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The Role of GDNF/Ret Signaling in Ureteric Bud Cell Fate and Branching Morphogenesis

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Summary

While GDNF signaling through the Ret receptor is critical for kidney development, its specific role in branching morphogenesis of the epithelial ureteric bud (UB) is unclear. Ret expression defines a population of UB "tip cells" distinct from cells of the tubular "trunks," but how these cells contribute to UB growth is unknown. We have used time-lapse mosaic analysis to investigate normal cell fates within the growing UB and the developmental potential of cells lacking Ret. We found that normal tip cells are bipotential, contributing to both tips and trunks. Cells lacking Ret are specifically excluded from the tips, although they contribute to the trunks, revealing that the tips form and expand by GDNF-driven cell proliferation. Surprisingly, the mutant cells assumed an asymmetric distribution in the UB trunks, suggesting a model of branching in which the epithelium of the tip and the adjacent trunk is remodeled to form new branches.

Introduction

Branching morphogenesis is a basic process that underlies the development of the kidney as well as many other organs such as lung, pancreas, and salivary glands. In the kidney, the metanephric mesenchyme induces the growth and branching of the ureteric bud (UB), an outgrowth from the Wolffian duct (WD), which gives rise to the renal collecting system (Erickson, 1968; Grobstein, 1953, 1955). Factors secreted by the tips of the UB branches, in turn, induce the surrounding mesenchymal cells to condense into epithelial vesicles, which then differentiate into the various segments of the nephron (Grobstein, 1953, 1955; Saxen, 1970). The pattern and the extent of UB branching and elongation are critical for development of a normal kidney, and mutations that perturb this process result in a spectrum of defects ranging from renal agenesis to reduced kidney size and reduced nephron number (Al-Awqati and Goldberg, 1998; Cullen-McEwen et al., 2001; Pohl et al., 2002).

The control of UB growth and branching has been studied extensively in recent years, and several growth factors and receptors have been implicated in the control of this process (Carroll and McMahon, 2003; Shah et al., 2004; Vainio and Lin, 2002). Among those proteins demonstrated to play a crucial role are the secreted protein glial cell line-derived neurotrophic factor (GDNF), its receptor Ret, and its coreceptor Gfrα1. Ret is a receptor tyrosine kinase (RTK), also important for development of the peripheral nervous system, while Gfrα1 is a GPI-linked protein that binds GDNF and allows it to activate the Ret RTK (Takahashi, 2001). Ret is expressed throughout the WD and the primary evagination of the UB, but it soon becomes restricted to the distal tips of the branching UB (Pachnis et al., 1993; Tsuzuki et al., 1995). GDNF is expressed in the metanephric mesenchyme adjacent to the caudal WD, where the UB will form, and later in the peripheral mesenchyme of the kidney surrounding the UB tips (Durbec et al., 1996; Hellmich et al., 1996; Pichel et al., 1996). Gfrα1 is coexpressed with Ret in the UB tips, as well as in some cells derived from the metanephric mesenchyme (Sainio et al., 1997; Yu et al., 1998).

A requirement for GDNF signaling through Gfrα1 and Ret during kidney development has been clearly established by mouse knockout studies: inactivation of any one of these genes results in renal agenesis or severe hypodysplasia, resulting from failure of the UB to evaginate from the WD, or to grow and branch normally (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994, 1996). A variety of other studies, including treatment with exogenous GDNF or anti-GDNF antibodies in kidney organ culture, transgenic misexpression of Ret in ectopic locations in the kidney, and generation of hypomorphic alleles of *Ret*, have confirmed the importance of this signaling system to induce and maintain UB growth throughout kidney development (reviewed in Sariola and Saarma, 1999, 2003). While *Ret* is a proto-oncogene and has been implicated in the proliferation of other cell types, there are conflicting reports as to whether GDNF induces cell proliferation in the UB (Michael and Davies, 2004; Pepicelli et al., 1997; Sainio et al., 1997; Vega et al., 1996). GDNF may promote survival of UB cells (Towers et al., 1998), as it does for certain neurons (Sariola and Saarma, 2003). It has been proposed that GDNF is a ramogen, inducing branching of the UB, and that it may act as a morphogen, controlling the growth of UB tips in specific directions and thus patterning the kidney (Pepicelli et al., 1997; Sainio et al., 1997; Tang et al., 1998; Towers et al., 1998; Vega et al., 1996). It is also possible that GDNF signaling is required to maintain the ability of the UB to induce nephrogenesis by cells of the metanephric mesenchyme. Thus, the specific consequences of GDNF/Ret signaling for ureteric bud cells remain unclear.

Understanding the role of GDNF/Ret signaling in UB branching morphogenesis requires that we first understand the normal fate of the cells at the UB tips, which express Ret. However, little is known about how these cells contribute to UB growth. From an early stage of metanephric kidney development, the UB epithelium appears to differentiate into distinct "tip" and "trunk" populations, based on patterns of gene expression. In addition to *Ret* and *GFRα1*, several other genes such as *Wnt11* and *c-ros* are expressed specifically in cells at the UB tips (Kispert et al., 1996; Sonnenberg et al., 1991).

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Conversely, other genes such as *Wnt-7b*, *aquaporin-3*, and *collagen XVIII* are expressed in the tubular portions, or trunks, of the UB but not in tip cells (Ecelbarger et al., 1995; Kispert et al., 1996; Lin et al., 2001). This raises interesting questions about the lineage relationship between tips and trunks. Are the tip cells a distinct lineage, whose daughters remain at the tips of the growing and branching UB, while the trunks elongate by the proliferation of preexisting trunk cells? Or do some daughters of the tip cells stay behind and differentiate into trunk cells as the UB extends? To address these questions, we have analyzed the development of mosaic kidneys, some of whose cells carry a UB-specific GFP transgene that allows their fate to be followed by time-lapse analysis of growth in organ culture (Srinivas et al., 1999).

We next used a modification of this approach to investigate the specific role of GDNF/Ret signaling, by testing the ability of Ret-deficient cells to contribute to ureteric bud development in mosaic embryos. In such mosaics, the wild-type cells can form most of the UB epithelium, and the mutant cells have the opportunity to contribute, depending on the specific processes in which they are deficient. We could envision a variety of outcomes, ranging from failure of mutant cells to contribute to the UB at all (if Ret were very important for cell proliferation and/or survival during outgrowth of the primary UB) to an ability of mutant cells to contribute to all regions of the UB (for example, if GDNF were important primarily to induce branching or to provide positional information, and the wild-type UB cells were sufficient to initiate branch formation and direct the growth of the tips). The results of these studies provide new insight into the roles of tip and trunk cell populations in the branching morphogenesis of the ureteric bud and into the role of GDNF/Ret signaling in this process.

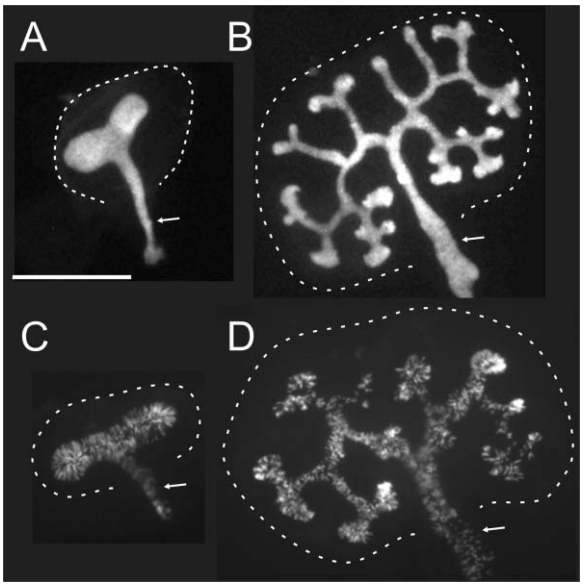

Figure 1. Examples of a *Hoxb7/GFP* Kidney and a *Hoxb7/GFP*↔Wild-Type Mosaic Generated by Embryo Aggregation

(A and B) *Hoxb7/GFP* transgenic kidney dissected at E11.5, the T-stage (A) and after several rounds of branching during 47 hr of culture (B). Note the ubiquitous expression of GFP throughout the epithelium of the ureteric bud. The dashed line shows the outline of the metanephric blastema, which is not visible in the GFP image. (C and D) Kidney from a mosaic embryo generated by aggregation of a wild-type and a *Hoxb7/GFP* transgenic morula, when dissected at E11.5 (C) and after 64 hr of culture (D). Note the extensive interspersion of GFP^+ and GFP^- (wild-type) cells at both time points. The arrows indicate the primary branch of the UB (presumptive ureter). The Wolffian duct was removed. Scale bar equals 0.5 mm. A QuickTime time-lapse movie of the growth of the kidney is available as Supplemental Movie S1. The Quick Time player can be downloaded at http://www.apple.com/quicktime/download/.

Results and Discussion

Continual Interspersion of Ureteric Bud Epithelial Cells during Normal Branching Morphogenesis

In this study, we have used time-lapse mosaic analysis to investigate the behavior of epithelial cells in different regions of the branching ureteric bud. *Hoxb7/GFP* transgenic mice express GFP throughout the WD and UB epithelium but not in the surrounding mesenchyme or its epithelial derivatives (Figures 1A and 1B) and thus allow UB branching morphogenesis to be visualized in real time during growth of the kidney in organ culture (Srinivas et al., 1999; Watanabe and Costantini, 2004). When mosaic embryos were generated by aggregation of *Hoxb7/GFP* morula-stage embryos with wild-type morulae, the UBs at E11.5 showed a fine interspersion of GFP-labeled (GFP^+) and unlabeled (GFP^-) cells (Figure 1C). This indicated that cells from each of the two parental embryos had contributed to the UB and that they had undergone extensive mixing during the initial outgrowth of the UB.

In an attempt to follow the normal fate of cells in different regions of the growing UB, we visualized the development of these mosaic kidney primordia in organ culture using time-lapse fluorescence microscopy. As the UB underwent branching morphogenesis, the GFP^+ cells contributed strongly to all branches of the UB, and the pattern of close interspersion with GFP^- wild-type cells was maintained (Figure 1D). The failure to form large coherent clones of GFP^+ or GFP^- cells indicated that, following a cell division in the UB epithelium, the daughter cells tend to move rapidly away from each other. Time-lapse analysis of these mosaic kidneys confirmed that there is a great deal of short-range cell motility in the UB epithelium, particularly at the growing tips and somewhat less so in the trunks (Supplemental Movie S1 at http://www.developmentalcell.com/cgi/content/full/8/1/65/DC1/). This suggests that regulation of cell adhesion and motility may contribute to elongation and branching of the epithelial tube.

Lineage Relationships between Normal Tip and Trunk Cells and the Mechanisms of Trunk Elongation

In the mosaic kidneys generated by morula aggregation, the large number of GFP^+ cells made it difficult to follow individual cells and their daughters for more than a few hours with the available resolution. Therefore, to generate mosaic kidneys with a lower proportion of GFP^+ cells in the UB, we used a different method of mosaic production, the injection of genetically marked ES cells into the blastocyst. ES cell lines were derived from the *Hoxb7/GFP* transgenic mouse strain. By injecting a

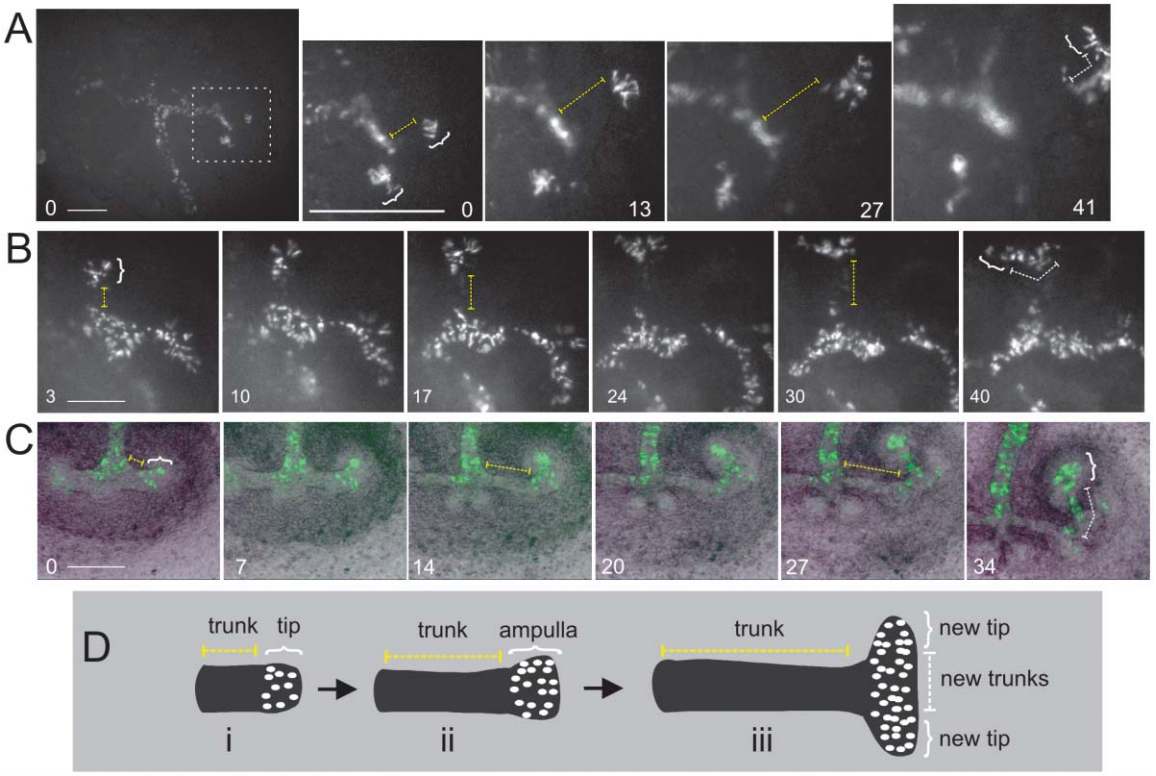

Figure 2. Tracing the Normal Fates of Ureteric Bud Tip Cells by Time-Lapse Analysis of *Hoxb7/GFP*↔Wild-Type Mosaic Kidneys

Mosaic embryos containing a minority of *Hoxb7/GFP* transgenic cells were generated by injection of *Hoxb7/GFP* ES cells into wild-type blastocysts. The kidney primordia were recovered at E11.5 and cultured for time-lapse photomicroscopy. Rows (A)–(C) show the growth of three different mosaic kidneys. In (A), the first panel shows the entire kidney, while the next four panels show a selected region (dashed box in the first panel) at the indicated number of hours of culture. In each of the three kidneys, a cluster of GFP$^+$ cells was initially located in the tip of a UB branch (white bracket), adjacent to a segment of UB trunk devoid of GFP$^+$ cells (yellow dashed line). As the kidney grew, the GFP$^+$ cells at first remained clustered in the tip and increased in number as the tip expanded to form an ampulla. Meanwhile, the trunk extended without incorporating any GFP$^+$ cells from the tip. This is schematized in diagram Di–Dii. Later, the GFP$^+$ cells started to become more widely distributed (beginning at 27 hr in [A], 24 hr in [B], and 20 hr in [C]), with some staying in the tips (white brackets) and others remaining behind in the new trunks (white dashed lines), as schematized in diagram Diii. (A) and (B) are GFP fluorescence images, while (C) shows GFP superimposed on brightfield images. Time-lapse movies of these three kidney cultures are available as Supplemental Movies S2–S4. Scale bars equal 0.25 mm.

small number (1–5) of these ES cells into wild-type blastocysts, we obtained several mosaic kidneys with a low enough proportion of GFP$^+$ cells that it was possible to follow isolated clusters of GFP$^+$ cells and their daughter cells over at least 1–2 days of culture.

The three kidneys shown in Figures 2A–2C and Supplemental Movies S2–S4 are examples in which we could identify a cluster of GFP$^+$ cells in the tip of a UB branch and immediately adjacent to a segment of trunk that was devoid of GFP$^+$ cells. This nonrandom distribution of GFP$^+$ cells was infrequently observed, but when it occurred it allowed us to trace the contribution of the tip cells to the subsequent growth of the branch. The origin of these clusters might be explained if the cells in the tip arise from the rapid proliferation of a small number of progenitor cells so that, by chance, a tip can sometimes be composed disproportionately of GFP$^+$ (or GFP$^-$) cells. This interpretation is supported by additional experiments using Ret mutant ES cells (see below).

During the subsequent extension of these mosaic branches, we could determine whether the GFP$^+$ cells in the tip later contributed to the growing trunk or remained confined to the tip region. In each case, there was an initial phase of growth in which the GFP$^+$ cells increased in number but remained in a localized cluster in the expanding tip or "ampulla." During this phase, the trunk elongated 2- to 3-fold (as indicated by the lengths of the dashed yellow lines in Figure 2) but did not incorporate any GFP$^+$ cells from the tip. Therefore, elongation of the trunk must have occurred by an internal mechanism, rather than by the recruitment of tip cells into the growing trunk. The mechanism of elongation is not clear, but could involve cell proliferation, changes in cell shape, or cell rearrangements such as convergent extension. We know that cell division occurs in the UB trunks, although the rate is lower than at the tips (Michael and Davies, 2004; our unpublished data).

During the later growth of each branch, the GFP$^+$ cells that had been confined to the tip (and/or their daughter cells) became more widely distributed, contributing both to the new tips and to the adjacent region of trunk (Figure 2A, 13–41 hr; Figure 2B, 24–40 hr; Figure 2C, 14–34 hr). The GFP$^+$ tip cells were not observed to migrate back

into the trunk, but rather, some of them were retained in the trunk as others moved forward with the growing tip. The time at which the tip cells started to contribute to the trunks coincided approximately with the initiation of the next generation of branching, as shown schematically in Figure 2D. Here, we use the terms "tip" and "trunk" to denote cell location and not necessarily gene expression profile. However, the tip-derived cells that contribute to the trunk must rapidly switch their pattern of gene expression—otherwise the observed segregation of tip and trunk markers would not occur. Our results imply that during UB branching morphogenesis, some of the tip cells are converted to trunk cells, and thus that tip cells are bipotential and not restricted to a tip cell fate. Whether trunk cells ever move into the tip was not clear from these experiments. However, the occurrence of lateral branching (the de novo formation of a new branch from the side of an existing trunk) during kidney development in organ culture (Watanabe and Costantini, 2004) implies that trunk cells can also be converted to tip cells.

Limited Ability of *Ret*$^{-/-}$ Cells to Contribute to Ureteric Bud Development in Mosaic Kidneys

To investigate the role of GDNF/Ret signaling in renal branching morphogenesis, we next applied this technology to the analysis of mosaic kidneys containing *Ret*$^{-/-}$ cells. In order to specifically examine the ability of the mutant cells to contribute to the Wolffian duct and ureteric bud, we generated *Ret*$^{-/-}$ ES cells that also carried the *Hoxb7/GFP* transgene. These ES cells were injected into wild-type blastocysts, and the kidneys were dissected from the resulting embryos at E11.5 and cultured. They were photographed periodically by fluorescence microscopy to follow the GFP^{+} cells (i.e., the mutant cells in the WD or UB) and by brightfield microscopy to follow the overall growth of the kidney and the formation of mesenchymal condensates.

The GFP^{+}, *Ret*$^{-/-}$ mutant cells were able to contribute extensively to the Wolffian duct and to the primary branch of the ureteric bud (i.e., the presumptive ureter) in many mosaic kidneys (Figures 3A–3C). This is consistent with the ability of all *Ret*$^{-/-}$ embryos to form a morphologically normal Wolffian duct and of some of them to form a primary ureteric bud (Schuchardt et al., 1994, 1996). However, in cases where there was a high proportion of *Ret*$^{-/-}$ cells in the Wolffian duct and UB (more than 50%, approximately), either the UB failed to elongate normally, even after 61 hr of culture (e.g., Figure 3A), or the kidney developed very poorly, based on overall kidney size and the number of mesenchymal condensates (e.g., Figure 3B). In contrast, those kidneys with a minority of mutant cells developed normally (Figure 3C). Therefore, in the presence of a majority of *Ret*-deficient UB cells, the few wild-type UB cells have a limited ability to compensate.

Judging by the expected positions of the UB tips within the mesenchymal condensates at the periphery of the kidney, it appeared that as the mosaic UB underwent several rounds of branching, the mutant cells contributed to the UB trunks in the central region of the kidney but not to the tips in the periphery (Figures 3B and 3C).

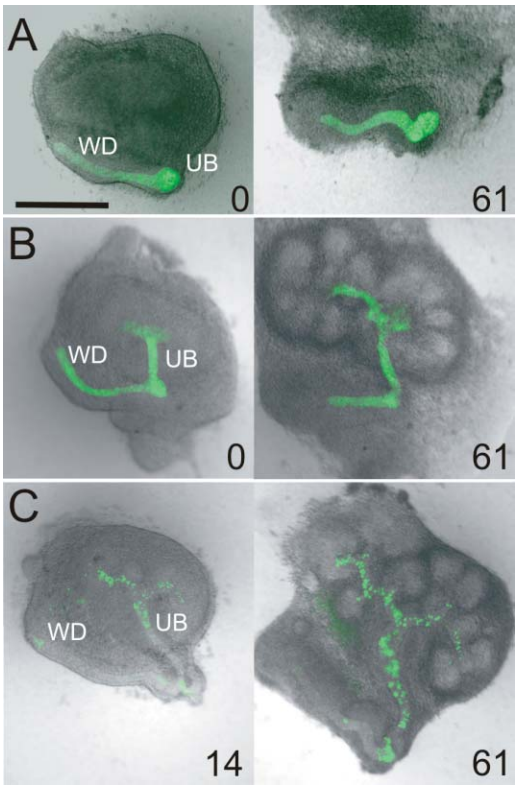

Figure 3. *Ret*$^{-/-}$ Cells in Mosaic Kidneys Can Contribute to the Wolffian Duct and the Primary and Secondary Branches of the Ureteric Bud, but when Abundant, They Impair Kidney Growth

Ret$^{-/-}$ ES cells carrying the *Hoxb7/GFP* transgene were injected into wild-type blastocysts, and the kidneys were recovered at E11.5 and cultured for 61 hr. For each kidney (A–C) at two time points, the GFP fluorescence image (pseudocolored green, representing mutant cells) was superimposed on the brightfield image (showing the entire kidney, including the mesenchymal condensates). In (A), there was a high proportion of GFP^{+} (i.e., mutant) cells in the Wolffian duct (WD), and the UB showed only minimal outgrowth by 61 hr. In (B), there was a somewhat lower proportion of mutant GFP^{+} cells in the WD, and thus a higher proportion of wild-type UB cells. The UB had grown out and branched by E11.5 (time 0), but by 61 hr of culture only a relatively small number of mesenchymal condensates was induced. The mutant UB cells were only found in the central region of the kidney and seemed to be absent from the most distal branches. In (C), there were fewer mutant GFP^{+} cells in the WD and UB, and the development of the kidney was essentially normal at 61 hr. GFP^{+} mutant cells were found in several UB branches, but not in the periphery of the kidney where the UB tips are located. Scale bar equals 0.5 mm.

To confirm this, we cultured additional mosaic kidneys, and after several days of observation, we stained them with anti-cytokeratin antibodies to visualize clearly the entire epithelium of the UB tree. This verified that the mutant cells failed to contribute to the UB tips or even to the most distal trunks (Figures 4C and 4D). In contrast, control GFP^{+} ES cells contributed to all regions of the UB (Figures 4A and 4B). To verify that the mutant cells displayed the same defects during in vivo development as they did in organ culture, we examined mosaic kidneys obtained in vivo at E14.5–E15.5. The mutant cells were found in the ureter and first generation branches

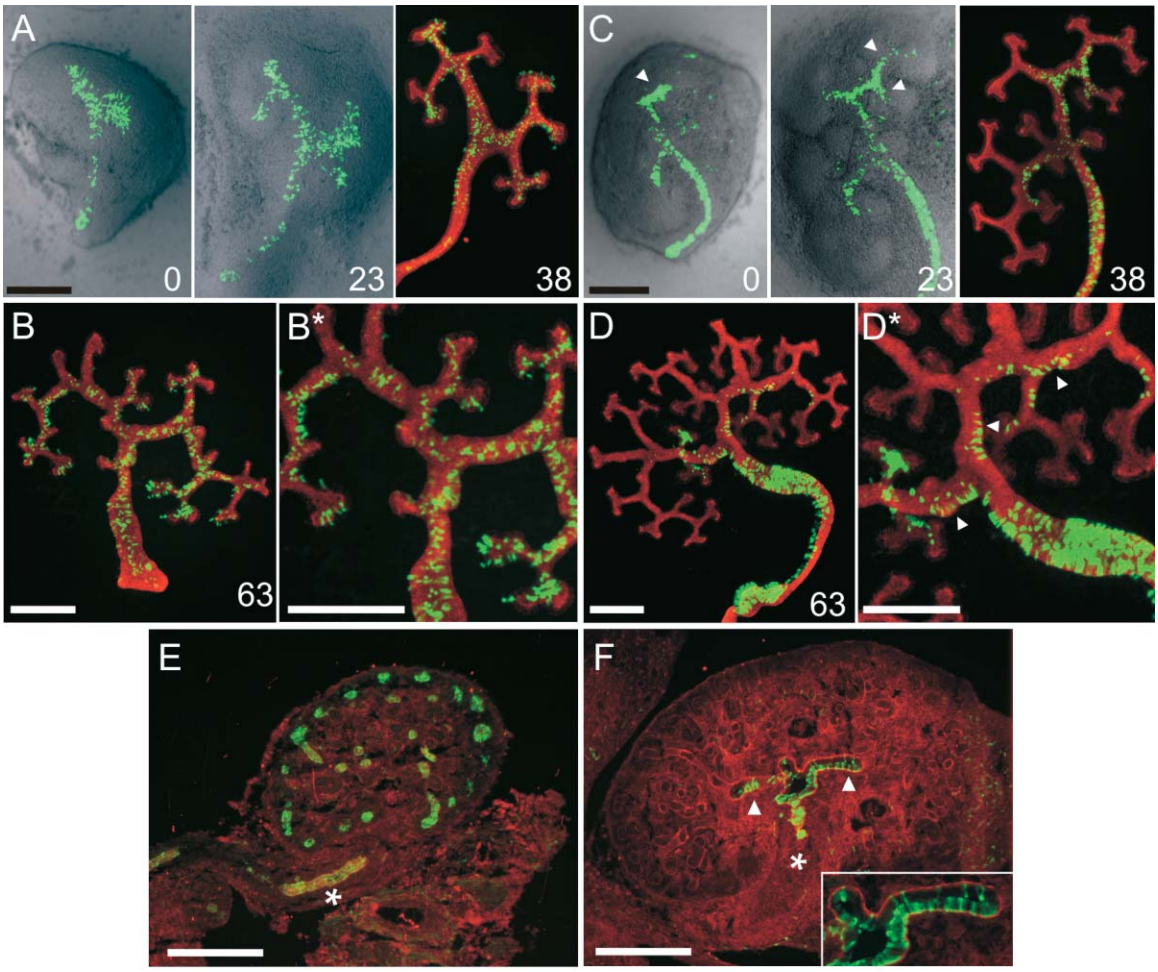

Figure 4. *Ret*$^{-/-}$ Cells in Mosaic Kidneys Are Excluded from the Distal Ureteric Bud Branches and Contribute Asymmetrically to Proximal UB Branches

(A) Culture of a control kidney from an E11.5 mosaic embryo made by injecting *Hoxb7/GFP* ES cells into a wild-type blastocyst. Composite GFP and brightfield images are shown at 0 and 23 hr, and an anti-cytokeratin-stained image (red) superimposed with the GFP image is shown at 38 hr.

(B) A different control mosaic kidney cultured for 63 hr and stained with anti-cytokeratin.

(B*) Enlargement of (B). Note the ability of the control GFP^{+} cells to contribute to all regions of the UB, including the tips, in an apparently random pattern.

(C) Culture of a mosaic kidney containing *Ret*$^{-/-}$, *Hoxb7/GFP* cells. GFP^{+} cells lacking Ret are found in several generations of UB branches and can be seen moving from a tertiary trunk (arrowhead at 0 hr) into two quaternary trunks (arrowheads at 23 hr). However, anti-cytokeratin staining at 38 hr shows that they are excluded from the UB tips and the more distal trunks.

(D) Kidney from a different mosaic embryo made with *Ret*$^{-/-}$, *Hoxb7/GFP* ES cells, recovered at E11.5 and cultured for 63 hr.

(D*) Enlargement of (D), showing the tendency of the mutant cells to contribute in an asymmetric pattern, often along the side of the UB epithelium closest to the ureter (arrowheads).

(E) Section of in vivo E15.5 control mosaic kidney, showing contribution of GFP^{+} cells to all regions of the UB, including the ureter (asterisk), central UB branches, and UB tips in the periphery.

(F) Section of in vivo E14.5 mutant mosaic kidney, showing contribution of GFP^{+} cells to the ureter (asterisk) and first generation UB branches (arrowheads) but not to distal branches or tips. Inset in (F), enlargement showing preferential contribution of mutant cells to the side of the UB closest to the ureter.

Scale bars equal 0.25 mm for (A)–(D) and 1 mm for (E) and (F).

but not in the peripheral branches (Figure 4F), while control GFP^{+} cells contributed throughout the UB (Figure 4E). These results establish that cells lacking Ret have a cell-autonomous defect in their ability to participate in ureteric bud morphogenesis.

One of the consequences of Ret signaling is believed to be the production by the UB of secreted paracrine signals: these include a yet to be identified signal that maintains and patterns the stromal cells (Batourina et al., 2001), as well as the expression of Wnt11, a secreted factor required for normal UB morphogenesis (Majumdar et al., 2003; Pepicelli et al., 1997). However, if the primary role of Ret signaling were to induce the expression of secreted factors, we would expect that

Ret$^{-/-}$ cells could survive and proliferate extensively throughout the UB, as long as enough wild-type cells were present to produce the paracrine signals. Our results argue against such a model: while Ret signaling may well contribute to the paracrine functions of the UB, it must have critical cell-autonomous functions as well.

Ret$^{-/-}$ Cells Are Specifically Excluded from the Ampulla but Can Contribute to Several Generations of Ureteric Bud Trunks

Despite their exclusion from the most distal branches at any given time, *Ret*$^{-/-}$ cells were found in the more proximal trunks of the UB (i.e., in the secondary and sometimes tertiary and quaternary branches) after several days of culture. Figure 4C shows an example in which mutant cells were present in a tertiary UB trunk at the beginning of the culture (time 0, arrowhead), and by 23 or 38 hr had populated the next generation of trunks (23 hr, arrowheads). Thus, the *Ret*$^{-/-}$ cells were able to persist and proliferate within the UB and to participate in the formation of new branches. This observation was at first difficult to reconcile with the prevailing view of renal branching morphogenesis: since nearly all new branches form at the tips of existing branches (Lin et al., 2001, 2003; Watanabe and Costantini, 2004), how did the mutant cells get as far as the quaternary trunks if they were never present in the tips during earlier generations of branching? Another unexpected finding was that the *Ret*$^{-/-}$ cells were often asymmetrically located on the "proximal" side of the secondary and tertiary UB trunks (i.e., on the side facing the parental branch; Figure 4D*, arrowheads), while control GFP^{+} cells were distributed apparently at random (Figure 4B*).

A potential explanation for both of these observations was obtained by examining additional mosaic kidneys at earlier stages of branching morphogenesis. When the primary UB had first grown out from the WD and formed a swollen ampulla at its tip (E11.0), in preparation for the first branching event, the mutant cells were present throughout the trunk of the UB but were excluded from the ampulla (Figure 5A). In ureteric buds at the "T-stage" (E11.5), when the UB had branched for the first time, to form the second generation of branches, the mutant cells now contributed to both of the second generation branches but did not extend all the way to the tips (Figures 5C, 5C*, 5D, and 5D*). Furthermore, the mutant cells were clearly restricted to the proximal side of each branch. In ureteric buds at a slightly more advanced stage, when each of the second generation tips had swollen to form an ampulla, in preparation for the next round of branching, the mutant cells were again present right up to, but not within, the expanded ampulla (Figures 5E and 5E*). In contrast, in mosaics made with control ES cells, the GFP^{+} cells were randomly distributed throughout the entire UB epithelium (e.g., Figure 5B). These observations show that cells lacking Ret are specifically unable to participate in formation of the ampulla, the region of the UB in which Ret is most highly expressed (Pachnis et al., 1993).

Exogenous GDNF is able to induce the outgrowth of ectopic UBs from the wild-type Wolffian duct, but not from *Ret* mutant Wolffian duct (Sainio et al., 1997). Consistent with this observation, when mosaic WDs were cultured in medium containing exogenous GDNF, ectopic UBs formed but they were composed only of wild-type cells and devoid of *Ret*$^{-/-}$ cells (Figure 6A). In contrast to the requirement for Ret for UB outgrowth from the WD, it has been observed that growth of the UB in kidney primordia isolated from *Ret*$^{-/-}$ embryos can be rescued to some extent by the addition of exogenous GDNF to the culture medium (Popsueva et al., 2003). This is thought to reflect the ability of GDNF to signal through GFRα1 in the absence of Ret, under some circumstances (Poteryaev et al., 1999; Trupp et al., 1999). This raised the possibility that the addition of high levels of exogenous GDNF to the medium might similarly rescue the ability of *Ret*$^{-/-}$ cells to contribute to the UB tips in mosaic kidneys. However, while addition of GDNF resulted in massive overgrowth and swelling of the UB ampullae, as previously observed (Pepicelli et al., 1997), only wild-type cells gave rise to the ampullae, and the mutant cells were excluded (Figures 6B and 6C). Thus, even though *Ret*$^{-/-}$ UB cells may be able to respond to GDNF (Popsueva et al., 2003), they are unable to compete efficiently with wild-type cells in mosaic kidneys.

Since cells lacking Ret can survive and proliferate in the UB trunks, why are they excluded from the tips? Presumably, there are signals other than GDNF acting on the trunks to induce cell proliferation and survival, so there is no requirement for the Ret RTK in trunk cells. One possibility is that *Ret*$^{-/-}$ cells in mosaic UBs are transiently incorporated into the tips, but they don't survive there, because the same signals and receptors that allow them to survive in the trunks may not be available in the tips. However, we never saw evidence of dying GFP^{+}, *Ret*$^{-/-}$ cells in the ampullae at any stage of UB branching. A second possibility is that Ret mutant cells have a migration defect and thus fail to migrate into the tip; however, such a model would require that in normal kidney development, Ret-expressing cells migrate from the UB trunk into the tip. Since Ret-expressing cells are not observed in the trunk during normal UB branching morphogenesis, this model appears unlikely. A third explanation, and one we favor, is that the *Ret*$^{-/-}$ cells are excluded from the ampulla because the ampulla is formed by a burst of GDNF-dependent cell proliferation at the UB tip. Even if some *Ret*$^{-/-}$ cells are close to the tip when the ampulla begins to form, they are quickly overgrown by the proliferation of wild-type cells. Consistent with this model, the rate of cell proliferation is normally much higher in the UB ampullae than in the trunks (Fisher et al., 2001; Michael and Davies, 2004; T.W. and F.C., unpublished data). Furthermore, the massive overgrowth of the UB tips induced by high levels of GDNF (Figure 6; Pepicelli et al., 1997) suggests that one normal role of endogenous GDNF is to induce formation of the ampulla. The GDNF family ligand neurturin is also expressed in the developing kidney (Davies et al., 1999), but it is apparently not critical for kidney development (Heuckeroth et al., 1999), so GDNF is apparently the relevant Ret ligand for this process.

The absence of the mutant cells from the most UB distal branches at later stages of kidney development might similarly be explained by an absolute requirement for Ret for proliferation of UB tip cells. It is striking that, rather than a gradual dilution of the mutant cells, there is a sharp demarcation between the trunk, where mutant

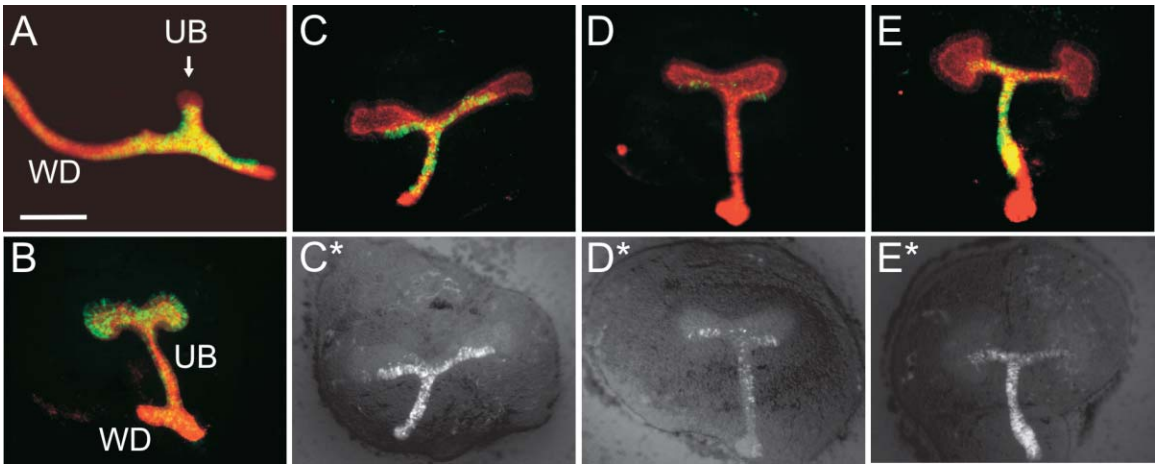

Figure 5. *Ret*$^{-/-}$ Cells Contribute to the Primary UB Trunk and to the Proximal Side of the Secondary UB Trunks but Are Excluded from the Ampullae that Form at the Tips

(A) Ureteric bud emerging from the Wolffian duct (WD) of an E11.0 mosaic embryo generated with *Ret*$^{-/-}$, *Hoxb7/GFP* ES cells (superimposed GFP and anti-cytokeratin images). The WD and trunk of the UB contain many mutant GFP^{+} cells, while the terminal ampulla of the UB (arrow) is devoid of mutant cells.

(B) Kidney from a control mosaic made with *Hoxb7/GFP* ES cells, at the T-stage (E11.5). The control GFP^{+} cells contribute throughout the UB epithelium.

(C–E) Two mosaic kidneys containing GFP^{+}, *Ret*$^{-/-}$ cells at the T-stage (C and D); (E) is a mosaic kidney containing GFP^{+}, *Ret*$^{-/-}$ cells at a slightly more advanced stage, when large ampullae have formed at the tips of the T. The upper image in each pair (C, D, E) is a superimposition of the GFP image with the anti-cytokeratin image, while the lower image (C*, D*, E*) shows GFP fluorescence superimposed with brightfield illumination. At the T-stage, the mutant cells are asymmetrically localized to the proximal side of the secondary UB trunks (the side facing the primary UB) and do not extend all the way to the tips. When the tips expand to form ampullae (E, E*), the mutant cells are excluded from the ampullae. Scale bars equal 0.25 mm.

cells can contribute, and the tips, where they are excluded, at the primary bud stage and the T-stage (Figure 5). We suggest that cells in the ampulla at any given stage will give rise to most of the UB epithelium that forms beyond this point, including all of the future tip cells and many of the trunk cells. Since the mutant cells are excluded from the ampulla, they cannot contribute to structures derived from this portion of the UB epithelium. This model is also consistent with the ability of normal tip cells to give rise to both tip and trunk cells, as shown in Figure 2.

Although the *Ret*$^{-/-}$ cells were excluded from the ampullae and from the most distal UB branches, nevertheless they frequently contributed to several generations of trunks. How did they get there? If the trunk cells were all derived from the ampullae of the previous branch generation, and *Ret*$^{-/-}$ cells were excluded from the ampullae, the mutant cells should never have proceeded past the first ampulla. This suggests that not only do cells of the ampulla give rise to trunk cells of the next generation, but cells from the trunk also contribute to the next generation of trunks. How might this occur? One clue comes from the observation that *Ret* mutant GFP^{+} cells (but not wild-type GFP^{+} cells) contribute preferentially to the surface of the branched UB epithelium closest to the parental branch. This was most obvious in the T-shaped UB at E11.5 (Figures 5C, 5C*, 5D, and 5D*) but also was apparent in some kidneys at a more advanced stage of development, after several rounds of branching had occurred (Figure 4D*). This observation suggests a mechanism of branching morphogenesis in which the epithelium of the ampulla, together with the adjacent region of trunk epithelium, is remodeled to form the next generation of branches.

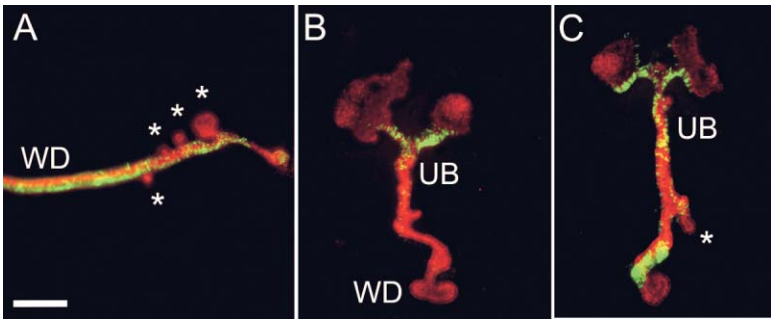

Figure 6. Exogenous GDNF Fails to Rescue the Ability of *Ret*$^{-/-}$ Cells to Contribute to the UB Ampullae

(A) Mutant mosaic Wolffian duct (E11.5) cultured with exogenous GDNF, which induced the outgrowth of several ectopic UBs (asterisks). The ectopic UBs are devoid of GFP^{+} mutant cells.

(B and C) Two mutant mosaic E11.5 kidneys cultured with exogenous GDNF, which induced the abnormal swelling of the UB ampullae, as well as the outgrowth of an ectopic UB (asterisk). The GFP^{+} mutant cells failed to contribute to the ampullae or the tip of the ectopic UB.

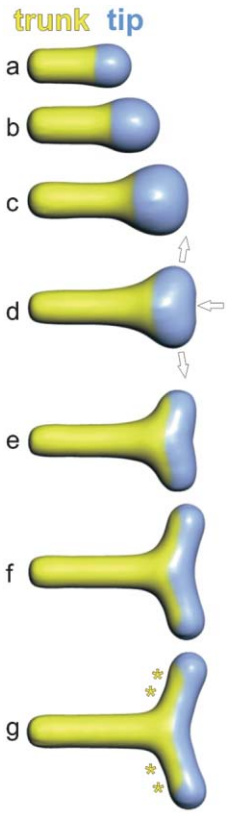

Figure 7. A Model for the Fate of Tip and Trunk Cells during Ureteric Bud Elongation and Branching

(a) A branch of the UB divided into trunk (yellow) and tip (blue). At subsequent stages of growth and branching, regions of the UB derived from the original trunk are shown in yellow and those derived form the original tip in blue. In (b) and (c), the trunk elongates through an internal mechanism, while the tip cells proliferate under the influence of GDNF/Ret signaling to form the enlarged ampulla. During the next branching event (beginning in d), the ampulla and the adjacent trunk epithelium are remodeled by hypothetical forces indicated by the three arrows in (d). This branching process causes the original trunk epithelium (yellow) to form the proximal side of two new trunks (yellow asterisks in g), while cells from the ampulla (blue) form the two new tips and the distal epithelium of the new trunks.

The mechanism we envision is illustrated in Figure 7. The first step in branching is the formation of the ampulla, driven by a burst of GDNF-induced proliferation of the Ret-expressing cells at the UB tip (colored blue in the Figure 7). In a mosaic UB containing wild-type as well as *Ret*$^{-/-}$ cells, the ampulla itself is devoid of mutant cells, but these mutant cells can be present in the trunk right up to the edge of the ampulla (e.g., Figure 5E). If the new branches were derived entirely from the epithelium of the ampulla, they would be devoid of mutant cells. Therefore, we suggest that branching involves the remodeling of a segment of trunk epithelium (colored yellow in Figure 7) together with the ampulla (colored blue) to form the two new branches. Because of the topology of tubular branching, the trunk epithelium gives rise only to the proximal surface of the new trunks, while the epithelium of the ampulla gives rise to the distal surfaces of the new trunks and to the two new tips. It is noteworthy that the asymmetric distribution of *Ret*$^{-/-}$ cells is not seen in the initial UB as it evaginates from the Wolffian duct (Figure 5A) or in the presumptive ureter that derives from this structure (Figures 5C and 5E), where the distribution appears random. This suggests that the mechanism of initial UB outgrowth from the WD is different from the subsequent expansion and branching of the ampulla.

The fluid nature of the UB epithelium envisioned by this model is consistent with recent time-lapse observations of UB branching morphogenesis using the *Hoxb7/GFP* transgenic mice. These studies showed that "branch points," the regions of the UB trunk where multiple trunks connect to each other, are not fixed structures but can be extensively remodeled during UB growth (Watanabe and Costantini, 2004). Whether the forces that cause this remodeling arise internally (generated by cytoskeletal changes within the epithelial cells, for example) or whether they are imposed externally (for example, by extracellular matrix proteins, as has been suggested for branching of the salivary gland epithelium; Sakai et al., 2003) is not yet clear.

Conclusions

We have used mosaic analysis with a GFP marker to follow the allocation of wild-type cells within the ureteric bud epithelium during normal branching morphogenesis, as well as the behavior of similarly marked cells lacking the Ret receptor tyrosine kinase. Our results provide new insight into the mechanisms of normal UB growth and the role of Ret signaling in this process. UB trunks can elongate transiently without incorporating cells from the tip, but over longer periods of time, the descendants of tip cells give rise to much of the trunk epithelium as well as to the new tips. Cells lacking Ret can contribute to the primary outgrowth of the UB, but they are excluded from the ampulla that forms at its terminus, apparently by a Ret-dependent proliferation of tip cells under the influence of GDNF. As a consequence, the *Ret*$^{-/-}$ cells also fail to contribute to the more distal UB branches, although some of these cells persist and proliferate in the trunks of more proximal branches. The importance of Ret for formation of the ampulla also explains why GDNF acts as a ramogen, since formation of the ampulla is the initial step in the generation of new branches. The ability of *Ret*$^{-/-}$ cells to contribute to several generations of UB trunks, often in an asymmetric distribution, suggests a branching mechanism in which the ampulla together with the adjacent UB trunk epithelium is remodeled to form the new branches. The methods of mosaic analysis employed here should be useful to define the developmental functions of other genes that cause an early failure in organogenesis.

Experimental Procedures

Derivation of Embryonic Stem Cell Lines

Mice heterozygous for a *Ret* null mutation (*Ret*$^{+/-}$; Schuchardt et al., 1994) were crossed with *Ret*$^{+/-}$ mice that carried the *Hoxb7/GFP* transgene (Srinivas et al., 1999) in a mixed genetic background. On day E3.5, blastocysts were seeded onto mitomycin C-treated primary embryonic fibroblast cells (feeder cells) and cultured in DMEM (GIBCO-BRL) containing 15% fetal calf serum (FCS), 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM nonessential amino

acids, 0.29 mg/ml glutamine, LIF (Leukemia Inhibitory factor), and 0.001% β-mercaptoethanol for 4–5 days until the blastocysts hatched and inner cell mass outgrowths formed. The outgrowths were dissociated to 2–6 cell aggregates in 0.25% trypsin and plated onto feeder cells in 4-well plates. After 6–8 days, potential ES cell colonies were picked and expanded in 96-well plates. Established ES cell lines were genotyped by Southern blotting with a GFP (Srinivas et al., 1999) and a *Ret* probe (Probe A; Schuchardt et al., 1994) to determine the presence of the *Hoxb7/GFP* transgene and the genotype at the *Ret* locus. One cell line thus obtained, ES(8), was $Ret^{+/-}$ and carried *Hoxb7/GFP* transgene. Line ES(8) was used as a control ES cell line for generation of mosaic mice, as the heterozygous *Ret* null allele has no effect on kidney development (Schuchardt et al., 1994). Subsequently, other ES cell lines that were $Ret^{+/+}$ and carried the *Hoxb7/GFP* transgene were obtained, and these gave identical results in mosaic analyses.

As no homozygous $Ret^{-/-}$ ES cell lines were found, the remaining wild-type *Ret* allele in line ES(8) was mutated by homologous recombination. The targeting vector was identical to that used to generate the original null allele (Schuchardt et al., 1994), except that it contained a pgk-hygromycinPA selectable marker in place of pMC1neoPA. The vector was linearized with SalI before electroporation into ES(8) cells, which were grown on hygromycin-resistant feeder cells and selected in 200 μg/ml hygromycin. Targeted clones were identified by EcoRI digestion of genomic DNA and hybridization of Southern blots with Probe A. The wild-type, pMC1neoPA, and pgk-hygromycinPA alleles yielded bands of 18 kb, 14 kb, and ~16 kb, respectively. Two independently selected $Ret^{-/-}$ clones (ES3D2 and ES3G8-11) were used for mosaic analyses and yielded identical results. All ES cell lines used had a normal karyotype.

Generation of Mosaic Mice

The mosaic embryo analyzed in Figures 1C and 1D and Supplemental Movie S1 was generated by aggregating a single *Hoxb7/GFP* transgenic morula with a single wild-type morula. For the experiments of Figure 2, 1–5 ES cells were injected into each blastocyst (Hogan et al., 1994). For all the other experiments, 10–15 ES cells were injected into each blastocyst. Blastocysts were derived from wild-type C57BL6/J, Swiss Webster, or B6CBAF1 mice.

Kidney Organ Culture and Time-Lapse Photography

Mosaic embryos were recovered at E11.5, and the kidney rudiments or entire urogenital systems were cultured on Transwell filters (Costar) in 5% CO_2 at 37°C, as described (Srinivas et al., 1999; Watanabe and Costantini, 2004) in DMEM with 10% fetal calf serum (Hyclone), 1× penicillin/streptomycin (GIBCO-BRL), and 0.29 mg/ml glutamine (GIBCO-BRL). For experiments with exogenous GDNF, the cultures were supplemented with rat recombinant GDNF (R&D Systems) at 100 ng/ml. Time-lapse imaging was performed as previously described (Watanabe and Costantini, 2004). Briefly, the Transwell filter units were enclosed in a culture chamber attached to a Prior automated stage on a Nikon TE300 microscope. The microscope, stage, and digital camera were controlled by a computer running SimplePCI (Compix, Inc.). Digital photographs (150 dpi, 1280 × 1024 pixel grayscale TIF files) of each kidney were taken at 20–40 min intervals, using an EGFP filter set (HYQ-GFP, Ex560/4, Chroma-41001) and a Hamamatsu Orca digital CCD camera.

Immunostaining

Anti-cytokeratin staining of organ cultures was performed as described (Ehrenfels et al., 1999; Vega et al., 1996) with a few modifications. Briefly, cultured kidneys were fixed on Transwell filter membranes in ice-cold 4% paraformaldehyde for 10 min followed by two brief washes in phosphate-buffered saline containing 0.1% Tween-20 (PBST). All subsequent washes and incubations were done in PBST. Cultures were blocked in 2% heat-inactivated goat serum (Sigma) for 1 hr at room temperature (rt), washed, and incubated with a 1:300 dilution of pan-cytokeratin monoclonal antibody (mAb) mix (Sigma) overnight at 4°C. After a 30 min wash, they were incubated with indocarbocyanine (Cy3)-conjugated donkey anti-mouse mAb (Jackson) at 1:800 dilution at rt for 1 hr. After a 30 min wash, the kidneys were visualized by fluorescence microscopy. Superimposition of GFP with brightfield or Cy3 images was performed using Adobe Photoshop.

For immunostaining of frozen sections, kidneys were fixed in ice-cold 4% paraformaldehye for 2 hr at 4°C. Tissues were washed in PBS, then incubated in 15% Sucrose/PBS and 30% Sucrose/PBS for several hours each, embedded in O.C.T. compound (Tissue-Tek), and frozen in dry-ice. 15 μm sections were cut on a cryostat, fixed in acetone for 5 min, and washed in Tris-buffered saline (TBS) and TBST (TBS with 0.025% Triton-X 100). Sections were blocked in 10% heat-inactivated horse serum (Sigma) with 1% BSA in TBS at room temperature for 2 hr and incubated at 4°C overnight in a mixture of primary antibodies (anti-GFP polyclonal, Abcam 6556-25, 1:500 and anti-pan cytokeratin) in TBS with 1% BSA. Sections were briefly washed in TBST and incubated in a mixture of secondary antibodies (FITC-conjugated donkey α-rabbit, Jackson ImmunoResearch, 1:200 and Cy3-conjugated α-mouse, see above) at room temperature for 2 hr. Sections were washed in TBST before being photographed by fluorescence microscopy.

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