Wnt6 regulates epithelial cell differentiation and is dysregulated in renal fibrosis

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The progressive increase in the incidence of patients with end-stage renal disease (ESRD) as a result of long-standing complications of diabetes is widely recognized as the most important problem facing clinical nephrology today (53). Significant efforts have been made to understand the molecular basis underpinning the pathogenesis of diabetic nephropathy and the currently accepted paradigm includes excessive production, deposition, and accumulation of extracellular matrix components such as collagens and fibronectin as a direct result of hyperglycemic stress (1).

Over the last decade improvements in methodologies and technology for high throughput genome screening have been applied to the analysis of the pathogenesis of diabetic nephropathy (4, 10, 16). One such approach by Cohen et al. (9) identified pathways activated in diabetic nephropathy. Subsequent studies both by ourselves and others have further implicated a role for Wnt signaling in progression of renal disease (24–26, 44).

The Wnt pathway is a highly conserved signaling network regulating diverse processes during nephrogenesis including cell fate decisions and morphogenesis; the Wnt ligands Wnt9b and Wnt4 are absolutely required during the commitment of mesenchymal cells to differentiated epithelia in the developing nephron (6, 27, 48), while ureteric bud elongation and branching morphogenesis require tightly regulated Wnt pathway deactivation by the endogenous antagonist Dickkopf-1 (Dkk-1) (42). The observation that these pathways are involved in adult kidney disease is intriguing from both pathogenic and therapeutic perspectives, with the prospect that recapitulation of developmental aspects of nephrogenesis might be manipulated to initiate and propagate organ regeneration and repair.

Several studies have put forward opposing theories explaining Wnt activation in kidney disease. Some studies report that upregulation of Wnts and their Frizzled (FzD) receptors are essential for reparative processes in tubular epithelia and the glomerular mesangium during acute kidney injury (AKI) through pathways directly linked to proliferation and regeneration (35, 51). Similarly, tubule-specific ablation of endogenous β-catenin in a mouse model of AKI was found to further aggravate kidney injury (57). Kato et al. (24) also studied the Wnt/β-catenin pathway in podocytes and found that the path-
way is essential for podocyte adhesion, differentiation and survival. In contrast, Dai et al. (12) have shown that Wnt/β-catenin signaling plays a critical role in podocyte injury and proteinuria. This suggests cellular responses to Wnts are both context and cell type specific; activation can be both protective and detrimental to the injured kidney (26).

To date little is known about the role of Wnt6 in the kidney. Here we demonstrate that Wnt6 is expressed in the mesonephros of the developing mouse embryo and that its expression is decreased in multiple models of renal fibrosis. We also show that Wnt6 induces de novo tubulogenesis in renal epithelial cells in vitro, suggesting it plays a role in epithelial differentiation and furthermore, we delineate the role of NF-κB in this process. We propose that manipulation of Wnt6 levels in the context of renal fibrosis has the potential to regulate epithelial differentiation and may therefore be of therapeutic relevance in renal disease.

EXPERIMENTAL PROCEDURES

Ethics statement. All animal studies were performed in accordance with ethical exemption approval granted by Trinity College Dublin and University College Dublin (UCD) animal research ethics committees.

In situ hybridization and GFP visualization of mouse embryos. Mouse embryos were collected from time-mated CD1 females at embryonic day (E) 9.5, 10.5, and 11.5 and were precisely staged using Thieler criteria (52). The Wnt6 and Fzd7 probes and the protocol used for in situ hybridization, optical projection tomography (OPT) scanning, and three-dimensional (3D) reconstruction was as described by Summerhurst et al. (49). GFP was detected indirectly using a specific antibody (Molecular Probes, Eugene, OR) in TCF/Lef:H2B-GFP reporter embryos before 3D imaging by OPT (17).

Animals. For unilateral ureteral obstruction (UUO), male C57Bl/6J mice aged 10–12 wk were placed in two groups: UUO (n = 3) and sham operated (n = 3). Animals were anaesthetized and the left kidney identified. In the UUO group, the ureter was ligated with sutures and in the sham group the ureter was manipulated with forceps but not ligated. Animals were sutured and day of surgery was deemed as day 0. On days 3 and 8, mice were culled and kidneys harvested.

For gentamicin rat model of immune-mediated kidney injury, male Sprague-Dawley rats weighing between 250 and 300 g were administered an intraperitoneal injection on day 0 (n = 4), or 120 mg/kg gentamicin (n = 4) daily for 8 days. Animals were anaesthetized and the left kidney identified. The UUO group, the ureter was ligated with sutures and in the sham group the ureter was manipulated with forceps but not ligated. Animals were sutured and day of surgery was deemed as day 0. On days 3 and 8, mice were culled and kidneys harvested.

For both models, kidneys were divided in two by vertical section. One half was fixed in formalin and then embedded in paraffin before sectioning. Collagen deposition was investigated by picro-sirius red staining. Sections were deparaffinized and rehydrated before incubation with 0.1% fast green and 0.1% direct red 80 for 2 h in the dark at room temperature. For immunohistochemical staining of Wnt6, rehydrated sections were incubated in citrate buffer and then placed in a pressure cooker for 8 min for antigen retrieval then incubated in anti-Wnt6 [1:900 primary antibody (1°Ab); R&D Systems, Oxon, UK] for 30 min at room temperature. Sections were incubated in biotinylated anti-sheep antibody [1:1000 secondary antibody (2°Ab); Vector Laboratories, Burlington, CA] for 10 min at room temperature and then incubated in 3,3-diaminobenzidine (DAB) to allow for protein detection and counterstained with hematoxylin and eosin. Sections were dehydrated, mounted and scanned using Scan-Scope (Aperio Technologies).

The other half of the kidney was halved again and then snap frozen in liquid nitrogen for isolation of protein or RNA. Protein was extracted using CellLytic M (Sigma-Aldrich, Wicklow, Ireland) with the addition of sodium orthovanadate (1 mM), phenylmethanesulfonyl fluoride (1 mM), sodium fluoride (1 mM), β-glycerophosphate (1 M), and additional protease and phosphatase inhibitor cocktails (Sigma-Aldrich).

Wnt6 plasmid and transfection. A Gateway compatible open reading frame express shuttle clone containing Wnt6 was obtained from Genecopoeia (Rockville, MD) and subcloned into pcDNA-DEST40 using a Gateway LR Clonase reaction as per manufacturer instructions (Invitrogen, Carlsbad, CA). This mammalian expression vector was used for all Wnt6 transfection experiments. Transient transfection of HKC8s was carried out using Fugene HD (Promega, Madison, WI) transfection reagent diluted in OptiMem (GIBCO Life Technologies, Carlsbad, CA) according to the following ratio: 2 μg Wnt6 plasmid DNA, 6 μl Fugene HD, and 94 μl OptiMem.

Cell culture. Human epithelial kidney cells (HKC8), wild-type mouse embryonic fibroblasts (MEFs), IKKα/β double knockout MEFs, and p65 NF-κB knockout MEFs were maintained in Dulbecco’s modified Eagle’s medium (Lonza, Basel, Switzerland) supplemented with 2 mM l-glutamine, 50 units/ml penicillin, 5 ng/ml streptomycin, and 10% fetal calf serum (GIBCO) at 37°C in an incubator with a high humidity atmosphere of 95% air:5% CO2. Madin-Darby canine kidney cells (MDCKs) were grown in 3D culture in matrigel essentially as described by Debnath et al. (13).

Recombinant TGF-β1 (Promokine, Heidelberg, Germany) reconstituted in DPBS (Lonza) was used to stimulate cells at a final concentration of 5 ng/ml. Wnt6 conditioned media were obtained from restricted media of Wnt6-overexpressing cells. Cells were treated with both growth factor and conditioned media separately and together following 24-h serum deprivation (0% FCS for HKC8s, 1% FCS for MEF cell lines). Unstimulated cells were treated with an equal volume of growth factor diluent (DPBS for TGF-β1) or empty vector transfected conditioned media. To determine early signal responses (canonical and noncanonical), cells were stimulated with recombinant TGF-B1 or Wnt6 conditioned media for 10, 30, 60, 90, and 180 min. For transfected cells with TGF-β1 and Wnt6, cells were transfected with a Wnt6 overexpression plasmid for 24 h followed by TGF-β1 as described above. For rescue experiment, cells were stimulated with TGF-β1 for 48 h followed by application of Wnt6 conditioned media for a further 24 h.

RNAi. To achieve gene knockdown, HKC8s were transfected with 2 μM siRNA targeting the frizzled 7 (Fzd7) gene (Qiagen, Manchester, UK) using Fugene HD. Nontargeting scrambled sequence (2 μM; Qiagen) was used as a negative control. RNA was extracted using the Trizol method and knockdown efficiency was evaluated by RT-PCR on a 1.5% agarose gel in Tris-acetate EDTA buffer (Invitrogen) using primers specific for Fzd7 and 18S and visualized with a Gene Genius gel documentation system. Primers were Fzd7: 5'-CAGACCGGAGACGTGAGAA-3', 3'-TGCGCCAGCATCGGATGCTA-5'; and 18S: 5'-GAGCCACCCGAGATTTAGGACA-3', 3'-TGATAGCCGCGGCGGTGTG-5'.

Preparation of cellular protein extracts including nuclear fractionation and Western blotting. Protein extracts were prepared in modified radioimmunoprecipitation (RIPA) lysis buffer consisting of 50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, and 100 mM ethylenediaminetetraacetic acid (Sigma-Aldrich) with the addition of inhibitors as described previously. For fractionated samples, cells were lysed with an NE-PER nuclear and cytoplasmic extraction kit according to the manufacturer’s guidelines (Pierce, Thermo Fisher Scientific, Waltham, MA). Protein was quantified by Bradford assay (Bio-Rad Laboratories, Richmond, CA). Samples were denatured in sample buffer containing sodium dodecyl sulfate and 20 μg of protein were loaded onto a 10% gel and subjected to electrophoresis, followed by transfer onto a polyvinylidene difluoride membrane. Membranes were blocked in 5% skimmed milk/0.1% PBS-Tween before incubation with primary antibody overnight at 4°C. Membranes were subsequently incubated in horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room tem-
perature. The binding of secondary HRP was detected and visualized using enhanced chemiluminescence (Advansta, Menlo Park, CA).

**Immunocytochemistry.** Cells were fixed in 3.7% paraformaldehyde (Bio-Rad) in PBS for 5 min followed by permeabilization with 0.01% TritonX-100 (Sigma-Aldrich)/PBS for 1 min. Slides were blocked in 5% normal goat serum (Sigma-Aldrich)/PBS for 1 h at room temperature and probed using specific primary antibodies in 5% goat serum/PBS overnight at 4°C. Slides were incubated with species-specific Alexa555-conjugated secondary antibodies diluted in 5% goat serum/PBS for 1 h at room temperature. F-actin was visualized with Alexa488-conjugated phalloidin (Invitrogen)/PBS (1:500) for 30 min. Cells were incubated in Hoechst 33342 (Invitrogen)/PBS (1:1,000) for 1 min to stain nuclei. Microscopic images were acquired using a Zeiss Imager.M1 upright microscope equipped with Axiovision 4.0 software.

**Antibodies.** For Western blotting, the following primary antibodies were used: monoclonal sheep anti-Wnt6 (1:500 1°Ab; 1:1,000 2°Ab; R&D Systems), monoclonal rabbit anti-GAPDH (1:10,000 1°Ab; 1:20,000 2°Ab; Cell Signaling Technology, Beverly, MA), monoclonal rabbit anti-phospho GSK3β (Ser9) (1:1,000 1°Ab; 1:2,000 2°Ab; Cell Signaling), monoclonal rabbit anti-GSK3β (1:1,000 1°Ab; 1:2,000 2°Ab; Cell Signaling), monoclonal rabbit anti-phospho β-catenin (Ser552) (1:1,000 1°Ab; 1:2,000 2°Ab; Cell Signaling), monoclonal rabbit anti-β-catenin (Ser552) (1:1,000 1°Ab; 1:2,000 2°Ab; Cell Signaling), monoclonal rabbit anti-phospho β-catenin (Ser552) (1:1,000 1°Ab; 1:2,000 2°Ab; Cell Signaling).

**Fig. 1.** Wnt6, frizzled 7 (FzD7), and TCF/lef GFP reporter gene expression in the mesonephros and metanephros of developing mouse embryos at embryonic days (E) 9.5, 10.5, and 11.5. Wnt6 expression was detected by whole mount in situ hybridization on mouse embryos at E9.5 (A), E10.5 (B–E), and E11.5 (F–I). At E9.5 expression is seen in the mesonephric duct (msd) and urogenital membrane (ug). An external view (volume rendered) of a 3-dimensional (3D) reconstruction of an optical projection tomography (OPT) scanned embryo (C), with a transverse section (D) and a coronal section (E) at the level of the hindlimb buds showing expression in the mesonephric duct. G–I: external views from different angles of a 3D OPT scanned and reconstructed embryo at E11.5, with areas of Wnt6 expression seen as white/light grey (white arrows), in the mesonephric ducts. J and K: TCF/lef GFP reporter gene expression shows canonical Wnt pathway activity in the mesonephric ducts and ureteric bud at E10.5; J is an external view of a volume rendered 3D image while K is a virtual sagittal section. L and M: FzD7 is coexpressed with Wnt6 in the mesonephric duct of the developing mouse kidney and is also expressed in the mesonephric tubules; L: sagittal section; M: coronal section. Representative scale bars (1 mm) are shown for each stage (A, B, and F). fl, Forelimb bud; hl, hindlimb bud; msd, mesonephric duct; mst, mesonephric tubule; ov, otic vesicle; s, somite; ub, ureteric bud (52).
### A. Wnt signaling pathway

- **Gene**: T Nelson, A Sm, D K, L T, etc.
- **Protein**: Wnt6, Wnt7a, Wnt7b, etc.

### B. Wnt superfamily

- **Gene**: Wnt6
- **Protein**: 40kDa, 37kDa

### B. Relative Wnt6 expression

- **Control**: Non-diabetic, eNOS-/db/db
- **Condition**: Non-diabetic eNOS-/db/db

### C. Relative Wnt6 expression

- **Condition**: Healthy living, Diabetic nephropathy

### D. Post-obstruction

- **Time**: 3 days, 8 days
- **Sample**: NM, M, NL, L

### E. Picro-sirius red

- **Region**: NL, L
- **Sections**: Cortex, Medulla

### F. Saline treated control, 40mg/kg gentamicin, 120mg/kg gentamicin

- **Gene**: Wnt6
- **Protein**: 40kDa, 37kDa

### F. Relative density (Wnt6/GAPDH)

- **Condition**: Saline treated control, 120mg/kg gentamicin

### G. ACR (ug/ml)

- **Condition**: Saline treated control, 40mg/kg gentamicin, 120mg/kg gentamicin
Fig. 2. Expression of Wnt6 is lost in tubulointerstitium of patients with diabetic nephropathy and in multiple animal models of renal fibrosis. The expression pattern of Wnt6 in the developing mouse kidney led us to speculate that it may be involved in both the specification and maintenance of epithelial cells. Preliminary analysis confirmed expression of Wnt6 in adult kidney and in primary cells from both the mesangium and the proximal tubular epithelium (results not shown). Gene expression analysis from the Hodgin study (publicly available at www.nephroseq.org) in diabetic mice (eNOS−/− db/db vs. control) demonstrated the activation of a program of gene expression centered on Wnt family ligands and Wnt signaling networks (Fig. 2A). Specifically, Wnt6 expression was significantly decreased in diabetic mice compared with control (P < 0.05, Fig. 2B). Increased urinary albumin creatinine ratio confirms eNOS−/− db/db mice have developed kidney disease (Fig. 2B). Samples from the tubulointerstitium of healthy living donor controls (n = 12) and diabetic nephropathy (n = 10) patients were taken and gene expression analysis/microarrays were performed to analyze differential expression of over 12,000 genes. Data were kindly provided by Katalin Suszta, University of Pennsylvania, through an ongoing collaboration with the UCD Diabetes Complications Research Centre, and have been made publicly available (www.nephroseq.org). Wnt6 expression in the tubulointerstitium is significantly decreased in patients with diabetic nephropathy compared with healthy living donor controls (P < 0.01, Fig. 2C). Decreased glomerular filtration rate confirmed patients had kidney disease (Fig. 2C). We subsequently analyzed the expression of Wnt6 in a model of tubulointerstitial fibrosis, the UUO mouse, and a gentamicin rat model of acute tubular injury. In the UUO model, Wnt6 expression is decreased at 3 and 8 days postobstruction compared with contralateral internal controls and hindlimb buds (Fig. 1, B–E) and at E11.5 (TS19) expression was still detected in the mesonephros (Fig. 1, G–I). Expression of the Wnt receptor encoding gene Fzd7 was detected at E10.5 and E11.5, coexpressed with Wnt6 in the mesonephric duct, although at a lower level (Fig. 1, L and M). Fzd7 expression was also detected in the mesonephric tubules. Notably, TCF/Lef reporter mouse embryos show GFP activity coincident with Wnt6 and Fzd7 expression in the developing kidney, indicating that canonical Wnt signaling is well established at this stage of development (Fig. 1, J–K; GFP is detected, grey/white) in the mesonephric ducts and tubules and the ureteric buds.

Wnt6 expression is decreased in the tubulointerstitium of patients with diabetic nephropathy and in multiple animal models of renal fibrosis. The expression pattern of Wnt6 in the developing mouse kidney led us to speculate that it may be involved in both the specification and maintenance of epithelial cells. Preliminary analysis confirmed expression of Wnt6 in adult kidney and in primary cells from both the mesangium and the proximal tubular epithelium (results not shown). Gene expression analysis from the Hodgin study (publicly available at www.nephroseq.org) in diabetic mice (eNOS−/− db/db vs. control) demonstrated the activation of a program of gene expression centered on Wnt family ligands and Wnt signaling networks (Fig. 2A). Specifically, Wnt6 expression was significantly decreased in diabetic mice compared with control (P < 0.05, Fig. 2B). Increased urinary albumin creatinine ratio confirms eNOS−/− db/db mice have developed kidney disease (Fig. 2B). Samples from the tubulointerstitium of healthy living donor controls (n = 12) and diabetic nephropathy (n = 10) patients were taken and gene expression analysis/microarrays were performed to analyze differential expression of over 12,000 genes. Data were kindly provided by Katalin Suszta, University of Pennsylvania, through an ongoing collaboration with the UCD Diabetes Complications Research Centre, and have been made publicly available (www.nephroseq.org). Wnt6 expression in the tubulointerstitium is significantly decreased in patients with diabetic nephropathy compared with healthy living donor controls (P < 0.01, Fig. 2C). Decreased glomerular filtration rate confirmed patients had kidney disease (Fig. 2C). We subsequently analyzed the expression of Wnt6 in a model of tubulointerstitial fibrosis, the UUO mouse, and a gentamicin rat model of acute tubular injury. In the UUO model, Wnt6 expression is decreased at 3 and 8 days postobstruction compared with contralateral internal controls and
nonmanipulated animals (Fig. 2D). The UUO model was confirmed fibrotic by marked accumulation of collagen in the tubulointerstitium (Fig. 2E) as evidenced by increased picrosirius red staining, with significant alterations in morphological appearance (glomeruli irregular in shape, tubules dilated).

Development of fibrosis was associated with decreased Wnt6 expression in the tubules of UUO mice kidneys 8 days postobstruction (Fig. 2E). In the gentamicin-treated rats, 40 mg/kg gentamicin resulted in a small increase in Wnt6 expression; however, this is completely lost in 120 mg/kg treated animals.
following 9 days of gentamicin administration (Fig. 2F). Increased urinary albumin creatinine ratio confirmed the 120 mg/kg treated rats had developed kidney injury (Fig. 2G).

\textbf{Wnt6 induces de novo tubulogenesis and activates canonical signaling in renal cells}. Developmental expression patterns taken together with the observation that Wnt6 expression is decreased in the tubulointerstitium of diabetic nephropathy patients and both animal models of renal fibrosis led us to speculate that it may play a role in regulating epithelial fate. Renal tubular epithelial cells (MDCK) were grown in 3D culture in matrigel for 10 days after which time they form polarized spherical cysts. Transfection of these spheres with Wnt6 and continuing culture led to the formation of giant spheroids exhibiting new tube-like protrusions indicative of de novo tubulogenesis (21) (Fig. 3A). Increased size and differentiation of these spheres were accompanied by cell proliferation as evidenced by increased staining of the proliferation marker Ki-67 (result not shown).

Previous studies have suggested that Wnt6 can activate both canonical (54) and noncanonical pathways (46) in a cell-specific manner. Developmental promoter reporter activity (Fig. 1, J and K) indicated a high level of TCF/LEF activity in the developing kidney coincident with Wnt6 expression. Wnt6 conditioned media was generated by transfecting renal epithelial cells (HKC8) with Wnt6 for 48 h. The presence of the V5 tag in the lysate and supernatant confirmed successful overexpression and secretion of Wnt6 (Fig. 3C). Epithelial cells were subsequently grown in this Wnt6 conditioned media for up to 3 h. Wnt6 increased the phosphorylation of GSK3\(\beta\) (Serine 9) and the phosphorylation of \(\beta\)-catenin (Serine 552), hallmarks of canonical Wnt signaling and nuclear translocation respectively (Fig. 3B).

Gene expression data also identified two potential receptors, Fzd6 and Fzd7, that were coexpressed with Wnt6 in patients with nephropathy (44). Similarly, both receptors were evident in the developing kidney; however, only Fzd7 had previously been implicated in activation of canonical signaling (39). siRNA knockdown of Fzd7 in renal epithelial cells led to the ablation of phosphorylation of GSK3\(\beta\) in response to Wnt6 identifying it as a putative receptor (Fig. 3D). Short interfering RNA (siRNA) knockdown of Fzd7 was confirmed by RT-PCR (Fig. 3E).

\textbf{Wnt6 regulates vimentin expression through inhibition of TGF-\(\beta\) induced p65 NF-\(\kappa\)B nuclear translocation}. The pathogenic microenvironment of the kidney during renal disease contains a number of growth factors that contribute to fibrosis including, TGF-\(\beta\), CTGF, and VEGF among others (30a). siRNA knockdown of Fzd7 in renal epithelial cells led to the ablation of phosphorylation of GSK3\(\beta\) and the phosphorylation of \(\beta\)-catenin (Serine 552), hallmarks of canonical Wnt signaling and nuclear translocation respectively (Fig. 3B).

\textbf{Wnt6 regulates vimentin expression through inhibition of TGF-\(\beta\) induced p65 NF-\(\kappa\)B nuclear translocation}. The pathogenic microenvironment of the kidney during renal disease contains a number of growth factors that contribute to fibrosis including, TGF-\(\beta\), CTGF, and VEGF among others (30a). siRNA knockdown of Fzd7 in renal epithelial cells led to the ablation of phosphorylation of GSK3\(\beta\) in response to Wnt6 identifying it as a putative receptor (Fig. 3D). Short interfering RNA (siRNA) knockdown of Fzd7 was confirmed by RT-PCR (Fig. 3E).

Wnt6 induced de novo tubulogenesis and activates canonical signaling in renal cells. A: Madin Darby canine kidney cells (MDCKs) were grown in 3D culture for 10 days to allow for spherical cyst formation. Cells were transfected for up to 72 h with an empty vector plasmid or a Wnt6 expression plasmid until tubulogenesis occurred. Cells were fixed with 3.7% paraformaldehyde and stained with Alexa488 conjugated phalloidin (to visualize filamentous actin: green) and Hoechst 33342 (to visualize nuclei: blue). In response to Wnt6 overexpression MDCK cells underwent differentiation to form de novo tubule structures. B: HKC8 renal epithelial cells were treated with Wnt6 conditioned media up to 3 h and activation of GSK3\(\beta\) and \(\beta\)-catenin (markers of canonical signaling) were noted. C: the presence of V5 tag in the lysate and supernatant confirmed the presence of Wnt6 for all media treatments. D: HKC8s were transfected with scrambled oligo negative control or Fzd7 targeting siRNA for 48 h followed by stimulation with Wnt6 conditioned media for 30 min. siRNA knockdown of Fzd7 inhibited Wnt6 induced GSK3\(\beta\) activation (lanes 3 and 4), suggesting Fzd7 as a putative receptor of Wnt6 signaling. Nonconditioned media (lanes 1 and 2) was used to control for other potential factors and mediators in conditioned media. Densitometry analysis confirms Wnt6 induced GSK3\(\beta\) activation is inhibited in Fzd7 knockdown cells. E: Fzd7 siRNA knockdown was validated by RT-PCR and densitometry analysis (normalized to 18S). EV, empty vector; Scr, scrambled oligo negative control; NC, nonconditioned media. *\(p < 0.01; n = 4\).

\textbf{DISCUSSION}

Wnt signaling is a fundamental pathway regulating cellular interactions and responses during embryonic development. During the process of nephrogenesis a number of Wnt ligands are expressed: Wnt-2b, -4, -5b, -6, -7b, -9b, and 11 and are expressed during ontogenesis, while Wnt-6, -7b, 9b, and 11 are expressed in the ureteric bud during organogenesis (26). The putative role of Wnt6 in tubulogenesis was first suggested by Itäranta et al. (21), who demonstrated expression in the ureteric bud (which goes on to form the collecting duct) in early stages.
of kidney development in Xenopus and provided evidence that Wnt6 derived from the ureteric bud could induce tubulogenesis in isolated metanephric mesenchyme in organ culture. More recently, comprehensive 3D analysis of Wnt and Fzd expression by whole mount in situ hybridization has delineated patterns of expression in the developing mouse embryo (www.emouseatlas.org) (49). Shown here is the first evidence of Wnt6 expression in the mesonephric ducts and tubules in the developing mouse kidney, supporting the hypothesis that it plays a role in tubulogenesis in vivo. Furthermore, analysis of expression data in the developing limb is informative with high levels of Wnt6 expression restricted to largely epithelial sites, suggesting a role in the establishment and/or maintenance of epithelial structures (49). Moreover, its putative receptor Fzd7 is coexpressed during these early stages of development while there is clear activation of canonical Wnt signaling in the developing kidney as evidenced in the TCF/Lef reporter mouse. Given that Wnt6 is required for maintenance of somite epithelial structures and is coincidentally found in zones where epithelialization processes occur, it is possible that Wnt6 regulates the transcription factors and signaling pathways associated with pluripotency and subsequent fate specification.

During progressive renal disease, the infiltration of inflammatory cells, such as monocytes and macrophages, and myofibroblasts is known to be a driver of chronic kidney disease (11, 55). Infiltrated macrophages in the renal tubular interstitium release proinflammatory factors thereby leading to the formation of a self-perpetuating insult. Increased levels of profibrotic cytokines such as TGF-β1 promote the accumulation of ECM but also regulate discrete biological processes leading to loss of epithelial integrity (3, 18, 37). It is now clear that progression of chronic kidney disease is closely correlated to the degree of renal interstitial fibrosis.

The origin of myofibroblasts during the development of renal fibrosis has been the subject of some controversy and debate over the last decade. Recent studies using fate tracing in mouse models of fibrosis have identified diverse sources including pericytes (20, 34), bone marrow (5, 30, 31), and both endothelial and epithelial to mesenchymal transition programs (14, 22, 30, 32, 56).

Regardless of the source, the pathogenic role of myofibroblasts during the progression of renal fibrosis is well accepted, as is the differentiation and loss of resident epithelial cells and structures. Many animal models have been proposed for the analysis of pathological insults that lead to tubulointerstitial fibrosis. Hemodynamic stress and nephrotoxic damage are commonly modeled using the UUO mouse and the gentamicin nephrotoxic rat, respectively. UUO manipulation generates a hydrostatic pressure within the kidney resulting in interstitial inflammation, myofibroblast accumulation, interstitial matrix accumulation, and tubular atrophy ultimately leading to renal fibrosis with loss of functional epithelia (8, 19). Onset of injury in this model is rapid with early signs of injury seen in a few days and more advanced damage seen as early as 10 days (8). Gentamicin, an aminoglycoside antibiotic known to be nephrotoxic (43), is preferentially taken up by proximal tubular cells of the nephron by binding to negatively charged phospholipids on the brush border (38); the capacity of gentamicin to subsequently alter mitochondrial respiration has been well documented in reports of both in vitro and in vivo studies (15, 40). Altered mitochondrial respiration enhances the generation of reactive oxygen species resulting in activation of an immune response with infiltration of mononuclear cells and subsequent tubular cell damage (28).

Both models develop pathologically relevant deposition of extracellular matrix and loss of epithelial integrity and, consequently, epithelial function (2, 8) with the gentamicin-treated rat a widely used model of acute renal failure (2). Decreased expression of Wnt6 is evident in the obstructed kidney, specifically in the tubules coincident with loss of epithelial integrity, compared with controls in the UUO model at both early (day 3) and late (day 8) stages of tubulointerstitial damage (Fig. 2E). Similarly, there is a clear decrease in Wnt6 levels in the high-dose gentamicin nephrotoxic rat kidneys compared with low-dose and noninjected control animals. Loss of Wnt6 in the tubulointerstitium of diabetic nephropathy patients and both animal models of renal fibrosis suggests that it is pathogenically important during loss of tubular epithelial specification, integrity, and function. Indeed, the regenerative potential of Wnt6 is well illustrated by its capacity to induce de novo tubulogenesis in renal epithelial cells grown in 3D culture, accompanied by increased proliferation (Fig. 3A).

Most of our understanding of fate specification comes from embryonic studies, which identified specific transcriptional hierarchies that regulate patterning of the anterior and posterior
mesoderm. Indeed the protocols developed by the Little laboratory show that tempo-selective inhibition of Wnt signaling using the GSK inhibitor CHIR99021 determines induction of collecting duct or renal mesenchyma (50). The subsequent self-organization of kidney organoids resulted in remarkable architecture that may be considered the functional equivalent of a nephron.

The question as to how Wnt6 signaling can be exploited to control fate specification and epithelialization is an intriguing one. Certainly the observation that Wnt6 is a canonical ligand, leading to nuclear accumulation of β-catenin and activation of TCF/Lef-mediated transcription, does not in itself explain the observation that it can inhibit/rescue epithelial differentiation in response to TGF-β. Interestingly, these effects appear to be driven primarily through the regulation of the expression of the intermediate filament vimentin, a well-established marker of epithelial to mesenchymal transdifferentiation.

The transient expression of vimentin filaments during the development of the nephron identifies cells that are undergoing fate specification (41). Similarly, the specification of induced pluripotent stem cells to mesenchymal stem cells is accompanied by increased expression of vimentin (7).

Increased expression of the intermediate filament vimentin is widely accepted as a marker of epithelial to mesenchymal transition, where it mediates cytoskeletal organization and focal adhesion maturation, while in contrast these transitions are characterized by repression of e-cadherin. These switches are mediated by critical transcription factors of the SNAIL, ZEB, and basic helix-loop-helix families. It is becoming increasingly apparent that TGF-β1 induces epithelial to mesenchymal transdifferentiation by acting at transcriptional, post-transcriptional, translational, and posttranslational levels through both SMAD-mediated and non-SMAD signaling (29). Our data suggest that partial rescue of TGF-β1-mediated epithelial to mesenchymal transdifferentiation can be altered by Wnt6 as evidenced by inhibition of vimentin, suggesting a potential role in epithelialization during renal development and highlighting that loss of Wnt6 likely has consequences for vimentin-mediated cytoskeletal organization and epithelial integrity. As Wnt6 conditioned medium contains other factors, careful consideration was given to the design and implementation of all experiments, using control conditioned medium from the parental cell line and protocols established for other members of the Wnt family.

Mechanistically, this appears to involve a degree of cross talk between Wnt pathways and TGF-β-mediated NF-κB. The requirement for NF-κB in TGF-β-mediated induction of vimentin is evident from our studies using p65/−/− and IKK−/− MEFs; cells lacking NF-κB activity (p65−/−) had no vimentin expression either basally or in response to treatment with TGF-β. Importantly, Wnt6 inhibited the nuclear translocation of p65 NF-κB.

Future studies will determine the level of interaction between TGF-β1 and Wnt signaling networks; however, we can speculate that the sequestering/inhibition of Erk activity by canonical Wnt6 signaling may result in a lack of availability for TGF-β/NF-κB noncanonical signaling resulting in inhibition of vimentin expression and preservation of epithelial characteristics.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


