

Developmental plasticity and regenerative capacity in the renal ureteric bud/collecting duct system

Derina Sweeney*, Nils Lindström* and Jamie A. Davies†

Branching morphogenesis of epithelia is an important mechanism in animal development, being responsible for the characteristic architectures of glandular organs such as kidney, lung, prostate and salivary gland. In these systems, new branches usually arise at the tips of existing branches. Recent studies, particularly in kidney, have shown that tip cells express a set of genes distinct from those in the stalks. Tip cells also undergo most cell proliferation, daughter cells either remaining in the tip or being left behind as the tips advance, to differentiate and contribute to new stalk. Published time-lapse observations have suggested, though, that new branches may be able to arise from stalks. This happens so rarely, however, that it is not clear whether this reflects true plasticity and reversal of differentiation, or whether it is just an occasional instance of groups of tip cells being 'left behind' by error in a mainly stalk zone. To determine whether cells that have differentiated into stalks really do retain the ability to make new tips, we have removed existing tips from stalks, verified that the stalks are free of tip cells, and assessed the ability of tip-free stalks to initiate new branches. We find stalks to be fully capable of regenerating tips that express typical tip markers, with these tips going on to form epithelial trees, at high frequency. The transition from tip to stalk is therefore reversible, at least for early stages of development. This observation has major implications for models of pattern formation in branching trees, and may also be important for tissue engineering and regenerative medicine.

KEY WORDS: Kidney, Regeneration, Stem cell, Ureteric bud, Branching

INTRODUCTION

Branching morphogenesis of epithelia is a common event in mammalian organogenesis. The process forms the airways of the lung, the milk ducts of the mammary glands, the exocrine ducts of the pancreas, the urine collecting ducts of the kidney, the seminiferous ducts of the prostate, and the ducts of salivary, lacrimal and uterine glands (Davies, 2005). Generally, these systems develop by dipodial branching, in which the ends of existing branches bifurcate and separate from one another as the tubule elongates. Although branching morphogenesis has been studied intensively for several years, significant gaps in our knowledge remain. One of the most important unanswered questions is whether the ability to initiate new branches is confined only to certain cells in a branching epithelium, for example those at the tip of an existing branch, or whether all parts of the epithelium can do it. The answer will have important implications for our basic understanding of how branched systems organize themselves and may also have implications for regenerative medicine. This report addresses this question in one of the most-studied branching epithelia, the renal collecting duct system.

The renal urinary collecting duct system arises from an initially unbranched epithelium, the ureteric bud, which invades the metanephric mesenchyme half way through mouse gestation and branches within it to produce approximately 1600 branches over approximately 10–11 rounds of bifurcation (Cebrian et al., 2004). Although much work has been done on this system, it is still not

clear whether the ability to branch is confined to just a subset of cells or whether it is spread generally throughout the system: there is circumstantial evidence on both sides of the argument.

The main arguments that the ability to produce new branches is restricted to the tip concern the normal pattern of branching, the normal pattern of cell differentiation, and a close correlation between the two. Detailed time-lapse observations of renal branching morphogenesis have shown that most branching events (94%) take place by bifurcation at the ends of existing branches (Watanabe and Costantini, 2004). Cells in the terminal 70 µm of branches ('tips') are the main zone of cell proliferation (Michael and Davies, 2004) and show patterns of gene expression that differ from those in the regions behind them ('stalks'). Tip-specific markers include *Wnt11* and *Sox9*, while stalk-specific markers include collagen XVIII, *Wnt9b* and a glycoprotein that binds *Dolichos biflorus* agglutinin (DBA) (Lin et al., 2001; Michael et al., 2007; Kent et al., 1996; Carroll et al., 2005; Kispert et al., 1996). Careful measurements suggest that the zone of proliferation, the zone of *Wnt11* expression, and the zone of absence of DBA and collagen XVIII seem to respect a common boundary (Table 1). The fact that most branching takes place in the tip zone, which shows different gene expression to the stalks, suggests that there may be a tip state of differentiation that makes cells capable of initiating branches.

One of the most persuasive arguments against the ability to form new tips being restricted to existing tips is the fact that new tips appear to form from stalk regions, albeit at very low frequency and accounting for only 6% of branch events (Watanabe and Costantini, 2004). The low frequency of these events makes their interpretation difficult. It is known from careful analyses of mosaic organs, a few cells of which express green fluorescent protein (GFP), that some cells get 'left behind' by the tips to contribute to the stalk (Shakya et al., 2005). It is therefore possible that the very infrequent lateral branches actually arise from small groups of such tip cells that have not yet differentiated into stalks. A second, circumstantial, argument, comes from the fact that cell lines from renal collecting ducts can

Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.

*These authors contributed equally to this work

†Author for correspondence (e-mail: jamie.davies@ed.ac.uk)

Table 1. Evidence that tip and stalk markers described to date respect a common boundary

Marker*	Expressed in	Boundary position	Source of information
DBA binding	Stalk	67 μm ($\sigma=18 \mu\text{m}$)	Michael et al., 2006
Collagen XVIII	Stalk	70 μm ($\sigma=25 \mu\text{m}$)	Our measurements from figure 1F,H of Lin et al., 2001
Wnt11	Tip	71 μm ($\sigma=18 \mu\text{m}$)	Michael et al., 2006
Sox9	Tip	89 μm ($\sigma=38 \mu\text{m}$)	Our measurements from figure 6A of Kent et al. 1996
High proliferation	Tip	Within the first 100 μm (low spatial resolution data)	Michael and Davies, 2004

*Some markers (Wnt9b) are missing from this list because no scale bars were provided in the micrographs presented by the authors who described their expression patterns.

produce branching tubules in three-dimensional culture systems without – as far as is known – requiring branch-producing cells to be in a separate state of differentiation (Santos and Nigam, 1993). A third argument is that various physical models of branching morphogenesis, such as viscous fingering, have no need for the ability to initiate branches to be restricted to specific cells (Fleury and Watanabe, 2002; Fleury et al., 2004). A fourth possible argument is that the Wolffian duct, from which the ureteric bud normally emerges as a single side branch, can be induced to produce supernumerary side branches by the focal application of ramogens, such as GDNF (Sainio et al., 1997; Davies et al., 1999). The problem with this argument is that the production of a side branch is an essential property of the amniote Wolffian duct, so extra side-branching from it does not necessarily imply that side-branching is a normal ability of the ureteric bud itself.

Establishing whether the ability to initiate branching is restricted or distributed within the ureteric bud/collecting duct system is important, because it carries major implications for understanding patterning mechanisms and for creating strategies to promote regeneration. We have therefore directly tested the ability of stalk regions to generate new branching tips. Our results support a model in which the ability to initiate branches is distributed widely, and not restricted to cells that already express genetic markers characteristic of branch tips.

MATERIALS AND METHODS

Dissection and organ culture

Metanephric rudiments were dissected from E11.5–E17.5 CD1 mouse embryos, the ureteric bud being cut close to its junction with the Wolffian duct/bladder. Ureteric ‘stalks’ were removed from tip regions by cutting just below the ‘T’ junction of E11.5 kidneys, and the remaining tip regions were retained for staining for Wnt11 or with *Dolichos biflorus* agglutinin (DBA). Deliberate injuries to ureteric bud stalks or mesenchyme, for the experiments that needed them, were achieved by stabbing with 0.5×16-mm needles. Where surrounding stroma had to be removed from ureters (see main text), this was achieved by trypsinization in 2× trypsin-EDTA for two minutes followed by manual separation of the stroma and stalk. Organs were cultured on Isopore filters (Millipore) on Trowell-type grids in 35-mm petri dishes in MEM (Sigma M5650), with 10% fetal calf serum and penicillin-streptomycin solution in 5% CO₂ at 37°C.

RT-PCR for Wnt 11

For determination of the maximum possible extent of contamination of stalk numbers by tip cells, we used conventional end-point PCR to detect *Wnt11* in various dilutions of kidney cDNA that represented known numbers of tip cells. In this way, we established that we could detect *Wnt11* cDNA derived from as few as 0.81±0.1 tip cells clearly (and very faintly from reactions from smaller numbers of cells). At the same time, we used the same PCR technique (described below) to attempt to detect *Wnt11* from stalk-derived cDNA without dilution, and showed the signal in a reaction representing cDNA from 0.44 stalks (see below) to be barely detectable. This was used to conclude that 0.44 stalks included fewer than 0.81 contaminating tip cells, or that a stalk contained fewer than two contaminating tip cells.

In detail, total RNA was isolated from 28 whole kidneys, or from 35 stalks-plus-surrounding mesenchyme, using the SV total RNA isolation kit (Promega), and 200 ng of each type of RNA was used to make cDNA using the MLV-RT kit (Promega). One twentieth of the cDNA was then used for each normal PCR reaction. The actual volumes and dilutions of each stage were recorded accurately for subsequent calculations of the number of tip cells and stalks represented in PCR reactions (these calculations also used the fact that each tip consists of 117±18 cells, the measurement of which is described in the immunofluorescence section below). Tracking the dilutions of the samples as they were processed indicated that each PCR reaction from stalk cDNA represented the RNA of about 0.44 stalks and that each normal PCR reaction from kidney included RNA from a mean of 81±12 tip cells (together with many non-tip cells). Primers for β -actin were used in the normal PCR reactions to provide a further check that the dilutions used to create the normal stalk and kidney PCRs were correct and represented the same total number of cells. In addition to standard PCR reactions, reactions were also performed in which the kidney cDNA from the reverse transcription (RT) step was diluted 1/10, 1/100, 1/500, 1/1000 and 1/5000: these therefore represented RNA from 8.1, 0.81, 0.16, 0.081 and 0.016 tip cells. This dilution series was run in lanes adjacent to the normal PCRs from kidney and stalk to establish a threshold of clear detection.

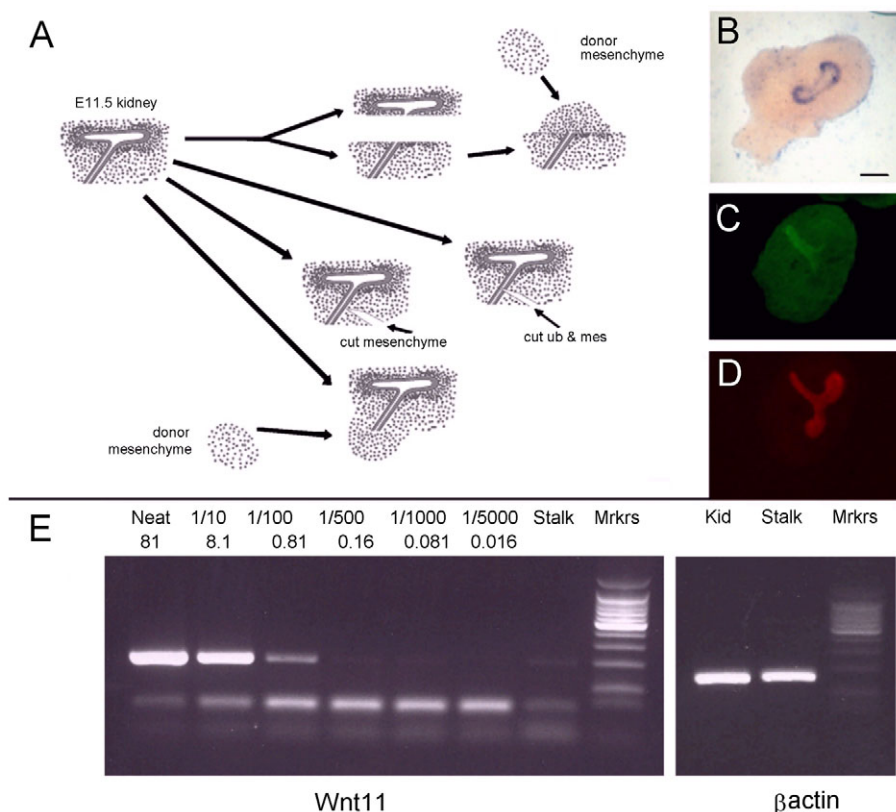
For detection of *Wnt11* expression in tips growing from ureteric bud stalks, total RNA was isolated from four stalks that had been allowed to generate new tips by surrounding them with fresh E11.5 mesenchyme, and cDNA was synthesized using the same techniques and concentrations as are described above.

Fixation and immuno/lectin-fluorescence

Kidneys/recombinants intended for immuno- or lectin-fluorescence were fixed in methanol, washed in PBS with 4% milk powder and incubated in 1/100 mouse anti-calbindin-D_{28k} (Abcam) and/or 1/200 rabbit anti-laminin (Sigma) in PBS overnight at 4°C. They were then washed in PBS, and transferred to donkey anti-mouse IgG-Texas Red (Abcam) diluted 1:100 and lectin from *Dolichos biflorus* (horse gram)-FITC (Sigma) diluted 10 ng/ml (1:100 of 1 mg/ml PBS stock) or 1/100 FITC anti-rabbit (Sigma) in 4% milk powder in PBS overnight at 4°C. A final wash for 30 minutes was carried out in PBS at room temperature while agitating gently. For determination of the mean number of cells in a tip, staining with *Dolichos biflorus* lectin was used to define (negatively) the tip, as described by Michael et al. (Michael et al., 2007), and confocal microscopy was used to measure the mean volume of the cellular part of a tip ($4.8 \times 10^4 \pm 6 \times 10^3 \mu\text{m}^3$) and the mean volume of tip cells ($413 \pm 37 \mu\text{m}^3$). The ratio was used to determine the mean number of cells per tip (117±18).

Culture of stalks in Matrigel

Culture in Matrigel was performed according to the methods of Sakurai et al. and Qiao et al. (Sakurai et al., 2001; Qiao et al., 1999). Briefly, stalks were isolated and cultured in a 1:1 mix of Growth Factor Reduced Matrigel (BD Biosciences) and kidney culture medium with 125 ng/ml recombinant human GDNF (Promega), 250 ng/ml recombinant human FGF1 (R&D Systems) and 625 ng/ml recombinant human pleiotrophin (R&D Systems). The stalks were cultured for 144 hours, fixed for two hours in 4% paraformaldehyde in PBS (pH 7.0), washed in 1% Triton X-100 in PBS for 30 minutes, stained overnight in FITC-phalloidin (Sigma P5282) at 4°C and washed in PBS for 1 hour at room temperature.

**Fig. 1. Dissections and recombinations.**

(A) Diagram of the tissue manipulations used for these experiments. The top arrows indicate separation of tips and stalk, and the culture of the amputated stalk with fresh mesenchyme, the middle arrows indicate injury to mesenchyme (mes) alone, or to stalk (ub) and mesenchyme, and the bottom arrows indicate culture of the 'wrong' end of the stalk with fresh mesenchyme. (B-D) Discarded portions stained with an in situ probe show the complete *Wnt11*-expressing tip regions (B), in addition to short regions of *Wnt11*-negative stalks; those stained with the stalk-specific stain DBA again show that the tips and a short length of stalk are present in the discarded region (C). Staining the same specimen with calbindin-D28_k, which stains both tips and stalks (D), shows where the tips are. Scale bar: 100 μ m. (E) Dilution PCR analysis of *Wnt11* expression in kidneys, including tips, and in de-tipped stalks. The numbers below the dilutions are the number of tip cells represented in the PCR. The signal in de-tipped stalks is far dimmer than the 1/100 dilution of kidney (with 0.81 tip cells). The actin bands demonstrate that the undiluted samples of kidney and stalk cDNA represent equal amounts of total cells, as intended.

In situ hybridization

The plasmid used to generate *Wnt11* probes for in situ hybridization has been used elsewhere (Kispert et al., 1996) and was kindly donated by S. Vainio. It consisted of a 2.1 kb cDNA of *Wnt11* in pSKII. Antisense DIG-labelled probes were generated by cutting the plasmid with *XhoI* and using T3 RNA polymerase; sense 'probes' were generated by cutting the plasmid with *XbaI* and using T7 polymerase. Cultures were first fixed in cold methanol to enhance their adhesion to their filters, then fixed overnight in 4% paraformaldehyde in PBS, incubated in 0.1% Tween 20 in PBS ('PBT') for 10 minutes, treated with 10 μ g/ml proteinase K in PBT for 15 minutes at room temperature, washed for 3 \times 5 minutes in PBT and post-fixed for 40 minutes in 4% formaldehyde in PBT. They were then incubated for 2-4 hours at 65°C in 50% deionized formamide, 25% 20 \times SSC, 2% Roche blocking powder, 0.1% Tween 20, 0.5% CHAPS, 1 mg/ml yeast tRNA, 0.5 M EDTA and 0.05% heparin. Probe, pre-heated to 80°C for 3 minutes, was added at 250 ng/ml and left overnight at 60°C. Samples were then washed in post-hybridization solution (50% formamide, 25% 20 \times SSC, 0.1% Tween 20, 0.5% CHAPS) for 2 \times 10 minutes, then in 75% post-hybridization solution (2 \times SSC), then in 50%, then in 25%, each for 10 minutes. They were then washed in 2 \times SSC, 0.1% CHAPS for 2 \times 30 minutes, and 0.2 \times SSC, 0.1% CHAPS for the same amount of time. They were then blocked in TBST with 10% sheep serum, incubated overnight in 1:200 alkaline phosphatase-conjugated anti-DIG (Roche) and developed the next day with NBT/BCIP solution. All buffer solutions used for in situ hybridization were treated with diethyl pyrocarbonate, and ProtectRNA (Sigma) was used in all solutions after proteinase K digestion. Sense controls were performed to support antisense experiments, and were negative.

RESULTS AND DISCUSSION

De-tipped ureteric bud stalks regenerate tips and undergo branching

In principle, ureteric bud stalks may refrain from producing new tips because they are intrinsically incapable of doing so, because they are inhibited by existing tips, or because the mesenchyme surrounding

them has been rendered unsupportive of branching by the previous passage of the tip. To test the intrinsic ability of stalks to produce new tips, we removed them from the influence of existing tips, by amputating those tips, and we provided fresh mesenchyme (Fig. 1A). To confirm that the entire tip region had been removed, each amputated tip region was stained either for *Wnt11* mRNA or with fluorescent DBA, to ensure that it contained the tip-stalk boundary (Fig. 1B-D). These are the same markers that we have previously used to study stalk/tip boundaries (Michael et al., 2007), and they define the tip with much more precision than other alleged tip markers, such as Ret and Ros, as explained by Michael et al. (Michael et al., 2007). In any (rare) case that complete removal of the tip could not be confirmed, the corresponding stalk was discarded. To ensure that the fresh mesenchymes did not contain ureteric tips, they were used only if a complete ureteric bud could be recovered from the donor kidney. As an additional check on the efficiency of dissection, samples of mesenchyme were also stained with anti-calbindin-D_{28K}, a marker for ureteric buds (Davies, 1994); they were negative, as expected.

As an additional check that stalks meeting the above criteria for purity really were free of contaminating cells, a dilution-series RT-PCR was performed to set an upper limit on the possible number of tip cells that could be present in an allegedly pure stalk sample. The details of the RT-PCR and the calculations made from it are explained in the Materials and methods. It showed that *Wnt11* in as few as 0.81 ± 0.12 tip cells, represented by the 1/100 dilution of kidney cDNA in Fig. 1E, could be detected clearly. The *Wnt11* in a PCR reaction representing the undiluted cDNA from 0.44 stalks shows a barely detectable band (Fig. 1E). Therefore, each stalk was contaminated by fewer than $0.81/0.44 = 1.8$ tip cells. This is far fewer than those needed to make even one tip (117 ± 18 cells), even after a few cell cycles. These PCR data

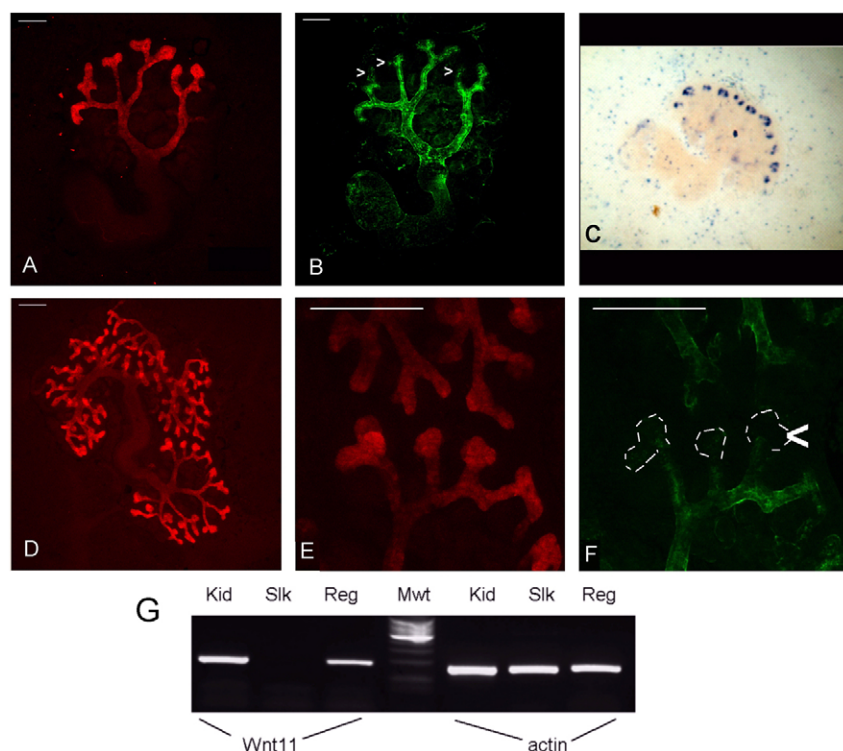


Fig. 2. Regeneration of new branching epithelia from E11.5 ureteric bud stalks.

(A,B) New branching tree generated from an isolated E11.5 stalk surrounded by fresh mesenchyme, stained with (A) anti-calbindin-D_{28k} and (B) the stalk marker DBA. Comparison of the images reveals that at least some of the new tips are DBA negative (arrowheads). (C) Another example, stained for *Wnt11* by in situ hybridization; the new tips have acquired *Wnt11* expression. (D-F) Generation of a new ureteric bud tree from the 'wrong end' of the ureteric bud. (D) Low-power view of a 'double-ended' kidney formed by branching from the cut end of the ureteric bud. (E,F) Higher-power view of the 'wrong end' tree, stained with anti-calbindin-D_{28k} (E) and DBA (F): a significant number of tips (outlined in F) have greatly reduced DBA expression compared with stalk (arrowhead). (G) These stalk-derived tips (Reg) express the tip marker *Wnt11* by RT-PCR at levels similar to those in whole kidneys (kid), whereas the stalks not allowed to generate new tips (Slk) do not express it at detectable levels. Scale bars: 100 μ m.

therefore support the in situ hybridization and immunostaining data in the paragraph above, and suggest that the stalks are not significantly contaminated by tip cells.

The majority (71%) of de-tipped stalks provided with fresh mesenchyme produced branched epithelial trees of an appearance broadly similar to those of a normal ureteric bud, albeit smaller (Fig. 2A). This fraction is approximately twice the number of stalks that could have contained even one contaminating tip cell, as calculated in the paragraph above, so cannot be due to contamination by tip cells. The other 29% simply expanded in a cyst-like manner, probably because they were damaged during handling. The branches produced in the 71% of cultures that produced trees terminated in ampulla-shaped tips that were indistinguishable from those of normal kidneys. What is more, in situ hybridization and RT-PCR for *Wnt11* showed that this tip marker was expressed at the tips of the branches generated by the stalk (Fig. 2C,G), and DBA lectin staining showed that this stalk marker was reduced in or absent from most new tips (Fig. 2B). The de-tipped stalks were therefore capable of regenerating tips that had normal marker expression, as well as normal morphology. The proportion in which this occurred, just over 70%, is much higher than the 6% of branches that seem to arise laterally from stalks in normal renal development (Watanabe and Costantini, 2004), and it is not reasonable to assume that these could have arisen from 'lost' clusters of tip cells left behind: if there were that many 'lost' tip cells, we and others would have seen them in *Wnt11* in situ stains.

Branching and tip formation can be induced even from the wrong end of the ureteric bud

To determine whether the ability to initiate branches was still present even in the most distal regions of the ureteric bud stalk, we left the existing tips of ureteric buds alone and instead packed fresh mesenchyme around the distal end of the ureter that was severed when the kidney was isolated from the embryo (Fig. 1A). Forty

percent of the E11.5 kidneys so treated showed prolific branching from the severed ureter to produce 'double-ended' trees (Fig. 2D). These tips lost DBA-binding activity (Fig. 2E,F) and also induced the formation of nephrons in the surrounding mesenchyme (Fig. 3C,D). This ability is retained by ureters from both E11.5 and E12.5 kidneys (Fig. 3A).

These results demonstrate that the ability of the ureteric bud to initiate new branch tips is not restricted to existing tips but is instead distributed widely, at least for the first few days of the bud's existence. This possibility has been suspected recently from time-lapse studies of ureteric branching (Shakya et al., 2005; Watanabe and Costantini, 2004), but, as pointed out in the recent review of Costantini and Shakya, it has not been directly examined before (Costantini and Shakya, 2006). The finding also implies that the specialized state of gene expression at the tips (*Wnt11*-positive, DBA-negative, etc) might be required for the proper organization of branching morphogenesis, but it cannot be needed for cells to make their first response to ramogenic signals. If it were, the *Wnt11*⁻, DBA⁺ stalks could not have responded. Expression of molecules such as *Wnt11* must therefore be secondary to the events that first induce new branches to form.

Although the distal ends of ureters of E11.5 and E12.5 kidneys could produce new branches when provided with fresh mesenchyme, those of E13.5, E14.5 and E15.5 kidneys failed to do so. These epithelia are surrounded by a sleeve of stroma that might, conceivably, inhibit tip formation. To address this possibility, we removed the stroma enzymatically before applying fresh E11.5 mesenchyme to the ureter epithelium. It was possible to remove 100% of stromal cells from ureters up to and including E13.5, but from E14.5 only about 90% of the cells could be removed (leaving significant uncovered areas of epithelium); further extending the enzymatic incubations resulted in the tissue losing structure completely. The E13.5 ureters freed completely from stroma were able to produce new tips when

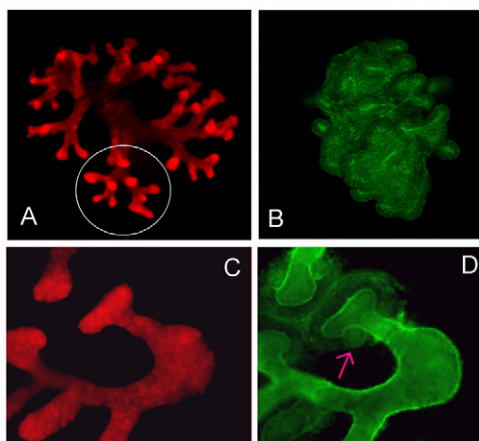


Fig. 3. Generation of branched ureteric systems from the 'wrong end' of the ureters of more mature kidneys. (A) A cut ureteric stalk of an E12.5 kidney capped with E11.5 metanephric mesenchyme ramified through the mesenchyme to generate a branched collecting duct system (circled). (B) Branching morphogenesis of an E11.5 de-tipped stalk transferred to matrigel with GDNF, FGF1 and pleiotrophin, incubated for 144 hours and stained with FITC-phalloidin to reveal its anatomy. It is interesting to note that the phalloidin stain is particularly strong in the apical regions of cells at the branch tips, as described for normal ureteric buds developing in whole kidneys (Michael et al., 2005). (C,D) Tips formed from the 'wrong end' of the ureter, as in A, induce the formation of nephrons in the surrounding mesenchyme. These are not detectable in the ureteric bud-specific anti-calbindin stain (C) but are visible in the anti-laminin stain (D, arrow).

provided with fresh E11.5 mesenchyme, and these tips went on to induce nephrons in that mesenchyme, suggesting that the failure of E13.5 ureters surrounded by stroma to produce new tips was due to an inhibitory influence of the stroma. Later ureters that could be freed substantially but not completely from stroma still failed to form tips. A simple mechanical influence of stroma, for example that it forms a diffusion barrier to molecules such as GDNF from the fresh mesenchyme outside it, is unlikely to explain this effect, as even the older enzyme-treated ureters had lost enough stroma to make the epithelium accessible. Bmps such as Bmp4 and Bmp5 are expressed strongly in this stroma (Dudley and Robertson, 1997), and are known to be inhibitors of branching (Hartwig et al., 2008; Gupta et al., 1999; Miyazaki et al., 2000; Michos et al., 2007). Gremlin 1 is a powerful antagonist of Bmps, particularly Bmp2 and Bmp4, and treatment of cultured kidney rudiments with exogenous gremlin 1 is sufficient to antagonize Bmp activity and alter ureteric branching in intact kidneys (Michos et al., 2007). To test whether the secretion of Bmps by the remaining peri-ureteric stroma might account for the repression of tip formation in our system, we applied the Bmp antagonist gremlin 1 at 5 µg/ml to the cultures. This concentration was the same as that used by Michos et al., and, in our hands, it had a modest effect on increasing the amount of branching in E11.5 kidneys, by 14% ($P=0.073$), suggesting that the molecule was active. It failed, however, to induce tip formation from the enzyme-treated E14.5 ureter/ fresh mesenchyme combinations. This suggests that the stroma secretes an inhibitor other than Bmps, or that the ability to produce new tips is lost as the epithelium itself matures.

New tip formation is a response to fresh mesenchyme, not to tissue injury

The process of setting up the cultures described above necessarily involved cutting mesenchymal and epithelial tissues. It was therefore possible that the production of new branches was simply a response to injury. To test this, two types of cutting experiment were performed without any transplantation of mesenchyme. In the first, a syringe needle was used to cut a slit in the mesenchyme adjacent to one side of the ureteric bud stalk but with no injury to the stalk itself and in the second, the cut passed through the stalk itself, as well as the surrounding mesenchyme (Fig. 1A). The injured kidney rudiments were then incubated for 6 days, uninjured kidneys being used as controls. None of the kidneys in either control or cut groups showed any evidence of branching from the stalk. Conversely, when mesenchyme was removed from the side of the stalk of the ureteric bud, without injuring the bud itself, and replaced by a clump of fresh metanephric mesenchyme, 75% of kidneys demonstrated emission of new branches from the side of the stalk. Injury alone was not therefore a sufficient trigger for production of new tips; fresh mesenchyme was required.

De-tipped stalks branch when placed in a three-dimensional matrix

Intact ureteric buds will grow and branch when placed in a three-dimensional gel matrix, consisting of Matrigel supplemented with GDNF, FGF1 and pleiotrophin (Sakurai et al., 2001). Isolated, de-tipped stalks transferred to this culture system, grow and branch in a manner similar to that of intact ureteric buds (Fig. 3B). This demonstrates that ramogenic factors already characterized in normal mesenchyme (GDNF, FGF1 and pleiotrophin) are sufficient to promote the regeneration of tips. It is notable that the density of tips is much higher in this system than in normal kidneys.

Ureteric stalks, then, are capable of forming new tips if provided with fresh mesenchyme or with a Matrigel artificially loaded with ramogens, such as GDNF and FGF1, known to be manufactured by fresh mesenchyme (Sainio et al., 1997; Sakurai et al., 2001). It is known that GDNF is not expressed by mesenchyme cells after they have been induced, by contact with the ureteric bud, to form nephrons and stroma (Sainio et al., 1997). FGF1 persists a little longer, but is still lost as nephrons mature beyond the 'S'-shaped stage (Cancilla et al., 1999). Indeed, not only do maturing nephrons and stroma cease to produce ramogens, they also begin to secrete anti-ramogenic factors, such as Bmp2 and Tgfβ (Lyons et al., 1995; Ritvos et al., 1995; Davies and Fisher, 2002; Dudley and Robertson, 1997; Bush et al., 2004; Gupta et al., 1999; Piscione et al., 1997). This suggests a model in which stalks are normally prevented from branching because the mesenchyme that surrounds them has already ceased to express ramogens. The likely importance of the mesenchyme in modulating the production of tips by the stalks is supported by the behaviour of stalks in ramogen-enriched Matrigel. The density of tips formed by the stalk is much higher than that seen in normal kidney development, suggesting that in the normal organ the mesenchyme surrounding the stalk must be non-permissive for tip formation. Indeed, it is the source of factors, such as heregulin α (neuregulin 1 – Mouse Genome Informatics), that support growth and maturation of the bud without inducing branching (Sakurai et al., 2005).

This system described above would, under normal circumstances, tend to restrict branching to the existing tips because these are the only cells that meet uninduced mesenchyme. Only if mesenchymal cell mixing, and/or inefficient branching of the bud throughout the mesenchyme, brought a population of uninduced mesenchyme cells

near to a stalk would production of a new tip by the stalk occur. A system organized according to these principles would be robust against errors, because any zones of the kidney 'missed out' by the branching of the tree would be able to induce secondary branches from stalks until they were adequately served. This presumably accounts for the very low, but non-zero (6%), frequency with which lateral branches have been observed to occur in culture (Watanabe and Costantini, 2004).

Understanding that the whole of the ureteric bud is capable of producing a branching tree, at least until it has matured too far, may have implications beyond the need to revise models for the control of pattern formation in this system. There is increasing interest in using the techniques of stem cell biology and tissue engineering to repair kidneys made defective by congenital disease or infection (Hayashi, 2006; Rookmaaker et al., 2004). Most current effort is aimed at using transplanted progenitor cells to create areas of kidney in which new nephrons, free of genetic defects, develop. The absence, in a fully formed kidney, of active ureteric bud tips to provide these areas with a collecting duct system has been seen as a potential problem of the technique. If, however, the stalks of the cortical bud/collecting duct system can generate new tips anyway, either at once or as a result of minor treatment, the entire enterprise becomes much more hopeful. For this reason, our observation that stalks can regenerate tips may have implications for regenerative medicine, as well as for basic developmental biology.

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References

- Bush, K. T., Sakurai, H., Steer, D. L., Leonard, M. O., Sampogna, R. V., Meyer, T. N., Schwesinger, C., Qiao, J. and Nigam, S. K. (2004). TGF-beta superfamily members modulate growth, branching, shaping and patterning of the ureteric bud. *Dev. Biol.* **266**, 285-298.
- Cancilla, B., Ford-Perriss, M. D. and Bertram, J. F. (1999). Expression and localization of fibroblast growth factors and fibroblast growth factor receptors in the developing rat kidney. *Kidney Int.* **56**, 2025-2039.
- Carroll, T. J., Park, J. S., Hayashi, S., Majumdar, A. and McMahon, A. P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell* **9**, 283-292.
- Cebrian, C., Borodo, K., Charles, N. and Herzlinger, D. A. (2004). Morphometric index of the developing murine kidney. *Dev. Dyn.* **231**, 601-608.
- Costantini, F. and Shakya, R. (2006). GDNF/Ret signaling and the development of the kidney. *BioEssays* **28**, 117-127.
- Davies, J. (1994). Control of calbindin-D28K expression in developing mouse kidney. *Dev. Dyn.* **199**, 45-51.
- Davies, J. (2005). *Branching Morphogenesis*. Georgetown, Texas, USA: Landes Biomedical.
- Davies, J. A. and Fisher, C. E. (2002). Genes and proteins in renal development. *Exp. Nephrol.* **10**, 102-113.
- Davies, J. A., Millar, C. B., Johnson, E. M., Jr and Milbrandt, J. (1999). Neurturin: an autocrine regulator of renal collecting duct development. *Dev. Genet.* **24**, 284-292.
- Dudley, A. T. and Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev. Dyn.* **208**, 349-362.
- Fleury, V. and Watanabe, T. (2002). Morphogenesis of fingers and branched organs: how collagen and fibroblasts break the symmetry of growing biological tissue. *C. R. Biol.* **325**, 571-583.
- Fleury, V., Watanabe, W., Nguyen, T.-H., Unbekandt, M., Warburton, D., Dejmeck, M., Nguyen, M., Lindner, A. and Schwartz, L. (2004). Physical mechanisms of branching morphogenesis in animals. In *Branching Morphogenesis* (ed. by J. Davies). Georgetown, Texas, USA: Landes Bioscience.
- Gupta, I. R., Piscione, T. D., Grisaru, S., Phan, T., Macias-Silva, M., Zhou, X., Whiteside, C., Wrana, J. L. and Rosenblum, N. D. (1999). Protein kinase A is a negative regulator of renal branching morphogenesis and modulates inhibitory and stimulatory bone morphogenetic proteins. *J. Biol. Chem.* **274**, 26305-26314.
- Hartwig, S., Bridgewater, D., Di Giovanni, V., Cain, J., Mishina, Y. and Rosenblum, N. D. (2008). BMP receptor ALK3 controls collecting system development. *J. Am. Soc. Nephrol.* **19**, 117-124.
- Hayashi, M. (2006). Development of new therapies, including regeneration of the kidney, for chronic kidney diseases. *Clin. Exp. Nephrol.* **10**, 99-101.
- Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H. and Koopman, P. (1996). A male-specific role for SOX9 in vertebrate sex determination. *Development* **122**, 2813-2822.
- Kispert, A., Vainio, S., Shen, L., Rowitch, D. H. and McMahon, A. P. (1996). Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* **122**, 3627-3637.
- Lin, Y., Zhang, S., Rehn, M., Itaranta, P., Tuukkanen, J., Heljasvaara, R., Peltoketo, H., Pihlajaniemi, T. and Vainio, S. (2001). Induced repatterning of type XVIII collagen expression in ureter bud from kidney to lung type: association with sonic hedgehog and ectopic surfactant protein C. *Development* **128**, 1573-1585.
- Lyons, K. M., Hogan, B. L. and Robertson, E. J. (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech. Dev.* **50**, 71-83.
- Michael, L. and Davies, J. A. (2004). Pattern and regulation of cell proliferation during murine ureteric bud development. *J. Anat.* **204**, 241-255.
- Michael, L., Sweeney, D. and Davies, J. A. (2005). A role for microfilament-based contraction in branching morphogenesis of the ureteric bud. *Kidney Int.* **68**, 2010-2018.
- Michael, L., Sweeney, D. E. and Davies, J. A. (2007). The lectin Dolichos biflorus agglutinin is a sensitive indicator of branching morphogenetic activity in the developing mouse metanephric collecting duct system. *J. Anat.* **210**, 89-97.
- Michos, O., Goncalves, A., Lopez-Rios, J., Tiecke, E., Naillat, F., Beier, K., Galli, A., Vainio, S. and Zeller, R. (2007). Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. *Development* **134**, 2397-2405.
- Miyazaki, Y., Oshima, K., Fogo, A., Hogan, B. L. and Ichikawa, I. (2000). Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J. Clin. Invest.* **105**, 863-873.
- Piscione, T. D., Yager, T. D., Gupta, I. R., Grinfeld, B., Pei, Y., Attisano, L., Wrana, J. L. and Rosenblum, N. D. (1997). BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. *Am. J. Physiol.* **273**, F961-F975.
- Qiao, J., Sakurai, H. and Nigam, S. K. (1999). Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. *Proc. Natl. Acad. Sci. USA* **96**, 7330-7335.
- Ritvos, O., Tuuri, T., Eramaa, M., Sainio, K., Hilden, K., Saxen, L. and Gilbert, S. F. (1995). Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mech. Dev.* **50**, 229-245.
- Rookmaaker, M. B., Verhaar, M. C., van Zonneveld, A. J. and Rabelink, T. J. (2004). Progenitor cells in the kidney: biology and therapeutic perspectives. *Kidney Int.* **66**, 518-522.
- Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V. et al. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-4087.
- Sakurai, H., Bush, K. T. and Nigam, S. K. (2001). Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogenesis. *Development* **128**, 3283-3293.
- Sakurai, H., Bush, K. T. and Nigam, S. K. (2005). Heregulin induces glial cell line-derived neurotrophic growth factor-independent, non-branching growth and differentiation of ureteric bud epithelia. *J. Biol. Chem.* **280**, 42181-42187.
- Santos, O. F. and Nigam, S. K. (1993). HGF-induced tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF-beta. *Dev. Biol.* **160**, 293-302.
- Shakya, R., Watanabe, T. and Costantini, F. (2005). The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev. Cell* **8**, 65-74.
- Watanabe, T. and Costantini, F. (2004). Real-time analysis of ureteric bud branching morphogenesis in vitro. *Dev. Biol.* **271**, 98-108.