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Tailbud-derived mesenchyme promotes urinary tract segmentation via BMP4 signaling

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Urinary tract morphogenesis requires the sub-division of the ureteric bud (UB) into the intra-renal collecting system and ureter, two tissues with unique structural and functional properties. In this report we investigate the cellular and molecular mechanisms that mediate their differentiation. Fate mapping experiments in the developing chick indicate that the UB is surrounded by two distinct mesenchymal populations: nephrogenic mesenchyme derived from the intermediate mesoderm and tailbud-derived mesoderm, which is selectively associated with the domain of the UB that differentiates into the ureter. Functional experiments utilizing murine metanephric kidney explants show that BMP4, a paracrine factor secreted by tailbud-derived mesenchyme, is required for ureter morphogenesis. Conversely, ectopic BMP4 signaling is sufficient to induce ureter morphogenesis in domains of the UB normally fated to differentiate into the intra-renal collecting system. Collectively, these results indicate that the border between the kidney and ureter forms where mesenchymal tissues originating in two different areas of the early embryo meet. These data raise the possibility that the susceptibility of this junction to congenital defects in humans, such as ureteral-pelvic obstructions, may be related to the complex morphogenetic movements that are required to integrate cells from these different lineages into a single functional structure.

KEY WORDS: Cell lineage, Tailbud, Urogenital system

INTRODUCTION

The urinary tract is composed of two distinct domains: the kidneys and a conduit system, which includes the ureters, bladder and urethra. The kidneys filter and modify blood in order to rid the body of excess fluids, solutes and metabolic wastes. These byproducts are then propelled through the ureters to the bladder for storage prior to excretion via the urethra. As would be expected based on their specialized functions, the renal tubules and the conduit system tissues exhibit vastly different structures. The renal tubules are comprised of a selectively permeable epithelial monolayer that is intimately associated with the vasculature to facilitate filtration and the exchange of solutes and ions between urinary space and systemic circulation. By contrast, the conduit system tissues are composed of an impermeable, multilayered or stratified epithelium invested with a well-developed smooth muscle coat that provides the peristaltic movements required for urine excretion.

The junction between the kidneys and the conduit system is a common site for congenital defects in humans, including obstructions that compromise renal function (Becker and Baum, 2006). This border lies between the intra-renal collecting tubule system and ureter, both of which derive from an epithelial primordium called the ureteric bud (UB). Cells at the proximal UB tip are induced to form the intra-renal collecting system via their interactions with the mesenchymal progenitors of the nephrons (Fig. 1), which secrete factors such as glial cell-derived neurotrophic factor (GDNF) (for a review, see Costantini and Shakya, 2006). The distal or tubular domain of the UB differentiates into the ureter and is surrounded by a mesenchymal cell population that can be distinguished from nephron progenitors by mRNA expression patterns. Several genes, including the transcription factor *Tbx18*, which are selectively expressed by this mesenchyme, are required for ureter morphogenesis (Airik et al., 2006; Miyazaki et al., 2000; Oshima et al., 2001). Thus, the different mesenchymal populations surrounding the proximal and distal UB are essential for collecting system and ureter morphogenesis, respectively.

The developmental process that establishes these distinct mesenchymal tissues has yet to be determined and can occur by at least two different mechanisms (Fig. 1C,D). The distal and proximal domains of the UB may be inherently different and direct a homogeneous population of intermediate mesoderm to either maintain a nephrogenic fate or differentiate into the unique population of mesenchyme surrounding the distal UB (Fig. 1C). Alternatively, the entire UB epithelium may be a homogeneous population of cells competent to differentiate into either collecting tubule or the ureter epithelium (Fig. 1D). In this model, distinct mesenchymal populations surrounding the proximal and distal UB are specified at a stage in development prior to their association with the UB; cell migration or tissue movements are required to establish the distinct mesenchymal populations around the UB. Discrimination between these two models is dependent on determining the origin of the mesenchymal cell populations surrounding the UB.

In this report we use fate mapping techniques to show that the tailbud, a population of undifferentiated cells that is located at the most caudal aspect of the embryo after the completion of gastrulation, contributes cells to the developing urinary tract. Specifically, our data show that tailbud-derived mesenchyme associates with the distal UB but not its proximal domains, which form the intra-renal collecting system. We found that tailbud-derived mesenchyme, including those derivatives that invest the distal UB or nascent ureter, expresses abundant levels of *Bmp4* mRNA and our

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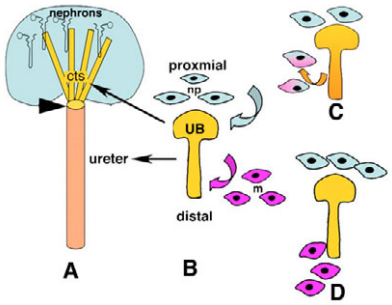

Fig. 1. The renal collecting system and ureter derive from the ureteric bud. (**A**) Schematic of mature urinary tract with arrowhead marking the junction between the kidney and conduit system. (**B**) The proximal ureteric bud (UB) gives rise to the collecting tubules, whereas the distal UB gives rise to the ureters. (**C**,**D**) Two models describing the process of UB sub-division into the ureters and renal collecting system. Cts, collecting tubules; np, nephron progenitor; m, periureteral mesenchyme.

functional experiments show that the BMP4 secreted by this mesenchyme is required for ureter differentiation. Furthermore, our data demonstrate that ectopic BMP4 signaling can induce ureter differentiation in domains of the UB normally fated to form the intra-renal collecting system. Thus, cells located at the proximal UB tips are multipotent and competent to differentiate into either ureter or collecting tubule epithelia.

Collectively, these results indicate that the ureter epithelial fate is controlled by the association of UB epithelia with tailbud-derived mesenchyme. These findings suggest that the complex morphogenetic processes required to bring tailbud-derived mesenchyme into close contact with the developing urinary tract may contribute to the high incidence of human congenital defects localized to the border between the kidneys and the ureters.

MATERIALS AND METHODS

Animals

Embryonated chick eggs were purchased from CBT Farms (Chestertown, MD), incubated at 38°C for the given times and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). All mice were housed in the Weill Medical College of Cornell University Animal Facility and treated according to the Research Animal Resource Center guidelines. Timed pregnant Swiss Webster female mice were purchased from Taconic Farms (Germantown, NY). Mice heterozygous for a *Bmp4*^{lacZ} reporter allele (Lawson et al., 1999), and mice homozygous for the *Bmp4* conditional allele created by the introduction of Cre recombinase (*loxP*) sites into the BMP4 locus (Kulessa and Hogan, 2002), were provided by Brigid Hogan (Duke University, Durham, NC). The *lacZ* transgene was detected by using the following primer pair: forward, 5′-ACCCAACTTAATCGCCTTGC-3′; reverse, 5′-AACAAACGGCGGCGGATTGACC-3′. The following primers were used to genotype animals carrying the *loxP* sites in the *Bmp4* allele: #79, 5′-AGACTCTTTAGTGAGCATTTTCAAC-3′; #80, 5′-AGCCCAATTTCCACAACTTC-3′; #24, 5′-AGGTGAGCAGAGCT-AAGATG-3′. The *Bmp4*$^{flox/+}$ versus *Bmp4*$^{flox/flox}$ genotype was detected using primer pair #79 and #80. The #79 and #24 primer pair was used to detect the occurrence of a recombination event. *Bmp4*$^{flox/flox}$ females were mated with *Cre/Esr1* (estrogen receptor 1) heterozygous male mice (strain name: B6.Cg-Tg(Cre/Esr1)5Amc/J; The Jackson Laboratory, Bar Harbor, ME) (Hayashi and McMahon, 2002). To check for the presence of the *Cre* recombinase transgene the following primer pair was used: forward, 5′-GTGAAACAGCATTGCTGTCACTT-3′; reverse, 5′-GCGGTCTG-GCAGTAAAAACTATC-3′. *Bmp4*$^{flox/+}$ male offspring carrying the *Cre* transgene were selected and mated with *Bmp4*$^{flox/flox}$ females to generate *Bmp4*$^{flox/flox}$*;Cre/Esr1* embryos. Timed pregnant dams and their subsequent litters were normal and did not appear to be affected by the presence of the *Cre* transgene. *Bmp4*$^{flox/flox}$ embryos carrying the *Cre* transgene died in utero approximately 24 hours after timed pregnant dams were injected with 5 mg tamoxifen (Sigma, St Louis, MO) (Hayashi and McMahon, 2002).

Lineage analyses

Chick embryos at given stages were injected in ovo with ≈1 nl of either CM-DiI (Molecular Probes) or 0.5×10^{7}-10^{8} ml^{-1} virions of concentrated SNTZ retrovirus (Hyer and Mikawa, 1997; Obara-Ishihara et al., 1999). Retroviral stocks were routinely assayed for replication competence as described (Obara-Ishihara et al., 1999). Eggs were resealed and incubated for given times before fixation with 4% paraformaldehyde. Transverse vibratome sections or whole mounts of DiI-injected embryos were examined using epifluorescence optics. SNTZ-injected embryos were processed for β-galactosidase activity (Lobe et al., 1999), examined as whole mounts, and then embedded in paraffin. Serial paraffin sections were prepared and then stained with Hematoxylin and Eosin. Unless otherwise stated, all fate mapping data are representative of a minimum of four embryos analyzed for each experimental condition described.

Metanephric explant cultures

Kidney rudiments were isolated from wild-type mouse embryos at the gestational stages noted; the day of vaginal plug was designated as E0.5. Rudiments were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomyocin on polycarbonate membrane filters (0.4 μm pore size, Transwell®; Corning, Wilkes Barre, PA). Recombinant BMP4, BMP7, Noggin and Gremlin were purchased from R&D Systems (Minneapolis, MN) and added to the culture medium daily. Timed pregnant *Bmp4*$^{flox/flox}$ females that were mated with *Bmp4*$^{flox/+}$*;Cre-Esr1* males were injected with tamoxifen at E12 and sacrificed 6 hours later. Kidney rudiments were isolated from embryos and cultured with 50 pM 4-hydroxytamoxifen (Sigma) to ensure complete *Bmp4* excision. The heads of isolated embryos were used for genotyping.

In situ hybridization detection of mRNA expression

Embryos were fixed with 4% paraformaldehyde and processed for whole-mount in situ hybridization according to standard protocols (Henrique et al., 1995). Briefly, samples were treated with 10 μg/ml of proteinase K for 30 minutes at 25°C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Reactions were developed with NBT/BCIP (Roche, Indianapolis, IN). *Bmp4* plasmids were kindly provided by Brigid Hogan's laboratory. Detection of *Bmp4* and *Pax2* mRNAs in the same sample was performed according to the double in situ hybridization techniques of Hurtado and Mikawa (Hurtado and Mikawa, 2006).

Immunofluorescent protein detection

Frozen sections, prepared according to standard protocol (Stern, 1993), or whole explants were blocked in 1% normal donkey serum (NDS)/PBS for 1 hour and primary antibodies diluted to appropriate concentrations in the same solution. Primary antibodies were incubated with samples for up to 3 hours at 37°C and secondary antibodies at described dilutions were applied after samples were thoroughly washed. Samples were examined with a Hamamatsu C4742-95 digital camera using a Metamorph Image capturing system (Universal Imaging of Molecular Devices, Downingtown, PA). Primary and secondary antibodies and their dilutions were as follows: uroplakin (rabbit anti-serum against total bovine uroplakins, a gift from the Tung-Tien Sun laboratory, New York University, 1:3000 dilution; secondary is donkey anti-rabbit, Molecular Probes, Carlsbad, CA, 1:200 dilution); smooth muscle actin (SMA) (Cy3-conjugated mouse monoclonal, Sigma, 1:400 dilution); E-cadherin (R&D Systems, 1:200 dilution; secondary is donkey anti-goat, Molecular Probes, 1:200); DAPI (Sigma, 1:1000 dilution).

RESULTS

Mesenchyme surrounding the proximal and distal UB derive from different sources

The early chick embryo was used to analyze the lineage of mesenchyme surrounding the UB because it is easily accessible to direct fate mapping techniques. We first demonstrated that the avian

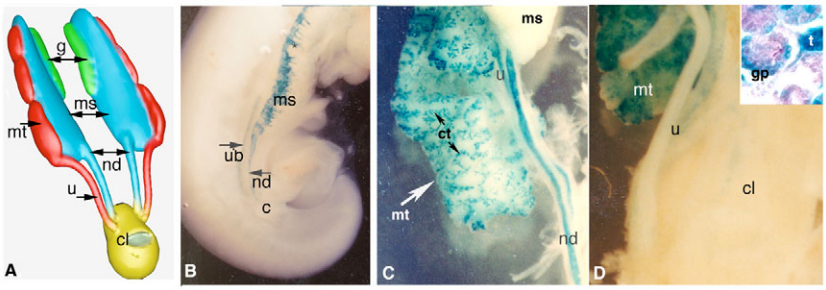

Fig. 2. Intermediate mesoderm gives rise to the collecting system and nephron epithelia. (**A**) Model of the developing avian urinary tract showing the position of the gonads (g), mesonephros (ms), nephric duct (nd), metanephros (mt), ureter (u) and cloaca (c). (**B**,**C**) The ureter and intra-renal collecting system derive from rostral intermediate mesoderm. Images of representative chick embryos with replication-defective retrovirus encoding *lacZ* injected into the intermediate mesoderm between the axial levels of somites 6-10. Injection performed at HH st10. After 6 days (B), blue *lacZ*-expressing lineage-tagged cells are present in short tubules (*) extending into the mesonephros (ms), the nephric duct (nd) and the ureteric bud (ub), which has yet to undergo branching morphogenesis. Lineage-tagged cells are not present in the cloaca (c), the tissue that gives rise to the terminal portions of the conduit system. After 14 days (C) β-gal-positive, lineage-tagged cells are present in the nephric duct (nd), the ureter (u) and the branched, metanephric (mt) intra-renal collecting system (ct). (**D**) Metanephric nephron epithelia derive from caudal intermediate mesoderm as determined by *lacZ* transfer into this tissue at HH st14. Examination of representative urinary tract 14 days after *lacZ* transfer caudal to somite 27 demonstrates that large populations of lineage-tagged cells are present in the metanephros (mt). Few, if any, tagged cells were present around the ureter (u) and cloaca (c). Paraffin sections of the metanephros (D, inset) demonstrate that lineage-tagged cells exhibit the morphological properties of podocytes (gp), the specialized nephron epithelia surrounding the glomerular capillary tuft, and epithelia of the tubular nephron segments (t).

ureter and intra-renal collecting system both derive from the UB. SNTZ, a replication-defective retrovirus derived from spleen necrosis virus, was used to transfer the *lacZ* gene into cells for lineage tracing (Hyer and Mikawa, 1997). Replication-defective viral stocks were injected into the intermediate mesoderm of Hamburger-Hamilton (HH) st10 embryos at the axial level of somites 6-10 (Fig. 2A-C); we have previously shown that this domain of intermediate mesoderm gives rise to the nephric duct. After 24 hours of incubation, the only lineage-tagged, β-galactosdiase-positive cells detected in the caudal aspect of the embryo are localized to the nephric duct (data not shown) (Obara-Ishihara et al., 1999). After 6 days of incubation, lineage-tagged cells were detected within the nephric duct, short tubules extending into the mesonephros, and the UB (Fig. 2B). Epithelial cells of the cloaca, which gives rise to the terminal portion of the conduit system, were unlabeled (Fig. 2B). After 14 days of incubation, lineage-tagged cells were localized to the nephric duct, ureter and intra-renal collecting tubules. These data demonstrate that the intra-renal collecting tubule system and the ureters of the avian embryo, as in the mouse, derive from the UB. By contrast, the epithelia of the cloaca derive from another tissue present in the early chick embryo (Fig. 2C).

We next determined whether the mesenchymal cells surrounding the proximal and distal UB derive from a common embryonic tissue. Classical embryological studies indicate that nephron progenitors surrounding the proximal UB derive from the intermediate mesoderm (Saxen, 1987). Therefore, we transferred *lacZ* into this tissue to test whether it gives rise to both mesenchymal nephron progenitors surrounding the proximal UB as well as the mesenchyme surrounding the distal UB. Intermediate mesoderm from the axial levels of somites 25-27 gave rise to mesonephric nephrons (n=6; data not shown). Metanephric nephron progenitors were efficiently labeled when SNTZ was injected into the unsegmented mesoderm caudal to somite 28, approximating the position of the intermediate mesoderm (n=3). Targeted *lacZ* transfer into this site resulted in the presence of large populations of lineage-tagged, β-galactosidase-positive nephron epithelia at later stages of development (Fig. 2D). However, lineage-tagged cells were not observed in tissues surrounding the ureter or cloaca in any of the embryos analyzed. These data suggest that the proximal and distal UB are associated with mesenchyme derived from different embryonic sources.

To further test this hypothesis, we used two independent fate mapping techniques to determine the origin of the mesenchymal cells surrounding the distal UB or nascent ureter. DiI, a carbocyanine dye, was injected into HH st14-16 embryos in ovo (Fig. 3A-F). Dye injections were targeted to focal domains of mesoderm from the axial level of somite 23 through to the axial level of somite 27, and unsegmented mesoderm rostral to the posterior neuropore. In addition, DiI was injected into the medial aspect of the embryo caudal to the closed neuorpore, which can be defined as the tailbud (Catala et al., 1995; Krenn et al., 1990; Wilson and Beddington, 1996). Embryos were analyzed 2-4 days post-injection and only the six embryos that were injected with DiI into the tailbud exhibited large populations of tagged cells immediately adjacent to the cloaca and the distal domain of the UB or the nascent ureter (Fig. 3E,F). Most importantly, DiI-tagged cells derived from the tailbud were not present around the proximal domain of the UB (Fig. 3D). Instead, the proximal UB tips or nascent collecting tubules were surrounded by unlabeled *Pax2*-expressing nephrogenic mesenchyme, which derives from the intermediate mesoderm (Fig. 3D) (Dressler et al., 1990). We also transferred *lacZ* into tailbud mesenchyme to mark cells derived from this compartment in a heritable fashion as DiI can be diluted by successive rounds of cell division (Fig. 3G-I). These fate mapping experiments, which were then analyzed at later stages of development, confirm and extend DiI fate mapping results. Cells deriving from the tailbud differentiate into sacral connective tissues, including muscle, and are present in the connective tissue surrounding the cloaca and the distal-most domain of the ureter (Fig. 3G-I). Tailbud-derived cells were not observed in the metanephros of any of the seven embryos analyzed (data not shown). Collectively, these data raise the possibility that signals secreted by tailbud-derived mesenchyme surrounding the distal UB may play a role in controlling ureter morphogenesis.

Bmp4 is expressed in the tailbud and the tailbud-derived mesenchyme surrounding the cloaca and distal UB

We used a candidate gene approach to test whether tailbud mesoderm expresses genes required for ureter morphogenesis. In both the developing chick and mouse embryo, several genes, including *Fgf8*, *Tbx6*, *Wnt5a* and *Bmp4*, are abundantly expressed in the tailbud (Gofflot et al., 1997; Knezevic et al., 1998). The spatial and temporal expression patterns of one of these genes, *Bmp4*, parallels the movement of tailbud-derived mesenchyme to a position

surrounding the epithelial tissues that give rise to the conduit system. As can be seen in Fig. 4, *Bmp4* is abundantly expressed in the avian tailbud at HH st14 (Fig. 4A). Approximately 2 days later, abundant *Bmp4* expression is detected around the caudal-most aspect of the developing urinary tract, including the cloaca and distal UB. By contrast, the proximal UB is surrounded by *Pax2*-expressing nephron progenitors (Fig. 4B-D).

Similarly, in the developing mouse *Bmp4* is abundantly expressed in the tailbud (Gofflot et al., 1997) and later, from E11.5-E13.5 by mesenchyme surrounding the distal UB and urogenital sinus (Fig. 4E,F). Thus, the spatial and temporal expression patterns of *Bmp4* mRNA are consistent with the movement of tailbud-derived cells to the developing conduit system and a possible role in ureter morphogenesis. Moreover, by E15.5, overt ureter differentiation, as determined by the upregulated expression of uroplakins in the UB epithelium and presence of an organized, SMA-positive connective tissue coat, occurs only in the domain of the UB invested with *Bmp4*-expressing mesenchyme (Fig. 5A-C). Uroplakins are not expressed by collecting tubule epithelia and although SMA-positive cells are interspersed between collecting tubules in the medullary

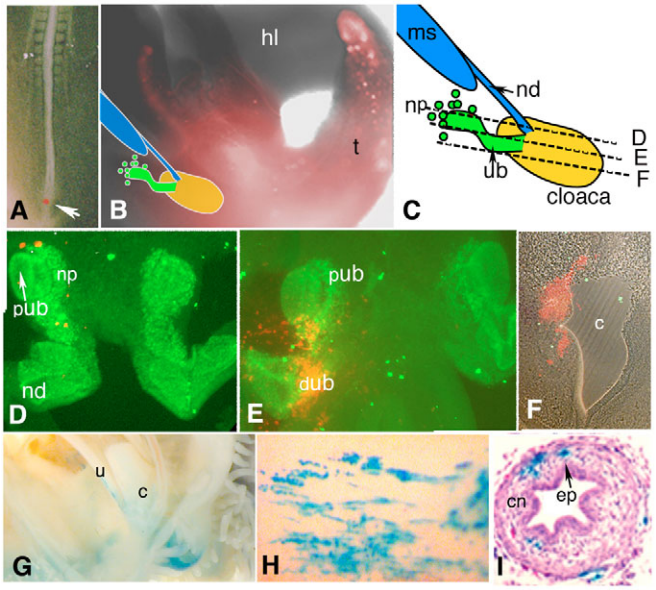

Fig. 3. Tailbud-derived mesenchyme associates with the developing conduit system. (**A**) Whole-mount image of HH st14 embryo immediately after DiI injection into the tailbud (arrowhead). (**B**) The same embryo after 48 hours of incubation, including a schematic of the urinary tract. Cells deriving from the tailbud (t) are localized to the sacral aspect of the embryo and the tail. Few, if any, lineage-tagged cells were observed rostral to the hindlimb (hl). (**C**) Details of the schematic. At this stage, the mesonephros (ms) has formed and the nephric duct (nd) is integrated into the cloaca. The UB has not yet branched and its proximal tip is surrounded by nephron progenitors (np). Locations of transverse sections in D-F are noted. (**D**,**E**) Tailbud-derived mesenchyme associates with the distal UB as revealed by staining for Pax2 (green). The proximal UB tip (pub in D) can be identified by Pax2 expression (green), its epithelial structure and its association with Pax2-expressing nephron progenitors. Few DiI-tagged cells are present around the proximal UB tip. The nephric duct, which also expresses Pax2, is also visible (nd). The distal, tubular portion of the UB (dub in E) is associated with large populations of DiI-tagged cells (red). Few tagged cells deriving from the tailbud are observed near the proximal-most domain of the UB (pub). (**F**) The cloaca is also surrounded by large populations of DiI-tagged cells deriving from the tailbud. (**G-I**) The heritable lineage marker, *lacZ*, was transferred into the tailbud at HH st14-15 and embryos were incubated for an additional 10 days. In whole-mount preparations (G), β-gal-expressing cells (blue in G) derived from the tailbud are present in sacral tissues, which include the cloaca (c) and the distal UB (dUB). (H) High-power examination of the cloaca indicates that lineage-tagged cells are spindle shaped and dispersed, characteristic of connective tissue. (I) Paraffin section of the ureter shows β-gal-tagged cells derived from the tailbud in the ureteral connective tissue coat (cn). Cells deriving from the tailbud are not detected within the ureter epithelium (ep, I) or metanephros (data not shown), which are derived from rostral tissues.

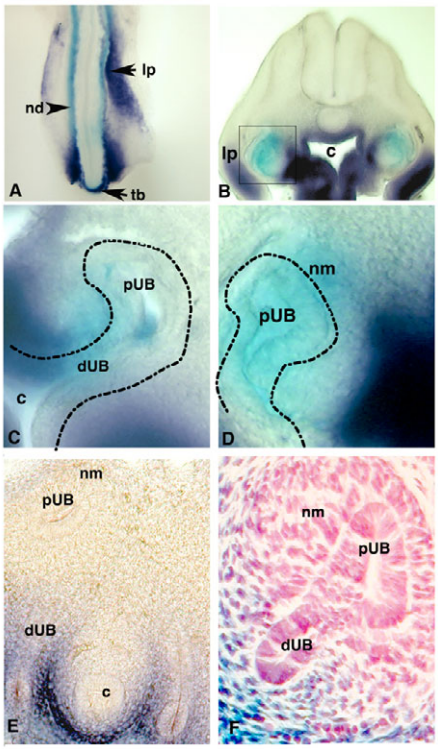

Fig. 4. *Bmp4* mRNA is expressed in the tailbud and its derivatives. Whole-mount image (**A**) and transverse vibratome sections (**B-D**) of chick embryos processed for the detection of *Pax2* (green) and *Bmp4* (purple) mRNAs. At HH st14 (A), *Pax2* mRNA is detected in the nephric duct (nd) and at the caudal-most aspect of the embryo. *Bmp4* mRNA is abundantly expressed in the lateral plate (lp) and the tailbud (tb). By HH st21 (B), *Bmp4* mRNA is abundantly expressed by mesenchyme surrounding the cloaca (c) and the lateral plate mesenchyme (lp), whereas *Pax2* mRNA is expressed in the developing metanephros (boxed area). High-power images of developing metanephros (C,D) demonstrate that the UB (outlined by broken line) and nephrogenic mesenchyme (nm) surrounding the proximal UB tip express *Pax2* mRNA. *Bmp4* mRNA is expressed around the distal (dUB) but not the proximal UB (pUB). The pattern of *Bmp4* expression in the developing chick urinary tract (C,D) is markedly similar, if not identical, to the pattern of *Bmp4* expression in the developing murine urinary tract (**E**,**F**). Whole-mount image (E) of the urinary tract isolated from an E11.5 mouse embryo demonstrates that abundant levels of *Bmp4* mRNA are expressed by mesenchyme surrounding the distal UB (dUB) and the cloaca (c). *Bmp4* is not detected in the nephrogenic mesenchyme surrouding the proximal UB tip (pUB). (F) Similar results are seen in sections of E11.5 mouse metanephric kidney rudiments isolated from *Bmp4*$^{+/lacZ}$ mouse embryos.

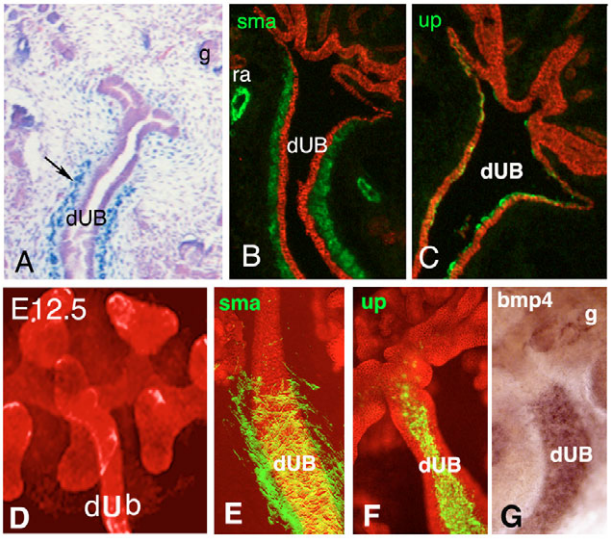

Fig. 5. Ureter differentiation occurs in zones of the UB surrounded by *Bmp4*-expressing mesenchyme. (**A**) Sections of E14.5 *Bmp4*$^{+/lacz}$ embryos show abundant β-gal staining, indicating that *Bmp4* expression persists in the mesenchyme (arrow) surrounding the distal UB (dUB). β-gal activity can also be detected in glomerular cells (g). (**B**,**C**) Immunofluorescent analysis of E15.5 frozen sections demonstrates that this zone of the UB network (red, E-cadherin staining) differentiates into the ureter, as determined by the presence of an SMA-positive connective tissue coat (B, green) and upregulated uroplakin expression (C, green). SMA-positive cells (B) are also detected around renal arteries (ra). At E12.5 (**D**), the E-cadherin-positive UB network lacks both UP-positive (D) and SMA-positive cells (data not shown). However, after E12.5 rudiments were cultured for 4 days (**E**,**F**) the distal-most domain of the E-cadherin-labeled UB (red) acquires a thick, SMA-positive connective tissue coat (E, green) and exhibits upregulated expression of uroplakins (F, green). (**G**) In situ hybridization detection of *Bmp4* in cultured rudiments indicates that ureter morphogenesis occurs only in domains of the UB bounded by *Bmp4*-expressing mesenchyme. *Bmp4* mRNA can also be detected in glomerular podocytes (g).

interstitium at later stages of development, these SMA-positive cells are not organized into a thick, circumferentially oriented connective tissue investment. Finally, we show that ureter differentiation occurs in metanephric kidney explants (Fig. 5D-F). Neither uroplakins (Fig. 5D) nor SMA-positive mesenchyme (data not shown) can be detected in freshly isolated E12.5 metanephric kidney rudiments, whereas these markers of ureter differentiation are abundantly expressed after 4 days of culture (Fig. 5E,F). Importantly, the domain of the UB that displays the ureteral phenotype in vitro parallels the spatially restricted expression of *Bmp4* in adjacent mesenchyme (Fig. 5G). Thus, this explant system successfully recapitulates ureter differentiation.

Bmp4-expressing mesenchyme is required for ureter morphogenesis

The role of BMP signaling in controlling ureter morphogeneis was analyzed in vitro using metanephric rudiments isolated from mice in which *Bmp4* alleles had been deleted by Cre recombinase-mediated excision (Fig. 6A). Female *Bmp4*$^{flox/flox}$ mice were bred with male *Bmp4*$^{flox/+}$ mice harboring a transgene containing sequence encoding a fusion protein of Cre recombinase and estrogen receptor 1 under the control of a chicken beta-actin promoter/enhancer coupled with the cytomegalovirus (CMV) immediate-early enhancer (Hayashi and McMahon, 2002). The Cre/ESR1 fusion protein can enter the nucleus only when bound to an estrogen receptor 1 ligand. Therefore, Cre-

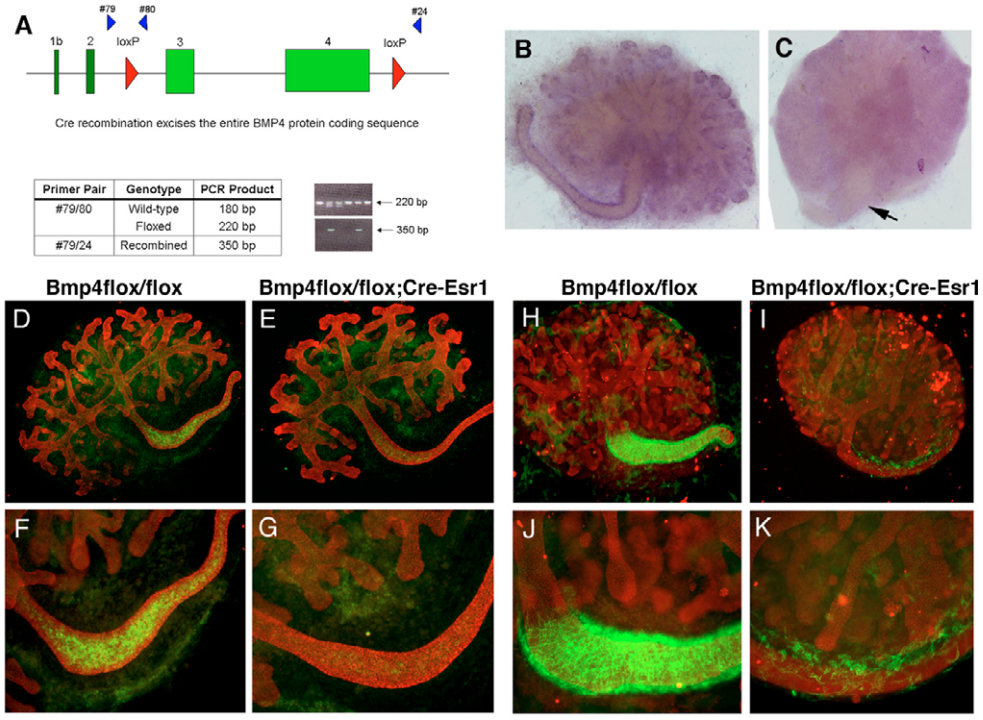

Fig. 6. BMP4 signaling is required for the differentiation of the distal UB into the ureter. (**A**) The Cre-flox system was used to conditionally remove exons 3 and 4 of the *Bmp4* gene. The presence of the *loxP* sites flanking the *Bmp4* allele and the occurrence of the recombination event were confirmed through genotyping using specific PCR primers. (**B**,**C**) In situ analysis of *Bmp4* expression was performed on rudiments isolated from E12.5 embryos and cultured for 4 days. Expression of *Bmp4* mRNA is detected in the *Bmp4*$^{flox/flox}$ rudiments (B), whereas no *Bmp4* is detectable in *Bmp4*$^{flox/flox}$;*Cre-Esr1* mutant rudiments (C). (**D-K**) Metanephric rudiments from E12.5 *Bmp4*$^{flox/flox}$ or *Bmp4*$^{flox/flox}$;*Cre-Esr1* mutant embryos were cultured for 4 days with 4-OH tamoxifen. Rudiments were analyzed as whole mounts using E-cadherin expression to visualize the UB network (red) and uroplakin (green, D-G) or SMA (green, H-K) to detect ureter differentiation. Uroplakin expression can readily be detected in *Bmp4*$^{flox/flox}$ cultures (D,F), but is difficult to detect in the *Bmp4*$^{flox/flox}$;*Cre-Esr1* rudiments (E,G). *Bmp4*$^{flox/flox}$ rudiments develop an SMA-positive coat (H,J) that condenses around the distal domain of the UB. In the *Bmp4*$^{flox/flox}$;*Cre-Esr1* rudiments, only a few unorganized SMA-positive cells are detected (I,K).

mediated excision of the floxed *Bmp4* alleles is inducible by maternal administration of the synthetic estrogen, tamoxifen. Timed pregnant mice were injected with tamoxifen at E12 and embryos harvested 6 hours post-injection because $Bmp4^{flox/flox}$ embryos carrying the *Cre* recombinase transgene begin to die in utero 24 hours after tamoxifen-induced *Bmp4* excision. Metanephric kidney rudiments were isolated and cultured in vitro in the presence of tamoxifen to ensure complete gene excision and *Bmp4* expression levels were assessed after 4 days of growth by in situ hybridization. Abundant *Bmp4* mRNA expression was seen in rudiments from $Bmp4^{flox/flox}$ embryos lacking the *Cre* transgene (Fig. 6B), whereas *Bmp4* mRNA expression was not detected in rudiments isolated from $Bmp4^{flox/flox}$, *Cre/ESR1* embryos (Fig. 6C). After 4 days of culture, uroplakin and SMA expression were detected in the distal-most domain of $Bmp4^{flox/flox}$ rudiments lacking the *Cre* transgene (Fig. 6D,F,H,J), whereas the expression of these markers indicative of ureter differentiation was dramatically reduced when *Bmp4* alleles were excised at E12 (n=4; Fig. 6E,G,I,K). Excision of *Bmp*4 alleles at developmental stages later than E12.5 did not perturb ureter differentiation, as determined by upregulated uroplakin expression and the formation of a SMA-positive connective tissue coat (n=4; data not shown). Collectively, these data demonstrate that BMP4 signaling prior to E12.5 is required for ureter morphogenesis.

Spatially restricted BMP signaling determines the site at which the UB is divided into ureter and collecting system domains

Because *Bmp4* expression is restricted to the distal UB at the time that this tissue is sensitive to BMP-dependent ureter induction, we tested whether ectopic BMP4 signaling can induce ureter differentiation in proximal domains of the UB normally fated to differentiate into the collecting tubules. E12.5 metanephric kidney rudiments were cultured in the absence or presence of increasing concentrations of recombinant, soluble BMP4 for 4-5 days (Fig. 7A-F). Explants were assayed for ureter differentiation by immunofluorescent techniques.

Recombinant BMP4 markedly alters the growth of metanephric kidney rudiments (Bush et al., 2004; Cain et al., 2005; Raatikainen-Ahokas et al., 2000). E12.5 rudiments cultured with increasing concentrations of recombinant BMP4 exhibit a dose-dependent decrease in UB tip and nephron number as compared with control cultures (Fig. 7C-F). Most importantly, in this report we show that exogenous BMP4 converted the proximal UB tips into tubular structures that exhibit a ureteral phenotype, in a dose-dependent manner (Fig. 7C-F). The proximal UB tips exhibit upregulated uroplakin expression (Fig. 7C,E) and become invested with a condensed, SMA-positive smooth muscle coat (Fig. 7D,F). Developmental plasticity persists in the UB, as similar results were obtained when E14.5 rudiments were cultured in vitro with high concentrations of recombinant BMP4 (Fig. 7G-I). As expected, in both control and BMP4-treated cultures, the distal domain of the UB differentiated into the ureter (Fig. 7G,H). Strikingly, the proximal UB tips also acquired a ureteral phenotype when older rudiments were cultured with recombinant BMP4, whereas large portions of the UB network between the definitive ureter and the UB tips did not develop ureteral characteristics (Fig. 7I,J). These data demonstrate that the proximal UB tips, normally fated to differentiate into the collecting system, acquire the ureteral phenotype in the presence of ectopic BMP4 signaling.

By contrast, high concentrations of BMP7, a TGFβ family member expressed by the UB and by nephrogenic mesenchyme around its proximal tips (Dudley and Robertson, 1997), were unable

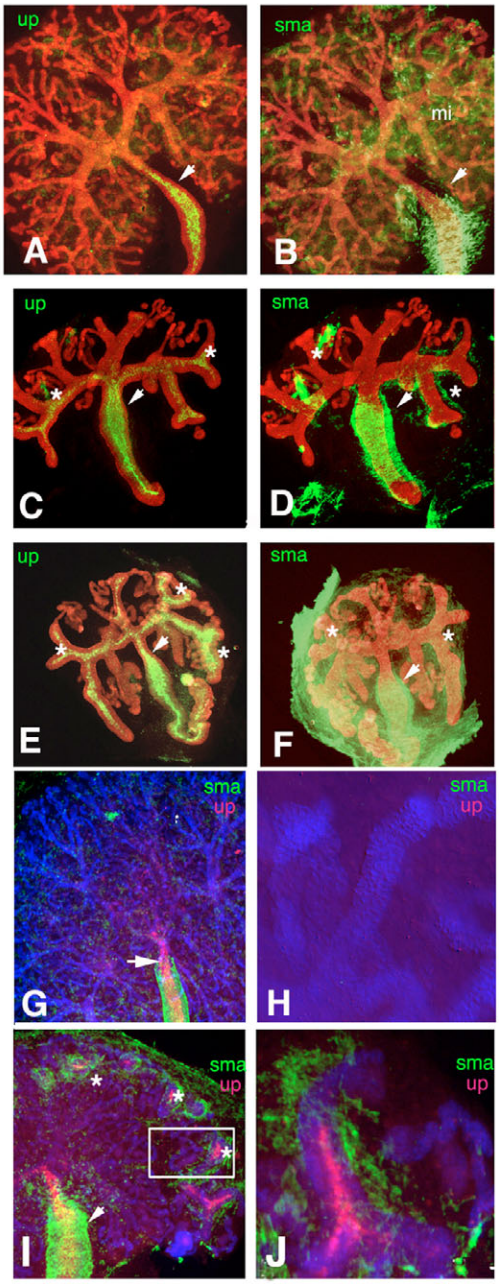

Fig. 7. Ectopic BMP signaling induces ureter morphogenesis in zones of the UB normally fated to differentiate into collecting tubules. Whole-mount images of E12.5 (**A-F**) or 14.5 (**G-J**) metanephric explants cultured for 4 days in the absence (A,B,G,H) or presence (C-F,I,J) of recombinant BMP4. The UB network is labeled with antibodies directed against E-cadherin (red, A-F; blue, G-J), differentiated ureter epithelia identified by uroplakin expression (A,C,E, green; G-J, red) and the ureteral smooth muscle coat visualized by SMA expression (B,D,F,H, green). (J) Enlargement of framed area in I. In control cultures (A,B,G,H), ureter differentiation occurs only in the distal-most domain of the UB (arrowheads) as determined by upregulated uroplakin expression (A,G) and formation of a well-organized SMA-positive (B,G) coat. SMA-positive cells can also be detected between medullary collecting tubules (ml), but are not organized into a dense connective investment around the tubules in this location. Both uroplakin-positive cells (B,E,I,J) and an SMA-positive connective tissue investment can be detected in more proximal zones of the UB (pUB) network (* referring to proximal domains of UB network) when cultures are maintained with 25 ng/ml (C,D) or 100 ng/ml BMP4 (E,F,I,J).

to induce ureter morphogenesis in the proximal domains of the UB normally destined to form the collecting system in vitro (data not shown). Together, these data demonstrate that ectoptic signaling by BMP4, a paracrine factor normally present in mesenchyme surrounding the distal UB, is capable of inducing ureter morphogenesis in domains of the UB fated to differentiate into the intra-renal collecting system. Thus, spatially restricted *Bmp4* expression plays a role in determining the location at which the UB is subdivided into the ureter and the collecting system.

DISCUSSION

The patterning of the UB into the intra-renal collecting system and ureter has significant functional consequences, as these segments of the urinary tract are essential for the regulation of body fluid volume and the excretion of wastes, respectively. Moreover, the junction between the collecting system and ureter is highly prone to congenital defects in humans. Here, we have shown that this patterning event is dependent on the association of the UB with two distinct mesenchymal cell populations: tailbud and intermediate mesenchyme. Tailbud-derived mesenchyme selectively associates with the distal UB and induces ureter morphogenesis, in part, via expression of BMP4.

Mesenchymal-to-epithelial signaling mediated by *Bmp4* patterns the UB

BMP signaling is likely to play a role in multiple processes required for ureter morphogenesis. Our results, combined with previous studies, implicate BMP signaling in the control of ureteral smooth muscle formation (Airik et al., 2006; Miyazaki et al., 2003).

Moreover, targeted deletion of *Shh*, which is expressed in the nascent ureter epithelium, results in a deficit of *Bmp4*-expressing peri-ureteral mesenchyme and an abnormally thin ureteral smooth muscle coat (Yu et al., 2002). Collectively, these data indicate that formation of the ureteral smooth muscle coat is dependent on signals from the nascent ureter epithelium, similar to what has been shown by Baskin in the developing bladder, and signaling within the mesenchymal population surrounding the nascent ureter (Baskin et al., 1996).

In this study we show that *Bmp4* is also required for the upregulated expression of uroplakins in the differentiating ureter. Uroplakins are a family of transmembrane proteins selectively expressed by the ureter epithelium and required for its water-impermeable properties (Hu et al., 2001). BMP4 secreted by peri-ureteral mesenchyme may signal directly to the underlying epithelium, as UBs isolated from the surrounding mesenchyme cease branching when exposed to recombinant BMP4 in vitro (Bush et al., 2004). However, targeted deletion of SMAD4 in the UB epithelium does not grossly affect ureter morphogenesis in vivo (Oxburgh et al., 2004). Because SMAD4 is an obligatory component of SMAD-dependent BMP signaling, if BMP4 directly signals to the UB epithelium, it is likely to do so via SMAD-independent mechanisms such as activation of LIM1 kinase (Lee-Hoeflich et al., 2004). Alternatively or additionally, *Bmp4* may upregulate the expression of unknown mesenchymal signaling factors that then directly induce ureter epithelial differentiation. Future experiments examining ureter differentiation in animal models lacking BMP receptors within the UB will be required to further dissect the mechanisms mediating BMP-dependent ureter epithelial differentiation.

We have also shown that ectopic BMP4 signaling induces ureter differentiation. Raatikainen-Ahokas et al. demonstrated that the large portions of the UB network acquire a dense connective tissue coat characteristic of the ureter when metanephric kidney explants are cultured with recombinant BMP4 (Raatikainen-Ahokas et al., 2000). Our experiments confirm and extend these observations. We show that ectopic BMP4 signaling at the early stages of renal development converts the entire UB network into tubules exhibiting a ureteral phenotype as determined by the upregulated expression of uroplakins and the acquisition of a thick connective tissue coat. This phenotypic conversion may be due to BMP-dependent migration or overproliferation of connective tissue and uroplakin-expressing cells from the nascent ureter to more proximal domains of the UB network. However, experiments analyzing UB differentiation in E14.5 kidney rudiments suggest that ectopic BMP4 signaling actually changes the fate of the UB from a collecting tubule to ureteral phenotype. High concentrations of recombinant BMP4 induce the most proximal UB tips to acquire a ureteral phenotype, whereas large portions of the UB network located between these abnormal UB tips and the definitive ureter do not develop ureteral characteristics. This result strongly suggests that ectopic BMP4 signaling changes the fate of the proximal UB from a collecting tubule to ureteral phenotype, because it is extremely unlikely that large populations of both uroplakin- and SMA-positive cells move from the distal-most domain of the UB to its most proximal tips without populating the intervening UB segments. These results are consistent with earlier studies demonstrating that the differentiated phenotype of epithelia derived from the UB is controlled, in part, by factors secreted by surrounding mesenchyme (Lipschutz et al., 1996).

Modulation of the timing and level of BMP signaling may be essential for the proper patterning of these tissues. Our results suggest that portions of the UB network become refractory to BMP-induced ureter differentiation or committed to a collecting tubule phenotype by E14.5. This early specification of the UB appears to allow BMP4 signaling to mediate additional developmental processes at later stages. For example, BMP4 expressed in the medullary interstitium and glomerulus may pattern the renal vasculature, as signaling by this TGFβ family member has been implicated in blood vessel formation, and antagonists of BMP signaling compromise the integrity of the glomerular capillary tuft (Miyazaki et al., 2006; Nimmagadda et al., 2005).

Sensitivity to both specific ligands and the total level of BMP signaling in the target tissue may also allow the BMPs to promote the formation of different cell types even in the absence of temporal restriction. Dose-response experiments included in this study demonstrate that high concentrations of recombinant BMP4 induce ureter morphogenesis in domains of the UB normally fated to differentiate into the renal collecting system whereas BMP7 does not. BMP7 is expressed by the UB and by nephrogenic mesenchyme surrounding its branching tips in vivo and is essential for maintaining the viability of nephron progenitors within the developing kidney (Dudley et al., 1995; Dudley and Robertson, 1997; Oxburgh et al., 2005). Since the receptor specificity of BMP4 and BMP7 differ, it is possible that the receptors mediating BMP4-dependent ureter morphogenesis do not respond to BMP7 signaling. However, despite the distinct receptor specificity and functions of BMP4 and BMP7 in the developing kidney, *Bmp4* can substitute for *Bmp7* in supporting nephron progenitor viability in vivo (Oxburgh et al., 2005). We hypothesize that the levels of ectopic *Bmp4* mRNA expressed around the UB tips when the gene is knocked into the *Bmp7* locus are not sufficient to induce ectopic ureter morphogenesis in vivo (Oxburgh et al., 2005). This hypothesis is consistent with data demonstrating that different levels of BMP

signaling promote diverse responses in many embryonic cell types (James and Schultheiss, 2005; Oxburgh et al., 2005; Wilson et al., 1997).

Generation of restricted BMP expression

Collectively, our data indicate that spatially restricted BMP4 signaling is essential for normal urinary tract patterning. Restricted BMP4 expression in the developing urinary tract may be imposed by several different mechanisms. Fate mapping experiments combined with mRNA expression analyses presented in this study show that the mesenchyme surrounding the distal UB derives from the tailbud and expresses *Bmp4* prior to associating with the developing urinary tract. By contrast, the mesenchyme surrounding the domain of the UB fated to form the renal collecting system does not express *Bmp4* and derives from the intermediate mesoderm. Therefore, one of the mechanisms that confines BMP4 signaling to the domain of the UB fated to become the ureter is the association of tailbud-derived mesenchyme with the most caudal segments of the developing urinary tract, including the distal UB.

Tailbud-derived mesenchyme also associates with the cloaca. The cloaca derives from endoderm and its ventral wall forms the bladder, the structure into which the ureter drains (Carlson, 1996). The bladder epithelium has different in vitro growth characteristics from the ureter epithelium, which derives from mesoderm (Liang et al., 2005). Despite these differences, the ureter and bladder epithelium share many similar features, including morphology and gene expression patterns (Liang et al., 2005). We suggest that the association of a common population of tailbud-derived, BMP4-expressing mesenchyme with both the distal UB and cloaca accounts for these similarities.

Our fate mapping results address the origin of the mesenchyme surrounding the distal UB in the developing chick embryo and several lines of evidence suggest that tailbud-derived mesenchyme plays a role in conduit system formation in a variety of species. Sustained BMP signaling within the tailbud is essential for the formation of the terminal portions of the zebrafish urinary tract (Pyati et al., 2006). Similarly, targeted deletion of the vitamin A degradation enzyme cyp26, which is localized to the murine tailbud, results in both massive tailbud apoptosis and gross ureter and bladder defects (Sakai et al., 2001).

Mesenchyme from the tailbud remains poorly understood. As this tissue does not arise from primary gastrulation, it is possible that it has a distinct gene expression profile and developmental potential compared with other embryonic mesenchyme (Catala et al., 1995; Gofflot et al., 1997; Knezevic et al., 1998; Wilson and Beddington, 1996). Our data raise the possibility that this mesenchyme is required for the formation of multiple structures within the murine urogenital system. In support of this hypothesis, a recent study indicates that mesenchyme surrounding the cloaca, which we show derives from the tailbud, plays a fundamental role in the differentiation of the bladder, urethra and genitalia (Haraguchi et al., 2007). Thus, tailbud mesenchyme may be required for the formation of multiple structures, both in the urogenital system and throughout the lumbar and sacral regions of the embryo. We suggest that the fate and function of tailbud mesenchyme merits further investigation.

Formation of the junction between the kidney and ureter is a complex, multi-stage process

Our data shows that a sharp boundary between the ureter and the branched collection system can be detected in the developing murine urinary tract by E15.5. This initial boundary undergoes further differentiation to create the renal pelvis, which does not fully mature until birth and shares properties with both the renal collecting system and ureter (Miyazaki et al., 1998). The mechanisms regulating renal pelvis formation are currently unclear, but it is likely that a failure to properly form an initial boundary at this site will ultimately result in general defects at the urteral-pelvic junction.

In conclusion, our results indicate that formation of the ureteral-pelvic junction, the border between the kidney and conduit system in the mature animal, is a complex, multi-stage process that requires the integration of cells from at least three different embryonic lineages into a single, composite structure. These aspects of ureteral-pelvic junction formation are reminiscent of cardiac outflow tract morphogenesis, which requires the integration of cells derived from the neural crest, lateral and cardiac mesoderm into a single, functional structure (Carlson, 1996). Quite possibly, as a result of the complex tissue interactions involved, the outflow tract is a major site of congenital malformations in humans. Our experiments analyzing ureter morphogenesis suggest that the high incidence of congenital defects localized to the ureteral-pelvic junction may also be related to the complexities of integrating cells from a variety of lineages into a single composite site.

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