Diversification of Cell Lineages in Ureter Development

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ABSTRACT

The mammalian ureter consists of a mesenchymal wall composed of smooth muscle cells and surrounding fibrocytes of the tunica adventitia and the lamina propria and an inner epithelial lining composed of layers of basal, intermediate, and superficial cells. How these cell types arise from multipotent progenitors is poorly understood. Here, we performed marker analysis, cell proliferation assays, and genetic lineage tracing to define the lineage relations and restrictions of the mesenchymal and epithelial cell types in the developing and mature mouse ureter. At embryonic day (E) 12.5, the mesenchymal precursor pool began to subdivide into an inner and outer compartment that began to express markers of smooth muscle precursors and adventitial fibrocytes, respectively, by E13.5. Smooth muscle precursors further diversified into lamina propria cells directly adjacent to the ureteric epithelium and differentiated smooth muscle cells from E16.5 onwards. Uncommitted epithelial progenitors of the ureter differentiated into intermediate cells at E14.5. After stratification into two layers at E15.5 and three cell layers at E18.5, intermediate cells differentiated into basal cells and superficial cells. In homeostasis, proliferation of all epithelial and mesenchymal cell types remained low but intermediate cells still gave rise to basal cells, whereas basal cells divided only into basal cells. These studies provide a framework to further determine the molecular mechanisms of cell differentiation in the tissues of the developing ureter.


The mammalian ureter has a compartmentalized tissue architecture that is functionally adapted to mediate efficient urinary drainage from the renal pelvis to the bladder. The outer mesenchymal compartment harbors a thick layer of peristaltically active smooth muscle cells (SMCs), the tunica muscularis, that is ensheathed by flexible and anchoring fibroelastic material, the tunica adventitia on the outside and the lamina propria on the inside. The inner compartment is a highly distensible epithelium that provides sealing to the luminal space. This urothelium features a single layer of cuboidal basal cells (B cells) that is attached to the lamina propria via a basement membrane, one or two layers of intermediate cells (I cells) that resemble B cells in shape and size, and a luminal layer of large squamous superficial cells (S cells) that exert a barrier function at least partly due to expression of uropilakins (UPKs) that form crystalline plaques on the apical surface.1,2

The differentiated cell types of the two ureteric tissue compartments arise from multipotent precursors during embryonic development. In the mouse, these precursor pools are established around embryonic day (E) 11.5 when the distal aspect of an epithelial diverticulum of the nephric duct, the ureteric bud, and its surrounding mesenchyme adopt a distal ureteric rather than a proximal renal fate. For the next days, the mesenchymal and epithelial progenitors multiply to support ureter elongation. At E16.5, i.e., shortly after onset of urine production in the kidney, expression of smooth muscle (SM) structural proteins and of UPKs testifies that SMC and S cell differentiation

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has been initiated. Around birth, the three epithelial and mesenchymal cell layers can be histologically clearly distinguished. Embryologic experiments have shown that the survival, patterning, and subsequent differentiation of the primitive ureteric epithelium and its surrounding mesenchyme depend on each other. Genetic analysis has identified some of the trans-acting signals and the downstream transcription factors that regulate these cellular programs. How ever, how the different cell types arise in time and how they relate to each other has been poorly studied.

Here, we set out to probe the developmental origin and relationship of the different epithelial and mesenchymal cell types of the mouse ureter. We describe the temporal profile of cell differentiation and proliferation in the ureter, and trace the fate of the two progenitor pools. We provide evidence that I cells are precursors for both B and S cells in development.

RESULTS

Cell Differentiation Occurs in a Temporally Controlled and Coordinated Manner in the Epithelial and Mesenchymal Tissue Compartments of the Embryonic Ureter

Previous work reported expression of cell-type–specific genes at selected stages of ureter development but did not address the precise temporal profile of the mesenchymal and epithelial differentiation programs. We therefore wished to correlate histologic changes with expression profiles of cell-type–specific marker sets in either tissue compartment at all stages of embryonic ureter development.

In the mature ureteric mesenchyme, adventitial fibrocytes are marked by expression of periostin (POSTN), whereas SMCs can be identified by transgelin (TAGLN) and actin, alpha 2, smooth muscle, aorta (ACTA2) expression. For the cells of the lamina propria no specific protein marker has been described, but they can be identified as mesenchymal cells negative for SMC markers adjacent to the ureteric epithelium (Figure 1, A–C, P40 panel). We have recently shown that the T-box transcription factor gene Tbx18 is expressed in the undifferentiated ureteric mesenchyme, and that the descendants of this expression domain constitute the ureteric mesenchymal wall throughout development and in adulthood. To detect and quantify cell differentiation in the ureteric mesenchyme, we therefore analyzed coexpression of cell-type–specific markers with a membrane-bound GFP reporter by immunofluorescence on proximal ureter sections in mice double heterozygous for a cre knock–in in the Tbx18 locus and the Rosa26<sup>Cre<sup>TM1G<sup> reporter line (Tbx18<sup>cre<sup>+/R26<sup>cre<sup>TM1G<sup>/+<sup>) (Figure 1, A–E).<sup>10,11</sup>

At E12.5, two histologically distinct cell populations were present in the GFP<sup>+</sup> ureteric mesenchyme. Cells adjacent to the ureteric epithelium were densely packed and spherical, whereas the outermost layers contained loosely organized, radially oriented, and spindle-shaped cells. Differentiation markers were not expressed at this stage. At E13.5, the number of GFP<sup>+</sup> mesenchymal cells was strongly increased and the outermost spindle-shaped cells started to express POSTN. POSTN expression expanded to cover most cells of the outer mesenchymal compartment at E14.5. At E15.5, few TAGLN<sup>+</sup>ACTA2<sup>+</sup> SMCs appeared in the dense inner mesenchyme that was surrounded by POSTN<sup>+</sup> adventitial fibrocytes. At E16.5, a contiguous SMC layer had developed that was separated from the urothelium by a TAGLN<sup>+</sup> layer of lamina propria cells. Most of the spindle-shaped outer fibrocytes were POSTN<sup>+</sup>. At E18.5, the ureteric mesenchyme was fully patterned and differentiated in TAGLN<sup>+</sup>GFP<sup>+</sup> lamina propria cells, a dense TAGLN<sup>+</sup>ACTA2<sup>+</sup>GFP<sup>+</sup> SMC layer, and radially organized POSTN<sup>+</sup>GFP<sup>+</sup> adventitial fibrocytes. The ratio of these cell types was dramatically changed at P40 by relative expansion of the lamina propria and the SMC layer and reduction of the tunica adventitia (Figure 1, A–E, Supplemental Table 1A).

Work in the bladder has shown that combinatorial expression of cytokeratin 5 (KRT5), of an isoform of the transcription factor p63 (ΔNP63), and of UPKs distinguishes the three urothelial cell types in this tissue. We confirmed for the adult ureter that B cells are KRT5<sup>+</sup>ΔNP63<sup>+</sup>UPK1B<sup>+</sup>, I cells are KRT5<sup>+</sup>ΔNP63<sup>+</sup>UPK1B<sup>+</sup>, and S cells are KRT5<sup>+</sup>ΔNP63<sup>+</sup>UPK1B<sup>+</sup>, with levels of UPK1B being significantly lower in I than in S cells (Figure 1, F and G, P40 panel); hence, that these markers can be used to profile urothelial differentiation in this organ (Figure 1, F–J). At E12.5, the mono-layered urothelium was negative for all of these differentiation markers. ΔNP63 expression occurred in single cells at E13.5 but was expressed in increasing cell numbers at E14.5 and labeled the majority of cells of the now double-layered urothelium at E15.5. Expression of Upk1b mRNA was weakly found throughout the urothelium at these stages as well characterizing these cells as I cells. At E15.5, single cells on the luminal side were negative for ΔNP63 which correlated with the advent of superficial UPK1B expression. At E16.5, the urothelium was composed of two to three layers. Coexpression of KRT5 and high levels of ΔNP63 indicated the presence of B cells that were intermingled with I cells (KRT5<sup>−</sup>ΔNP63<sup>+</sup>UPK1B<sup>+</sup>) in the basal layer. UPK1B expression was strong in the intermediate and apical layer. The abundance of B cells significantly increased from E16.5 to E18.5, and B cells constituted the majority of urothelial cells at P40. The relative number of KRT5<sup>−</sup>ΔNP63<sup>+</sup>UPK1B<sup>+</sup><sup>low</sup>) I cells significantly decreased from E16.5 to E18.5 and remained at low abundance in the adult. KRT5<sup>−</sup>ΔNP63<sup>−</sup>UPK1B<sup>+</sup><sup>high</sup>) S cells were clearly identifiable at E16.5 and E18.5, but decreased in abundance until P40 (Figure 1, F–J, Supplemental Table 1B). Interestingly, the relaxed adult urothelium showed a pyramidal topology on sections with large binucleated S cells at the tip, small I cells in the middle, and numerous B cells at the base (Figure 1, F–J, Supplemental Figure 1).

Comparative analysis of mesenchymal and epithelial differentiation markers at proximal, medial, and distal levels of the ureter revealed that the expression patterns of differentiation
Figure 1. Cell differentiation in ureter development occurs in a temporally and spatially controlled manner. (A–E) Time course of cell differentiation in the ureteric mesenchyme. (A) Hematoxylin and eosin (H&E) staining on transverse sections of the proximal ureter. At E12.5, the inner and outer mesenchymal region (IM and OM) and the ureteric epithelium (UE) are indicated. At P40, the three subregions of the mesenchyme, the lamina propria (LP), the smooth muscle (SM), and the tunica adventitia (TA) are marked adjacent to the urothelium (U). (B and C) Coimmunofluorescence analysis of expression of the SMC marker TAGLN and the lineage marker GFP (B) and of the SMC marker ACTA2 and the adventitial marker POSTN (C) on transverse sections of the proximal ureter in Tbx18<sup>cre/+</sup>;R26<sup>mTmG/+</sup> mice. Nuclei are counterstained.
markers are spatially conserved but are activated with a delay of 1–2 days at more distal levels (Supplemental Figures 2 and 3).

We conclude that differentiated cell types arise in a distinct order and in a proximal to distal wave along the ureteric tube. In the mesenchyme adenital fibrocytes precede SMCs and lamina propria cells, whereas in the epithelial compartment I cells differentiate before B and S cells.

**Proliferation Rates Explain Differential Cell-Type Expansion in the Developing Ureter**

Our analysis has shown that the different epithelial and mesenchymal cell types of the ureter start to differentiate at different time points and change in abundance over time. To determine whether this subdivision represents a compartment boundary between prospective adenital fibrocytes and SMCs, and to define the origin of the lamina propria cells, we performed genetic lineage tracing experiments using cre lines that are specific to the outer or inner mesenchymal domain. To detect the descendants of outer mesenchymal cells, we took advantage of the fact that Foxd1 is expressed in this domain exclusively at E12.5 and E14.5 (Supplemental Figure 4A).

Marker analysis on sections of proximal E12.5 and E18.5 ureters double heterozygous for a Foxd1

**Adventitial Cells are Separated Early in Mesenchymal Development Whereas Lamina Propria Cells Derive from SMC Precursors**

Our expression analysis showed that the homogenous mesenchymal progenitor pool is separated into a histologically and molecularly distinct inner and outer domain as early as E12.5. To determine whether this subdivision represents a compartment boundary between prospective adenital fibrocytes and SMCs, and to define the origin of the lamina propria cells, we performed genetic lineage tracing experiments using cre lines that are specific to the outer or inner mesenchymal domain. To detect the descendants of outer mesenchymal cells, we took advantage of the fact that Foxd1 is expressed in this domain exclusively at E12.5 and E14.5 (Supplemental Figure 4A). Marker analysis on sections of proximal E12.5 and E18.5 ureters double heterozygous for a Foxd1

To confirm the region-specificity of the labeling reaction, we treated Axin2

**Axin2 is expressed at E12.5 and subsequent stages in the inner ring of mesenchymal cells in the ureter as a response to WNT signals emitted from the adjacent epithelium (Supplemental Figure 4B).** To trace the descendants of this Axin2

To con

When Axin2

For embryos tamoxifen-treated at E12.5 and E13.5, which received tamoxifen injection on E12.5, the number of labeled cells was 73% and 17%, whereas only 10% of labeled cells were

with DAPI (blue). (D) Schematic representation of cell differentiation in the ureteric mesenchyme. Fibrocytes of the tunica adventitia (yellow) are defined as POSTN

For numbers see Supplemental Table 1B.
detected in the tunica adventitia (Figure 3, B and C, Supplemental Table 3). We conclude that Axin2+ inner mesenchymal cells are precursors of SMCs, and that lineage restriction to adventitial fibrocytes occurs around E12.5 to E13.5. Lamina propria cells derive from inner mesenchymal SMC progenitors by switching off the SMC program at E14.5 to E16.5.

I Cells are Progenitors of B and S Cells in Development

The ureteric epithelium derives from the distal aspect of the ureter bud that emerges as an outgrowth of the nephric duct around E10.5. To confirm that the distal ureter stalk indeed harbors precursors for all epithelial cell types of the mature ureter, we performed genetic lineage tracing on the basis of cre lines mediating recombination in all of these precursors. Pax2 and Shh are genes that are expressed throughout the ureteric epithelium at E12.5 and at subsequent stages (Supplemental Figure 4, C and D). A transgenic line, in which cre was expressed under Pax2 regulatory elements (Pax2-cre),15,16 mediated full recombination of the R26mTmG reporter allele in the ureteric epithelium at E12.5; a line harboring a cre knock-in into the Shh locus17 did so partially (Figure 4, A and B). In Pax2-cre/+;R26meanTmG/+ and Shhcre/++;R26meanTmG/+ ureters analyzed at E18.5, GFP+ cells populated all three urothelial cell layers, whereas a contribution to the ureteric mesenchyme was never observed (Figure 4, A and B).

Our expression analysis further demonstrated that the epithelial compartment of the ureter remains undifferentiated until E13.5 and expresses markers for I cells from E14.5 onwards. S cells and B cells were only detected from E15.5 and E16.5 onwards, respectively, suggesting that I cells are precursors for both S and B cells in development. To validate this hypothesis, we used cell-type–specific creERT2 lines and traced their descendants in ureter explant cultures after 4-hydroxytamoxifen–induced stage-specific recombination of the R26meanTmG reporter allele.

To trace the fate of B cells we used a creERT2 lineage tracing system on the basis of the B cell–specific expression of the Krt5 gene.12,18 When ureters from Krt5creERT2/+;R26meanTmG/+ embryos were explanted at E14.5, treated with 4-hydroxytamoxifen, and analyzed after 10 days GFP+ cells were not detected, compatible

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**Figure 2.** Proliferation rates explain differential cell-type expansion in the developing ureter. (A) Coimmunofluorescence analysis on proximal ureter sections at E12.5 and E14.5 for BrdU and the nuclear counterstain DAPI (blue). White dotted lines demarcate the ureteric epithelium (UE), and the inner and outer mesenchymal cell populations (IM and OM). (B) Quantification of cell proliferation by the BrdU index in the areas indicated in (A). (C)
with the fact that KRT5 expression only occurs around E16.5. When ureters from E16.5 and E18.5 embryos were treated and analyzed after 8 and 6 days, respectively, GFP+ cells were present and unambiguously expressed the B cell marker KRT5, suggesting a lineage restriction of the B cell population (Figure 4, C and D, Supplemental Table 4A).

Because cre lines specific for I cells are not established, we used a Upk3a-GCE/+ line,12,19 that labels I and S cells but not B cells (Supplemental Figure 5), to trace I cell descendants. Irrespective of whether ureters of Upk3a-GCE/+;R26mTmG/+ embryos were explanted at E14.5 and analyzed after 10 days, explanted at E16.5 and analyzed after 8 days, or explanted at E18.5 and analyzed after 6 days, GFP+ cells differentiated in roughly equal ratio into KRT5+ B cells and KRT5− UPK1B+ I and S cells, suggesting that I cells are B cell progenitors in development (Figure 4, E and F, Supplemental Table 4B).

DISCUSSION

Early Lineage Restriction in the Ureteric Mesenchyme Separates Adventitial Fibrocytes from a Common Precursor of SMCs and Subepithelial Fibrocytes

Our previous work has shown that all mesenchymal cell types of the ureter derive from a Tbx18+ mesenchymal progenitor pool that surrounds the distal portion of the ureter bud and separates the metanephric blastema from the nephric duct around E11.5.9 Shortly after, at E12.5, this homogeneous cell population is subdivided into an inner compartment of densely packed, spherical cells and an outer compartment of loosely organized, spindle-shaped, and radially oriented cells, implying that mesenchymal cells of the two domains have acquired different fates. Our findings support such an early lineage restriction in the ureteric mesenchyme. First, differential gene expression occurs around E12.5 in the two mesenchymal subregions. The
outer domain expresses POSTN and Foxd1, whereas the inner region is positive for Axin2. Second, and more importantly, lineage tracing of Foxd1+ cells showed that these cells exclusively contribute to adventitial fibrocytes at E18.5, whereas Axin2+ cells differentiate into cells of the lamina propria and the SM layer. Our marker and proliferation analyses argue that adventitial fibrocytes arise first and become terminally differentiated before the other mesenchymal cell-types around E16.5 to E18.5. In contrast, Axin2+ cells exist as bipotent precursors from E12.5 to E15.5, and differentiate into SMCs and subepithelial fibrocytes around E16.5. The descent of lamina propria and SMCs from a common precursor is also supported by the observation that lamina cells initially also express ACTA2 but downregulate it afterward. Lamina cells and SMCs maintain high proliferation rates at least until E18.5, arguing that they only terminally differentiate postnatally. Given the maintenance of cell division at low rates in both fibrocyte populations in adults, it is tempting to speculate that adventitial fibrocytes replenish themselves whereas lamina cells serve as precursors for themselves and for SMCs.

Tissue separation experiments provided compelling evidence that SMC differentiation in the ureter, and in many other visceral tubular organs, depends on signals from the adjacent epithelium. Our own work has recently shown that canonical WNT signaling is required to initiate SM precursor development and to suppress an adventitial fate in the ureter. Because Axin2 is a direct transcriptional target of this signaling pathway, this suggests that only cells adjacent to the ureteric epithelium that perceive a WNT signal escape from the default program to become adventitial fibrocytes, and differentiate into SM progenitors instead. It is interesting to note that Axin2 expression persists after E14.5 only in cells directly in contact with the ureteric epithelium, i.e., in lamina propria cells, supporting our idea that these cells represent a persisting pool of SM progenitors. Whether downregulation of WNT signaling is a prerequisite for SM progenitors to differentiate

Figure 4. I cells are progenitors of B and S cells in ureter development and homeostasis. (A and B) Coimmunofluorescence analysis on transverse sections of the proximal ureter of the lineage marker GFP with the epithelial marker CDH1 at E12.5, and the B cell marker KRT5, the B and I cell marker ΔNP63, and the S cell markers UPK1B/UPK2 in Pax2-cre/+;R26mTmG/+ embryos (A) and in Shh+ve/+;R26mTmG/+ embryos (B) shows that Pax2+ and Shh+ cells of the early ureteric bud contribute to all urothelial cell types in the ureter but not the surrounding mesenchyme. (C and D) Coimmunofluorescence analysis of the lineage marker GFP with the B cell marker KRT5 (upper panel) and the S cell marker UPK1B (lower panel) on transverse sections of ureters isolated at E14.5, E16.5, and E18.5, induced with 500 nM 4-hydroxytamoxifen for the first 24 hours and cultured for 10, 8, and 6 days; and in adult ureters 4 weeks after tamoxifen administration in Krt5creERT2/+;R26mTmG/+ mice (C) and Upk3a-GCE/+;R26mTmG/+ mice (E). (D and F) The bar diagrams display the percentage of lineage-labeled (GFP+) cells contributing to KRT5+UPK1B2 B cells or to KRT52 UPK1B+ I and S cells in the ureter of Krt5creERT2/+;R26mTmG/+ mice (D) and of Upk3a-GCE/+;R26mTmG/+ mice (F) at the indicated stages and conditions. The number of counted GFP+ cells (n) is given. For additional numbers see Supplemental Table 4, A and B. (G) Schematic representation of the lineage relations of urothelial cell types. KRT5+ cells only give rise to B cells whereas UPK3A+ cells give rise to B, I, and S cells. BC, basal cell; IC, intermediate cell; SC, superficial cell; UE, undifferentiated epithelium.
I Cells Are Progenitors for B and S Cells in Urothelial Development and Homeostasis

Here, we have shown that all cell types of the mature urothelium derive from a common Pax2\(^+\) Shh\(^+\) precursor in the ureter bud. Differentiated cell-types arise from this precursor in a strict temporal sequence. At E14.5, all epithelial cells bear the molecular signature of an I cell. With the onset of stratification at E15.5, single apical cells express S cell markers. At E16.5, KRT5\(^+\) cells appear in the basal layer but subsequently expand and constitute the main cell-type of the mature urothelium. The sequential appearance of differentiated cell types suggests that I cells are a common precursor for S and B cells. This hypothesis is further supported by our genetic lineage tracing experiments. Whereas Upk3a\(^+\) cells contributed to all cell-types throughout development and in adult tissue homeostasis, KRT5\(^+\) cells only replicated themselves. Interestingly, proliferation rates of I cells were low compared with that of B and S cells, suggesting that they first differentiate along their respective lineages and then expand. Of course, our analysis cannot exclude that I cells are heterogeneous and harbor distinct subpopulations that give rise to B and S cells, respectively. Identification of additional biomarkers, possibly by single cell transcriptome analysis, may help to resolve such an issue in the future.

So far, urothelial progenitors have only been studied in the bladder, mostly under conditions of regeneration. Even though the urothelium of the bladder and the ureter are derived from different germ layers, our study and a recent report suggest that stratification and differentiation follow a similar time course in both organs, thus, might be controlled by a common molecular program.\(^{12}\) However, the nature of the urothelial progenitors in the bladder has been controversially discussed. Although initially B cells were thought to be progenitors for the other urothelial cell types,\(^{22}\) Gandhi and colleagues suggested I cells to be S cell progenitors only and defined B cells as a unipotent, self-renewing population of unknown descent.\(^{12}\) A recent work identified a KRT14\(^+\) subpopulation of B cells to give rise to all urothelial cell types after injury.\(^{23}\) Whether the injury conditions ablate S and I cells so much that B cells are reprogrammed to compensate for their loss remains to be seen. Unlike the bladder, where KRT14 expression occurs in a large fraction of KRT5\(^+\) B cells both in the embryonic and adult urothelium, KRT14 is not expressed in the embryonic ureter and is found at very low levels in few B cells in the mature ureteric urothelium (Supplemental Figure 6). This indicates that B cells in both organs are of different heterogeneity, and that a KRT14\(^+\) subpopulation of B cells might be of less relevance for urothelial homeostasis and regeneration in the ureter.

The molecular pathways that regulate the specification, differentiation, and maintenance of the urothelium remain obscure. Retinoic acid signaling has been implied in the specification of the bladder urothelium from an endodermal precursor,\(^{12}\) whereas BMP4 was reported to be involved in the maintenance of basal cell differentiation.\(^{24}\) Whether these signaling pathways are involved in urothelial development of the ureter as well should be a focus of future research.

CONCISE METHODS

Animals

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Histologic Analysis

Embryos, urogenital systems, and ureters were paraffin-embedded and sectioned to 5 μm. Hematoxylin and eosin staining was performed according to standard procedures.

In Situ Hybridization Analysis

Nonradioactive in situ hybridization analysis of gene expression was performed on 10 μm paraffin sections of the proximal ureter with digoxigenin-labeled antisense riboprobes.\(^{25}\) At least three embryos of each genotype were used for each analysis.

Immunofluorescence Detection of Antigens

For immunofluorescence analysis on 5 μm paraffin sections polyclonal rabbit–anti-TAGLN (1:250, ab14106; Abcam), monoclonal mouse–anti-GFP (1:250, 11814460001; Roche), polyclonal rabbit–anti-GFP (1:250, sc-8334; Santa Cruz), monoclonal mouse–anti-ACTA2 (1:250, A5228; Sigma-Aldrich), polyclonal rabbit–anti-αNTP63 (1:250, 619001; BioLegend), polyclonal rabbit–anti-KRT5 (1:250, PRB-160P; Covance), monoclonal mouse–anti-KRT14 (1:250, ab7800; Abcam), monoclonal mouse–anti-UPK1B (1:250, WH0007348M2;
Sigma-Aldrich), polyclonal rabbit–anti-POSTN (1:250, ab14041; Abcam), monoclonal mouse–anti-BrdU (1:250, 1170376; Roche), or polyclonal rabbit–anti-CDH1 (1:250, gift from Rolf Kemler) were used as primary antibodies. Biotinylated goat–anti-rabbit IgG (1:250, 111065033; Dianova), Alexa488-conjugated goat–anti-rabbit IgG (1:500, A11034; Molecular Probes), and Alexa555-conjugated goat–anti-mouse IgG (1:500, A21422; Molecular Probes) were used as secondary antibodies. The signals of BrdU, ANP63 and POSTN primary antibodies were amplified using the Tyramide Signal Amplification system (NEL702001KT; Perkin Elmer). For coimmunofluorescence with primary antibodies from the same host (ANP63 and KRT5) detection was performed sequentially and epitopes of the first antibody were blocked with goat–anti-rabbit Fab (1:250, 111007003; Dianova). Before staining, paraffin sections were deparaffinized and cooked for 15 minutes in antigen unmasking solution (H-3300; Vector Laboratories). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). At least three embryos of each genotype were used for each analysis.

Three-Dimensional Reconstruction of S cells

For three-dimensional reconstruction of S cells in the adult ureter 28 consecutive 5 μm transverse paraffin sections were stained for CDH1 and DAPI as described above. CDH1 and DAPI signals were used to highlight cell boundaries and nuclei that were reconstructed with the Amira 5.3.3 software (FEI).

Cell Proliferation Assay

Cell proliferation rates in wildtype ureters (n=3 per stage) were investigated by the detection of incorporated BrdU on 5 μm paraffin sections similar to published protocols.26 For embryonic stages the BrdU incorporation time was 1 hour; for adult ureters incorporation time was 10 hours due to low cell division rates. For each specimen 12 sections of the proximal ureter were assessed. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to the total number of nuclei as detected by DAPI counterstaining in arbitrarily or molecularly defined compartments of the ureter. Data were expressed as mean±SD.

Organ Cultures

Ureters were dissected from the embryo, explanted on 0.4 μm polyester membrane Transwell supports (Corning), and cultured at the air-liquid interface for 24 hours with DMEM/F12 (Gibco) supplemented with 10% FCS (Biochrom), 1× penicillin/streptomycin (Gibco), 1× pyruvate (Gibco), and 1× Glutamax (Gibco). 4-Hydroxytamoxifen (H7904; Sigma-Aldrich) was added to the medium at a final concentration of 500 nM for the first 24 hours of culture to induce recombination. Culture medium was replaced every day.

Image Analysis

Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera or a Leica DM6000 microscope with Leica DFC500X digital camera and afterward processed in Adobe Photoshop CS4.

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