MINI REVIEW

The role of TGF-beta and epithelial-to mesenchymal transition in diabetic nephropathy

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Abstract

Transforming Growth Factor-beta (TGF-β) is a pro-sclerotic cytokine widely associated with the development of fibrosis in diabetic nephropathy. Central to the underlying pathology of tubulointerstitial fibrosis is epithelial-to-mesenchymal transition (EMT), or the trans-differentiation of tubular epithelial cells into myofibroblasts. This process is accompanied by a number of key morphological and phenotypic changes culminating in detachment of cells from the tubular basement membrane and migration into the interstitium. Ultimately these cells reside as activated myofibroblasts and further exacerbate the state of fibrosis. A large body of evidence supports a role for TGF-β and downstream Smad signaling in the development and progression of renal fibrosis. Here we discuss a role for TGF-β as the principle effector in the development of renal fibrosis in diabetic nephropathy, focusing on the role of the TGF-β1 isoform and its downstream signaling intermediates, the Smad proteins. Specifically we review evidence for TGF-β1 induced EMT in both the proximal and distal regions of the nephron and describe potential therapeutic strategies that may target TGF-β1 activity.

Introduction:

Diabetic nephropathy (DN) is the single commonest cause of entry into the renal replacement therapy programme, and with the incidence of the disease doubling in the past decade DN now accounts for approximately 50% of those patients presenting with end-stage renal failure [1]. Although the aetiology of Type I and Type II diabetes is notably distinct, glucose-evoked changes in renal physiology are almost indistinguishable and often lead to complete destruction of kidney function prompting the need for dialysis or transplantation therapy [2]. Multiple structural and functional changes are associated with the disease, specifically in the glomerulus, tubulointerstitium and vasculature [3], where glycaemic injury includes structural abnormalities ranging from hypertrophy, thickening of the glomerular basement membrane, tubular atrophy and interstitial fibrosis [4]. These changes contribute to increased glomerular filtration rate, proteinuria, systemic hypertension and the loss of renal function [4]. Histologically, DN is characterised by an accumulation of extracellular matrix (ECM) in both the glomerular mesangium and tubular interstitum, culminating in excessive renal scarring and a decline in excretory function [4-7]. Renal fibrosis is evidenced by glomeruosclerosis, tubulinterstitial fibrosis (TIF), infiltration of inflammatory mediators and the activation of alpha-smooth muscle actin (α-SMA)-positive myofibroblasts [6][8]. Of these fibrotic changes, tubulointerstitial fibrosis is the key underlying pathology and represents the final common pathway for End-Stage Renal Disease (ESRD). Understanding those mediating signals which regulate deposition of fibrotic material in the interstitium is critical to future identification and development of site specific therapeutics which may alleviate this damage. Central to TIF is tubular epithelial-to-mesenchymal transition (EMT), or the transdifferentiation of tubular epithelial cells into myofibroblasts [9-10]. In DN, EMT occurs as cells attempt to evade apoptosis as a consequence of exposure to pathophysiological stimuli [11-12]. Cells undergo transition in a process that involves the generation of active myofibroblasts, excessive deposition of ECM and destruction of normal tissue architecture [13-14]. The ability to switch phenotype stems from a unique plasticity of epithelial cells that has, up until recently, been universally accepted as the sole driving force behind the generation of interstital fibroblasts in kidney disease. EMT of proximal tubule cells (PTC) has been clearly

documented in DN, with overwhelming evidence implicating the cytokine Transforming Growth Factor-beta 1 (TGF-β1) as the key mediator [15-16]. Our knowledge of the pathology of TGF-β1 in the kidney is extensive however, the majority of this research is dedicated to exploring the consequences of TGF-β1 signalling in glomerular cells, mesangial cells or podocytes. The aim of the current review is to discuss a role for TGF-β1 in DN, specifically in the proximal tubule and the collecting duct, where potential therapeutic strategies to inhibit renal fibrogenesis in diabetes may be unearthed.

TGF-β1 as a molecular mediator of renal fibrosis in Diabetic Nephropathy:

Deposition of ECM is required for normal wound healing in response to renal injury, but the excessive deposition of matrix is the pathological hallmark of renal fibrosis and occurs when cells of the nephron over compensate in an attempt to maintain both cell integrity and function. A tight balance between synthesis and breakdown of matrix proteins is therefore required to maintain normal function. One way to control this balance is via the release of mediators from inflammatory or connective tissue cells which act to regulate collagen and matrix metalloproteinase (MMP) production. Loss of this regulation can ultimately tip the balance from repair to injury culminating in a build up of fibrotic material and scar formation.

Diagnosed by glomerulosclerosis, tubulointerstitial fibrosis, inflammatory filtration, and loss of renal architecture [17], renal fibrosis develops in response to an accumulation of ECM. In DN, progressive tubulointerstitial fibrosis represents the final common pathway of chronic renal failure and is a consequence of increased production and altered degradation of ECM components. In diabetes, hyperglycaemia is the driving force behind the majority of these pathways and the inevitable build up of fibrotic material [16][18]. A decline in the number of nephrons parallels increased fibrosis as interstitial scarring replaces the spaces left by nephrons lost in this pathological process; the net result is impaired renal function.

Although more than a dozen fibrogenic factors affect renal function, it is widely recognised that the pro-sclerotic cytokine TGF-β1 and its downstream Smad signalling cascade, represents the predominant pathway orchestrating renal fibrosis [19][20]. Abnormalities in TGF-β1 have been linked to a variety of disorders, including autoimmune diseases, malignancies, and chronic renal disease [21]. Indeed, the up-regulation of TGF-β appears prevalent in all forms of chronic kidney disease (CKD) in both animal models and humans. Increased expression of TGF-β receptors have been described in experimental models of renal disease including; membranous nephropathy, obstructive nephropathy, and DN [21], whilst animal models of spontaneous diabetes (BB rat and NOD mouse) demonstrate increased TGF-β1 mRNA expression within 3-7 days of the onset of hyperglycaemia [22]. In the streptozotocin (STZ)-induced diabetic rat, mRNA expression for both TGF-β1 and the type II TGF-β receptor are increased within 3days following exposure to hyperglycamia [22]. Initiated in response to the actions of numerous circulating signalling molecules, hyperglycaemia drives the production of various downstream mediators of TGF-β. These downstream effector molecules include Advanced Glycation End products (AGEs), protein kinase C (PKC) and diacylglyerol (DAG). Together they instigate excess deposition of ECM [16][18][23]. Glucose-induced TGF-β1 mediated increases in ECM have been reported in cultured mesangial cells [24], podocytes [25] and tubular epithelial cells [26], the cumulative effect of which is destruction of the renal parenchyma. A recent study by Reiniger *et al* (2010) demonstrated that removal of the AGE receptor in the diabetic OVE26 mouse, attenuated the degree of glomerulosclerosis with a concomitant improvement in renal function [27], whilst over-expression of PKC, has been shown to exert fibrotic effects in human proximal tubular cells [28]. Patient studies also implicate TGF-β1 with fibrosis in DN and individuals with both type I and type II diabetes mellitus exhibit enhanced production of TGF-β1 in their kidneys, expression of which closely correlates to the degree of glycaemic control [29-30].

The role of TGF-β1 signalling in renal fibrosis:

The TGF-β superfamily is comprised of secreted peptides subdivided into four main subgroups: the mullerian inhibitory substance (MIS) family, the inhibin/activin family, the bone morphogenetic protein (BMP) family and the TGF-β family [31]. Of these secreted peptides, all but the MIS family; are commonly associated with mammalian development, homeostasis and pathobiology. A broad spectrum cytokine, TGF-β regulates many fundamental biological processes including cell growth, differentiation, adhesion, proliferation tissue repair and apoptosis [32-33]. Of the five distinct isoforms which have been identified in vertebrates, only three have been shown to be expressed in mammals [34]. These isoforms exhibit ubiquitous levels of expression and act to evoke a response via the initiation of 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 [35]. More recently, a study performed by Nyhan and colleagues demonstrated a role for a separate signalling cascade. Using proximal tubule cell line models, HK2 and RPTEC cells, they demonstrated that the Jagged/Notch signalling pathway is activated in response to TGF-β1. Furthermore, the cytokine-evoked changes in Jagged 1 expression preceeded EMT associated gene changes in both E-cadherin and vimentin, effects which appeared to be dependent on Smad3 activity [36]. Therefore, TGF-β1 can modulate transcription of multiple target genes through the activation of at least one, or a cross talk of multiple signaling pathways.

The TGF-β1 gene encodes a 390 amino acid precursor molecule that is composed of a signal peptide, the active TGF-β1 molecule and a latency associated peptide (LAP). Only when the signal peptide is proteolytically cleaved from the TGF-β1 gene product is an inactive latent TGF-β1, covalently bound with the LAP, released [37]. Dissociation of the covalent bond depends on environmental changes in pH, but ultimately TGF-β1 is released from the latent complex, enabling mature, active TGF-β1 to bind to its cell surface receptors [38]. The TGF-β receptor is a heteromeric trans-membrane complex consisting of both the TGF-β receptor II (TβRII) and TGF-β receptor type I (TβRI), the latter includes activin-like kinase (ALK) receptors. Unlike many membrane bound complexes which possess tyrosine kinase activity, the TGF-β receptors possess serine/threonine

kinase activity. Binding of TGF-β1 to the TBRII receptor is accompanied by phosphorylation and subsequent activation of the type I receptor within its cytoplasmic domain. This association results in the downstream phosphorylation and activation of its classic signaling mediators, Smad2 and Smad3 [39-40]. 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) [39]. 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 (see figure 1) [39].

TGF-β/Smad signaling is stringently controlled within the cell and is regulated at both prereceptor and postreceptor stages through multiple levels of modulation. However, in many cell lines, TGF-β1 has the ability to regulate its own transcription through binding of an AP-1 transcription factor complex to the TGF-β1 promoter [41-42] or through the involvement of a Smad3-dependent signalling pathway [43]. Autoinduction of TGF-β1 is a well recognized phenomenon which has been described in a variety of cell types [42]. However, the significance of this may be tissue-specific. The effect of auto-induction, whether beneficial or detrimental to the cell, depends on a scenario in which expression is upregulated. For example, TGF-β1 mRNA auto-induction is thought to have a beneficial role in cardiac wound healing after ischemic injury [44] , whilst auto-induction of TGF-β1 at the site of injury in the proximal tubule may result in a positive feedback, where sustained cytokine production may accelerate EMT and increase the generation of myofibroblasts thus promoting and exacerbating fibrosis. A correlation between Smads and AP-1 has also been suggested following elegant studies performed in a mouse model of unilateral ureteric obstruction and in fibroblasts obtained from Smad2 and Smad3 knockout animals [45-46]. These studies confirmed not only a correlation between Smad signalling and TGF-β1 auto-induction, but further demonstrated that the relationship depended on the presence and activation of Smad3. These observations agree with previous studies and support a role for Smad signalling in the development of renal fibrosis [47].

The majority of TGF-β1 targeted genes regulated in EMT rely on Smad3-dependent transcriptional regulation [45]. Recent studies in renal proximal tubule cells demonstrated angiotensin II-induced tubular EMT was Smad3-dependent [48], whilst (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 a Smad3-dependent mechanism [49]. Despite the predominant involvement of Smad3, a role Smad2 should not be discounted [50]. Differential roles for both Smad2 and Smad3 have been identified [51]. Smad3-dependent reduction of the cell adhesion protein E-cadherin in human proximal tubular cells (hPTC) is paralleled by a Smad2-dependent induction of metallomatrix proteinase 2 [52]. Microarray analysis of TGF-β1-induced EMT in mouse and human epithelial cells demonstrates a critical requirement for Smad signalling in the regulation of all tested target genes [52] and an acute need for stringent control of Smad signalling in order to protect the cells from unwanted responses to TGF-β. Indeed, a safeguard mechanism exists in the form of inhibitory Smads and transcriptional co-repressors [53]. However, the level of hyperactive Smad signalling observed in certain types of renal disease reflects abberant levels of both Smad co-repressors and their subsequent regulators [54-55]. 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 [56-57]. Smad6 appears to play a more specific role in the Bone Morphogenic Pathway (BMP) [58-60].

The co-repressors SnoN (Ski-related novel gene, non Alu-containing), Ski (Sloan-Kettering Institute proto-oncogene), and TGIF (TG-interacting factor) prevent gene transcription through inhibition of R-Smads [61]. Since these antagonists are critical in ensuring the regulation of Smad-mediated gene transcription, a fine balance must be achieved in order to match cellular demands. It is of no surprise that diminished levels of co-repressors are observed in animal models of obstructive nephropathy and diabetes [67]. Smad ubiquitination regulatory factor-2 (Smurf2) is an ubiquitin ligase that specifically targets certain members of Smad proteins for degradation, including Ski, SnoN and TGIF [63]. The close association between Smurf2 expression and enhanced SnoN degradation [62] suggests that the dysregulation of Smurf2 is most likely to affect profibrotic TGF-β/Smad signalling and may contribute to the development and progression of renal fibrotic diseases in humans [63]. A

role for Smurf2 in the degradation of inhibitory Smad7, an event paralleled by increased tubular EMT has recently been reported [48].

EMT in the kidney

Renal tubules are developmentally derived from the metanephric mesenchyme through a process termed mesenchymal-to-epithelial transdifferentiation (MET). This cellular differentiation is fluid and cells retain the ability to revert back to their original mesenchymal phenotype through EMT. Commonly associated with epithelia of embryonic origin, this plasticity is critical in early stages of development and is commonly referred to as type I EMT [64]. There are two further subtypes of EMT, type II and type III, the latter of which occurs in epithelial cancer cells that differ genetically and epigenetically from untransformed epithelial cells. In adults, EMT is associated with tissue injury and repair; a process instigated as the demand for fibroblasts and wound healing increases [68]. Type 2 EMT is now used to categorise events that occur in epithelial cells following injury. As cells undergo Type 2 EMT they provide the interstitium with a new supply of fibroblasts. Furthermore, in an attempt to restore a healthy population of cells lining the tubular basement membrane (TBM) some epithelial cells are able to employ EMT as a means to migrate into damaged areas of the nephron prior to reconversion into an epithelial phenotype [64].

In EMT, the loss of epithelial characteristics coincide with the acquisition of proteins associated with a mesenchymal phenoptye (See figure 2) These morphological and phenotypic changes occur at four different stages: (i) the loss of epithelial cell adhesion molecules such as Epithelial (E)-cadherin and zonula occludens protein ZO-1 are replaced by the (ii) mesenchymal markers α-SMA and the intermediate filament protein vimentin. The loss of cell adhesion is accompanied by (iii) cytoskeletal remodelling and morphological changes resulting in tubular basement membrane disruption. (iv). As myofibroblasts exhibit enhanced motility and increased proliferative and contractile capacity, these cells possess the ability to migrate from TBM into the interstium. This migratory capacity leads to increased deposition of the ECM and makes EMT pivotal

in the pathology of tubulointerstitial fibrosis. Normally, tubular epithelial cells form a highly coupled epithelial sheet held together by the adhesion molecule E-cadherin. Loss of E-cadherin expression occurs in the early stages of EMT and results in the dissociation of cells within the epithelial sheet [66]. Whether the loss of E-cadherin is a consequence or a cause of EMT remains unknown. However, TGF-β1 is unable to initiate EMT without disrupting the integrity of cell-to-cell contact, indicating involvement of E-cadherin in this TGF-β1-mediated process [67]. The loss of cell adhesion represents the beginning of a series of events culminating in transition from an epithelial-to-mesenchymal phenotype. Changes in E-cadherin are rapidly accompanied by an up-regulation of mesenchymal markers. Re-organisation of the actin cytoskeleton into stress fibres containing *de novo* expression of α-SMA is accompanied by an exchange of cytokeratin for vimentin filaments and the expression of fibroblast-specific-protein-1 (FSP1), a Ca^{2+}-binding protein involved in motility, invasion, and tubulin polymerization. These morphological and phenotypic changes support matrix remodelling and migration across the TBM into the interstitial environment [68].

The migratory phenotype, used to define EMT remains controversial, especially in the context of fibrosis [69]. Loss of an epithelial phenotype can be clearly identified by a loss in the expression of specific epithelial proteins, in particular E-cadherin. Acquisition of a mesenchymal phenotype is more difficult to assign [70] and may explain why EMT is often over-looked in studies of renal disease. Classic markers used to define EMT include vimentin and α-SMA [71]. Whilst vimentin is not specific for fibroblastoid cells [72], staining of both vimentin and tubular β-catenin is currently being used clinically to assess early renal injury (clinical trial NCT#01079143) [73]. Although α-SMA represents the most commonly used marker in EMT, heterogeneity of expression means that this protein is not a definitive marker [74], and clarification of EMT in fibrosis should be regarded with caution, since confirmation of this phenotypic transformation appears to depend upon a complex interplay of events. The concept that cells of an epithelial origin are able to undergo a phenotypic transformation in response to pathophysiological stimuli and ultimately traverse the TBM has, up until recently, been universally accepted. However, there is now clinical evidence from biopsies of renal allografts suggesting that the changes in epithelial phenotype precede fibrosis [75].

Consequently, the contribution of EMT to renal fibrosis *in vivo* and, more importantly, the exact origin of the myofibroblasts [76-77] has become an area of intense debate [78].

Type 2 EMT is commonly defined as the ability of adult epithelial cells to undergo dedifferentiation, traverse the TBM into the interstitium and transdifferentiate into a myofibroblast phenotype capable of synthesising and increasing the deposition of ECM. Ultimately the epithelium is replaced by mesenchymal cells and fibrous scar tissue. Whilst these activated myofibroblasts are commonly thought of as key effector cells in the pathogenesis of renal fibrosis, it is becoming increasingly clear that they originate from multiple lineages. This has subsequently led many to cast doubt over the contribution of EMT to renal fibrosis *in vivo* and more importantly, question the exact cell type involved [76-77]. An accumulating body of evidence suggests that cells contributing to this pool, may include local interstitial fibroblasts [79], pericytes [80][81] local mesenchymal stem cells [82] or the injured epithelium itself [10]. As a result of these studies, there is now considerable debate both for and against a role of EMT in renal fibrosis. These arguments are eloquently summarised in a recent article published in JASN where eminent leaders in the field (Michael Zeisberg and Jeremy Duffied) debate the case for EMT in fibrosis [83].

Until recently, support for the role of EMT in fibrosis was based upon the following observations: (1) Tubular epithelial cells transform into activated myofibroblasts in response to pathophysiological stimuli, e.g. TGF-β1, Connective Tissue Growth Factor (CTGF) and AGE products (2) Characteristic features of this phenotypic transition include the loss in expression of epithelial markers including E-cadherin, Zo-1 and cytokeratin, with subsequent acquisition of mesenchymal markers, e.g. α-SMA and FSP1. (3) The use of lineage tracking to confirm the epithelial origin of cells within a myofibroblast pool in various models of fibrosis [10] [84-88]. (4) Amelioration of fibrosis using therapies designed to target EMT [87]. Of these different types of studies, the most persuasive is genetic fate mapping, which allows for cells of the epithelium to be genetically tagged and mapped throughout a disease process. Pioneering work by Iwano *et al* employed fate mapping to establish a role for EMT in unilateral ureteral obstruction (UUO) [10]. However, there has been a dearth of follow up work of this type and the established mainstay criteria supporting a role for EMT in fibrosis

is usually based around determining morphological changes and establishing whether there are alterations in the expression of key epithelial/mesenchymal markers. Surprisingly, these studies fail to demonstrate complete EMT in which fibroblasts fully migrate and traverse the TBM. More commonly observed is the phenomenon known as partial EMT (p-EMT) in which cells express both epithelial and mesenchymal markers. This intermediate phenotype is of relevance because the acquisition of mesenchymal markers and expression of ECM proteins that occur early in EMT may be transient, occurring in cells exposed to the diabetic milieu and contributing to fibrosis [71][88]. The argument against the 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. Furthermore, they suggest that epithelial cells contribute to fibrosis through paracrine cell signalling [81][89].

TGF-β1, EMT and fibrosis in diabetic nephropathy:

The original link between fibrosis and EMT was suggested 15years ago in a model of mouse anti-tubular membrane disease where it was observed that renal tubular epithelial cells were capable of expressing the FSP1 [87]. A role for EMT in the progression of CKD was later confirmed in a model of UUO using genetically tagged (Lac Z) proximal tubule cells. The authors demonstrated that of those matrix producing cells resident within the tubulointerstitial space, 36% were of epithelial origin and thus derived from renal tubular epithelium through EMT [10]. The underlying pathology of EMT has since been observed in renal biopsies from diseased kidney where the proportion of cells undergoing transition correlated to both the level of serum creatinine and the degree of interstitial fibrosis [88]. Evidence for EMT *in vivo* has since been described in various forms of of CKD, including diabetic nephropathy [89][90]. In diabetes, glomerular fibrosis is observed in the progression from incipient to overt nephropathy [6]. Whilst tubulointerstitial fibrosis can also present itself in these early stages, a build up of fibrotic material in the tubular interstitium tends to accompany disease progression, correlating with a gradual decline in renal function [6].

Consequently, progressive tubulointerstitial fibrosis, in part mediated by EMT, represents the final common pathway leading to renal failure in diabetic nephropathy [7].

In models of renal disease, EMT occurs in response to hypoxia, reactive oxygen species, AGE and numerous pro-fibrotic cytokines, growth factors and metalloproteinases. Of these TGF-β1 represents the principle candidate in the development and progression of fibrotic complications. A role for TGF-β1 induced EMT in the pathology of tubulointerstitial fibrosis in DN has been reported and histological examination of kidneys from diabetic animals and biopsies from patients with DN where phenotypic changes associated with EMT have been observed [89][91]. In a study by Burns *et al*, EMT was studied in a rat tubular epithelial cell line (NRK-529) and the STZ-induced Sprague Dawley rat [89]. Following incubation with TGF-β1, EMT in NRK-529 cells was confirmed by the altered expression of α-SMA, Vimentin and E-cadherin, whilst Sprague Dawley rats exhibited increased renal gene expression of TGF-β1 at 32 weeks and enhanced α-SMA and collagen with a concomitant decrease in E-cadherin expression compared to control [89]. Similar markers of EMT have also been observed in a variety of animal models, including STZ-treated Wistar Kyoto rats, Sprague Dawley rats and the STZ-Ren-2 rat [89-90][92].

The existence of FSP1 in podocytes from patients with diabetes is most likely associated with induction of podocyte detatchment through EMT [91]. Depletion of glomerular podocytes is an important feature of progressive diabetic nephropathy. Although the most plausible explanation for this podocyte depletion is detachment from the glomerular basement membrane after cellular apoptosis, the mechanism is unclear. FSP1 is constitutively expressed in the cytoplasm of tissue fibroblasts or epithelial cells converted into fibroblasts by EMT. In a study performed by Yamaguchi *et al*, of 109 patients presenting with both Type 2 diabetes and diabetic nephropathy 35% were normoalbuminuric, 15% had microalbuminuria, 7% had macroalbuminuria and 43% had decreased kidney function. In biopsies from 43 of these patients it appeared that the number of FSP1 positive podocytes was significantly higher in individuals with macroalbuminuria as opposed to those presenting with normoalbuminuria ($p=0.03$). Furthermore, the number of FSP1 positive podocytes was larger in those glomeruli exhibiting diffuse mesangiopathy than in those with focal

mesangiopathy. FSP1-positive podocytes selectively expressed Snail and integrin-linked kinase, a known trigger for EMT.

Recently, a role for microRNAs in regulation of EMT in the diabetic kidney has been established. MicroRNAs (miRNAs) are endogenous non-coding RNA molecules, which function as negative regulators of gene expression [93]. miRNA 192 is expressed in both glomerular and tubular human kidney sections. Interest in this exciting area of research stems from the fact that miRNA 192 is down-regulated in DN in response to TGF-β, an event that correlates to increased fibrosis [94]. The link between a loss in miRNA 192 and the development of fibrosis in DN is thought to be a consequence of a loss of regulation of those genes involved in EMT, a concept recently supported by Wang *et al* [95]. Using rat proximal tubule cells, they demonstrated that both TGF-β1 and TGF-β2 were able to induce EMT and fibrogenesis as a consequence of a loss in mir-200a expression. This loss in expression was shown to augment EMT induced fibrosis through alleviating mi2-200a inhibition of Smad 3 activity [95].

The collecting duct in diabetic nephropathy: a role for TGF-β1?

Until recently, EMT in the kidney was associated with more proximal regions of the nephron. However, pioneering studies by Butt *et al* utilizing a fetal primate model of obstructive nephropathy confirmed the capacity of those cells within the colletcing duct to undergo EMT [96]. Furthermore, both insulin growth factor 1 (IGF1) and TGF-β1 have been shown to induce classic EMT-like changes in mouse inner medullary collecting duct cells [97], despite initial studies suggesting that these cells could not undergo phenotypic conversion in response to TGF-β1 [98]. How TGF-β1 induced EMT contributes to fibrosis in the distal nephron is unclear. In 2009 Aldehni F *et al*. demonstrated that TGF-β1 induced EMT in the mouse renal collecting duct increased Bestrophin 1 (Best1), a protein known to regulate the concentration of intracellular Ca^{2+} and increase cellular proliferation [99]. Suppression of Best1 by RNAi inhibited proliferation and down-regulated markers of EMT, suggesting that Best1 may function as a downstream mediator of TGF-β1 induced EMT and renal

fibrosis in the collecting duct. Deletion of the TβRII in cultured collecting duct cells evoked an increase in TGF-β activation with a subsequent exacerbation in renal fibrosis [100].

TGF-β1 also promotes increased expression of the serum and glucocorticoid inducible kinase-1 (SGK1), a serine/threonine kinase that regulates sodium re-absorption through control of the epithelial sodium channel (ENaC) [101-103]. SGK1 is elevated in models of diabetes [104], suggesting that it may contribute to the development of secondary hypertension. Interestingly SGK1 is expressed in numerous tissues that exhibit fibrosis including cases of Crohn's disease, lung fibrosis, liver cirrhosis, fibrosing pancreatitis, DN and glomerulonephritis [105]. These studies suggest that SGK1 may also have a role in mediating the fibrotic effects of TGF-β1 in the collecting duct.

THERAPEUTIC INTERVENTION

The pro-fibrotic actions of TGF-β make it an ideal therapeutic target for reno-protective agents. Although TGF-β1 is regarded as the major isoform involved in fibrosis, improved renal function coincides with reduced expression of both TGF-β1 and TGF-β2 [106]. In the STZ-rat, renal expression of TGF-β2 is markedly increased and parallels ECM deposition in early stages of the disease [107]. By contrast, TGF-β1 protein levels remain unchanged during this initial period despite increased mRNA levels [107] Consequently, recent studies have focused on the antifibrotic potential of selectively targeting TGF-β2 for the prevention of progressive renal disease [108]. However, in diabetes, the pro-fibrotic role for all 3 isoforms [109] supports efforts to neutralize TGF-β1, TGF-β2 or TGF-β3, to reduce renal scarring and improve overall kidney function [110]. Inhibition of TGF-β in this way also prevents glomerular enlargement and suppresses the expression of genes encoding for the ECM in models of chemically induced diabetes [110]. These findings were corroborated in *db/db* mice, where both TβRI and TβRII receptor mRNA expression were increased [111]. Chronic treatment of these animals with TGF-β-neutralizing antibodies markedly diminished the expression of collagen and fibronectin and reduced mesangial matrix expansion. Several anti-fibrotic and reno-

protective agents have also been shown to partially alleviate TGF-β induced fibrosis including, Bone Morphogenic Protein-7 (BMP-7) and Hepatocyte Growth Factor (HGF).

An osteogenic factor, BMP-7 plays an important role in kidney development and regulation of nephrogenesis. Renal fibrosis in diabetes is inhibited by BMP-7. Following EMT, increased levels of TGF-β are paralleled by a reduction in expression of BMP-7, as a potential consequence of increased gremlin levels [112]. Gremlin limits BMP-7 availability and is markedly elevated in humans with DN [60]. This reciprocal relationship accounts for low BMP-7 concentrations in models of acute and chronic renal injury [113], and explains how exogenous BMP-7 restores renal function through blockade of EMT [114] Aside from the glomerular reno-protective effects observed in both STZ-rats and *db/db* mice, BMP-7 is capable of intercepting at the level of TGF-β signalling. In the adult, BMP-7 alleviates TGF-β-induced renal fibrosis [115] and antagonises TGF-β-induced Smad3-dependent EMT [116]. However, the mechanism remains elusive since BMP-7 is unable to negate TGF-β-induced EMT in human PTC, suggesting that the effects are region specific [117]. The extent by which BMP-7 blocks EMT therefore requires further clarification.

A key anti-fibrotic cytokine, HGF prevents renal tissue fibrosis after chronic injury [118]. Administration of HGF reduces loss of kidney function, whilst blockade of HGF signalling further exacerbates the extent and progression of renal fibrosis [119]. As with BMP-7, HGF and TGF-β have a reciprocal relationship. HGF inhibits TGF-β-induced EMT, and ameliorates renal fibrotic lesions in numerous models of renal disease [119-120]. The underlying molecular mechanisms mediating these reno-protective effects are unknown. The anti-fibrotic activity of HGF appears to stem from up-regulation of the transcriptional co-repressor SnoN. Binding of this co-repressor to Smad2 results in formation of a transcriptionally inactive complex, preventing the activation of Smad-mediated genes thereby blocking TGF-β-induced EMT [121]. Administration of HGF has been shown to alleviate renal complications in DN, including the reversal of glomerulosclerosis [122], a reduction in albuminuria [123] and blockade of fibrosis with a concomitant improvement in renal function [124]. However, a number of studies contradict these findings, suggesting that chronically elevated HGF promotes the progression of nephropathy in *db/db* mice [125]. Furthermore, potential proto-oncogenic

actions of HGF raises questions as to the potential therapeutic use of this growth factor in alleviating EMT induced complications in DN. Further studies are essential if HGF is to be considered as a future therapeutic means of intervening in fibrosis in DN.

Recent candidates identified upstream of HGF include, 9-cis retinoic acid, 1,25-dihyrdoxyvitamin D3, the PPARγ agonist troglitazone and C-peptide [120-124][126]. A cleavage product of pro-insulin, C-peptide exerts a number of protective affects against the micro-vascular and macro-vascular complications associated with hyperglycaemia in type I diabetes [127] and in those patients with DN, C-peptide is reno-protective [128]. Recently, the Connecting-peptide has been shown to negate TGF-β induced EMT in cells of the proximal tubule [126] and as an adjunct to insulin therapy, C-peptide could be used to alleviate some renal complications of diabetes. However, like BMP-7 and HGF these findings are preliminary and whilst encouraging, need to be fully corroborated in the clinical scenario.

Studies of miRNA expression in diabetic nephropathy have predominantly been performed in animal models of diabetes and suggest that miRNAs may represent potential future therapeutic targets of renal fibrosis. Their role within the kidney appears to be region specific, e.g. miR-192 has been shown to exhibit elevated levels of expression in glomeruli isolated from the STZ mouse [129]. In this study, the function of miR-192 was shown to be of regulating repression of TGF-β1-induced changes in extracellular matrix proteins. Contrary to this, in proximal tubule cells reduced levels of mir-192 have been described [93]. The loss of miR-192 has been shown to correlate with the degree of tubulointerstitial fibrosis in diabetic nephropathy. In Madin Darby canine kidney epithelial cells undergoing TGF-β1-induced EMT, a decrease in mir-200 and mir-205 suggests a link between miRNAs and the regulation of EMT [130]. Although these early studies suggest that miRNAs may represent reliable future biomarkers, the area of research demands considerable further attention,

Conclusion:

Although rigorous glycaemic control may reduce the burden of diabetes-associated morbidity, the Diabetes Control and Complications Trial (DCCT) showed that even with intensive insulin treatment, a substantial proportion of patients still develop complications. According to the 2007 Renal Registry Report, diabetic nephropathy (DN) accounts for a significant proportion of the 40,000 patients that require dialysis or transplantation in the UK each year and Worldwide, DN is the single commonest cause of entry into the renal replacement therapy programme. The increasing prevalence of diabetic nephropathy further highlights the urgency for discovery of successful therapeutic agents aimed at alleviating renal fibrosis. TGF-β-induced EMT is a key contributor to fibrotic scar formation as seen in DN; therefore manipulating downstream TGF-β signalling represents a viable therapeutic target in an attempt to restore renal function. Administration of the anti-fibrotic growth factors HGF or BMP-7 can reverse the fibrogenic response and evidence supports the development of these types of reno-protective agents to alleviate complications of CKD, including DN. However, before a future treatment can ultimately arrest/reverse chronic kidney disease, further studies are required to fully understand how TGF-β1 exerts its plethoric effects.

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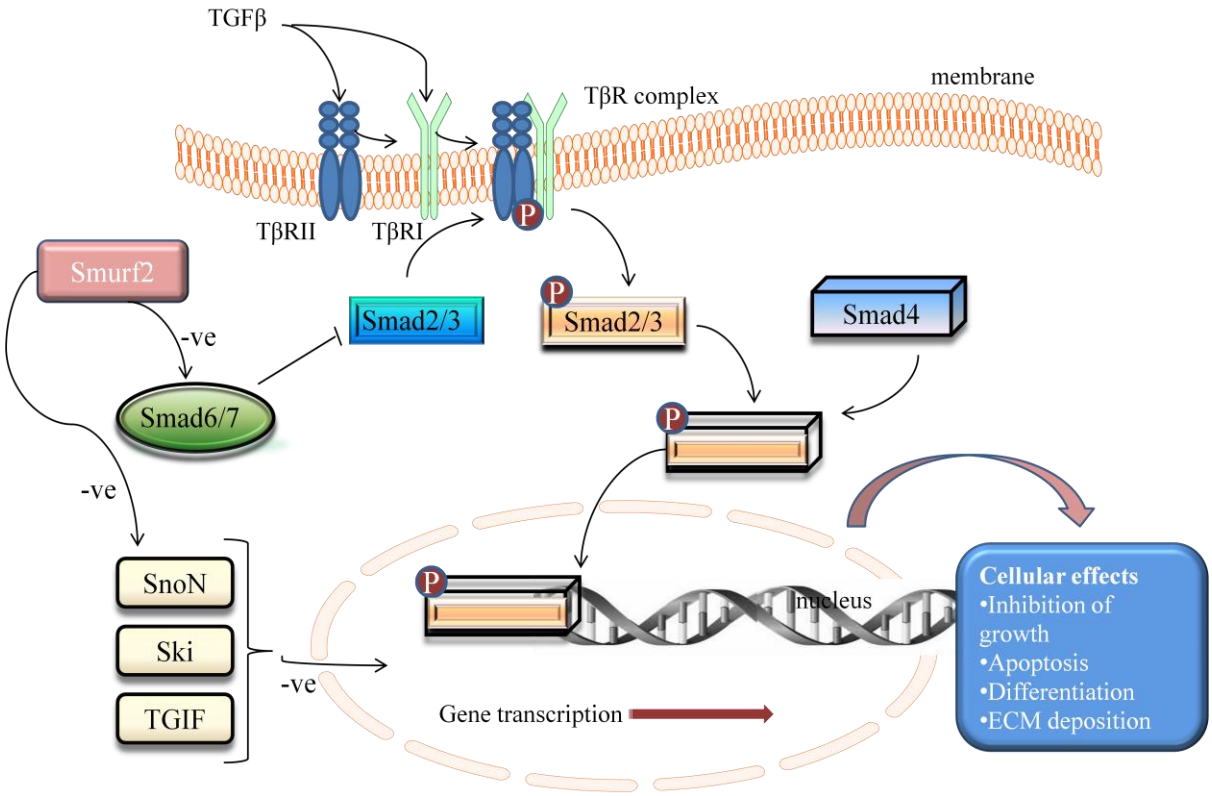
FIGURE LEGENDS:

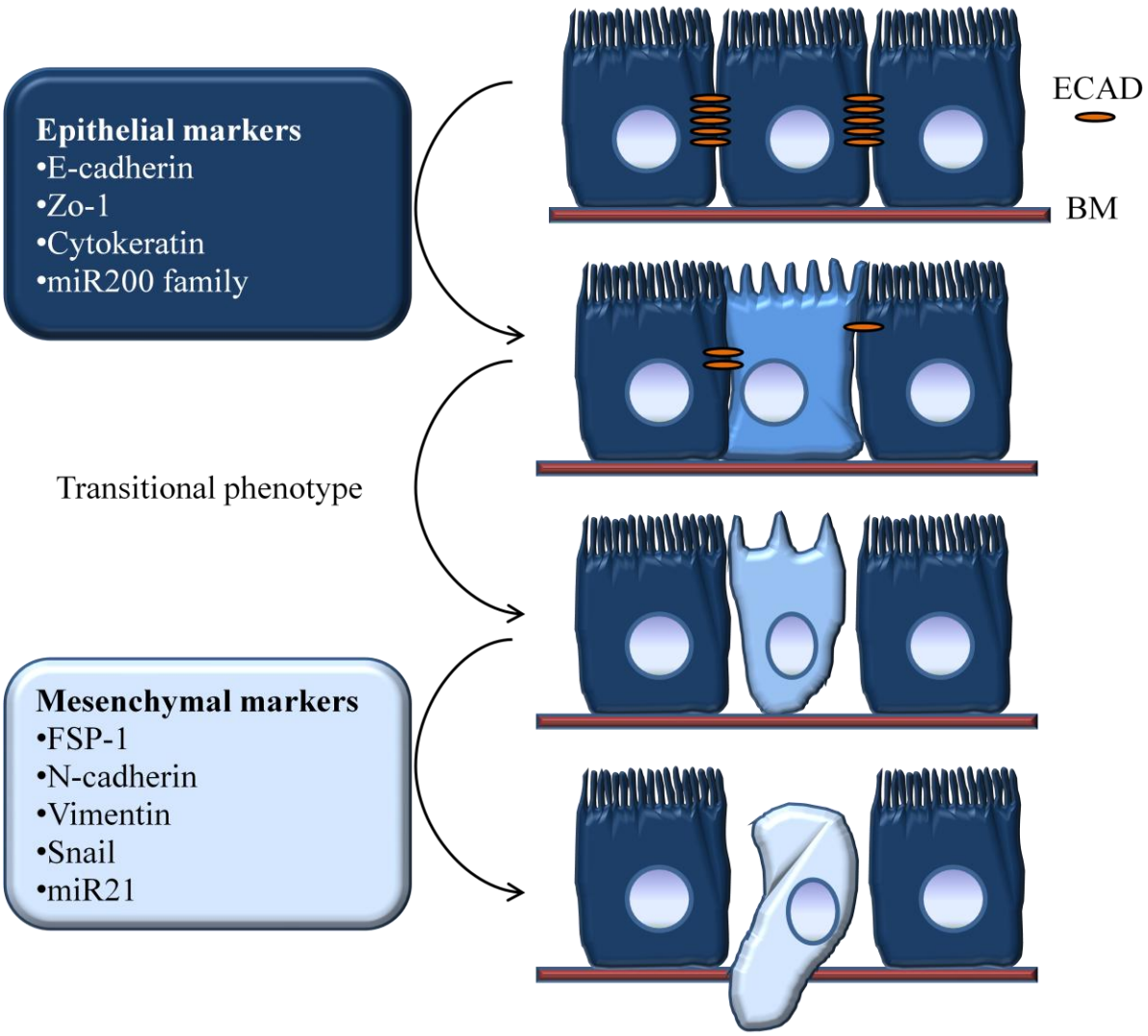
Figure 1: Schematic depicting TGF-β1 signaling

TGF-β binds to its type II serine/threonine kinase receptor and instigates autophophorylation permitting the recruitment of the type I receptor. This interaction forms an activated heteromeric complex and facilitates phosphorylation of the receptor-regulated Smad2/3, promoting interaction with common Smad4. The active Smad2/3/4 complex translocates to the nucleus where it regulates the transcription of TGF-β1 target genes. TGF-β1 signaling is stringently regulated, and depends on both inhibitory Smads6/7 and transcriptional co-repressors that include SnoN, Ski, TGIF that help modify net cellular effects.

Figure 2: Type 2-EMT in the kidney

Loss in cell adhesion through the down-regulation of key epithelial markers, including E-cadherin (ECAD) and Zona-occludins (Zo-1), represents an initial step in type 2-EMT. Disassembly of the adherens junction complex is accompanied by a number of morphological and phenotypic changes. These gross changes are accompanied by alteration in the expression profile of key EMT proteins that include: up-regulation of vimentin, α-smooth muscle actin and fibroblast specific protein (FSP-1). Ultimately, cells detach from the tubular basement membrane (BM) and migrate into the interstitium where they exacerbate renal fibrosis.


TGFβ
membrane
TβR complex
TβRII
TβRI
P
Smurf2
-ve
Smad2/3
P
Smad2/3
Smad4
Smad6/7
P
-ve
SnoN
Ski
TGIF
-ve
P
nucleus
Gene transcription
Cellular effects
•Inhibition of growth
•Apoptosis
•Differentiation
•ECM deposition


Epithelial markers
•E-cadherin
•Zo-1
•Cytokeratin
•miR200 family
Transitional phenotype
Mesenchymal markers
•FSP-1
•N-cadherin
•Vimentin
•Snail
•miR21
ECAD
BM