Canonical WNT signaling during kidney development

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The WNT family is comprised of 19 secreted glycoproteins which act as short-range intercellular signaling molecules, recognizing one of the 10 frizzled receptors expressed at the surface of nearby target cells. The canonical WNT signaling pathway is activated by WNTs which bind to cognate frizzled receptors and heterodimerize with LRP5 or LRP6 coreceptors (2). Activated receptors recruit dishevelled protein (Dvl) and inhibit degradation of cytoplasmic β-catenin via the GSK3β/axin/β-catenin-mediated WNT signaling pathway. The activities of the canonical WNT signaling pathway are in proximity to the known sites of renal WNT signaling in kidneys of mice bearing a β-catenin-responsive TCF/Gal transgenic reporter. In metanephric kidney, intense canonical WNT signaling was evident in epithelium of the branching ureteric bud and in nephrogenic mesenchyme during its transition into renal tubules. WNT signaling activity is rapidly downregulated in maturing nephrons and becomes undetectable in postnatal kidney. Sites of canonical WNT signaling activity are in proximities of the many FRZ and LRP5/6 receptors in fetal kidney, where WNTs activate the canonical pathway in cultured MK4 (derived from nephrogenic mesenchyme) cells and inner medullary collecting duct (IMCD-3, mouse collecting duct) cells (ATCC number CRL-2123) expressing NIH3T3 cells were also able to induce tubule formation in the coculture assay, suggesting that the canonical pathway inhibitor, Dickkopf-1, arborization of the ureteric bud was significantly reduced. We conclude that restricted zones of intense canonical WNT signaling drive branching nephrogenesis in fetal kidney. Thus it has been difficult to decipher the function of canonical WNT signaling in other contexts; these include WNT5A (19), WNT2b (15), and WNT7b (35). Additional WNTs (WNT6, WNT9, and WNT11) have been shown to activate the canonical signaling pathway in developing kidney. We also show that when fetal kidney explants are exposed to the canonical WNT signaling pathway inhibitor, Dickkopf-1, branching nephrogenesis is significantly suppressed.

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lottsville, VI), mouse WNT7b cDNA (kindly provided by E. Morrissey), mouse WNT2b cDNA (kindly provided by I. Drummond), TOPFLASH and FOPFLASH reporter vectors (Upstate Biotech), and pGL2 basic (Promega, Madison, WI; as a control). All transfections included the renilla luciferase expression vector, pRL-SV40 (Promega), as a control for sample-to-sample variation in transfection. Transfections were performed in triplicate in 24-well plates; each experiment was performed three times. At 60% confluence, cells were transfected with 200 ng of the various plasmids using FuGENE 6 Transfection Reagent (Roche, Penzberg, Germany) according to the protocol recommended by the manufacturer. Firefly luciferase and renilla luciferase reporter activities were determined using Dual Luciferase Assay System reagents (Promega) and quantified in a Microlumat Plus Luminometer (EG&G Berthold). Reporter activity was expressed as the ratio of luciferase to renilla values. Statistical analysis was performed using Student’s t-test.

For study of the canonical signaling inhibitor, DKK1, cells were plated at 60% confluence in a 24-well plate; recombinant mouse DKK1 protein (R&D Systems, Minneapolis, MN) was added (0 or 500 ng/ml) to the wells. After 24 h, the cells were transfected with either TOPFLASH or control vector and pRL-SV40 renilla as a transfection efficiency control. Fresh DKK1 was added to the media 1 h after the transfection was performed. Firefly luciferase and renilla luciferase reporter activities were determined after 24 h. The experiment was performed two times in triplicate.

Reporter mice. Animal procedures followed the guidelines established by the Canadian Council of Animal Care and were approved by the Animal Care Committee from McGill University. CD1 mice bearing a β-catenin-responsive lacZ reporter gene have been previously described (22). Briefly, this transgene contains six TCF/LEF response elements cloned upstream of a minimal Hsp68 promoter bearing a lacZ reporter gene. C3H mice (provided by F. Costantini) were described elsewhere (30).

Immunohistochemistry. Paraffin-embedded sections (7 μm) of embryonic kidneys were incubated in 5% H2O2 to quench endogenous peroxidase activity, followed by a 30-min incubation with normal horse serum. Tissue sections were then incubated with anti-nonphosphorylated β-catenin antibody (Upstate, Lake Placid, NY), washed, and incubated with a universal biotinylated secondary antibody (Vector Laboratory, Burlingame, CA). Staining was developed using DAB (Vector Laboratory) and counterstained with Gill’s hematoxylin.

Analysis of LacZ activity in transgenic mice. The protocol for β-galactosidase staining has been described elsewhere (23). Kidneys from mice bearing the TCF/lacZ transgene and wild-type mice were removed and fixed in PBS containing 2 mM MgCl2, 0.02% NP-40, 0.01% deoxycholate, 1% formaldehyde, and 0.2% glutaraldehyde, rinsed in washing buffer (PBS with 2 mM MgCl2, 0.02% NP-40, 0.01% deoxycholate), and stained in the dark overnight in washing buffer supplemented with 1 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide. After being stained, kidneys were washed in PBS and visualized directly or embedded in paraffin for sectioning and standard counterstaining with hematoxylin and eosin.

Immunofluorescent microscopy. Frozen sections of embryonic kidneys (10 μm) from transgenic mice were blocked in normal serum and incubated with rabbit anti-β-galactosidase antibody (1:250, Chemicon International, Temecula, CA). Sections were washed and incubated with rhodamine-tagged secondary anti-rabbit IgG antibody (1:50, Chemicon International), washed, and incubated with fluorescein-tagged dolichos biflorus lectin (1:200, Vector Laboratory) before microscopic examination under fluorescent light.

For WT1 staining of GFP explants, kidneys were fixed for 10 min in methanol, washed in PBS 0.1% Tween 20 (PBST), and incubated overnight at 4°C with rabbit polyclonal anti WT1 antibody (C19, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in PBST/2% BSA. After washes in PBST, kidneys were incubated with anti-rabbit Alexa Fluor 594 (Invitrogen) 1:400 at 22°C for dual immunofluorescent microscopy.

Kidney explant culture. Kidneys from HoxB7/GFP mice were dissected from embryonic day 13.5 (E13.5) embryos and placed on a filter in six-well plates in DMEM with 10% BSA (control) or medium containing 2 μg/ml recombinant mouse DKK1 protein (R&D Systems). The explants were cultured in a humidified 37°C incubator under 5% CO2. Kidneys were supplemented every 24 h with fresh medium (control) or medium containing DKK1. Pictures were taken every 24 h under fluorescent light. UB tips were counted using Image J software. Statistical analysis was performed using Student’s t-test.

RESULTS

Canonical β-catenin signaling activity is progressively restricted to UB tips and newly formed S-shaped bodies in developing kidney. To examine the pattern of canonical β-catenin pathway signaling activity, E11.5 embryos (E11.5) or microdissected kidneys (E14-P21) from TCF/β-galactosidase mice were assessed for transgene activity. The TCF signal is first seen in the nephric duct at E11.5 (Fig. 1, A and B). By E13-E15, the TCF reporter signal is seen throughout the arborizing UB but is somewhat stronger in UB tips than in its trunk (Fig. 1, C, D, E). By E16.5 the TCF signal is highly restricted to the UB tips and distal S-shaped body but is suppressed in UB trunks (Fig. 1F).

To confirm that transgene expression reflects endogenous canonical WNT signaling activity, sections from X-gal-stained E15 transgenic kidney (Fig. 2A) were compared with sections from E15 wild-types stained with an antibody against active (nonphosphorylated) β-catenin (Fig. 2B). Active β-catenin staining is, like the TCF signal, intense at the tips of the UB and in distal portions of the S-shaped body. To confirm that canonical WNT signaling becomes restricted to the tips (vs. trunks) of the UB, cryosections of E16.5 kidney were co-stained with antibody for β-galactosidase (red) and a UB marker dolichos biflorus agglutinin (green). At this stage, the fused image shows restricted TCF signaling at UB tip but is disappearing in the UB trunk (Fig. 2, C–E).

As kidney development proceeds, the TCF signal is sustained only in the nephrogenic zone (Fig. 3, A and B). All canonical signaling activity is extinguished as nephrogenesis comes to an end in the early postnatal period (Fig. 3, C and D).

WNT2b, WNT4, and WNT7b stimulate canonical β-catenin signaling. Since our observations in the TCF/LacZ mouse indicate intense activity of the canonical WNT pathway at the UB tips and in the distal S-shaped bodies, we used cell lines derived from these two lineages, MK4 (murine nephrogenic mesenchyme) and IMCD (murine collecting duct), to screen various WNTs for canonical activity. Six WNTs have been reported in developing kidney, but only four of these (WNT2b, WNT4, WNT7b, and WNT11) are expressed in sustained fashion near the branching UB tips and emerging S-shaped bodies. Cells were grown to 60% confluence and transiently transfected with pcDNA expression vectors containing various full-length murine WNT cDNAs (or empty vector) in the presence of either TOPFLASH or FOPFLASH (mutant) reporter vectors. Cotransfection with SV40/renilla vector was used as a control for transfection efficiency. In the absence of any WNT vectors, TOPFLASH had significant basal activity above a promoterless luciferase vector in both cell lines. TOPFLASH activity reflects β-catenin/TCF signaling in these
cells, since mutation of one of the three sense TCF response elements (FOPFLASH) reduced activity by 40%. TOPFLASH activity was unaffected by cotransfection with a (noncanonical) WNT11 expression vector (data not shown). In the presence of WNT2b, basal TOPFLASH activity was stimulated 5.6-fold in MK4 cells ($P < 0.03$) and 6.6-fold ($P < 0.05$) in IMCD cells above the empty expression vector controls (Fig. 4, A and B). WNT7b stimulated TOPFLASH 7.4-fold ($P = 0.09$) and 4.3-

Fig. 1. Ontogeny of β-catenin signaling during early development in TCF/lacZ reporter transgenic mouse kidney. Mouse embryonic (A and B) and kidney (C, D, E) sections were stained for β-galactosidase activity. At embryonic day 11.5 (E11.5), β-galactosidase is seen in the nephric duct (arrows; A and B). At E13, strong signal is observed throughout the arborizing ureteric bud (UB; C). At E14.5, strong staining is seen in the branching UB, particularly at its tips, and in distal segments of S-shaped bodies (arrows; D). Low-power view of E15.5 kidney shows strong β-galactosidase staining in UBs, whereas maturing collecting ducts show reduced staining (E). At E16.5, the β-galactosidase signal is restricted to the tips of the branching UBs (F). Scale bar = 100 μm for A, B, D, E, and F and 1 mm for C.

Fig. 2. β-Catenin signaling is restricted to the UB tips at later stages in development. Higher-power view of E15 kidney shows β-galactosidase staining at the tips of the UB and in the distal portion of S-shaped bodies (arrow); there is reduced β-galactosidase staining in the maturing trunk of the collecting duct (A). E15 wild-type mouse kidney probed with anti-unphosphorylated β-catenin antibody shows active β-catenin (arrows) at the tips of the branching UB and in the S-shaped bodies (B). Cryosections from E16.5 kidneys were stained with the fluorescent UB marker dolichos biflorus agglutinin (green; C) and a rhodamine-labeled antibody raised against β-galactosidase (red; D); the merged image demonstrates colocalization at the UB tip (E). Scale bar = 100 μm.
fold ($P < 0.01$), in the two cell lines, respectively. Although WNT4 had no effect on MK4 cells, it stimulated TOPFLASH 2.7-fold ($P < 0.01$) in IMCD cells (Fig. 4, A and B).

**DKK1 inhibition of the canonical β-catenin pathway suppresses branching nephrogenesis.** Since β-catenin/TCF signaling was associated with UB tips, we considered the possibility that the canonical WNT pathway might be involved in branching nephrogenesis. To first confirm that DKK1 inhibits the canonical WNT signaling pathway in cells derived from the UB, as it does in other cell types (1), we transiently cotransfected IMCD cells with TOPFLASH and an expression vector containing the full-length murine DKK1 cDNA or an empty vector control. Cells were harvested after 48 h and assayed for luciferase activity. Luciferase activity was reduced to 23% of control in the presence of DKK1 plasmid ($P < 0.05$; Fig. 5A).

IMCD cells were also exposed to recombinant murine DKK1 protein (0 or 500 ng/ml) for 24 h and then transiently transfected with TOPFLASH reporter vector. Fresh DKK1 was added 1 h after transfection and luciferase was measured 24 h later. As seen in Fig. 5B, recombinant DKK1 suppressed TOPFLASH activity by ~40% ($P < 0.01$).

To examine the effect of DKK1 on branching morphogenesis, E13.5 kidneys were isolated from HoxB7/GFP mice and placed in explant culture for 24–48 h. From each embryo ($n = 4$), one kidney was cultured in the presence of recombinant DKK1 protein (2 μg/ml) to inhibit the canonical pathway, while the contralateral kidney served as a control. At 0, 24, and 48 h, explants were photographed under fluorescent light to assess the number of terminal UB tips; the extent of arborization was expressed as the percent increase in UB tip number compared with baseline for each kidney. At 24 h, UB tip number increased by 40% in controls but by only 18% in kidneys exposed to DKK1 ($P < 0.005$; Fig. 6, A and B). At 48 h, UB tip number had increased by 70% of baseline in controls vs. 40% of baseline in the presence of DKK1 ($P < 0.01$; Fig. 6, A and B).

To ascertain whether DKK1 blockade of canonical signaling affects structure of individual nephrons derived from induced mesenchyme, we visualized the expression pattern of Wilms Tumour protein (WT1) in E13 kidney explants isolated from HoxB7/GFP mice after 24-h exposure to DKK1. In normal explants, WT1 protein is seen in condensing mesenchyme capping UB tips and in podocyte layers of emerging glomeruli; this pattern is well-preserved in DKK1-treated explants (Fig. 6C).

**DISCUSSION**

In this study, β-catenin/TCF signaling activity was first noted at E11.5 in cells of the nephric duct cells during its caudal descent. This was also noted by Maretto et al. (20) in E13.5 kidney of a similar β-catenin/TCF reporter mouse. The
nephric duct lineage expresses a unique panel of genes such as Lim-1, c-Ret, Pax2, Pax8, and Gata3. Conceivably, canonical WNT signaling may specify nephric duct cell fate as it does in a variety of other developmental settings. In zebrafish, the canonical effects of WNT2b in lateral plate mesoderm are required for liver specification; liver organogenesis fails in prtr(−/−) mutants bearing Wnt2b null alleles (25). Canonical WNT signals propel differentiation of bone cell precursors along the osteoblast pathway (10). In the inner ear, canonical WNT signals drive precursor cells toward the otic placode fate (26). Canonical WNT signaling is important for organogenesis of lung, pancreas, and mammary gland as well (5, 7, 24).

In developing metanephric kidney, we noted that β-catenin signaling activity becomes progressively focused at the tips of UB branches and gradually disappears from maturing UB trunks. Costantini has shown that UB tip cells express a unique panel of genes such as Ret and Wnt11, distinguishing them from sister cells which comprise the UB trunk (28). Davies and Michael (21) have also drawn attention to the fact that cell division at UB tips is especially intense at UB tips compared with the UB trunk. The β-catenin/TCF pathway activates transcription of specific gene targets including Myc and cyclin D1 (9) and can negatively regulate transcription of the potent cell cycle inhibitor, p21, in HEK293 cells (14). It seems plausible therefore that the β-catenin pathway could be involved in cell fate specification and proliferation of UB tip cells.

Strong β-catenin/TCF signaling activity was observed in the mesenchymal clusters capping each UB tip. As mesenchymal condensates progressed to the S-shaped body stage, β-catenin/TCF signaling was restricted to the distal portion of the structure, comprising the anlage of renal proximal and distal tubules; WNT signaling was conspicuously absent in the emerging glomerulus. This could suggest an additional role for canonical WNT signaling in cell fate specification and rapid growth of the nephron’s tubular segments derived from mesenchyme. Interestingly, mice with a conditional knockout of β-catenin in lung epithelial cells exhibit fairly normal proximal airways but lack the distal portions of the pulmonary tree (24).

Since β-catenin/TCF signaling was especially intense in the nephrogenic zone of developing kidney, we considered the possibility that the pathway might be required for branching morphogenesis. To test this hypothesis, we blocked the canonical WNT signaling in kidney explants using recombinant DKK1. DKK1 specifically blocks the canonical pathway by binding to the frizzled coreceptor, LRP5/6, and interfering with WNT ligand binding (1). When E13.5 kidney explants from HoxB7/GFP fetal mice were exposed to DKK1, we found that the number of UB branch tips was significantly reduced (40% of controls) within 24 h. If we take into account that UB branching is reiterated many times before nephrogenesis comes to an end, the impact of a moderate inhibition of branching is amplified many times over. The effect of DKK1 is comparable to the effect of Pax2 haploinsufficiency which produces renal insufficiency in mutant mice (8). This is the first demonstration
that canonical WNT signaling is required for branching morphogenesis in the kidney. De Langhe et al. (6) observed similar inhibition of pulmonary branching in murine fetal lung explants exposed to DKK1.

Our studies identify the primary sites of canonical β-catenin/TCF pathway activity during kidney development. This begs the question as to which WNT ligands might be driving this focused pathway activity. Several canonical WNTs (WNT7b, WNT6, and WNT9b) are expressed in the nephric duct and the early UB. However, WNT7b is evident in the UB stalk by E13.5 (16) but is not evident at UB tips or in S-shaped bodies. WNT6 is expressed in the UB at early stages but is downregulated after E14.5 (13). WNT6 can induce tubulogenesis in tissue culture assays but was unable to support UB branching in vitro. Similarly, WNT9b is expressed in the nephric duct and UB stalk from E9.5 to E14.5, but is downregulated thereafter (4). While each of these three WNTs could contribute to canonical signaling in early stages, none could account for the intense TCF signaling activity associated with UB tips or S-shaped bodies during later stages of nephrogenesis.

WNT11, WNT2b, and WNT4 are all expressed at sites that might account for the observed TCF signaling activity. However, WNT11 has been consistently associated with noncanonical signaling pathway activity in other reports and had no effect on TOPFLASH in our assays. On the other hand, WNT2b is expressed as early as E11.5 in metanephric mesenchyme (18) and has been shown to activate the β-catenin pathway in other settings (17). Lin et al. (30) showed that WNT2b supported growth of isolated mouse UB. In our studies, WNT2b activated the canonical pathway in both MK4 (mesenchymal) and IMCD (UB lineage) cells.

WNT4 is expressed in the condensing mesenchyme and in the S-shaped bodies as they differentiate (31) and can activate the canonical pathway in Madin-Darby canine kidney cells derived from the renal collecting duct (19). Similarly, in our assays, WNT4 activated the canonical TOPFLASH reporter in IMCD cells. However, there was no apparent effect on mesenchymally derived MK4 cells. Furthermore, we found no apparent effect of DKK1 on WT1 expression in glomeruli of fetal kidney explants. Thus the restricted canonical signaling activity at the distal end of the S-shaped body may reflect stimulation by mesenchymal WNT2b. WNT4 derived from the condensing mesenchyme might contribute to other canonical ligands affecting the UB tip, but the profound effects of WNT4 on progression of condensing mesenchyme to the S-shaped body stage must involve its capacity to activate noncanonical signaling pathways (19).

As murine kidney development progresses, canonical WNT signaling activity disappears from maturing segments of the nephron and is restricted to the nephrogenic zone. In the perinatal period, as nephrogenesis comes to an end, WNT signaling activity is completely extinguished. The inhibitory mechanism is not entirely clear, but Simons et al. (29) proposed that onset of tubular flow through mature nephron segments induces signals from luminal cilia which may suppress the canonical WNT pathway. When IMCD cells were exposed to laminar flow in vitro, expression of the cilial protein, inversin, increased and cytoplasmic levels of β-catenin fell. Inversin appears to suppress TOPFLASH activity by inducing degradation of the key signal transduction pathway molecule, dishevelled.

In summary, intense canonical WNT signaling pathway activity is evident throughout the nephric duct and early UB, where it is required for normal branching morphogenesis. When canonical WNT activity is blocked by exogenous Dickkopf-1 protein, arborisation of the UB is diminished. As development proceeds, WNT signaling is progressively restricted to UB tips and distal portions of the S-shaped body but is suppressed in the emerging glomerulus and in maturing trunks of the UB and is globally downregulated as nephrogenesis comes to an end. We hypothesize that suboptimal WNT signaling could result in renal hypoplasia, whereas failure of mechanisms that normally suppress WNT signaling might contribute to aberrant budding growth of tubular cells in polycystic kidney disease.

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REFERENCES