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GDNF/Ret signaling and renal branching morphogenesis

From mesenchymal signals to epithelial cell behaviors

Frank Costantini

Department of Genetics and Development; Columbia University Medical Center; New York, NY USA

Signaling by GDNF through the Ret receptor tyrosine kinase is required for the normal growth and morphogenesis of the ureteric bud (UB) during kidney development. Recent studies have sought to understand the precise role of Ret signaling in this process, and the specific responses of UB cells to GDNF. Surprisingly, the requirement for *Gdnf* and *Ret* was largely relieved by removing the negative regulator *Spry1*, revealing unexpected functional overlap between GDNF and FGF10. However, the kidneys that developed without *Gdnf*/*Ret* and *Spry1* displayed significant branching abnormalities, suggesting a unique role for GDNF in fine-tuning UB branching. GDNF/Ret signaling alters patterns of gene expression in UB tip cells, and one critical event is upregulation of the ETS transcription factors Etv4 and Etv5. Mice lacking *Etv4* and *Etv5* fail to develop kidneys. Thus, these genes represent key components of a regulatory network downstream of *Ret*. Studies of chimeric embryos in which a subset of cells lack either *Ret*, *Etv4/5* or *Spry1* have revealed an important role for this pathway in cell movement. Ret signaling, via Etv4 and Etv5, promotes competitive cell rearrangements in the nephric duct, in which the cells with the highest level of Ret signaling preferentially migrate to form the first ureteric bud tip.

Introduction

The kidney is a complex organ, whose basic components include blood vessels, thousands of nephrons (the excretory units, each consisting of a proximal tubule, loop of Henle, distal tubule and connecting tubule) and a tree-like collecting system (including cortical and medullary collecting ducts, calyces, papilla and ureter) that conveys the urine from each nephron to the bladder. The renal collecting system in some ways resembles the highly branched epithelia of other organs, such as the lung, but also has many distinct features, such as the functions and properties of the differentiated epithelial cells, specific branching patterns and some of the signals and genes that regulate its development. Development of the collecting system has been investigated extensively because of its attractive features as a basic experimental system to study morphogenesis, the many types of birth defects that can affect it in humans and the possibility of eventual repair or regeneration to treat human kidney disease. This review will describe some of the molecular and cellular mechanisms underlying the formation of the collecting duct system, focusing on recent experiments in our laboratory that have addressed these issues.

The nephrons and the collecting system both originate in the intermediate mesoderm (IM) (**Fig. 1A**). The dorsal portion of the IM forms a tube, called the nephric duct (ND) or Wolffian duct, whose tip elongates caudally between about E8.0 and E9.5, until it contacts and joins the cloaca. The ventral IM remains as a mesenchymal cell population called the nephrogenic cord (NC). The rostral ND and NC together generate the pronephros and mesonephros, primitive renal tubules that are transient organs in higher vertebrates and will not be further discussed here. The metanephric (or adult) kidney forms at the level of the hindlimb, through inductive interactions between the ND and a specialized region of the NC, called the metanephric mesenchyme (MM) (**Fig. 1B and C**). The MM contains progenitor cells that will later form the epithelia of the metanephric nephrons,[1-3] and it also produces the inductive signals that promote and position the outgrowth of the ureteric bud (UB) from the ND and its subsequent branching (**Fig. 1D–F**).

Outgrowth of the UB is a crucial initiating event of kidney development, which depends on the prior differentiation of the MM from the IM. The UB gives rise to the epithelium of the collecting ducts, calyces, pelvis and ureter. Failure to make a UB invariably leads to renal agenesis. The correct rostral-caudal positioning of the UB is very important, as buds that form in the wrong position results in a ureter that fails to connect correctly to the bladder.[4] After the UB penetrates the metanephric mesenchyme (E11.0), it begins to branch repeatedly, mostly by bifurcation at the tips.[5] Other modes of branching (lateral budding and terminal trifurcation) also occur less frequently, at least in organ culture;[5] the patterns of branching that occur in vivo have not been well documented in the mouse kidney, as they have been in the lung.[6] Consistent with a specific role in growth and branching, the cells at the UB tip express many genes that are not

Correspondence to: Frank Costantini; Email: fdc3@columbia.edu
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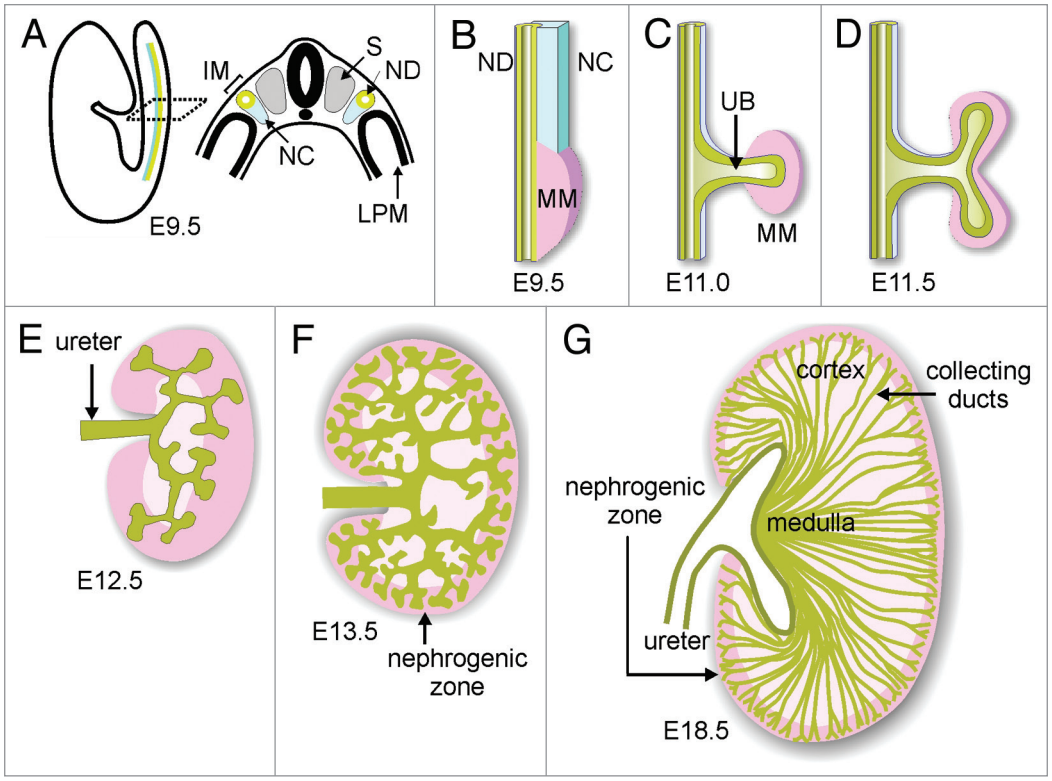

Figure 1. Schematic diagram of development of the renal collecting duct system. (A) Diagram illustrating location of intermediate mesoderm (IM), including the nephric duct (ND) and nephrogenic cord (NC) in the mouse embryo. S, somite; LPM, lateral plate mesoderm. (B) Formation of metanephric mesenchyme (MM). (C) Outgrowth of ureteric bud (UB). (D) First branching of the UB. (E and F) Continued UB branching and formation of the nephrogenic zone (dark pink). (G) Elongation of collecting ducts to form the medulla. Modified from reference 13.

expressed by cells in the trunks (i.e., the tubular portions) and vice versa.[7,8]

The UB branches for about 10 generations over the next 3–4 days as the kidney grows, then it enters a phase in which the UB trunks elongate extensively, followed by 1–2 more rounds of terminal branching[9] (**Fig. 1G**). Since the UB tips continually induce new nephrons as they branch, mutations that even subtly reduce UB branching can lead to renal hypoplasia with reduced nephron number. As low nephron number is thought be a risk factor for hypertension and kidney failure,[10-12] the control of UB branching morphogenesis is not only a fascinating biological problem, but a medically significant one.

Ureteric bud branching morphogenesis is known to be largely controlled by signals from the surrounding mesenchyme, and also probably by signaling within the epithelium. But how do these signals result in changes in the size and shape of the UB epithelium? Presumably, they lead to changes in gene expression patterns in UB cells (as well as to more direct effects on the cell, e.g., via the cytoskeleton), and these in turn cause alterations in cellular behavior. Ultimately, in order for morphogenesis to occur, the properties of UB cells (e.g., cell size, shape, adhesion to other cells) or their behaviors (e.g., survival, division, migration) must be altered (**Fig. 2**). I will next describe some recent experiments that have sought to elucidate each of these steps (extracellular signals, gene expression changes and cellular behaviors) in the control of UB morphogenesis.

Signals that Control Ureteric Bud Morphogenesis

Several signals from the MM, which together control UB outgrowth and branching, have been described (reviewed in ref. 13–15). GDNF is apparently the most important inducer of UB outgrowth, as the UB fails to form in most *Gdnf*$^{-/-}$ mutant embryos, as in those for *Ret*, the receptor tyrosine kinase (RTK) for GDNF or *Gfra1*, the GDNF co-receptor (reviewed in ref. 16 and 17). Similarly, mutations in many other upstream genes that reduce or eliminate *Gdnf* expression (e.g., *Npnt*, α8β1 integrin, *Gdf11*, *Six2*, *Eya1*, *Pax2* or *Hox11* paralogs) also cause UB outgrowth to fail. Conversely, most of the mutations in mice that cause multiple UBs (leading to duplex ureters and kidneys) do so by expanding rostrally the domain of *Gdnf* expression (*Slit2, Robo2, FoxC2*) or failing to negatively regulate the response to GDNF (*Spry1, Bmp4*).

Several other growth factors that signal through receptor tyrosine kinases also play a role in UB branching, but their importance for UB outgrowth from the ND remained unclear. Studies of the ND in organ culture show that several members of the FGF family are competent to induce ureteric budding.[18] Lack of *Fgf7* or *Fgf10* in vivo causes reduced UB branching leading to renal hypoplasia, as does deletion of *Fgfr2* in the UB epithelium.[19] Similarly, VEGF-A,[20] HGF and EGF signaling promote UB branching.[21,22] However, none of these mutations in mice interfered with UB

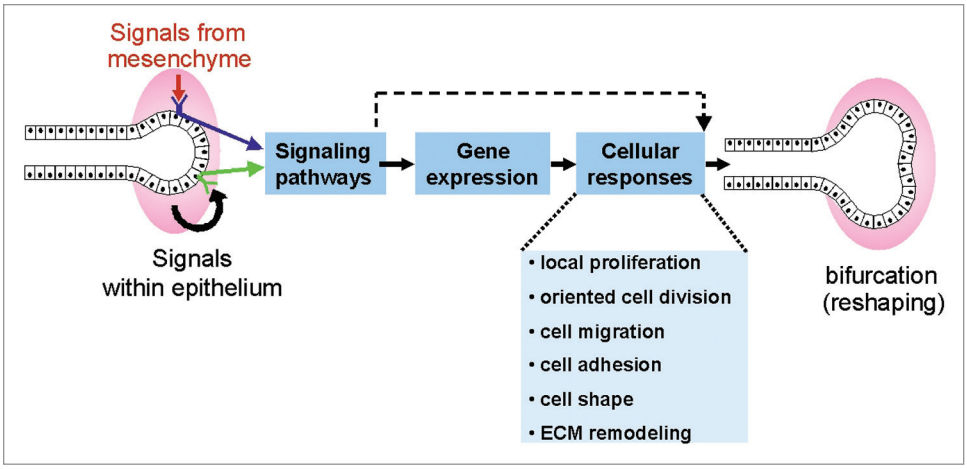

Figure 2. Working model for some of the events underlying branching morphogenesis. Ureteric bud branching morphogenesis is controlled by signals (both promoting and inhibiting branching) from the metanephric mesenchyme and probably other signals within the epithelium. The receptors trigger intracellular signaling pathways, which lead to changes in gene expression, as well as transcription-independent changes in the cell. Some of the cellular responses that may underlie branching (i.e., changes in the shape of the epithelium) are listed.

outgrowth from the ND, and their affects on renal branching were relatively minor. Thus, the prevailing view was that GDNF has a unique role in UB outgrowth in vivo, and that it then plays a continuing role, together with several other factors, to control the more complex growth and branching of the UB during renal development.

However, genetic studies in our lab have revealed an unexpected degree of overlap in function between *Gdnf* and *Fgf10* (and perhaps other factors) in UB outgrowth in vivo. These experiments were designed to better understand the specific role GDNF/Ret signaling in UB branching. It had been postulated that GDNF acts as a chemoattractant, analogous to the role of Fgf10 in lung bud branching,[23] to attract the two new tips of a branching UB.[24] We first tried to test this model by generating mice that expressed GDNF ectopically in the UB epithelium, and not in its normal site, the MM.[25] Our reasoning was that this was likely to eliminate any spatial gradients of GDNF that might normally provide positional information to the UB. These mice developed grossly normal kidneys, with typical T-shaped branching UB tips,[25] showing that the mesenchymal expression of GDNF is not critical for UB branching. However, several caveats remained. First, for technical reasons we were unable to analyze the branching pattern in detail, to see if it was entirely normal. Second, while GDNF was not expressed in the MM, it remained conceivable that GDNF expressed by UB cells might bind to extracellular matrix in a way that regenerated the same (hypothetical) extracellular gradients as in wild-type kidneys.[26]

More recently, we have avoided these caveats by seeking conditions in which kidneys can develop in the complete absence of GDNF, and also by using better imaging techniques to visualize branching patterns. Sprouty1 (*Spry1*) is a negative regulator of RTK signaling whose expression is induced in the UB tips by GDNF/Ret signaling, and which acts in a negative feedback loop to modulate GDNF/Ret signaling.[27] It was previously shown that heterozygosity for *Spry1* could rescue the renal hypoplasia that occurs in *Gdnf*$^{+/-}$ heterozygotes,[28] but double homozygotes had not been examined. While *Gdnf*$^{-/-}$ mice rarely make a ureter or a kidney (and the kidneys that sometimes develop are extremely rudimentary), surprisingly, we found that the absence of the negative regulator *Spry1* restored ureter and kidney development in >90% of *Gdnf*$^{-/-}$;*Spry1*$^{-/-}$ mice (**Fig. 3A–C**).[29] The kidneys were somewhat reduced in size and nephron number (~50–70% of normal), but quite normal in shape and histology. To exclude the possibility that another Ret ligand, such as neurturin,[30] was able to substitute for GDNF, we also examined *Ret*$^{-/-}$;*Spry1*$^{-/-}$ mice and these had kidneys similar to those of *Gdnf*$^{-/-}$;*Spry1*$^{-/-}$ double mutants. These results suggested that a major role of GDNF/Ret signaling is to "balance" the negative regulation provided by *Spry1* and when both are absent, other endogenous factors can replace GDNF as the inducer of UB outgrowth from the ND and continued branching.

These findings left several questions: First, what other signals can replace GDNF (but only when *Spry1* is absent)? Fgf10 was a strong candidate, as it is expressed in the MM, similarly to GDNF and is able to induce ectopic buds from cultured NDs, in the absence of any GDNF.[18,29] When *Fgf10* was eliminated in *Gdnf*$^{-/-}$;*Spry1*$^{-/-}$ mice, UB outgrowth failed completely in the triple mutants. Thus, *Fgf10* must be responsible, at least in part, for promoting and correctly positioning, UB outgrowth in *Gdnf*$^{-/-}$;*Spry1*$^{-/-}$ mice. It is not yet clear if other factors (e.g., HGF, EGFs, VEGF-A) are also required under these conditions (to a greater extent than when *Gdnf* and *Spry1* are present).

A second question was whether the specific pattern of branching was affected in *Gdnf*$^{-/-}$;*Spry1*$^{-/-}$ or *Ret*$^{-/-}$;*Spry1*$^{-/-}$ mice. Examining the UB at various stages of kidney development, with the aid of fluorescent protein-encoding transgenes[31,32] and 3-D image-rendering, revealed that the UB branched extensively, but in a distinctly abnormal pattern. In a normal kidney at E15.5, the UB tips are evenly spaced, uniform in size and mostly branching in the "orthogonal bifurcation" pattern,[6] in which each new pair of branches forms at a

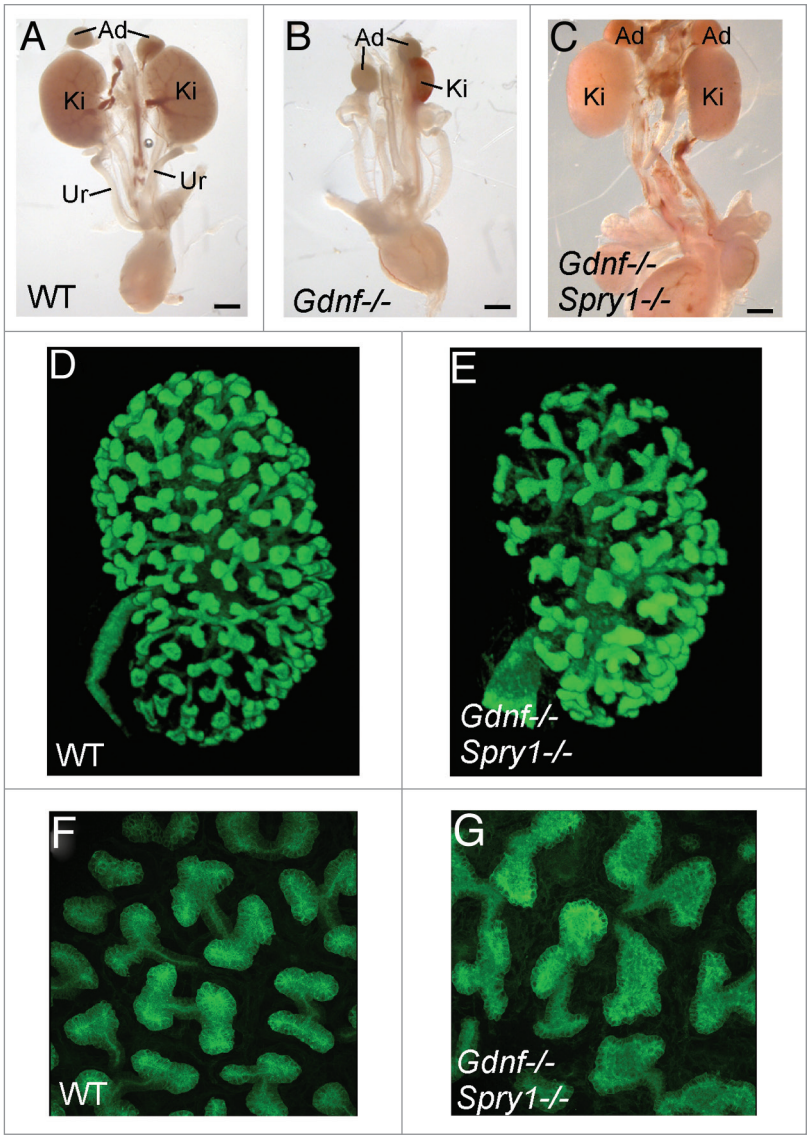

Figure 3. Loss of *Spry1* rescues kidney development in *Gdnf*$^{-/-}$ mice, but with an abnormal UB branching pattern. (A–C) excretory systems dissected from newborn mice of the indicated genotypes. Ad, adrenal; Ki, kidney; Ur, ureter. (D–G) 3D reconstruction of the branched UB in E15.5 wild-type and double-mutant kidneys (also carrying a *Hoxb7/myrVenus* transgene to allow the UB to be imaged). While the wild-type UB (D and F) shows a reiterative pattern of orthogonal bifurcation (F), with tips growing at right angles to their predecessors, the UB tips in the double mutant (G) instead display a variety of abnormal tip shapes and branching patterns. The 3D images were generated from confocal Z-stacks, using Volocity (D and E) or ImageJ (F and G). Modified from reference 29.

~90 degree angle from the parental branch (**Fig. 3D and F**). In contrast, the double mutant UB tips were irregular in size, shape and branch angle (**Fig. 3E and G**). This was not due to simply the absence of *Spry1* (in *Spry1*$^{-/-}$, the tips were swollen but otherwise appeared normally patterned; not shown) and instead seems to reflect a specific role of *Gdnf* in branch patterning.

The mechanism of this effect remains unclear. It is possible that gradients of GDNF normally pattern the UB tips, but if so, the GDNF protein distribution must be regulated by some mechanism other than gene expression,[26] as GDNF mRNA is very diffusely expressed in the MM.[16] Alternatively, GDNF signaling to the UB tips may stimulate a unique program of gene expression and cellular behavior that leads to the normal pattern of UB morphogenesis. A key question now is whether GDNF provides positional information in this context. Answering it will require better methods to visualize the normal spatial distribution of GDNF and its interaction with the ECM, as well as methods to subtly alter its spatial expression pattern in vivo.

Signaling Pathways and Gene Expression Changes Downstream of Ret

How does GDNF/Ret signaling induce UB growth and branching? Ret activates a number of intracellular signaling pathways, including Ras-Erk MAP kinase, PLCγ/Ca^{+} and PI3K-Akt pathways, as well as others.[33] The importance of these three pathways has been studied using specific chemical inhibitors in organ cultures, or by making knock-in mice with mutations in Ret tyrosine residues whose phosphorylation upon GDNF signaling activates different downstream pathways. Inhibiting PI3K entirely blocks UB growth and branching,[34] while, conversely, deleting PTEN (the PI3K phosphatase) causes irregular UB branching.[35] Inhibiting MEK (Erk Map kinase-kinase) slows UB branching with a smaller effect on overall elongation.[5,36] A substitution for Ret tyrosine 1062, which is linked to both the PI3K and Erk pathways (as well as several others), results in renal agenesis.[37,38] Blocking the activation of PLCγ by mutating Ret tyrosine 1015 has complex effects, leading to multiple ureters but also smaller kidneys,[38] apparently because this pathway is required for normal UB branching, but also for normal *Spry1* expression. For the most part, it is not clear how these signaling pathways are coupled to the alterations in cellular properties and behaviors that underlie morphogenesis.

Ret signaling also leads to changes in gene expression in the UB,[27,39,40] but compared to the large number of upstream genes known to regulate *Gdnf* or *Ret* expression,[41,42] very few downstream genes were known. To address this question, we performed a microarray screen to identify genes whose expression in isolated ureteric buds (iUBs) was altered by culture in the presence/absence of GDNF.[43] In addition to the three genes previously known to be upregulated by GDNF (*Wnt11*, *Spry1*, *Ret*), the screen identified many novel genes upregulated by GDNF, including

the chemokine receptor *Cxcr4*, the cytokine *Crlf1*, the signaling inhibitors *Dusp6* and *Spred2* and the transcription factors *Myb*, *Etv4* and *Etv5*. *Crlf1* is believed to have a role in the induction of nephrogenesis by the UB tips.[8] *Cxcr4*, the receptor for Cxcl12/Sdf-1, is important for cell migration in several developing systems and in the immune system.[44,45] In the kidney, it is important for development of the glomerular tuft and renal vasculature,[46] and inhibitor studies suggest a role in UB branching and nephrogenesis,[47] but its role in UB morphogenesis in vivo is currently being investigated. The possible roles of *Myb*, *Spred2* and *Dusp6* in UB branching remain to be determined. The most important finding resulting from this screen was the crucial role of *Etv4* and *Etv5* in kidney development, downstream of Ret (and probably other RTKs).[43]

Etv4 and *Etv5* encode two very closely related transcription factors of the ETS family, which are involved in neuronal development, spermatogenesis and limb development, where their expression is induced by GDNF, FGFs or HGF.[48-51] In developing kidneys, *Etv4* and *Etv5* are both expressed strongly in the UB tips and more weakly in the MM and nascent nephrons; their expression in the UB requires normal levels of Ret signaling, via the PI3K pathway, as it is abolished in mice with a hypomorphic *Ret* mutation, or in wild-type kidneys cultured with a PI3K-inhibitor.[43] Mice lacking either *Etv4* or *Etv5* had only rare defects in kidney development, but *Etv4*/*Etv5* double mutants were severely affected, establishing a redundant role for these two genes. $Etv4^{-/-};Etv5^{+/-}$ mutants had either renal agenesis or hypodysplasia, while $Etv4^{-/-};Etv5^{-/-}$ newborn mice lacked kidneys entirely, although they often had ureters. The hypoplastic kidneys in $Etv4^{-/-};Etv5^{+/-}$ mutants had severe branching defects and UBs isolated from these kidneys branched very poorly in Matrigel cultures compared to controls,[43] suggesting a cell-autonomous role in the UB. As *Etv4* and *Etv5* expression requires normal Ret signaling levels, their lack of expression likely contributes significantly to the failure of kidney development in *Ret*, *Gdnf* and *Gfrα1* mutant mice.

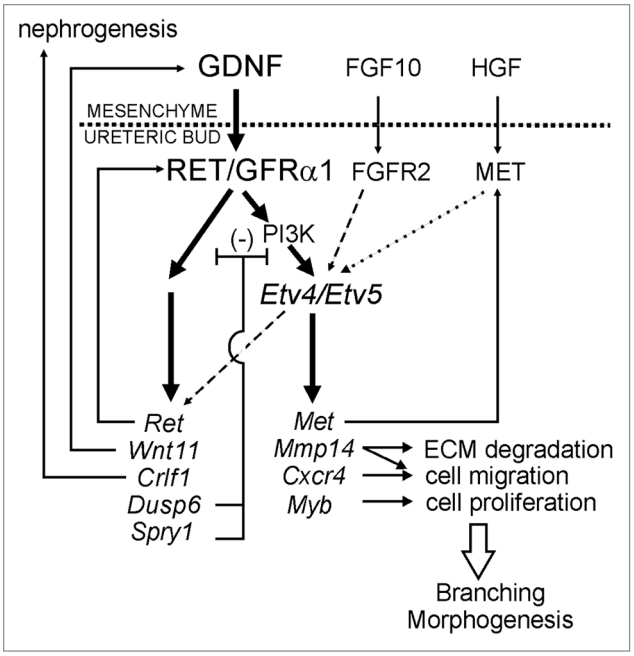

Figure 4. Model for role of *Etv4* and *Etv5* in a gene network controlling ureteric bud branching morphogenesis. GDNF from the metanephric mesenchyme signals to the UB via GFRα1 and RET, thus upregulating a set of genes (bold arrows), including *Etv4* and *Etv5*. *Etv4* and *Etv5* expression requires PI3-kinase activity, while the signaling pathways regulating other downstream genes are unknown. FGF10 signaling normally has a lesser role, if any, in regulating *Etv4/Etv5* expression in the UB (dashed arrow). Several of the genes downstream of *Ret* (e.g., *Met*, *Mmp14*, *Cxcr4*, *Myb*) are apparently regulated via *Etv4* and *Etv5*, as their expression is extinguished in $Etv4^{-/-};Etv5^{+/-}$ kidneys. Other GDNF-induced genes (e.g., *Ret, Wnt11, Crlf1, Dusp6, Spry1*) are relatively insensitive to *Etv4/Etv5* levels (dashed arrow) and are likely regulated via other transcription factors. Collectively, these downstream genes compose part of a regulatory network that promotes UB branching morphogenesis and other aspects of kidney development. In three positive feedback loops, Ret signaling positively regulates its own expression, *Wnt11* upregulates *Gdnf*,[39] and *Met* encodes the receptor for HGF, which may also upregulate *Etv4/Etv5* (dotted arrow). *Spry1* and *Dusp6* are inhibitors of RTK signaling that participate in negative feedback loops; Dusp6 is a MAP kinase phosphatase, while the signaling pathway(s) inhibited by Sprouty1 is unclear. *Crlf1* may participate in the induction of nephrogenesis. *Mmp14*, *Cxcr4* and *Myb* are likely to contribute to cellular processes important for UB morphogenesis. Reproduced from reference 43.

Unlike a lack of *Gdnf* or *Ret*, the requirement for *Etv4* and/or *Etv5* in kidney development could not be overcome by removing *Spry1*.[43] This suggests that *Etv4* and *Etv5* mediate the effects of other growth factors besides GDNF and while these individual growth factors are to some degree redundant, they all signal through Etv4 and Etv5. Thus, the two ETS transcription factors are key components of a gene network downstream of receptor tyrosine kinases that promotes and controls renal branching morphogenesis (**Fig. 4**).

To identify some of the target genes of *Etv4* and *Etv5* in the kidney, we first tested several of the genes that are regulated by GDNF/Ret signaling. Expression of *Cxcr4* and *Myb* was greatly reduced in $Etv4^{-/-};Etv5^{+/-}$ mutant kidneys (as in *Ret*-hypomorphic kidneys), suggesting that these genes are normally upregulated by GDNF/Ret signaling via *Etv4* and *Etv5*. However, *Ret*, *Crlf1*, *Dusp6* and *Wnt11* were expressed at normal levels in $Etv4^{-/-};Etv5^{+/-}$ kidneys, indicating that their expression is relatively insensitive to *Etv4/Etv5* (or at least that a single *Etv5* allele is sufficient for their normal expression).

We also examined *Met* and *Mmp14*, two genes known to regulated by Etv4 in other cell types and found that their expression requires *Etv4/5* in the UB. Upregulation of *Met* (encoding the receptor tyrosine kinase for HGF) by Ret signaling is probably important to render the UB responsive to HGF, and thus to promote further growth and branching.[52]

MMP14 (MT1-MMP) is a membrane-type matrix metalloproteinase, which has been implicated in UB branching in culture,[53,54] as well as by analysis of *Mmp14*$^{-/-}$ mice (Zent R and Riggins K, personal communication). MMPs may promote branching morphogenesis in several ways: through their enzymatic activity, they may remodel the extracellular matrix (ECM) to allow UB growth and also release bound growth factors.[55] MMP14 may also function via an autocrine signaling mechanism that promotes cell migration in vitro.[56]

While these candidate gene studies yielded some insight into the possible effector genes downstream of *Etv4/Etv5*, the full set of target genes remains to be defined by more comprehensive approaches. It is also important to address the phenotypic effects of *Etv4/5* expression at the cellular level, a issue that has been investigated by some of the experiments described below.

The identification of many genes whose expression is altered by Ret signaling in the UB and of Etv4 and Etv5 as key transcription factors in this regulatory network, provides a large set of candidate genes that may play some role in UB morphogenesis. The most straightforward genetic approach to examine their functions (i.e., examining the phenotypes of individual knockout mice) is expensive and time consuming and better methods are needed to screen large numbers of genes for effects on kidney development. Hopefully, a combination of organ culture systems (whole kidneys or isolated UBs) and improved methods to manipulate gene expression in these systems (such as viral vectors and RNA interference) can provide a higher throughput assay for gene function in the developing kidney.

Cellular Events in UB Morphogenesis

Signals to the UB, and the resulting changes in intracellular signaling and gene expression patterns, must ultimately be translated into changes in the behavior of UB epithelial cells in order for the UB to grow and branch in specific ways. Some of the cellular events likely to be relevant include cell proliferation, oriented cell division, changes in cell shape, changes in adhesive properties, cell migration and remodeling of the ECM (**Fig. 2**). While several cell culture models for UB branching are available, they do not appear to accurately reflect developmental events in vivo. Explanted kidneys or isolated UBs developing in organ culture[57] provide a more authentic view of the cellular events that occur in vivo. Several additional methods and experimental "tools" have been developed that are useful for this purpose.

One such tool we have recently described is a mouse strain that expresses a membrane-bound form of yellow fluorescent protein myrVenus in the ND and UB under the *Hoxb7* promoter, allowing individual cell outlines to be seen clearly in live specimens with a confocal microscope.[32] This allowed us to observe a change in cell shape in the caudal ND preceding ureteric budding, which to our knowledge had not been previously reported. At E10.0, the caudal portion of the ND thickens and forms a pseudostratified epithelium[58] (**Fig. 5**), i.e., one in which the nuclei lie at different levels along the apical-basal axis, but every cell contacts the basement membrane. Locally pseudostratified regions were known to occur in several types of epithelia before they produce outgrowths, including the mammary line,[59] otic placodes,[60] and the liver and thyroid buds.[61,62] The significance of the pseudostratified domain of the ND remains unclear, as do the signals that induce its formation. We speculate that the high density of nuclei generated in the pre-budding ND epithelium during this process might permit rapid expansion of a localized region of the ND to form the UB. An examination of the literature revealed that the ND apparently fails to become pseudostratified in *Osr1* mutant mice (see Fig. 7 in ref. 63). Since *Osr1* is expressed in the IM and MM but not the ND, this suggests the possibility that a signal from the MM causes the caudal ND to undergo this alteration. However, GDNF/Ret signaling is not required, as *Ret*$^{-/-}$ NDs become pseudostratified similarly to wild-type.[58] By E14.5, after the bud has grown out and branched a few times, the UB reverts to a simple cuboidal epithelium,[54,58] while continuing to branch. Therefore both pseudostratified and simple epithelia can undergo similar branching events.

A valuable method we have developed to study cell behaviors during kidney development is the generation of chimeras using embryonic stem (ES) cells carrying transgenes driven by the *Hoxb7* promoter, which express fluorescent reporter proteins (e.g., GFP) specifically in the ND/UB lineage.[58,64] When injected into blastocysts of a different strain, the ES cells contribute to all tissues in the resulting chimeric embryos, but because the GFP is only expressed in ND and UB cells, it is possible to clearly visualize the ND/UB cells without interference by the overlying mesenchyme. We first examined the development of chimeric kidneys made with *Hoxb7/GFP* cells that were wild-type (except for the transgene), which were injected into wild-type (unmarked, GFP-negative) embryos.[64] The behaviors of the GFP^{+} and GFP^{-} cells showed that there was extensive cell movement in the UB epithelium, although whether it was random or directed was not apparent. They also showed that some GFP^{+} UB tip cells divided to yield both GFP^{+} tip cells (self-renewal) and GFP^{+} trunk cells (differentiation). This suggested that tip cells are the bipotential progenitors of new tips and trunks,[64] a model supported by recent inducible genetic fate mapping studies (Paul Riccio and F. Costantini, unpublished data).

We next generated chimeras using ES cells that were *Ret*$^{-/-}$ and carried *Hoxb7/GFP*, allowing us to examine the specific cellular defects caused by lack of *Ret*. In the early UB (E11.0), the tip was devoid of mutant (GFP^{+}) cells, while the trunk contained both wild-type and mutant cells; as the UB branched, the mutant cells failed to contribute to any of the new tips, and were soon "diluted out" by the wild-type cells.[58,64] To examine the earlier events that lead to the exclusion of *Ret*$^{-/-}$ cells from the UB tips, we analyzed additional chimeras in which the host embryo expressed cyan fluorescent protein (CFP) in the ND and UB, while the ES-derived (*Ret*$^{-/-}$) cells expressed GFP (**Fig. 6A**).[58] This allowed us to perform time-lapse imaging of bud formation and outgrowth. We found that at E10.0 the mutant and wild type cells

were randomly arranged in the ND, but before budding, wild type cells moved together into a cluster in the dorsal ND (E10.5, **Fig. 6B and B'**), which next emerged as the tip of the UB (E11.0, **Fig. 6C**), while mutant cells were largely excluded from this region. We called this region the "primary UB tip domain" because it gives rise to the first UB tip, as well as to later tips.[58] Thus, the reason that *Ret*$^{-/-}$ cells fail to contribute to the primary UB tip domain is apparently a defect in cell movement in the ND. There was no difference in proliferation or survival between the *Ret*$^{-/-}$ and wild-type cells in the ND.[58]

Further studies with chimeric embryos made using ES cells and host embryos with different levels of Ret signaling revealed that ND cells can compete, based on their level of signaling, to contribute to the UB tip domain. For example, cells lacking *Spry1* (which have elevated signaling), prevailed over wild-type cells to form the UB tip domain (**Fig. 6E**). This competition is reminiscent of that observed in the developing fly respiratory epithelium, where cells compete based on FGF-receptor signaling levels to form the tip of the air sac or the tracheal branch.[65,66] In normal, non-chimeric mouse embryos, all ND cells express the *Ret* gene at similar levels, but the level of Ret signaling (as reflected by di-phosphorylated Erk MAP kinase, a downstream indicator of Ret signaling) was very heterogeneous. Furthermore, time-lapse imaging showed that normal ND cells can move independently of their neighbors during bud formation. Together, the data suggest a model in which a subset of ND cells have elevated Ret signaling and these cells migrate past their neighbors to form the first UB tip.[58] Why might similar competitive cell behaviors occur in branching epithelia in both flies and mammals? Perhaps competition limits the number of cells involved in branch formation or budding, thus forming a small, discrete bud rather than a massive swelling.

As several of the genes we had identified downstream of *Ret* and *Etv4/5* are implicated in cell migration (in particular, *Cxcr4, Met* and *Mmp14*), we asked whether *Etv4/Etv5* mutant cells would also show a defect in the chimeric assay.[67]

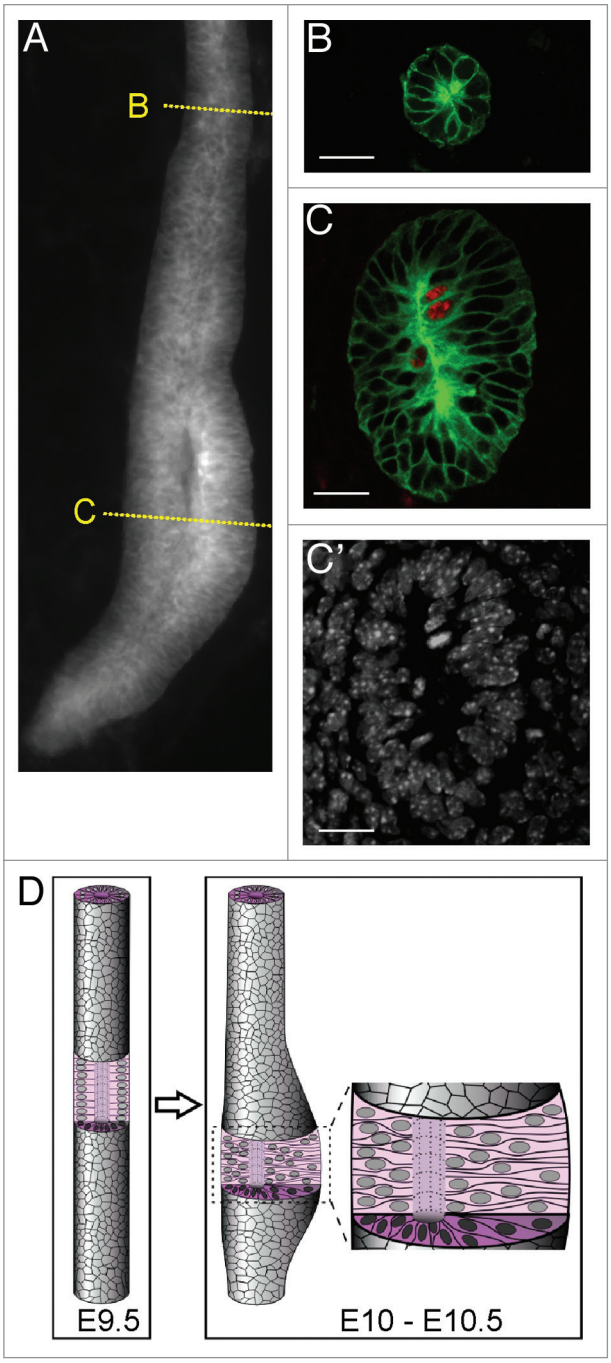

Figure 5. The nephric duct forms a (transiently) pseudostratified epithelium before ureteric bud formation. (A) Whole mount image of wild-type Nephric duct carrying *Hoxb7/myr-Venus* transgene at E10.0. Yellow lines indicate approximate planes of the sections at right, which show rostral ND in (B) and pseudostratified caudal ND in (C). (B and C) were stained with anti-GFP to detect myr-Venus (green) and anti-pH3 (red). C', Hoechst nuclear stain of the section in (C). (D) Schematic diagram of the formation of pseudostratified epithelium preceding ureteric budding. The pseudostratified epithelium persists during early UB branching, but reverts to a simple epithelium before E14.5.[58] Scale bars 20 μM. Modified from reference 58.

Indeed, *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ cells behaved identically to *Ret*$^{-/-}$ cells, failing to contribute to the UB tip domain in chimeras with wild-type cells (**Fig. 6D**). *Etv4*$^{-/-}$*;Etv5*$^{-/-}$ cells were even more defective, contributing mainly to the rostral ND, less often to the caudal ND and almost never to the tip or trunk of the UB.[67] Thus, the effects of

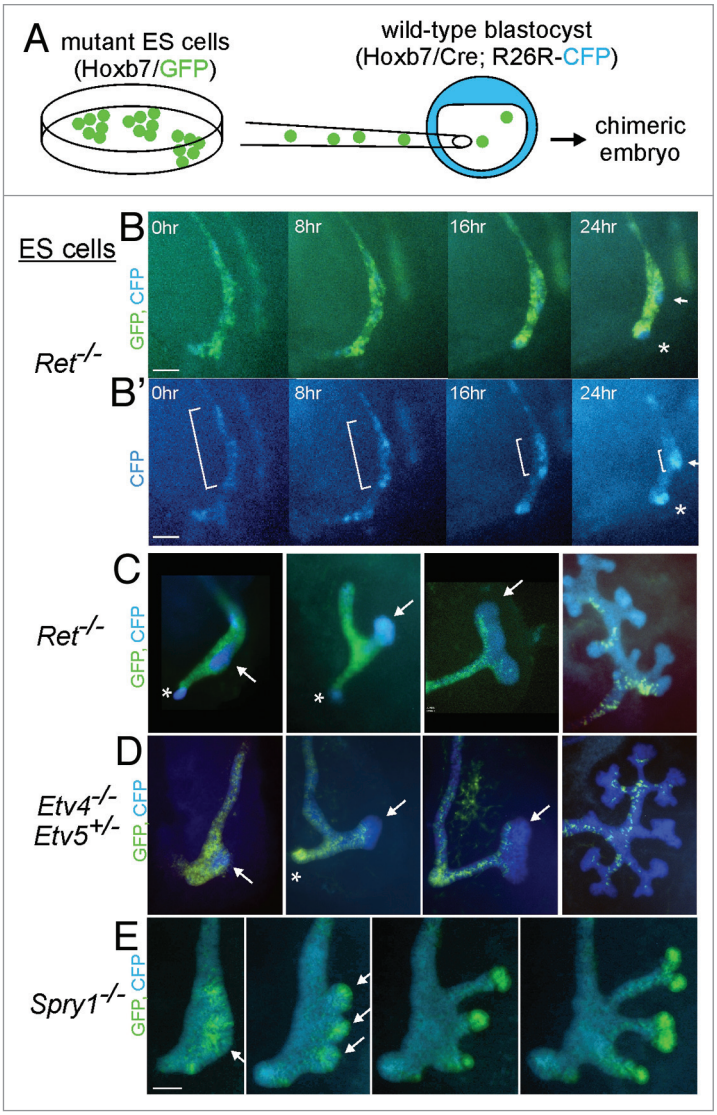

Figure 6. Time-lapse chimera analysis of the behavior of cells with mutations affecting Ret signaling. (A) ES cells were derived from mouse embryos with mutations in *Ret*, *Etv4* and *Etv5* or *Spry1* and carrying a *Hoxb7/GFP* transgene expressed in the ND and UB lineage. The ES cells were injected into blastocysts carrying *Hoxb7/Cre*[68] and R26R-CFP, a Cre-reporter strain that expresses CFP in any cells expressing Cre and their descendants.[69] Thus, in the ND and UB of the chimeric embryos, mutant cells (derived from ES cells) appear "green" and wild-type cells (derived from host embryo) appear "blue." Images from left to right show successive stages of ND development (B and B') and UB outgrowth and branching (C–E). (B and B') time-lapse imaging of development of a $Ret^{-/-}$↔wild-type chimera starting at ~E10.0. (B) shows CFP and GFP images merged, revealing interspersion of wild-type (CFP^+) and $Ret^{-/-}$ (GFP^+) cells in the nephric duct at 0 hr, but enrichment of wild-type cells at the primary UB tip domain (arrow) and the CND (*) by 24 hr. (B') shows only the CFP channel, revealing that wild-type cells undergo rearrangements to form the UB tip domain (brackets) and the CND (*). (C) additional $Ret^{-/-}$↔wild-type chimeras at successive stages (starting at E10.5) of UB formation, outgrowth and branching. When the UB grows out and branches, the $Ret^{-/-}$ cells contribute to the trunks, but not to the UB tips. (D) $Etv4^{-/-}$, $Etv5^{+/-}$ cells behave virtually identically to $Ret^{-/-}$ cells in the UB, but they can contribute to the CND (*). (E) $Spry1^{-/-}$ cells behave oppositely to $Ret^{-/-}$ or $Etv4^{-/-}$, $Etv5^{+/-}$ cells, preferentially contributing to the primary UB tip domain and then to the UB tips. Note that formation of multiple UBs is a common property of $Spry1^{-/-}$ embryos.[27] Modified from references 58 and 67.

GDNF/Ret signaling on ND cell movement appear to be mediated by *Etv4* and *Etv5*; and the more severe defect in $Etv4^{-/-}$; $Etv5^{-/-}$ cells than $Ret^{-/-}$ cells supports the model that *Etv4/5* also mediate additional signals, most likely from FGFRs or other RTKs.

While this work illustrates the power of chimera analysis to reveal the roles of specific genes in cell behaviors, it raises as many questions as it answers. Why are signaling levels heterogeneous among ND cells? What guides the movement of some ND cells towards the site where the UB tip domain forms—chemoattraction by GDNF or some other guidance mechanism that is activated downstream of Ret signaling? Does this process involve cell sorting due to differences in cell adhesion? Do similar cell rearrangements play a role in continued UB branching, or is this a phenomenon specific to the initial bud formation from the ND? A variety of experimental approaches will be required to address these issues.

Questions and Answers

Dr. David Ornitz, Alumni Endowed Professor of Developmental Biology, Washington University School of Medicine: What do you make of the GDNF expression pattern?

Dr. Frank Costantini, Professor of Genetics and Development, Columbia University Medical Center: The distribution of GDNF mRNA appears very diffuse throughout the metanephric mesenchyme. So if there is any truth to the idea that GDNF is localized and can pattern the UB by chemoattraction, as FGF10 is thought to do in lung development, it has to be occurring at the protein level, perhaps by its ability to bind to extracellular matrix molecules such as heparan sulfate proteoglycans.

Dr. Ornitz: Do you think the regulation of epithelial branching patterning is intrinsic to the mesenchyme?

Dr. Costantini: Well, we know that if you culture the ureteric bud in Matrigel, without mesenchyme (Sanjay Nigam's work, primarily), it will branch with a certain pattern, but the pattern doesn't really look like that in a normal kidney.

Dr. Ornitz: Does the mesenchyme pattern epithelial branching or is the branching intrinsic to the epithelium.

Dr. Costantini: The epithelium has an intrinsic ability to branch as shown by its ability to branch in Matrigel in the presence of GDNF and other soluble factors, but the pattern seems to be different, so I think that the mesenchyme fine-tunes the pattern of branching. While this depends on GDNF signaling through Ret, whether it does it through chemoattraction by GDNF or by another mechanism, is not yet clear.

Dr. Jianghui Hou, Assistant Professor of Medicine, Washington University School of Medicine: From a cell biology point of view, tubular cell proliferation or tubular elongation requires the mitotic spindles of proliferating cells to align in the same direction as the elongation angle. Defects in aligning the mitotic spindle may affect tubular branching. Have you found any gene related to this process downstream of the Ret signaling pathway that might cause tubular branching defects?

Dr. Costantini: That is a very good question. In later kidney development it is established that mitotic orientation is involved in the elongation of the collecting duct, so I think it could have a role in branching, but we don't know.

Dr. Hila Barak, Postdoctoral Fellow in Developmental Biology, Washington University School of Medicine: Is it known whether cells in the stroma play a role in ureteric bud branching?

Dr. Costantini: We know the stoma is important for normal ureteric bud branching, based mainly on studies with *Foxd1* (*Bf2*) mutant mice, but to my knowledge the specific factors that are made by the stroma are not really known.

Dr. Barak: In your presentation you showed us a list of genes that have the most elevated expression in the ureteric buds which were cultured with GDNF. I saw that this list includes genes that have a role in proliferation. Have you examined the role of those genes in the ureteric bud?

Dr. Costantini: If you go further down on the list of genes upregulated by GDNF (than what I showed in my slide) there are a lot of genes that are proliferation-associated. This is consistent with other evidence that one of the effects of GDNF is to promote ureteric bud cell proliferation.

Dr. Robert Heuckeroth, Associate Professor of Pediatrics and Developmental Biology, Washington University School of Medicine: I was struck by these beautiful positive and negative feedback loops. What is known about what turns this process off? Why does the kidney not keep growing forever and what happens in postnatal life? As a child gets bigger, does the ureteric bud continue to branch or do ducts just elongate?

Dr. Costantini: One paper that addresses this question was from Larry Patterson in Cincinnati.[70] They looked at markers of nephrogenesis and ureteric bud branching and found that kidney development continues for three days after birth in the mouse. Then the nephrogenic mesenchyme, which produces GDNF, disappears and markers of Ret signaling in the UB, like Wnt11, are also lost.

So it appears that branching and nephron induction continue a little bit after birth and then they stop and you mainly get duct elongation and nephron maturation thereafter.

Dr. Heuckeroth: What is the underlying mechanism that stops branching?

Dr. Costantini: I don't think that is known, but the result just mentioned suggests that it may be the loss of GDNF-producing mesenchyme.

Dr. Raphael Kopan, Professor of Medicine and Developmental Biology Washington University School of Medicine: Mouse cells heterozygous for Ret are behaving like wild type. Can I then assume that human conditions are due to homozygous recessive mutations?

Dr. Costantini: In humans, heterozygous *RET* loss of function mutations cause Hirschsprung's disease, a developmental defect of the enteric nervous system and sometimes kidney defects. In contrast, *Ret* heterozygous mice are essentially normal and only homozygotes have both severe kidney and enteric nervous system defects.

Dr. Kopan: So in humans, heterozygotes have a phenotype and in mice, they do not. Do you think you could expose this if you were to lower *Etv4* by one copy, then also eliminate one copy of Ret.

Dr. Costantini: We did that experiment and didn't see any genetic interaction. But when Cristina Cebrian in the lab combined a *Gdnf* heterozygote (which has an incompletely penetrant kidney phenotype) with either *Etv4* homozygosity or *Etv4/Etv5* double heterozygosity (neither of which had much of an effect alone), we did see a big effect.

Dr. Kopan: The *Fgf10* rescue was incomplete. Do you think that if you were to delete *Spry1* only in the ureteric bud you would repair the effect. How do you feel about it? Have you tried recombination of normal mesenchyme?

Dr. Costantini: The problem with that is when you do the recombination, in the best of circumstances, wild type-wild type, you don't really get a very nice branching pattern, so that would not be a good way to look at it. Combining the *Spry1* ureteric bud specific knockout and a *Gdnf* knockout could be done, but we haven't. So you are suggesting that the Spry1 might have an effect in mesenchyme? Albert Basson and Jonathan Licht did a UB-specific Spry1 knockout as well as the complete *Spry1* knockout and the phenotypes were the same. So based on that, there is no evidence as yet for a role of *Spry1* in mesenchyme.

Dr. Susan Kiefer, Assistant Professor of Medicine, St. Louis University School of Medicine: I was struck by the behavior of ETV4 and 5 cells in chimeric experiments being identical to Ret in the ureteric bud region, but it didn't look like it was identical in the common nephric duct.

Dr. Costantini: I didn't go into that, but that is exactly right. In the *Ret* chimera, the $Ret^{-/-}$ cells don't contribute to the common nephric duct. But the $Etv4^{-/-}$, $Etv5^{+/-}$ cells do. So although that process is *Ret* dependent, it is less dependent on *Etv4* and *Etv5* ("less" because the cells still have one *Etv5* allele).

Dr. Kiefer: Do you think there is going to be a totally different set of *Ret* dependent genes in the common nephric duct region?

Dr. Costantini: Even in the kidney, there are some genes that are *Ret* dependent but expressed normally in $Etv4^{-/-}$, $Etv5^{+/-}$ mutant kidneys. So the genes regulated by Ret in the common nephric duct might be some of these genes or could be

different genes than those regulated by Ret in the kidney.

Dr. Sanjay Jain, Assistant Professor of Medicine and Immunology and Pathology, Washington University School of Medicine: Regarding Spry1 and Ret, it seems that in the double knock out there is rescue of the individual phenotypes, at least morphologically. Have you seen any differentiation problems in the branching tips.

Dr. Costantini: As I showed, we looked at several ureteric bud tip markers and all we looked at (Ret, Wnt11, Etv4) were normal. We looked at only one trunk marker, Wnt7b and it was also normal.

Dr. Jain: What I mean is in differentiation markers of the collecting duct.

Dr. Costantini: We did not look at markers of the differentiated collecting ducts.

Dr. Marc Hammerman, Chromalloy Professor of Medicine, Washington University School of Medicine: Do you know what renal function is like in those animals?

Dr. Costantini: Well they don't live, because the removal of *Spry1* doesn't rescue all of the other problems caused by loss of *Ret* or *Gdnf*, so they die within a day of birth.

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