001$.