Canonical Wnt signaling regulates smooth muscle precursor development in the mouse ureter

Mark-Oliver Trowe1,*, Rannar Airik1,*, Anna-Carina Weiss1, Henner F. Farin1, Anna B. Foik1, Eva Bettenhausen1, Karin Schuster-Gossler1, Makoto Mark Taketo2 and Andreas Kispert1,‡

SUMMARY

Smooth muscle cells (SMCs) are a key component of many visceral organs, including the ureter, yet the molecular pathways that regulate their development from mesenchymal precursors are insufficiently understood. Here, we identified epithelial Wnt7b and Wnt9b as possible ligands of Fzd1-mediated β-catenin (Ctnnb1)-dependent (canonical) Wnt signaling in the adjacent undifferentiated ureteric mesenchyme. Mice with a conditional deletion of Ctnnb1 in the ureteric mesenchyme exhibited hydroureter and hydronephrosis at newborn stages due to functional obstruction of the ureter. Histological analysis revealed that the layer of undifferentiated mesenchymal cells directly adjacent to the ureteric epithelium did not undergo characteristic cell shape changes, exhibited reduced proliferation and failed to differentiate into SMCs. Molecular markers for prospective SMCs were lost, whereas markers of the outer layer of the ureteric mesenchyme fated to become adventitial fibroblasts were expanded to the inner layer. Conditional misexpression of a stabilized form of Ctnnb1 in the prospective ureteric mesenchyme resulted in the formation of a large domain of cells that exhibited histological and molecular features of prospective SMCs and differentiated along this lineage. Our analysis suggests that Wnt signals from the ureteric epithelium pattern the ureteric mesenchyme in a radial fashion by suppressing adventitial fibroblast differentiation and initiating smooth muscle precursor development in the innermost layer of mesenchymal cells.

KEY WORDS: Wnt, Ctnnb1, Ureter, Tbx18, Smooth muscle cell

INTRODUCTION

The mammalian ureter is a simple tube that mediates by unidirectional peristaltic contractions the efficient removal of urine from the renal pelvis to the bladder. The structural basis of the flexibility and contractile activity of this tubular organ is a two-layered tissue architecture of an outer mesenchymal wall composed of radially organized layers of fibroelastic material, contractile smooth muscle cells (SMCs) and adventitial fibroblasts, and an inner specialized highly expandable impermeable epithelial lining. Whether acquired or inherited, compromised drainage of the urine to the bladder by physical barriers or by functional impairment of the SMC layer results in fluid pressure-mediated dilation of the renal parenchyme (Chevalier et al., 2010; Rosen et al., 2008; Song and Yosypiv, 2011).

The three-layered mesenchymal coating of the mature ureter arises from a homogenized precursor tissue that is established in the metanephric field after formation of the ureter as an epithelial outgrowth of the Wolffian duct. In the mouse, this mesenchymal precursor pool remains undifferentiated from embryonic day (E) 11.5 to E15.5 and supports the elongation of the distal ureter stalk. From E15.5, i.e. shortly before onset of urine production in the developing kidney at E16.5, the mesenchyme in direct proximity to the ureteric epithelium differentiates in a proximal-to-distal wave into SMCs that will form layers with longitudinal and transverse orientations. Between the SMCs and the urothelium, a thin layer of stromal cells develops that contributes to elasticity of the ureteric tube. The outer mesenchymal cells remain more loosely organized and differentiate into adventitial fibroblasts (Airik and Kispert, 2007).

Despite its simple design and the relevance of congenital defects of the ureteric wall, only a small number of genes crucial for development of the ureteric mesenchyme have been characterized in recent years (Airik and Kispert, 2007; Uetani and Bouchard, 2009). Phenotypic analyses of mutant mice suggested that the T-box transcription factor gene 18 (Tbx18) specifies the ureteric mesenchyme (Airik et al., 2006); that Bmp4, a member of the family of secreted bone morphogenetic proteins, inhibits budding and branching morphogenesis of the distal ureteric epithelium, directs a ureteric fate and/or promotes SMC differentiation (Brenner-Anantharam et al., 2007; Dunn et al., 1997; Miyazaki et al., 2003); that the transcriptional regulators GATA binding protein 2 (Gata2), teashirt zinc finger family member 3 (Tshz3) and SRY-box containing gene 9 (Sox9) act as downstream mediators of Bmp4 function in the mesenchyme to activate expression of myocardin (Myocd), the key regulator of SMC differentiation (Airik et al., 2010; Caubit et al., 2008; Wang and Olson, 2004; Zhou et al., 1998); and that sonic hedgehog (Shh) signaling from the ureteric epithelium maintains Bmp4 in the mesenchyme and dose-dependently inhibits SMC fates (Yu et al., 2002).

The Wnt gene family encodes secreted growth and differentiation factors that have been implicated in numerous processes of vertebrate development and disease. Wnt proteins signal via at least three distinct pathways, of which only the canonical pathway has been implicated in transcriptional control of cell proliferation and differentiation. This pathway uniquely and critically involves the cytoplasmic protein β-catenin (Ctnnb1),...
which is stabilized upon binding of a Wnt ligand to a Frizzled (Fzd) receptor complex on the cell surface, and translocates to the nucleus to activate target gene transcription (Barker, 2008; MacDonald et al., 2009; Miller and McCrea, 2010).

Here, we study the functional involvement of Wnt signaling in the development of the (distal) ureter, particularly its mesenchymal component, in the mouse. We provide evidence for a crucial function of the Ctnnb1-dependent sub-branch of this pathway in the specification of the SMC lineage and radial patterning of the ureteric mesenchyme.

MATERIALS AND METHODS

Mouse strains and husbandry

For the production of a conditional misexpression allele of Tbx18, a knock-in strategy into the X-chromosomal hypoxianine guanine phosphoribosyl transferase (Hprt) gene locus was employed (Luche et al., 2007). Construction of the targeting vector, ES cell work and generation of chimeras followed exactly the procedure established for the generation of an Hprtlox allele (Airik et al., 2010). Beta-cateninlox (Ctnnb1<sup>fl/+</sup>, Ctnnb1<sup>fl/fl</sup> (Brault et al., 2001), beta-catenin<sup>cre/loxP</sup> (Ctnnb1f<sup>cre/fl</sup>, Ctnnb1f<sup>cre/fl</sup> (Harada et al., 1999), Hprt<sup>lox</sup>RE2<sup>fl</sup>/GFP (Muzumdar et al., 2007) and Tbx18<sup/+</sup>/Hprt<sup>lox</sup>RE2<sup>fl</sup>/GFP mice (Trowe et al., 2010) were maintained on an NMRI outbred background. Embryos for Wnt (pathway) gene expression analysis were derived from matings of NMRI wild-type mice. Tbx18<sup>18<sup>+/</sup></sup>;Ctnnb1<sup>fl/fl</sup> were obtained from matings of Tbx18<sup>18<sup>+/</sup></sup>;Ctnnb1<sup>fl/fl</sup> males and Ctnnb1<sup>fl/fl</sup> females. Tbx18<sup>18<sup>+/</sup></sup>;Ctnnb1<sup>fl/fl</sup> and Tbx18<sup>18<sup>+-</sup></sup>;Ctnnb1<sup>fl/fl</sup> littermates were interchangeably used as controls. Tbx18<sup>18<sup>+/</sup></sup>;Ctnnb1<sup>fl/fl</sup>;<R26<sup>T</sup>TmG/TmG> and Tbx18<sup>18<sup>+-</sup></sup>;Ctnnb1<sup>fl/fl</sup>;<R26<sup>T</sup>TmG/TmG> mice were obtained from matings of Tbx18<sup>18<sup>+/</sup></sup>;R26<sup>T</sup>TmG/TmG and Tbx18<sup>18<sup>+</sup></sup>;R26<sup>T</sup>TmG/TmG mice, respectively. For timed pregnancies, vaginal plugs were checked on the morning after mating and noon was taken as E0.5. Embryos and urogenital systems were dissected in PBS and fixed in 4% paraformaldehyde (PFA) in PBS and stored in methanol at −20°C. Genomic DNA prepared from yolk sacs or tail biopsies was used as E0.5. Embryos and urogenital systems were dissected in PBS and fixed in 4% PFA, paraffin embedded, and processed in Adobe Photoshop CS.

Organ cultures

Explant cultures of embryonic kidneys or urogenital systems were performed as previously described (Airik et al., 2010). The pharmacological Wnt pathway inhibitor IWR1 (Sigma, dissolved in DMSO) was used at final concentrations of 50 μM and 10 μM. Culture medium was replaced every 24 hours.

Morphological, histological and histochemical analyses

Ink injection experiments to visualize the ureteropelvic lumen were performed essentially as described (Moorman et al., 2001). For each marker, at least three independent specimens were analyzed.

Whole-mount in situ hybridization was performed using the two-tailed Student’s t-test. Data are expressed as mean ± s.d. Differences were considered significant when P<0.05. Apoptosis in tissues was assessed by TUNEL assay using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon) on 5-μm paraffin sections. All sections were counterstained with DAPI.

RESULTS

(Canonical) Wnt signaling in ureter development

To determine the involvement of Wnt signaling in ureter development, the expression of genes encoding Wnt ligands (Wnt1 to Wnt16) and Frizzled receptors (Fzd1 to Fzd10) was analyzed by in situ hybridization of whole ureters at E12.5 and E16.5, i.e. before and after cell differentiation has occurred in this tissue. This screen identified Wnt7b, Wnt9b and Fzd1 as Wnt components with specific expression in the ureter at these stages (Fig. 1). To better resolve the spatiotemporal expression profile of these genes, we performed an in situ hybridization analysis both in whole ureters and on transverse ureter sections from E11.5 to E18.5. Wnt7b and Wnt9b were co-expressed in the ureteric epithelium from E11.5 to E14.5. Expression of Wnt9b was downregulated after that stage, whereas mesenchymal expression remained detectable at subsequent stages, whereas mesenchymal expression was maintained and confined to the innermost cell layer at E12.5 and E14.5. Axin2 expression in this domain was markedly downregulated after the onset of SMC differentiation at E15.5 (Fig. 1G,H).
Together, this analysis suggests that Wnt7b and Wnt9b from the ureteric epithelium activate the canonical signal transduction pathway via Fzd1 in the adjacent undifferentiated mesenchyme.

**Conditional inactivation of Ctnnb1 in the ureteric mesenchyme results in hydroureter**

To investigate the role of canonical Wnt signaling in the ureteric mesenchyme, we employed a tissue-specific gene inactivation approach using a Tbx18cre line generated in our laboratory (Airik et al., 2010) and a floxed allele of Ctnnb1 (Ctnnb1fx), the unique intracellular mediator of this signaling pathway (Brault et al., 2001). We tested the efficiency of Tbx18cre-mediated recombination in the ureteric mesenchyme with the sensitive reporter line R26mTmG. In this line, recombination is visualized by bright membrane-bound GFP expression in a background of membrane-bound red fluorescence. Anti-GFP immunofluorescence analysis on sections provides additional cellular resolution of Cre-mediated recombination events (Muzumdar et al., 2007). In Tbx18<sup>cre</sup>∶R26<sup>mTmG</sup> mice, GFP activity was observed in a domain abutting the mesenchyme of the Wolffian duct and of the metanephric kidney at E11.5, as expected from the Tbx18 expression pattern (Airik et al., 2006). Analysis of ureter sections at E12.5, E14.5 and E18.5 revealed GFP expression throughout the entire mesenchymal compartment, confirming that Tbx18<sup>cre</sup> mediates recombination in precursors of all differentiated cell types of the ureteric mesenchyme, i.e. fibroblasts of the inner lamina propria and outer adventitia, and SMCs (supplementary material Fig. S1). The tissue-specific inactivation of the canonical Wnt signaling pathway in Tbx18<sup>cre</sup>∶Ctnnb1<sup>fx</sup> ureters was validated by the absence of Axin2 expression in the mesenchymal but not in the epithelial compartment at E11.5 and E12.5 (supplementary material Fig. S2).
At E18.5, urogenital systems of Tbx18<sup>cre/+;</sup>Ctnnb1<sup>fx/fx</sup> embryos displayed a prominent hydrourerter and hydronephrosis phenotype. These abnormalities were fully penetrant and occurred bilaterally in both sexes (Fig. 2A-D). In the female mutant, the uteruses appeared stretched, the translucent ovaries were attached more anteriorly to the kidneys, and the Wolffian duct had not regressed. In the male mutant, the testes and epididymides were tethered to the posterior pole of the kidneys. Adrenals were absent from the mutant urogenital systems of both sexes (Fig. 2B,D). Adrenogenital defects are compatible with a requirement of Ctnnb1 in female differentiation and adrenal development (Chassot et al., 2008; Kim et al., 2008). They most likely derive from Tbx18<sup>cre/+;</sup>Ctnnb1<sup>fx/fx</sup> embryos was not associated with morphological defects in the urogenital system, arguing against a dose-dependent requirement for Ctnnb1 and genetic interaction of Tbx18 and Ctnnb1 in ureter development (data not shown).

Histological analyses revealed dilation of the entire renal collecting system, including the collecting ducts, calyx and pelvis, and absence of the papilla in mutant kidneys (Fig. 2E,F). The ureter was strongly dilated and featured a flat and single-layered urothelium with a thin layer of surrounding mesenchyme (Fig. 2G,H).

Hydroureternephrosis can result from structural or functional deficits of the peristaltic machinery and from physical obstruction along the ureter and its junctions. To distinguish these possibilities, we analyzed the continuity of the ureteric lumen and the patency of the junctions by injecting ink into the renal pelvis. In all genotypes the ink readily passed into the bladder (Fig. 2I,J), excluding physical barrier formation as a cause of obstruction. To further analyze the nature of functional ureter impairment, we cultured E15.5 ureter explants for 4 days, monitoring daily for peristaltic activity and contraction patterns. Wild-type ureters elongated extensively in culture and initiated unidirectional peristaltic activity and contraction patterns. Wild-type ureters contracted and degenerated over time (Fig. 2K-L). To characterize the cellular changes that caused this behavior, we analyzed the expression of markers that indicate cell differentiation within the ureteric mesenchyme; the ureteric epithelium is outlined (dashed line).

Cytodifferentiation of the ureteric mesenchyme into SMCs and fibroblasts as shown by in situ hybridization analysis on transverse sections of the proximal ureter. (G,H) Arrows indicate the inner ring of mesenchymal cells; the ureteric epithelium is outlined (dashed line). adrenal gland; bl, bladder; k, kidney; p, pelvis; t, testis; u, ureter; ue, ureteric mesenchyme.

**Fig. 3. Early onset of kidney and ureter anomalies in Tbx18<sup>cre/+;</sup>Ctnnb1<sup>fx/fx</sup> mouse embryos.** (A) Morphology of whole urogenital systems. (B,C) Hematoxylin and Eosin stainings of sagittal sections of kidneys (B) and of transverse sections of proximal ureters (C). (D-H) Cytodifferentiation of the ureteric mesenchyme into SMCs and fibroblasts as shown by in situ hybridization analysis on transverse sections of the proximal ureter. (G,H) Arrows indicate the inner ring of mesenchymal cells; the ureteric epithelium is outlined (dashed line).

To define both the onset and progression of urogenital malformations in Tbx18<sup>cre/+;</sup>Ctnnb1<sup>fx/fx</sup> embryos, we analyzed urogenital systems from E12.5 to E16.5. On the morphological level, Tbx18<sup>cre/+;</sup>Ctnnb1<sup>fx/fx</sup> embryos were distinguished by the absence of the adrenals at E14.5. At E16.5, the failure of the testes to separate from the kidneys was apparent, and a mild dilation of the proximal ureter was observed in some mutant specimens (Fig. 3A). Histological analysis revealed the first hydronephrotic lesions (dilation of the pelvicalyceal space) in the mutant kidney at this stage, i.e. shortly after onset of urine production (Fig. 3B). In the Ctnnb1-deficient ureter, a mesenchymal compartment was established at E12.5 but all cells remained loosely organized with small cell bodies arranged in a tangential fashion at subsequent stages. This contrasted with the
situation in the wild-type ureteric mesenchyme, where loosely organized cells of typical fibroblast appearance with large protrusions were restricted to an outer layer, while cells adjacent to the epithelium were denser in appearance with large cell bodies from E12.5 onwards (Fig. 3C).

To examine whether these histological changes were accompanied or followed by changes in differentiation of the ureteric mesenchyme, we analyzed expression of the SM regulatory gene Myocd, the SM structural genes Tagln and troponin T2, cardiac (Tnnt2), and of markers of the adventitial fate collagen 1a2 (Col1a2) and fibulin 2 (Fbln2). In the wild type, Myocd was activated at E14.5 in the proximal region of the ureter (Fig. 3D), whereas Tagln and Tnnt2 were first expressed at E16.5. Col1a2 and Fbln2 expression was homogenous in the ureteric mesenchyme at E14.5, but was excluded from the inner mesenchymal layers comprising lamina propria fibroblasts and SMCs at E16.5. In the mutant ureter, Myocd and SM structural genes were never expressed. Col1a2 and Fbln2 expression, by contrast, was found throughout the ureteric mesenchyme (Fig. 3D-H).

In summary, morphological and histological analyses revealed the onset of ureter anomalies in Tbx18cre/+;Ctnnb1fx/fx embryos at E12.5, with a progression of phenotype severity during subsequent embryonic stages and onset of hydroureter at E16.5. This, together with the absence of SMC differentiation and expanded expression of adventitial fibroblast markers argues for a function of canonical Wnt signaling in ureteric mesenchymal patterning and/or in the initiation of the SM developmental program.

Defects in mesenchymal patterning precede SM differentiation defects

In order to analyze the molecular changes that caused the defective SMC differentiation in Tbx18cre/+;Ctnnb1fx/+ embryos, we screened for expression of a panel of genes that have been implicated in the early development of the ureteric mesenchyme and the initiation of the SM program by in situ hybridization analysis on ureter sections. In E12.5 wild-type ureters, Bmp4, Gata2, the target of Shh signaling patched 1 (Ptc1) (Ingham and McMahon, 2001), podocyte-expressed 1 (Pod1, also known as Tcf21), Tshz3, Tbx18, Sox9 and secreted frizzled-related protein 2 (Sfrp2) were expressed throughout the mesenchymal compartment with increased levels in cells adjacent to the epithelium. Expression of chemokine (C-X-C motif) ligand 12 (Cxcl12) and BMP-binding endothelial regulator (Bmpr) appeared uniformly high throughout the entire ureteric mesenchyme. In Tbx18cre/+;Ctnnb1fx/+ ureters, only the expression of Tbx18, Sox9 and Sfrp2 was altered, as these were no longer detected at this stage (Fig. 4A-J). The normal expression of Tbx18 in Tbx18cre/+;Ctnnb1fx/+ embryos excluded a gene-dosage effect as the cause for Tbx18 downregulation in the Ctnnb1 mutant ureter (supplementary material Fig. S4).

In E14.5 wild-type ureters, Bmp4, Gata2, Ptc1, Pod1 and Tshz3 were expressed in the inner layer of the mesenchymal cells from which SMCs will arise. Expression of Bmpr and Cxcl12 was restricted to the outer layer from which adventitial fibroblasts will differentiate. In the Ctnnb1-deficient ureter, mesenchymal expression of Bmp4 and Pod1 was absent, and Gata2 and Tshz3 were strongly reduced. Expression of Ptc1 was less affected, arguing that Shh signaling was still present to some degree (Fig. 4A-E). Expression of Bmpr and Cxcl12 was found throughout the mutant ureteric mesenchyme at this stage (Fig. 4F,G, arrows). To exclude the possibility that the Ctnnb1-deficient ureteric mesenchyme acquires the fate of an adjacent tissue, we additionally checked the expression of markers of the somitic mesoderm (Tcf13, also known as paraxis), the hindlimb mesenchyme (Tbx4), adrenogenital tissue (Nr5a1, also known as SF1), the cap mesenchyme of nephron progenitors (Osr1, Uncx4.1, Six2), and chondrocytes (Col2a1). None of these markers was ectopically activated in the ureteric mesenchyme of Tbx18cre/+;Ctnnb1fx/+ embryos (supplementary material Fig. S5). Together, this suggests that the initial specification of the ureteric mesenchyme occurs
Development 139 (17)

Fig. 5. Cellular defects in Ctnnb1-deficient ureteric mesenchyme. (A-C) Analysis of cell death by TUNEL assay (A) and of cell proliferation by BrdU incorporation assay (B,C) on transverse sections of mouse proximal ureter at E12.5 and E14.5. White dashed circles in A indicate the ureteric epithelium (ue); black dashed circles in B mark the ureteric epithelium and the inner and outer layers of ureteral mesenchymal cells (um) that were analyzed for proliferation. (C) Quantification of BrdU-positive cells. E12.5 (n=3), wild type versus mutant: ue, 0.217±0.113 versus 0.258±0.097, P=0.05; um (inner layer), 0.272±0.017 versus 0.202±0.017, P=0.008; um (outer layer), 0.172±0.009 versus 0.181±0.039, P=0.727. E14.5 (n=5), wild type versus mutant: ue, 0.204±0.024 versus 0.19±0.042, P=0.527; um (inner layer), 0.246±0.014 versus 0.211±0.022, P=0.034; um (outer layer), 0.196±0.021 versus 0.174±0.01, P=0.047. Error bars indicate s.d. *, P<0.05; **, P<0.01; two-tailed Student’s t-test. (D) Expression analysis of Cnd1 and Mycn by in situ hybridization on transverse sections of the proximal ureter at E12.5.

normally in Tbx18cre/+;Ctnnb1Icre/+ embryos but that during the subsequent differentiation step fibroblasts expand at the expense of (prospective) SMC fates.

As Tbx18-deficient ureters also exhibit a severe reduction of SMCs (Airik et al., 2006), we examined whether the early loss of Tbx18 expression contributed to the observed defects in Ctnnb1-deficient ureters. We tested this hypothesis by re-expressing Tbx18 in the Ctnnb1-deficient ureteric mesenchyme. We generated an HprtTbx18 allele by integrating a bicistronic transgene cassette containing the mouse Tbx18 ORF followed by IRES-GFP into the ubiquitously expressed X-chromosomal Hprt locus, similar to a previously published strategy for Sox9 (Airik et al., 2010) (supplementary material Fig. S6). To activate transgene expression, we used the Tbx18cre mouse line. Re-expression of Tbx18 in Tbx18cre/+;Ctnnb1Icre/+;HprtTbx18/+ embryos did not rescue hydroureter formation and loss of SMC differentiation at E18.5, nor did it reconstitute the expression of markers that were absent or reduced in Tbx18cre/+;Ctnnb1Icre/+ ureters at E14.5 (supplementary material Figs S7, S8). Since we found normal expression of Tbx18 in the ureteric mesenchyme of Tbx18cre/+;Ctnnb1Icre/+;HprtTbx18/+ embryos at E11.5 (supplementary material Fig. S9), we conclude that the loss of Tbx18 at E12.5 or after contributes only to a minor degree, if at all, to the observed molecular and histological changes in the Ctnnb1-deficient ureteric mesenchyme.

**Cellular changes in the ureteric mesenchyme of Tbx18cre/+;Ctnnb1Icre/+ mice**

The loss of expression of markers of the inner, and expansion of markers of the outer, mesenchymal cell layer in Ctnnb1-deficient ureters might reflect a role of canonical Wnt signaling in maintaining SMC precursors. The TUNEL assay did not detect apoptotic cells at E12.5 or E14.5 in the mutant ureteric mesenchyme (Fig. 5A) making it unlikely that Wnts simply act as survival factors for these cells. However, Wnt signaling contributed to some degree to the proliferation of the inner ring of Axin2-positive mesenchymal cells at E12.5 and E14.5, as detected by reduced BrdU incorporation in this domain of the Ctnnb1-deficient ureteric mesenchyme (Fig. 5B,C). Strong reduction of the cell-cycle regulator gene cyclin D1 (Cnd1) and slightly reduced expression of the pro-proliferative factor Mycn (Fig. 5D), which are regulated by canonical Wnt signaling in other contexts (Shutumman et al., 1999; ten Berge et al., 2008), might contribute to this finding.

**Canonical Wnt signaling is sufficient to induce SMC development in the ureteric mesenchyme**

Our loss-of-function analysis indicated an essential role of Ctnnb1-dependent Wnt signaling for SM development in the ureter, possibly by specifying an SMC precursor. To further test this hypothesis, we performed a complementary gain-of-function study with conditional (Tbx18cre-mediated) overexpression of a stabilized form of Ctnnb1 [Ctnnb1(ex3)Icre] in the ureteric mesenchyme in vivo (Fig. 6) (Harada et al., 1999). As shown above, Tbx18cre-mediated recombination is not restricted to the SMC lineage but occurs in the precursor pool of all mesenchymal cell types of the ureter (supplementary material Fig. S1), allowing ectopic activation of canonical Wnt signaling in prospective fibroblasts as well.

We validated this experimental strategy by comparative expression analysis of the lineage marker GFP and the target of canonical Wnt signaling Axin2 on adjacent sections. In control embryos (Tbx18cre+;R26tmG/+), GFP expression marked a band of mesenchymal cells between the metanephric mesenchyme and the Wolffian duct at E11.5, and labeled all mesenchymal cells surrounding the distal ureter stalk at E12.5. At both stages, Axin2 expression was barely detectable under the conditions used (we developed the color reaction for a shorter time than shown in Fig. 1) in the innermost layer of mesenchymal cells adjacent to the ureteric epithelium. In Tbx18cre+;R26tmG/+;Ctnnb1(ex3)Icre/+ embryos, Axin2 expression was strongly activated in almost all GFP-positive cells in the E11.5 and E12.5 ureter, confirming the premature and ectopic activation of canonical Wnt signaling at high levels in precursor cells for all differentiated cell types of the ureteric mesenchyme (Fig. 6A–H†). Histological analysis revealed that the enlarged GFP+ Axin2+ domain in E12.5 Tbx18cre+;R26tmG/+;Ctnnb1(ex3)Icre/+ ureters almost exclusively harbored densely packed large mesenchymal cells that were rhomboid in shape. In control embryos, this cell morphology was restricted to the innermost ring of the ureteric mesenchyme at E12.5 and E14.5, whereas the rest of the GFP+ ureteric mesenchyme featured cells that were slender and loosely packed.
as is typical for fibroblasts (Fig. 6I-J). The BrdU incorporation assay demonstrated that cell proliferation was actually decreased, correlating with the absence of Ccdn1 expression, while TUNEL staining detected increased levels of apoptosis in the GFP+ domain of the ureteric mesenchyme in Tbx18cre/+;R26mTmG/+;Ctnnb1(ex3)fx/+ embryos at E12.5 (Fig. 6K-N'). Together, these findings argue against a selective proliferative expansion of putative SM progenitors by enhanced Wnt signaling but point to a reprogramming of prospective fibroblasts toward an SMC fate.

We further tested this idea by analyzing the expression of a panel of marker genes associated with differentiation of SMCs and fibroblasts in the ureter. Bmp4, Gata2 and Tshz3, which are expressed throughout the entire ureteric mesenchyme of wild-type ureters at E12.5 and mark prospective SMCs of the inner ring in the wild type at E14.5, were expressed throughout the large GFP+ Axin2+ domain of the ureteric mesenchyme of Tbx18cre/+;R26mTmG/+;Ctnnb1(ex3)fx/+ embryos at E12.5. By contrast, Tbx18, Podl1 and Ptc1, which have similar expression patterns in the wild type, were severely downregulated in the mutants. Expression of Bmper and Cxcl12, which are restricted to the non-myogenic lineage of the ureteric mesenchyme in the wild type starting from E14.5, was clearly excluded from the GFP+ Axin2+ mesenchymal cells in Tbx18cre/+;Ctnnb1(ex3)fx/+ ureters. To our surprise, we detected weak expression of Myocd in the mutant, whereas no expression was found in the wild-type ureteric mesenchyme at this stage (Fig. 6O). These data indicate that stabilization of Ctnnb1/enhanced Wnt signaling prevents fibroblast differentiation and promotes premature SMC differentiation in the ureteric mesenchyme.

As Tbx18cre/+;Ctnnb1(ex3)fx/+ mice die at ~E12.5 due to cardiovascular lesions (Norden et al., 2011), we could not observe the long-term effect of our misexpression approach on cell differentiation in vivo. Therefore, we explanted whole urogenital systems at E12.5, cultured them for 4 days and examined the histology and expression of SM structural markers on adjacent transverse sections of the proximal ureter (Fig. 7). Analyses at later time points, which might have been more representative of the full differentiation potential of the ureteric mesenchyme, were not possible owing to subsequent degeneration of the mutant ureter by apoptosis (compare with Fig. 6N'). In control specimens, the E-cadherin (Cdh1)-positive ureteric epithelium was encircled by mesenchymal cells that expressed the lineage marker GFP but were Axin2- and Cxcl12-, whereas an dense region expressed the fibroblast marker Col1a2, whereas an
inner ring of highly condensed mesenchymal cells was positive for the SMC markers Acta2 and Tagln (Fig. 7C,G,I,K). In Tbx18<sup>cre<sup/>+;R26<sup>mTmG<sup>+</sup>;Ctnnb1<sup>lox3lox</sup>/+</sup> explant cultures, the ureteric epithelium appeared mildly hypoplastic, with cells showing enlarged nuclei. An accumulation of GFP-positive mesenchymal cells in proximity to the ureteric epithelium expressed high levels of Axin2, demonstrating continuous activity of the canonical Wnt signaling pathway in these cells (Fig. 7B,D). These GFP<sup>+</sup> Axin2<sup>+</sup> cells were not positive for the fibroblast marker Col1a2 but expressed Acta2 and Tagln, albeit at slightly reduced levels compared with the wild type (Fig. 7H,J,L). These data strongly suggest that forced activation of the Ctnnb1-dependent Wnt signaling pathway is sufficient to induce SMC differentiation in the ureteric mesenchyme.

**DISCUSSION**

**Specification of SMC precursors by epithelial Wnt signals in the ureteric mesenchyme**

The development of the distal ureter and its surrounding mesenchymal layer lacks the complex morphogenetic processes of the proximal part. Nonetheless, it is obvious that proliferation rates and differentiation waves require spatial and temporal coordination between the epithelium and the surrounding mesenchyme to achieve continuous elongation and to acquire functional integrity at the onset of urine production in the kidney. In fact, embryological experiments have shown that the urethelium is necessary for the survival of isolated ureteric mesenchyme and induction of SMCs (Cunha, 1976), and that the mesenchymal layer controls urothelial survival, proliferation and differentiation (Airik and Kispert, 2007). However, the molecular nature of the underlying signaling systems has remained largely obscure, with the exception of epithelial Shh signals that are required for patterning and proliferation of the adjacent SMC layer (Yu et al., 2002).

Our data suggest that epithelial signals of the Wnt family play a crucial role in SMC development in the ureteric mesenchyme. Our exhaustive in situ hybridization screen identified expression of Wnt7b and Wnt9b in the ureteric epithelium but failed to detect expression of Wnt family members in the adjacent mesenchyme. By contrast, we found expression of the Wnt receptor gene Fzd1 and of the target of canonical Wnt signaling Axin2 in the mesenchyme but only weakly and transiently (Axin2 at E11.5) in the epithelium, arguing that Wnt7b and Wnt9b act in a paracrine fashion on the mesenchymal tissue compartment to activate the canonical Wnt signaling pathway. Previous functional analyses of Wnt7b and Wnt9b have revealed their role as canonical Wnt ligands in the development of the renal medulla and as inducers of nephrogenesis but have not described ureter defects (Carroll et al., 2005; Yu et al., 2009), suggesting that Wnt7b and Wnt9b act redundantly in the ureteric epithelium. Axin2 expression was confined to a single layer of mesenchymal cells directly adjacent to the epithelial compartment, indicating that, in this context, epithelial Wnt signals are largely non-diffusible and do not act as morphogens, but act from cell to cell. To our knowledge, Axin2 is the earliest marker that molecularly distinguishes mesenchymal cells of an inner from that of an outer mesenchymal compartment. Shortly after the onset of Axin2 expression at E12.5, cells of the inner ring undergo characteristic cell shape changes that clearly distinguish them from outer mesenchymal cells. Although lineage tracing has not yet been performed to confirm this, it is very likely that SMCs arise exclusively from the (Axin2<sup>+</sup>) inner compartment, whereas the outer compartment gives rise to adventitial fibroblasts.

Our genetic experiments and supportive pharmacological inhibition studies showed that loss of Ctnnb1-dependent Wnt signaling results in absence of the SMC lineage. This phenotype is compatible with a number of functions for canonical Wnt signaling, including the aggregation, specification or survival of uncommitted precursor cells, proliferation and expansion of specified precursors and/or terminal differentiation of SMCs. Our analysis of Ctnnb1-deficient embryos has shown that the ureteric mesenchyme aggregated normally around the distal ureter stalk and exhibited reduced proliferation but survived throughout development. In turn, stabilization of Ctnnb1 did not lead to aggregation of cells but merely to shape changes of resident cells that had reduced proliferation and increased apoptosis, strongly arguing against a primary function of canonical Wnt signaling in the aggregation and survival of the ureteric mesenchyme. We suggest that proliferation of the ureteric mesenchyme, particularly increased proliferation of SMC precursors, is mediated both by Shh (Yu et al., 2002) and by epithelial Wnt signals. We deem it unlikely that canonical Wnts act late on as terminal differentiation signals for SMCs either, as Axin2 expression was activated early and dropped sharply after the onset of Myocd expression. In our opinion, our dataset is most compatible with a role of canonical Wnt signaling in initiating SMC development by specifying SMC precursors at the expense of the alternative fibroblast fate, for the following reasons. First, expression of Axin2 preceded the characteristic cell shape changes of the inner ureteric mesenchymal cell layer. Second, these cell shape changes were largely prevented when canonical Wnt signaling was absent, whereby SMC differentiation failed completely and the fibroblast layer was expanded instead. Third, expression of all markers of SMC precursors was completely lost by E14.5. Finally, canonical Wnt signaling was sufficient to induce the characteristic cell shape
changes and marker expression in unprogrammed precursor cells, which was followed by SMC development at the expense of fibroblast fates.

Mesenchymal cells expressing stabilized Ctnnb1, i.e. possessing enhanced Wnt signaling, expressed Myocd and SM structural genes at much lower levels than in the wild-type ureter. It is conceivable that additional signals emitted from the ureteric epithelium are required for increased proliferation and terminal differentiation of these cells. In fact, cells with ectopic Wnt signaling lacked the Shh signaling that has previously been shown to increase the proliferation of SMCs (Yu et al., 2002). Alternatively, or additionally, prolonged activation of canonical Wnt signaling might actually prevent terminal differentiation.

A requirement for Ctnnb1-dependent Wnt signaling in SMC development is not without precedence but the specific function and mode of action seem to vary in different tissues. Loss of Wnt7b from the pulmonary epithelium resulted in decreased mesenchymal differentiation and proliferation and, later in development, decreased vascular SMC integrity in the lung (Shu et al., 2002). Wnt4 was shown to be required in an autocrine fashion for SMC differentiation in the medullary stroma (Itäranta et al., 2006) and for SMC proliferation during intimal thickening in the vascular system (Tsaousi et al., 2011).

Canonical Wnt signaling is independent of Tbx18 but acts upstream of other factors required for SMC differentiation

To date, only a small number of factors have been identified as crucial for ureteric SMC development (Airik and Kispert, 2007): Tbx18 was implicated in the specification and cohesive aggregation of the ureteric mesenchyme (Airik et al., 2006), Bmp4 and Gata2 in control of ureter budding and SMC differentiation (Brenner-Anantharam et al., 2007; Dunn et al., 1997; Miyazaki et al., 2003; Zhou et al., 1998), Shh signaling in the proliferation and patterning of the ureteric mesenchyme (Yu et al., 2002) and Tshz3 and Sox9 in SMC differentiation (Airik et al., 2010; Caubit et al., 2008). Gata2 and Shh signaling are thought to regulate Bmp4, which in turn regulates Tshz3. Although only specifically shown for Sox9 and Tshz3, all of these genes are likely to act upstream of Myocd, the key regulator of SMC differentiation (Wang and Olson, 2004). Therefore, the loss of expression of all of these factors might collectively contribute to the loss of SMCs in the Ctnnb1-deficient mesenchyme. Downregulation of Tbx18 preceded that of the other genes relevant to SMC formation, suggesting a primary requirement of this transcription factor upstream of other molecular circuits. However, re-expression of Tbx18 in the Ctnnb1-deficient ureteric mesenchyme did not induce expression of any of these factors in this tissue, nor did it rescue SMC differentiation and hydroureter formation. Although this finding does not exclude the possibility that Tbx18 is relevant for SMC differentiation, it shows that Tbx18 is not sufficient to trigger the ureteric SMC differentiation program downstream of canonical Wnt signaling.

Early loss of expression of Tbx18, Sfrp2 and Sox9 at E12.5 and later loss of Bmp4, Tshz3, Gata2 and Pod1 in the Ctnnb1-deficient ureteric mesenchyme argue for differential regulation by Wnt signaling. In the first case, Ctnnb1-dependent Wnt signaling might be directly required to maintain the expression of Tbx18 [Sfrp2 and Sox9 depend on Tbx18 in turn (Airik et al., 2006)], whereas downregulation of the second set of genes might merely reflect a loss of specification of this cell type. The latter contention is supported by the concurrent expansion of markers for prospective fibroblasts. Interestingly, these markers are initially expressed uniformly as well, but become progressively restricted to the outer layer of prospective and definitive fibroblasts after E12.5. Hence, Wnt signaling may pattern the ureteric mesenchyme by inducing SMCs and/or by repressing a fibroblast fate.

Although all of these factors directly or indirectly depend on canonical Wnt signaling, they differentially respond to ectopic activation of this pathway in the prospective ureteric mesenchyme in Tbx18<sup>−/−</sup> / Ctnnb1<sup>lox/lox</sup> embryos: expression of Tbx18 and Pod1 is repressed, whereas Bmp4, Gata2 and Tshz3 are induced. We propose that this regulation reflects a differential cooperation of Wnt signaling with other signaling systems (e.g. Shh) in the ureteric mesenchyme.

It has recently been suggested that in the developing lung epithelial Wnt7b mediates, via the canonical pathway, the direct transcriptional activation of the extracellular matrix protein tenascin C (Tnc), which in turn is necessary and sufficient for expression of Pdgfra/b in the mesenchymal compartment (Cohen et al., 2009). We did not detect any changes in the expression of
these three genes in the Ctnnb1-deficient ureteric mesenchyme, arguing that the molecular pathways downstream of Wnt7b/Ctnnb1 differ in different developmental settings (supplementary material Fig. S10).

In summary, our analysis suggests that Wnt proteins from the ureteric epithelium act as paracrine signals to initiate SM precursor development in adjacent mesenchymal cells, and thus may pattern the mesenchyme in a radial fashion (Fig. 8). Our findings emphasize the importance of epithelial-mesenchymal signaling in ureter development. They will help to further dissect the molecular pathways that are important for SMC differentiation in the excretory system and to develop strategies for the directed formation of this important cell type for therapeutic purposes.

Acknowledgements
We thank Andrew P. McMahon, Brian Parr, Cath Mendelsohn, Charles J. Sherr, Christir Betholtz, Gregory Shackleford, Laurent Fasano, Liz Robertson, Luc Leysn, Matthew Scott, Richard Stump, Suzanne Cory and Yingzi Yang for probes; R. Adelstein for the rabbit SMMHC antiserum; Rolf Kemler for the anti-Cdh1 antibody and Ctnnb1 mice; and Achim Gossler and Aravind Shekar for technical help.

Funding
This work was supported by a grant from the Deutsche Forschungsgemeinschaft [DFG KI728/7 to A.K.].

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242dev.077388/DJC1

References

Development 139 (17)