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Wnt/β-catenin signaling regulates nephron induction during mouse kidney development

Joo-Seop Park*, M. Todd Valerius* and Andrew P. McMahon†

Mammalian nephrons form as a result of a complex morphogenesis and patterning of a simple epithelial precursor, the renal vesicle. Renal vesicles are established from a mesenchymal progenitor population in response to inductive signals. Several lines of evidence support the sequential roles of two Wnt family members, Wnt9b and Wnt4, in renal vesicle induction. Using genetic approaches to specifically manipulate the activity of β-catenin within the mesenchymal progenitor pool in mice, we investigated the potential role of the canonical Wnt pathway in these inductive events. Progenitor-cell-specific removal of β-catenin activity completely blocked both the formation of renal vesicles and the expected molecular signature of an earlier inductive response. By contrast, activation of stabilized β-catenin in the same cell population causes ectopic expression of mesenchymal induction markers in vitro and functionally replaces the requirement for *Wnt9b* and *Wnt4* in their inductive roles in vivo. Thus, canonical Wnt signaling is both necessary and sufficient for initiating and maintaining inductive pathways mediated by Wnt9b and Wnt4. However, the failure of induced mesenchyme with high levels of β-catenin activity to form epithelial structures suggests that modulating canonical signaling may be crucial for the cellular transition to the renal vesicle.

KEY WORDS: Canonical Wnt signaling, Nephrogenesis, Mesenchymal-to-epithelial transition, Tubulogenesis, Mouse

INTRODUCTION

Maintenance of an appropriate homeostatic balance of water and salt levels, and the removal of nitrogenous waste products of metabolism are crucial functions attributable to the nephrons of the mammalian kidney. The tubular epithelial network of the nephron derives from two initial embryonic sources: (1) the ureteric bud, a caudal outgrowth of the Wolffian duct that forms the collecting duct system; and (2) the adjacent metanephric mesenchyme, or blastema, that forms the renal tubules (Vainio and Lin, 2002; Yu et al., 2004). Reciprocal and repetitive inductive interactions between the invading ureteric bud and adjacent metanephric mesenchyme results in branching growth of the ureteric bud, elaborating the network of the collecting duct system for urine transport and induction of a mesenchymal-to-epithelial transition within the mesenchyme, establishing the renal vesicles, the precursors of the renal tubule (Mori et al., 2003; Vainio and Lin, 2002; Yu et al., 2004). Renal vesicle formation is preceded by the appearance of pre-tubular aggregates of induced mesenchyme beneath the tip of the branching ureteric epithelium. There, aggregates then transition to epithelial renal vesicles over a period of several hours. Subsequently, the renal vesicle undergoes extensive growth and complex morphogenesis and patterning that result in the establishment of mature nephrons (Mori et al., 2003; Vainio and Lin, 2002; Vize et al., 2003; Yu et al., 2004).

Two members of the Wnt family of lipid-modified secreted glycoproteins, Wnt9b and Wnt4, play central roles in the initial stages of the tubulogenic program (Carroll et al., 2005; Kispert et al., 1998; Stark et al., 1994). *Wnt9b* is expressed in the ureteric epithelium, whereas *Wnt4* is expressed in the pre-tubular aggregate, as one of the first molecular responses to inductive signaling mediated by the ureteric epithelium. Genetic analyses of *Wnt9b* and *Wnt4* mutant mice indicate that *Wnt9b* is a primary inductive signal required for all aspects of renal vesicle induction, including the activation of *Wnt4* (Carroll et al., 2005). Wnt4 appears to play an auto-inductive role within the induced mesenchyme; one that is essential for propagating the inductive process and completing the transition to epithelial renal vesicles (Kispert et al., 1998; Stark et al., 1994). Although the importance of these signals is clear, the molecular pathways through which these sequential signals operate have not been determined.

Wnts can activate several pathways (reviewed by Clevers, 2006; Nelson and Nusse, 2004). The best defined has been termed the canonical Wnt pathway. In this, Wnt-mediated signaling counters a phosphorylation-triggered pathway of proteosomal degradation, thereby enabling the stabilization of β-catenin in receiving cells. Elevated levels of β-catenin allow the formation of complexes between β-catenin and TCF/LEF DNA-binding proteins. The resulting activator complex directly regulates transcriptional targets (Nelson and Nusse, 2004). Wnt signaling has also been shown to regulate cell polarity and cell movement, at least in part through the JNK signaling pathway, to increase the intracellular concentration of calcium in various contexts, and through a PKA/CREB-based mechanism in muscle specification (Chen et al., 2005; Pandur et al., 2002; Veeman et al., 2003).

To explore the mechanism of action of Wnt9b and Wnt4, we investigated the potential role of β-catenin-dependent signaling in renal vesicle induction in the mammalian kidney. Our data provide strong evidence that β-catenin-dependent canonical Wnt signaling is the primary mode of both Wnt9b and Wnt4 signaling. Further, our findings suggest that β-catenin-mediated canonical signaling acts transiently to induce the mesenchyme, as the downregulation of β-catenin activity is essential for the transition to the fully epithelial state of the renal vesicle.

Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

*These authors contributed equally to this work
†Author for correspondence (e-mail: amcmahon@mcb.harvard.edu)

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MATERIALS AND METHODS

Mouse strains

β-catenin$^{c/c}$ (Brault et al., 2001), *Catnb*$^{ex3/ex3}$ (Harada et al., 1999), *β-catenin*$^{+/n}$ (Haegel et al., 1995), *Wnt4*$^{+/n}$ (Stark et al., 1994) and *Wnt9b*$^{+/n}$ (Carroll et al., 2005) mouse lines have been described previously. The *Six2TGC* mouse line will be described more fully elsewhere.

Histology

The first presence of a copulatory plug was considered embryonic day 0.5 (E0.5). Postnatal day 1 (P1) and E13.5 kidneys were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated in ethanol, embedded in paraffin wax and sectioned at 6 μm. Sections were stained with Hematoxylin and Eosin.

Explant culture

Metanephric mensenchyme was dissected from E11.5 kidneys as previously described (Carroll et al., 2005). Each explant was cultured on Nucleopore Track-Etch Membrane (pore size=1 μm, Whatman, Florham Park, NJ) in DMEM/10% FBS for 24 hours.

In situ hybridization

E12.5 kidneys and explant culture samples were fixed in 4% paraformaldehyde at 4°C overnight and dehydrated in methanol. Hybridized samples were developed in BM purple (Roche, Indianapolis, IN), stored in 80% glycerol and photographed using a Nikon DXM1200 digital camera.

Immunohistochemistry

Embryonic day 13.5 and 15.5 embryos were fixed in 4% paraformaldehyde at 4°C for 1 hour, incubated in 20% sucrose at 4°C overnight and imbedded in OCT. Sections (10 μm) were incubated with primary antibodies to β-catenin (Epitomics, Burlingame, CA), E-cadherin (Zymed, S. San Francisco, CA), β-galactosidase (Cappel, Cochranville, PA) and *Dolichos biflorus* DBA lectin (Sigma, St Louis, MO), and secondary antibodies conjugated to Cy2, Cy3 and Cy5. Sections were photographed on a Zeiss LSM510 Axioplan inverted confocal microscope.

RESULTS

β-catenin activity is required within mesenchymal progenitors for induction of renal vesicles

The renal vesicles are thought to arise from a mesenchymal progenitor population that caps the ureteric bud. In an independent study (M.T.V., unpublished), we developed a transgenic mouse line (*Six2TGC*) that expresses a GFP::Cre fusion protein controlled by the promoter of Six2, a transcriptional regulator present in a subpopulation of this mesenchyme. Recent studies indicate that Six2 acts to maintain these cells in a mesenchymal progenitor cell state (Self et al., 2006). Cell fate analysis with this line revealed that Six2GFP::Cre-positive cells give rise exclusively to renal vesicle derivatives in the mature kidney. Thus, the *Six2GFP::Cre* transgene enables specific modulation of gene activity directly within the renal vesicle progenitor cell compartment before normal induction.

To examine the role of canonical Wnt signaling in the initiation of nephrogenesis, we removed β-catenin activity from the renal vesicle progenitor component of the metanephric mesenchyme using the *Six2TGC* line in combination with a conditional loss-of-function (LOF) allele of β-catenin, *Catnb*^{c} (Brault et al., 2001; Harada et al., 1999) (*Catnb* is also known as *Ctnnb1* – Mouse Genome Informatics). β-catenin LOF mutants (*Catnb*$^{c/–}$*; Six2TGC*) reached term but died within 24 hours of birth. Dissection of newborn mutants revealed a greatly reduced kidney structure when compared with genetic control littermates that where phenotypically normal (Fig. 1A, compare to adrenal gland). In contrast to wild type, *Catnb*$^{c/–}$ and *Six2TGC* controls (Fig. 1B-D), β-catenin LOF mutants exhibited few mature nephron structures and entirely lacked an active nephrogenic zone (Fig. 1E). The presence of some epithelial structures with histologically identifiable renal tubule components (e.g. glomerulus, proximal convoluted tubule) indicated that some nephrogenesis did occur, although the number of nephrons was greatly reduced and their organization was abnormal. The absence of normal kidney architecture most likely accounts for the early postnatal lethality of mutants. Further histological examination of E13.5 mutant kidneys revealed that nephrogenesis was dramatically reduced at this early stage (Fig. 1F,G). In wild-type kidneys, nephrogenesis was active within the nephrogenic zone of the cortical region and several renal vesicles and S-shaped bodies could be observed ventral to the ureteric tips of the collecting duct (Fig. 1F). By contrast, β-catenin LOF mutants lacked a well-defined nephrogenic zone and few S-shaped bodies were apparent (Fig. 1G). As a secondary consequence of loss of the nephrogenic zone, ureteric branching was also greatly reduced (Fig. 2A,B). Together, these results demonstrate that β-catenin function is essential for normal nephrogenesis within the nephron-forming lineage of the mouse kidney.

We next assessed the molecular phenotype resulting from β-catenin LOF mutation. Several key genes have been described that demarcate the earliest molecular responses to inductive signaling within the metanephric mesenchyme and highlight the transition of

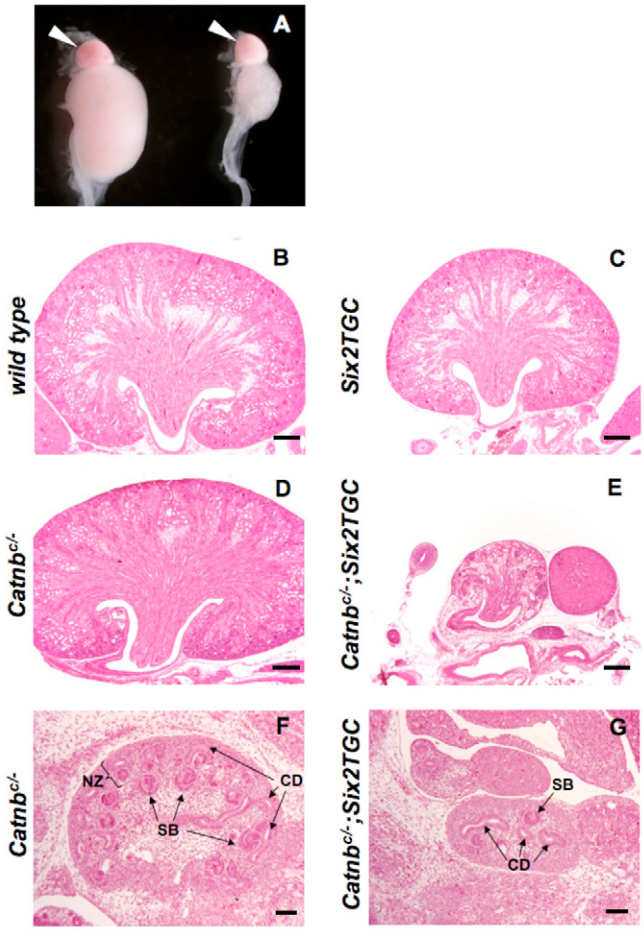

Fig. 1. Removal of β-catenin in renal vesicle progenitor cells causes reduced nephron formation in mice. (**A**) P1 kidneys from wild-type (left) and β-catenin LOF mutant (right). White arrowheads point to the adrenal glands. (**B-G**) Histology of kidneys from LOF mutants of β-catenin and proper controls at P1 (B-E) and at E13.5 (F,G). *Six2TGC* kidneys are slightly smaller than wild type. Scale bars: 0.5 mm in B-E, 0.1 mm in F,G. CD, collecting duct; NZ, nephrogenic zone; PA, pre-tubular aggregate; SB, S-shaped body.

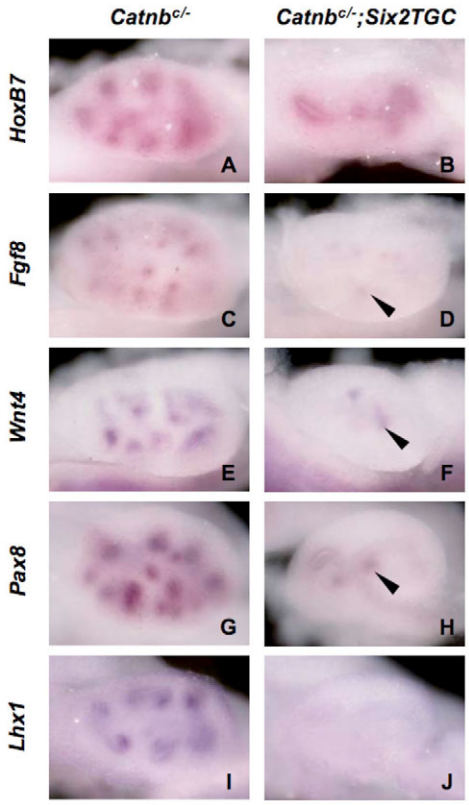

Fig. 2. Removal of β-catenin in renal vesicle progenitor cells in mice results in reduced tubulogenesis and branching.
(**A**,**B**) Whole-mount in situ hybridization of *HoxB7* (a marker for ureter) and (**C-J**) mesenchymal induction markers (*Fgf8*, *Wnt4*, *Pax8* and *Lhx1*) on E12.5 embryonic kidneys from control (A,C,E,G,I) and LOF mutants of β-catenin (B,D,F,H,J). Black arrowheads point to expression of mesenchymal induction markers in β-catenin LOF mutants.

responding cells to pre-tubular aggregates and renal vesicles. Furthermore, functional studies have defined roles for several of these in the tubulogenic program. *Fgf8*, *Pax8*, *Wnt4* and *Lhx1* are all expressed within the pre-tubular aggregate; each is also expressed at later stages in a subset of cells within tubular derivates of the renal vesicle. Genetic studies have placed *Fgf8* and *Pax8* upstream of *Wnt4* in metanephric mesenchyme induction, although continued expression of each depends on Wnt4 signaling. *Lhx1* lies genetically downstream of *Wnt4* (Kispert et al., 1998; Kobayashi et al., 2005; Stark et al., 1994). Loss of either *Fgf8* or *Wnt4* signaling results in a complete failure of the specification of early renal vesicles (Grieshammer et al., 2005; Stark et al., 1994). By contrast, *Lhx1* mutants establish a renal vesicle, but the early morphogenesis and patterning of these is defective (Kobayashi et al., 2005). Together, these markers enable a detailed molecular analysis of the initial steps of the tubulogenic program within induced mesenchyme before formation of overt epithelial structures.

By E12.5, the first pair of renal vesicles has formed and epithelialized in normal kidneys. At this stage, *Wnt4*, *Fgf8*, *Pax8* and *Lhx1* are all expressed in control littermates within pre-tubular aggregates and renal vesicles (Fig. 2C,E,G,I). By contrast, their expression was limited to a pair of centrally located structures in β-catenin LOF mutants (Fig. 2D,F,H,J). Thus only a very limited inductive response was observed. Loss of β-catenin within the nephron progenitor pool results in a rapid cessation of ureteric branching (Fig. 2A,B), a phenotype also observed in mutants that lack a primary Wnt9b inductive signal (Carroll et al., 2005). We also performed immunohistochemistry using anti-cleaved caspase 3 to address possible apoptosis of the nephron progenitor pool. We did not observe a marked increase in apoptosis in β-catenin LOF compared to controls (data not shown). This suggests the limited inductive response is not due to apoptosis of the nephron progenitors.

The formation of a reduced number of renal vesicles in the β-catenin LOF mutant suggests either that β-catenin is not strictly required for the initiation of tubulogenesis, or that the dynamics of Six2TGC-mediated recombination and/or β-catenin turnover does not remove all β-catenin activity before an initial inductive signal. To address this, we examined β-catenin by immunohistochemistry in E12.5 β-catenin LOF mutants. Consistent with the latter view, the renal vesicles and S-shaped bodies in LOF mutants retained β-catenin, suggesting that the earliest forming renal vesicles escaped Cre recombination in our experimental system (see Fig. S1 in the supplementary material). Importantly, no renal vesicles or mature nephron derivatives were observed in the mutants that lacked β-catenin at this later stage (see Fig. S1 in the supplementary material; data not shown). Collectively, these results are consistent with a cell-autonomous requirement for β-catenin activity within renal vesicle progenitors for the induction of a tubulogenic program.

Expression of a stabilized form of β-catenin in the cap mesenchyme initiates tubulogenesis

We further assessed the role of β-catenin by generating β-catenin gain-of-function (GOF) mutants (*Catnb*$^{ex3/+}$*; Six2TGC*), in which a conditional allele of stabilized β-catenin was expressed within the metanephric mesenchyme following Six2TGC-mediated excision of the third exon of β-catenin. This conditional recombination event generates a β-catenin protein lacking degradation target sequences (Harada et al., 1999); as a result, β-catenin accumulates and the canonical pathway is activated in a ligand-independent process (Harada et al., 1999).

β-catenin GOF mutants developed to term but died within 24 hours of birth, exhibiting marked renal agenesis (data not shown). At early stages (E12.5-13.5), β-catenin GOF mutants showed reduced epithelial branching (Fig. 3A-D) and an absence of epithelial stages of tubulogenesis, although GOF mutant kidneys were slightly larger than control genotypes (Fig. 3A,B). Strikingly, GOF mutants exhibited large clusters of highly condensed mesenchyme beneath the ureteric tips, where pre-tubular aggregates normally form, and variably sized clumps above the ureteric tips in the position, where Six2-positive metanephric mesenchyme is located (Fig. 3A,B).

To assess the progression of tubulogenesis, we examined *Wnt4*, *Fgf8*, *Pax8* and *Lhx1* expression in E12.5 β-catenin GOF mutants. Control littermates (*Catnb*$^{ex3/+}$) showed normal expression patterns, as expected for all markers tested (Fig. 3E,G,I,K). By contrast, β-catenin GOF mutants showed ectopic, disorganized expression of these early markers of the inductive response (Fig. 3F,H,J,L). Both the number and size of *Wnt4* and *Fgf8* expression domains were dramatically increased in β-catenin GOF mutants (Fig. 3F,H). Furthermore, ectopic expression of these markers was observed dorsal to the ureteric epithelium in GOF mutants, where uninduced mesenchymal progenitors normally reside (arrows, Fig. 3F,H, and data not shown). Expression of *Pax8* and *Lhx1* showed a generally similar pattern to that of *Fgf8* and *Wnt4*, although the expression levels of these genes were not markedly elevated (Fig. 3J,L). These data indicate that activation of β-catenin signaling in the cap

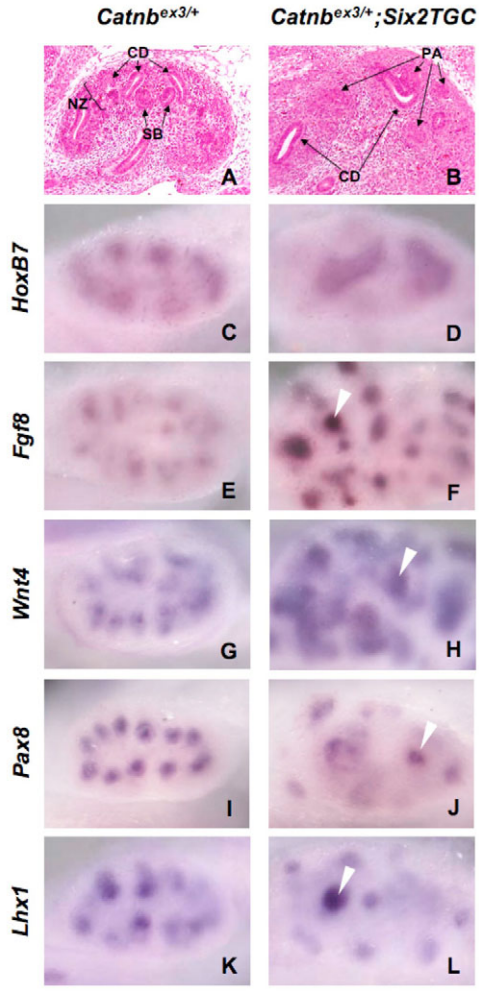

Fig. 3. Kidneys from GOF mutants of β-catenin show ectopic expression of early tubule markers and reduced branching in mice. (**A**,**B**) Histology of kidneys from wild type and GOF mutants of β-catenin at E13.5. Active nephrogenesis occurs in the cortex of wild-type kidneys (A), but neither S-shaped body nor nephron is formed in GOF mutants of β-catenin (B). (**C**,**D**) Whole-mount in situ hybridization of *HoxB7* and (**E-L**) the mesenchymal induction markers (*Fgf8*, *Wnt4*, *Pax8* and *Lhx1*) on E12.5 embryonic kidneys from wild type (C,E,G,I,K) and GOF mutants of β-catenin (D,F,H,J,L). White arrowheads indicate ectopic expression of mesenchymal induction markers dorsal to the ureteric epithelium. CD, collecting duct; NZ, nephrogenic zone; PA, pre-tubular aggregate; SB, S-shaped body.

mesenchyme population of nephron progenitors is sufficient to initiate an ectopic program of tubule induction. However, the failure to observe overt epithelial structures at later stages suggests that the mesenchymal-to-epithelial transition may be blocked in these cells.

Mesenchymal-to-epithelial transition of induced renal progenitors is blocked by continued β-catenin activity

To examine epithelial formation in β-catenin GOF mutants, we used an antibody against E-cadherin, a broad epithelial marker and a *Dolichos biflorus* lectin, DBA, that recognizes only the ureteric epithelial network (Dahl et al., 2002; Vize et al., 2003; Watanabe et al., 1981). In this, DBA-negative/E-cadherin-positive cells represent epithelial, metanephric mesenchyme derivatives (e.g. S-shaped body and mature tubules). To identify β-catenin GOF cells, a conditional reporter allele (R26R) (Soriano, 1999) was included in the crosses so that all cells undergoing Cre-mediated recombination also expressed β-galactosidase. In wild-type kidneys, DBA-negative/E-cadherin-positive cells were β-galactosidase-positive, as expected (Fig. 4G). In β-catenin GOF mutants, β-galactosidase-positive cells formed the large condensates (Fig. 4F). However, these β-galactosidase-positive cells were E-cadherin negative (Fig. 4H), indicating a failure of epithelial formation when stabilized β-catenin was continually present. Thus, whereas stabilization of β-catenin may be sufficient to initiate mesenchymal induction, a downregulation or loss of canonical Wnt activity may be necessary for cellular transition to epithelial structures.

Activation of β-catenin in metanephric mesenchyme is sufficient for initiating a tubulogenic program

The ectopic sites of induction following stabilization of β-catenin within the metanephric mesenchyme suggest that activation of β-catenin may be sufficient for this process. To address this issue, we isolated metanephric mesenchyme at E11.5 from genetic controls and from embryos that had undergone Six2TGC-mediated activation of β-catenin. We then compared the inductive response in vitro in these mesenchymal explants to that observed in intact kidney explants. As expected, all markers of mesenchymal induction were induced in intact explants (Fig. 5A-D) but were absent from isolated control metanephric mesenchyme explants following 24 hours of culture (Fig. 5E-H). By contrast, we observed a robust activation of all markers following β-catenin activation in isolated mesenchymal explants, independent of the normal inductive source, the ureteric epithelium (Fig. 5I-L).

Activation of early tubule markers by GOF β-catenin in cap mesenchyme does not require Wnt4 or Wnt9b

These explant studies raise the question of whether activation of β-catenin can functionally replace Wnt signals in the intact kidney in vivo. As discussed earlier (see Introduction), several studies point to the sequential action of Wnt signaling in metanephric mesenchyme induction, where *Wnt9b* activates *Wnt4*, *Fgf8* and *Pax8* in the pre-tubular aggregate, and *Wnt4* is subsequently necessary for maintaining the expression of these markers and for de novo induction of *Lhx1* (Kobayashi et al., 2005; Stark et al., 1994). To determine whether activated β-catenin could substitute for the inductive activity of these Wnts, we activated β-catenin signaling in *Wnt9b* null and *Wnt4* null mutant backgrounds (Figs 6 and 7, respectively).

As previously reported, *Wnt9b* is essential for all molecular readouts of the inductive response and for normal ureteric branching beyond the first bifurcation event that establishes the T-bud stage (Fig. 6A-H). However, activation of stabilized β-catenin in the cap mesenchyme of *Wnt9b* mutant at E12.5 was sufficient for mesenchymal induction, reflected by extensive ectopic expression of all these markers (Fig. 6I-P).

At E12.5, *Wnt4* mutants and control genotypes expressed *Pax8* and *Lhx1* weakly in the ureteric epithelium (Fig. 7C,D,G,H). However, in contrast to controls, neither marker was expressed in the mesenchyme of *Wnt4* mutants as expected (Fig. 7G,H). The small foci of *Fgf8* expression in *Wnt4* mutants (Fig. 7F) was consistent with evidence that initial *Fgf8* activation is independent of *Wnt4* and upstream of *Wnt4* itself (Grieshammer et al., 2005; Perantoni et al., 2005). When stabilized β-catenin was activated in metanephric mesenchyme of *Wnt4* mutants using *Six2TGC*, we observed ectopic

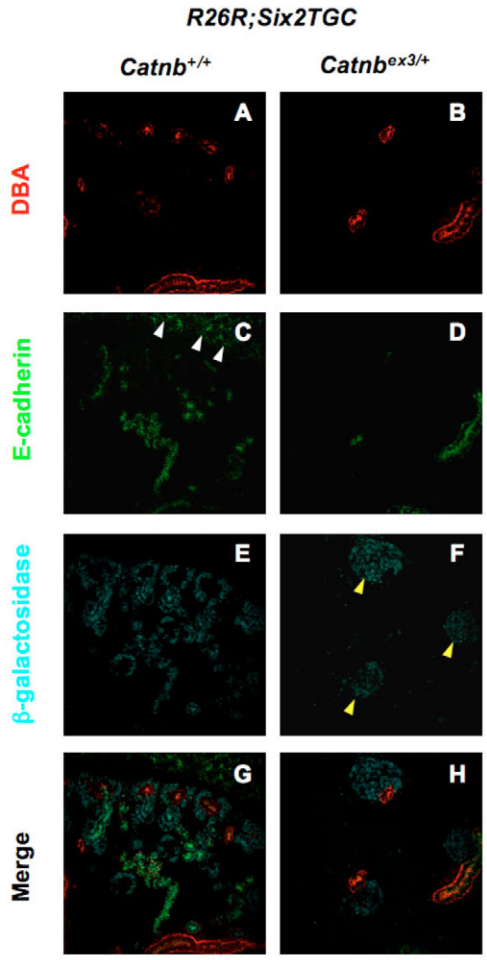

Fig. 4. Activated Wnt/β-catenin signaling in renal vesicle progenitor cells blocks the mesenchymal-to-epithelial transition in mice. (**A-H**) Analysis of epithelial formation by induced renal vesicle progenitors. (A,B) DBA-negative and (C,D) E-cadherin-positive cells represent epithelial tubules originated from renal vesicles. (E,F) β-galactosidase (cyan) labels *Six2TGC*-expressing cells and their descendants. In wild-type embryos (G), most cells in this population express β-galactosidase. However, in GOF mutants of β-catenin (H), E-cadherin expression does not overlap with β-galactosidase expression, indicating that Six2TGC-mediated activation of Wnt/β-catenin pathway blocks epithelialization. Yellow arrowheads indicate E-cadherin-negative clusters of cells that originate from the *Six2TGC*-expressing cell population. White arrowheads indicate epithelial structures in adrenal gland.

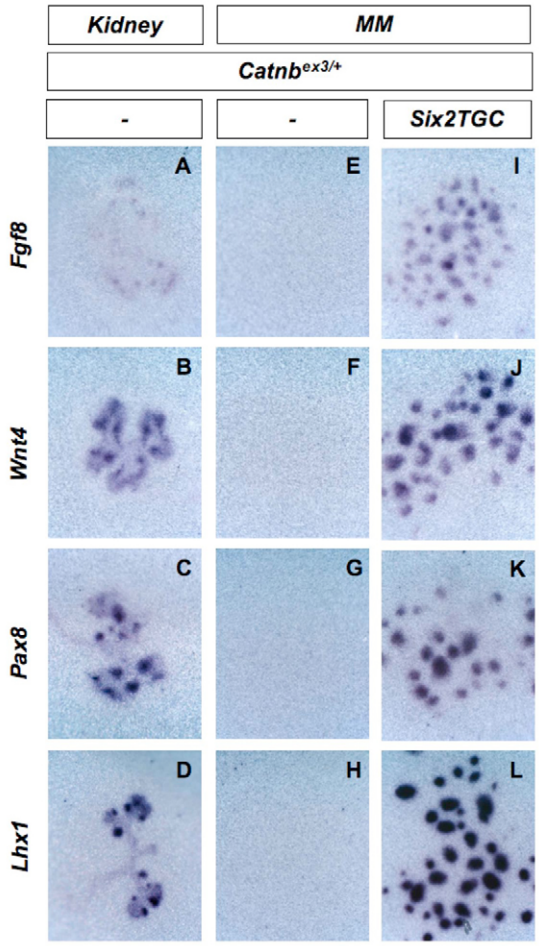

Fig. 5. Metanephric mesenchyme explants from GOF mutants of β-catenin express mesenchymal induction markers in mice. Metanephric mesenchyme explants dissected from E11.5 control (**E-H**) or β-catenin GOF mutants (**I-L**) were tested for expression of mesenchymal induction markers. Whole kidney explants from control embryos (**A-D**) were used as positive control. MM, metanephric mesenchyme.

expression of *Fgf8*, *Pax8* and de novo activation of *Lhx1* in the mesenchyme derivatives (Fig. 7N-P). Together, these results indicate that stabilization of β-catenin, the crucial regulatory event in transcriptional regulation of the canonical Wnt signaling pathway, functionally replaces the activities of *Wnt9b* and *Wnt4* in induction of metanephric mesenchyme.

DISCUSSION

Biochemical and genetic analyses of nephron induction in the mammalian kidney have highlighted the importance of several signaling pathways in initiating the transition of a mesenchymal progenitor population to an epithelial renal tubule precursor, the renal vesicle. Central to these are two Wnt signals, *Wnt9b* and *Wnt4*, which appear to act sequentially in the formation of the renal vesicle (Carroll et al., 2005; Kispert et al., 1998; Stark et al., 1994). Here we have used genetic approaches to directly manipulate β-catenin activity within these progenitors to address the potential role of canonical Wnt signaling in renal vesicle induction. Several lines of evidence point to a central role for the canonical Wnt pathway in these inductive events.

First, there is an absolute requirement for β-catenin activity within the metanephric mesenchyme to generate epithelial components of the renal vesicle and its derivatives. The loss of a normal inductive response suggests a crucial role for β-catenin in initiating the earlier aspect of the inductive response. Second, activation of β-catenin within the metanephric mesenchyme initiates this response, and moreover, functionally replaces the requirement for ureteric epithelial Wnt9b in vitro. Finally, activation of β-catenin in the metanephric mesenchyme functionally rescues the defective inductive responses in *Wnt9b* and *Wnt4* mutants in vivo. However, whereas molecular components of the early tubulogenic program are restored, the failure to form normal epithelial tubules when metanephric mesenchyme continues to produce activated β-catenin points to the need to attenuate canonical Wnt signaling to realize a normal epithelial transition.

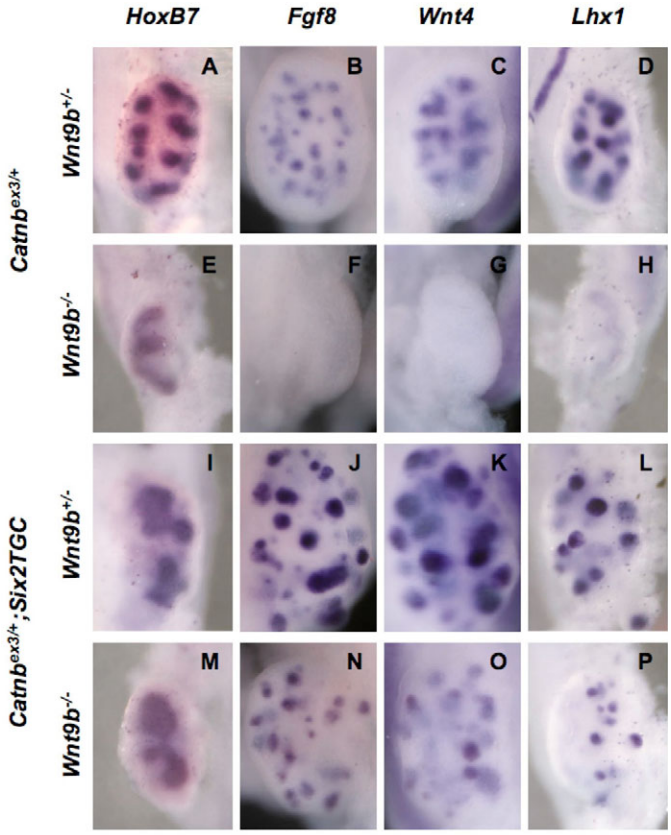

Fig. 6. Activated Wnt/β-catenin signaling in renal vesicle progenitor cells in mice is sufficient to induce mesenchymal induction markers in the absence of Wnt9b. (**A-P**) Whole-mount in situ hybridization at E12.5. (A,E,I,M) *HoxB7* and (B-D,F-H,J-L,N-P) the mesenchymal induction markers (*Fgf8*, *Wnt4* and *Lhx1*) on E12.5 embryonic kidneys. The mesenchymal induction markers are not detected in kidneys from *Wnt9b* mutants (F,G,H) and ectopically expressed when Wnt/β-catenin pathway is activated by Six2TGC (J,K,L). Six2TGC-mediated activation of Wnt/β-catenin pathway also causes ectopic expression of the mesenchymal induction markers in *Wnt9b* mutants (N,O,P).

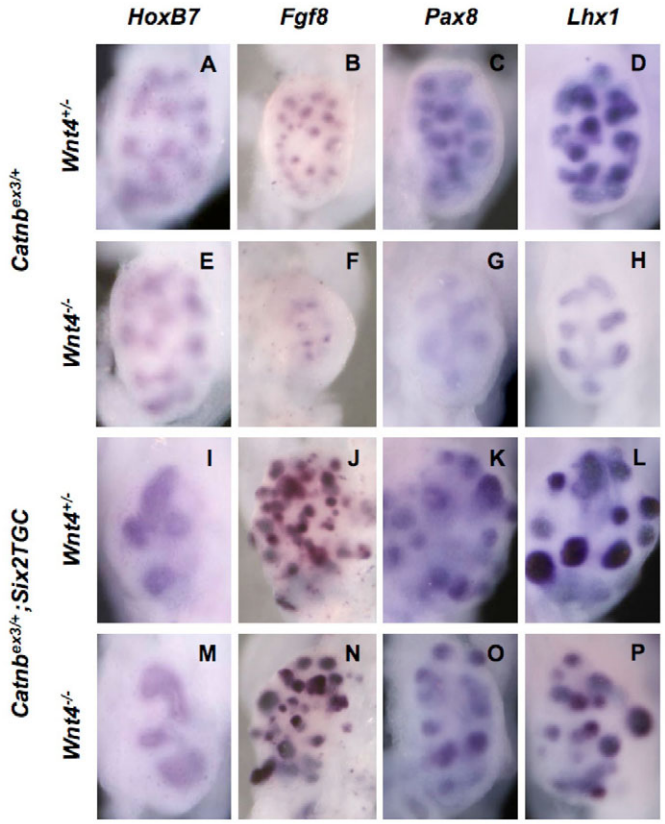

Fig. 7. Activated Wnt/β-catenin signaling in renal vesicle progenitor cells in mice is sufficient to induce mesenchymal induction markers in the absence of Wnt4. (**A-P**) Whole-mount in situ hybridization at E12.5. (A,E,I,M) Whole-mount in situ hybridization of *HoxB7* and (B-D,F-H,J-L,N-P) the mesenchymal induction markers (*Fgf8*, *Pax8* and *Lhx1*) to E12.5 embryonic kidneys. Although *Wnt4* mutant kidneys express *Fgf8* (F), they do not express *Pax8* and *Lhx1* in the mesenchyme (G,H). In *Wnt4* mutants, *Pax8* and *Lhx1* are expressed only in the collecting duct (G,H). Six2TGC-mediated activation of Wnt/β-catenin pathway causes ectopic expression of the mesenchymal induction markers in both *Wnt4* heterozygotes (J,K,L) and mutants (N,O,P).

Given the epistatic relationships between Wnt9b and Wnt4, distinguishing between a model wherein β-catenin mediates the inductive activity of both signals versus Wnt4 alone is not straightforward. However, several observations suggest that canonical Wnt action mediates Wnt9b and Wnt4 inductive responses. First, removal of β-catenin activity within the metanephric mesenchyme at E12.5 results in a severe branching deficiency (Fig. 1G, Fig. 2B) that is normally associated with loss of Wnt9b but not Wnt4 signaling. Thus, a Wnt9b-specific response within the metanephric mesenchyme, mediated through β-catenin, may be a component of the reciprocal interaction that coordinates ureteric epithelial branching with mesenchyme induction. *Fgf8* is completely absent in Wnt9b mutants and present, albeit at reduced levels, in *Wnt4* mutants. Further, Fgf8 signaling has been implicated in the regulation of epithelial branching in several contexts (Jaskoll et al., 2004; Shu et al., 2005). However, initial branching is not severely defective in *Fgf8* mutants (Grieshammer et al., 2005; Perantoni et al., 2005); thus the molecular mediators of this response remain to be determined.

Evidence that links β-catenin function most directly to Wnt4 signaling comes from the induction of *Lhx1* in *Wnt4* mutants following activation of stabilized β-catenin in the metanephric mesenchyme. Wnt4 is essential for *Lhx1* activation (Kobayashi et al., 2005); thus, the restoration of *Lhx1* expression indicates a direct role for β-catenin-mediated canonical Wnt signaling in the Wnt4 arm of the inductive response. Although our study suggests that both Wnt4 and Wnt9b act through β-catenin in their inductive roles, we cannot rule out the possibility that they may also activate non-canonical Wnt pathways in parallel.

While we observed ectopic induction dorsal to the ureteric bud in both Wnt4 and Wnt9b null mutants following Six2-mediated stabilization of β-catenin, the extent of the inductive response is reduced in the Wnt9b null mutant. This may reflect the more severe phenotype of the *Wnt9b* background, in which kidney development arrests after the first branching event (Carroll et al., 2005). By contrast, branching and expansion of Six2 progenitors continues for an additional 24 hours or more in the *Wnt4* mutant (Stark et al., 1994). Thus, the phenotypic differences most likely reflect differences in the number of Six2 progenitors that exist on each background.

Why do we fail to observe an epithelial transformation on activation of β-catenin despite the formation of aggregates and initiation of what appears to be a normal inductive response? The simplest explanation is that completion of a mesenchymal-to-

epithelial transition requires β-catenin signaling to be turned off. In previous studies in which lithium was used as a non-specific activator of canonical Wnt signaling, high doses blocked tubule formation (Davies and Garrod, 1995), consistent with our observation here. At present, our understanding of this crucial cellular event is rudimentary, so we are unable to directly address how canonical Wnt signaling may specifically inhibit epithelial formation. However, canonical Wnt signaling could interfere with other regulatory pathways. For example, several studies indicate that activation of canonical Wnt signaling can inhibit non-canonical Wnt responses (Veeman et al., 2003). Thus, if Wnt4 signaling bifurcates through a canonical (inductive) and a non-canonical (epithelialization) branch, activated β-catenin may block the latter.

The spatial dynamics of mesenchymal induction

Normal renal vesicle induction is first observed within pre-tubular aggregates beneath the tips of the ureteric bud. Recent evidence has highlighted the importance of *Six2* in regulating spatial induction of the metanephric mesenchyme (Self et al., 2006). *Six2*-expressing cells are restricted to the dorsal metanephric mesenchyme that caps the ureteric epithelium, and *Six2* is rapidly downregulated in induced pre-tubular aggregates. In the metanephric mesenchyme, Six2 acts to inhibit a tubulogenic program in renal vesicle progenitors. Consequently, renal vesicles are formed prematurely and ectopically on the dorsal side of the ureteric bud in *Six2* mutants (Self et al., 2006). As a result of the depletion of the progenitor population, kidney development ceases at an early stage. Our data here indicate that canonical Wnt signaling within the *Six2* population leads to a similar early inductive response to that observed in *Six2* mutants. Thus, within the renal vesicle progenitor pool, Six2 may act to inhibit canonical Wnt signaling, which is normally mediated through ureteric epithelial-derived Wnt9b. Conversely, downregulation of *Six2* is one of the earliest indicators of the induced mesenchyme. Thus, inhibition of *Six2* expression through canonical Wnt signaling may play a central role in modulating Six2 action. While the exact details of the molecular interactions remain to be determined, our data suggest a crucial regulation of canonical Wnt signaling within the tip/cap region of the kidney to ensure appropriate local activation of the inductive response and, importantly, the maintenance of a repopulating progenitor pool.

In summary, the studies herein further our understanding of the action of Wnt signaling in kidney development, demonstrating that the primary inductive activities of both Wnt9b and Wnt4 are likely to be mediated through canonical Wnt signaling. These findings have mechanistic implications for the maintenance of the progenitor cell compartment and for the mesenchymal-to-epithelial transition that is the cellular readout of these inductive processes.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/13/2533/DC1

Note added in proof

Kuure et al. have recently presented evidence lending further support to a linkage between beta-catenin stabilization within the metanephric mesenchyme and nephron induction (Kuure et al., 2007).

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