CHAPTER 8

Branching Morphogenesis in Mammalian Kidneys

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Introduction

Branching morphogenesis is an important mechanism for the development of the permanent kidneys of reptiles, mammals and birds. Branching of renal epithelia is similar to that seen in the other organs described in this book[1] but organogenesis of kidneys has unique features that, at the expense of some complication, offer an opportunity to address deep questions of both developmental and evolutionary biology. Understanding the development of branched epithelia in the kidney is also important medically because abnormalities of these epithelia are responsible for a number of serious diseases which, at an incidence of more than 1:800, are amongst the most common human congenital abnormalities.[2,3]

The branched epithelium of the kidney, the collecting duct system, exists mainly to channel urine to a common drainage duct, the ureter. This contrasts with the other organs described in this book, such as the mammary, salivary and prostate glands, in which cells derived from the branching epithelium are responsible for producing the secretions of the organ, as well as for channelling them to the outside world. In the mammary gland, for example, cells derived directly from the branching milk ducts produce mammary alveoli that secrete milk into the ducts (see Chapter 7). In kidneys, the main 'secretion' (urine) is made by nephrons, which are epithelial structures derived not directly from the collecting ducts but rather by a mesenchymal-epithelial transition in the tissue that surrounds them. These nephrons then connect to nearby ducts and drain in to them. The functions of 'secretion' and drainage are therefore almost separate in the kidney ('almost' because the collecting ducts do play a role in modifying the contents of urine, particularly acid-base balance).

The use of a branched drainage structure arose rather late in the evolutionary history of vertebrate excretory systems; the permanent kidneys of agnatha, fish and amphibians are unbranched (or show only rudimentary branching of the fusion type—see Chapter 1) and highly-branched kidneys arose only with animals whose entire life cycle can be spent out of water. This late acquisition of branching is not unique to the excretory system—the airways of lungs, for example, are highly-branched in mammals and birds but hardly branch at all in most reptiles.[4] What is unusual is that reptiles, mammals and birds still make the primitive forms of kidney (pronephroi and mesonephroi) during their embryonic lives and construct their branched kidneys (metanephroi) as completely new organs.

The first morphological sign of mammalian kidney development is the emergence of an epithelial tube called the ureteric bud. This forms as an outgrowth from an existing epithelial tube, the nephric duct, that runs down the cranio-caudal axis of the body and drains the temporary kidneys of the embryo (pro- and mesonephroi) (Fig. 1). The ureteric bud grows towards and invades an adjacent area of intermediate mesoderm, the metanephrogenic mesenchyme. Once in that mesenchyme, the bud begins to branch and continues to grow to create a

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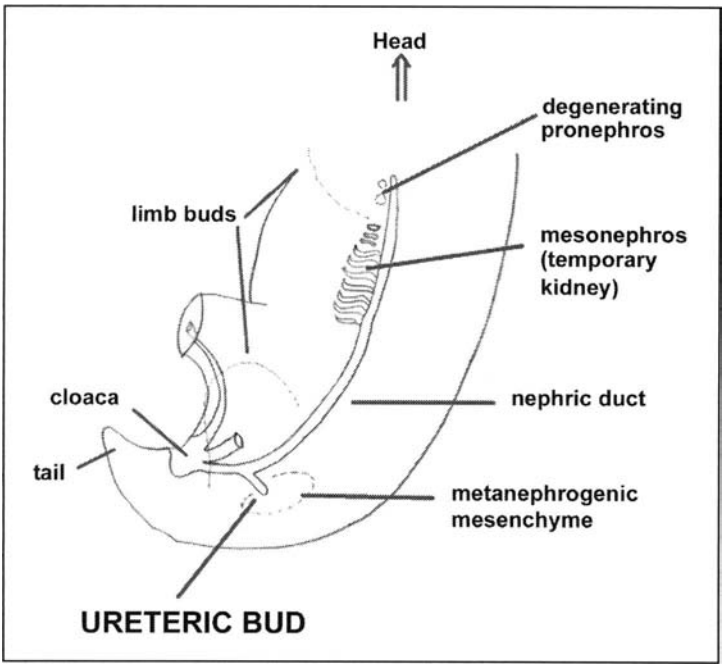

Figure 1. The general arrangement of the developing excretory system of an E10.5 mouse embryo.

tree-like arrangement of tubules (Fig. 2). As it grows, the epithelial tree induces nearby groups of mesenchymal cells to differentiate into nephrons which will later connect to it. The nephrons command a blood supply (to form the glomerular capillaries and the counter-current multiplication system) and also a nerve supply. Since the shape of the collecting duct controls the positions at which nephrons form, and the nephrons control the blood and nerve supplies, it is fair to say that the branching of the ureteric bud/ collecting duct determines the anatomy of the entire organ. The rest of this chapter will be dominated by discussion of the mechanisms and regulation of ureteric bud branching, in view of its importance, but I shall discuss the blood system at the end.

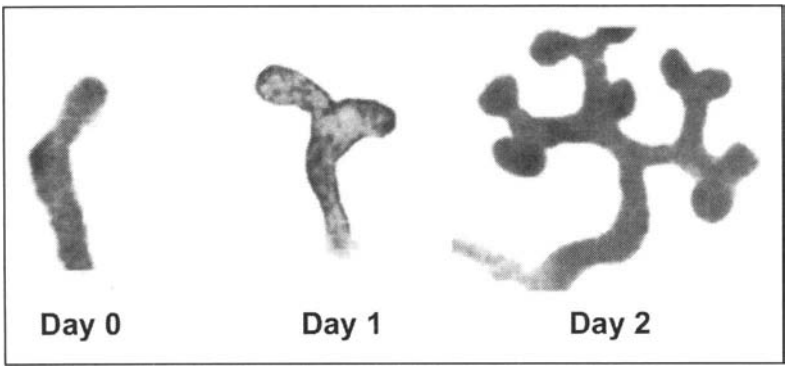

Figure 2. The branching of ureteric bud in organ culture (stained with anti-calbindin-D-28K, a marker for ureteric bud in this system[113]).

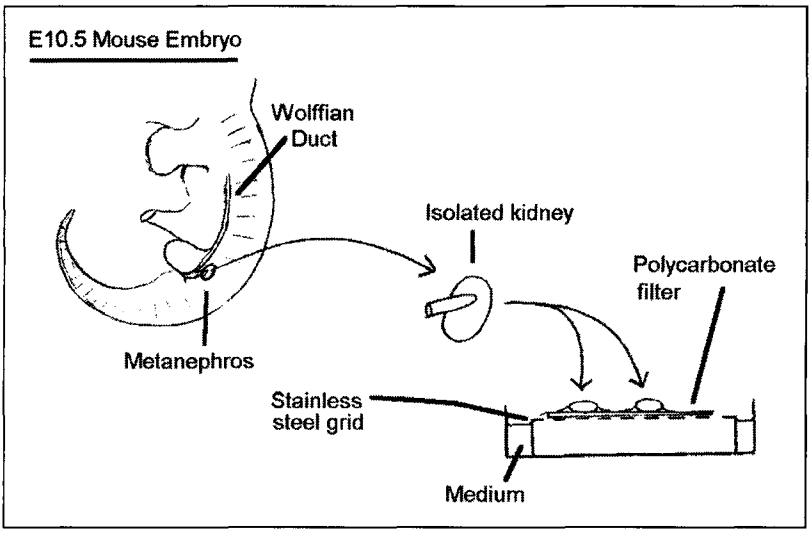

Figure 3. The standard method for mouse kidney organ culture.

Models for Studying Branching in the Kidney

Embryonic kidneys are unusually accessible for study, partly because they will grow well in organ culture and partly because mammalian embryos can rely completely on their mother's circulation for excretion so that renal abnormalities do not cause an early termination of foetal development.

One obvious 'model' for study of renal development is growth in vivo, of either completely normal animals or of mutants. This method has the advantage that development is seen in a realistic situation, but it has the disadvantages of poor access and of separating direct effects of a mutation on kidney development from indirect effects caused by abnormal development of other embryonic systems. It is, however, the only method suitable for the analysis of later events, because no culture system has yet managed to support kidney development up to mature stages.

Organ culture of isolated kidney rudiments has been a powerful model for studying mammalian organogenesis since the 1950s, when it was found that rudiments, isolated by microdissection at embryonic day 10.5 and cultured on a supporting filter at a gas-medium interface, would grow organotypically and would reproduce the first few days of renal development[5] (Fig. 3). Analysis of kidneys growing in culture can help to separate systemic effects of a mutation from local effects autonomous to the kidney.[6] Cultured kidneys are also accessible to antibodies, pharmacological reagents and exogenous growth factors, a fact that has been central to a large number of studies on the functions of particular molecules in renal development. Recently, we have developed a technique, based on small interfering RNAs (siRNAs), for inhibiting specific genes in cultured kidneys at a time of the experimenter's choosing;[7] this should greatly facilitate the molecular analysis of renal development. Isolated kidneys can be dissected into their component tissues (eg ureteric bud and mesenchyme), and these tissues can then be recombined and will develop normally—this allows experimenters to study the development of a ureteric bud from a mutant animal in the context of a wild-type mesenchyme and vice-versa. Such tissue recombination experiments have been valuable in determining precisely which tissues are affected by a mutation.[6]

Recently, a culture system has been developed in which isolated ureteric buds will grow and branch apparently normally in a 3-dimensional gel (in the presence of appropriate growth and

survival factors).[8] This allows experimenters to study the regulation of ureteric bud morphogenesis in the absence of feedback loops operating in the mesenchyme, which used to be thought to be essential for branching to occur normally.[9,10] Finally, there is a culture system in which cell lines generated from ureteric bud or from mature collecting ducts are suspended in a 3-dimensional collagen matrix, in which they multiply and form cysts with the same polarity (apical domain facing the lumen) as a normal ureteric bud. When these cysts are treated with appropriate growth factors (such as HGF), they produce processes which then branch in a manner alleged, by those who use this system, to be similar to that of a real ureteric bud.[11] A great advantage of the cell-line model is that the cells can be transfected before use so that advanced genetic manipulations can be performed on them, something that is difficult for intact kidneys.

There is, as in other systems, some tension between proponents of simple culture models and those who insist that only experiments carried out in vivo are truly informative. Progress is usually fastest, however, when a combination of all techniques can be used so that simple models can generate hypotheses quickly and these can then be tested in, for example, transgenic animals. Regulation of ureteric bud branching by the GDNF signalling system (described later) is an excellent example of a story that draws on cell lines, isolated ureteric buds, cultured kidneys and transgenic animals and is much stronger for the combination.[12-16]

There is also an occasional tendency for commentators to reject claims that a molecule expressed in kidneys and shown to have an effect in organ culture is a regulator of renal development, if knockout of that molecule in vivo has no detectable phenotype. This rejection is based on a misunderstanding of the likely properties of a regulatory network. The few biological networks to have been studied mathematically have been found to possess the same general 'scale-free' architecture as man-made networks such as the Internet.[17] They would therefore be expected to show the similar responses to damage. Deleting random elements of such a network leads to its 'graceful failure;' a gradual reduction in efficiency with increasing numbers of deleted elements rather than a cataclysmic collapse.[18] Even knocking out up to 5% of the components randomly makes little difference to such a network as a whole (this is why the Internet is tolerant of the random failures of hardware that happen all the time). Only by targeting the few very critical elements can single deletions bring about serious damage. The lack of an obvious effect when any one particular renal gene is knocked out does not therefore mean that that gene has no role in the regulation of kidney development, but only that the gene is question is not one of those few critical network elements. It is to be hoped that an increasing understanding of biological networks will lay this confusion to rest, particularly as genetic experiments identify more and more partially-penetrant phenotypes which may well just be the expression of the declining efficiency seen in 'graceful failure'.

The Ureteric Bud Tip As an 'Organizer' of the Kidney

In many developing systems, one specific component seems to play such an important role in regulating the behaviour of all of the others that it is considered to be an 'organizer'. That is not to say that the other components do nothing or that the organizer is completely autonomous, but rather that most of the important regulatory pathways, even those originating elsewhere, pass via and are integrated by that organizer. The first organizers to be described were those of gross body structure, such as the dorsal lip of the frog blastopore,[19] but subsequent studies have identified organizers of more local development, such as the enamel knots that control the development of teeth.[20,21]

The tips of the branching ureteric bud/ collecting duct system control renal development to such an extent that they too seem to be organizers. Their many activities will be described in more detail below, but in brief summary; they are the main site of cell division in the ureteric bud, they are responsible for branching of the bud, they receive and integrate mesenchyme-derived signals that control bud morphogenesis, they originate signals that control mesenchyme development, proliferation and apoptosis, they induce differentiation of

nephrons and they probably originate signals that ensure the correct spacing of collecting duct branches. In short, if the concept of an organizer has any validity in the kidney, then the bud tip has by far the strongest credentials for the role. It is of course possible that the whole language of 'organizing centres' is inappropriate and that further analysis of renal development will reveal it to be under the control of a much more distributed network, but studies of various types of real-world networks suggests that the presence of key integrating nodes is common. In networks as diverse as bacterial metabolism, interacting *Drosophila* proteins, littoral food webs, the World Wide Web hyperlinks and flight paths between airports, there are a few nodes through which very high traffic (/information/ energy) flows;[22-25] in networks controlling development, these would be called organizers.

In this chapter, I shall begin by describing the cell- and molecular-biological features of the ureteric bud tips, concentrating on those most closely connected to morphogenesis (reviews about other aspects of renal development may be found elsewhere[26,9,27]). I will then go on to explain how some of these features of the bud, combined with those of surrounding cells, might produce feedback systems that regulate branching morphogenesis.

Cell Biology of the Branch Tips

The tips of the ureteric bud/ collecting duct system are slightly bulb-shaped, when not actually branching. They are composed of a rather disorganised epithelium, which seems to have more than one layer (at least in rats) and which is not surrounded by the obvious, continuous basement membrane that can be seen around the stalks when examined by electron microscopy.[28] The tip cells also show rather few intercellular junctions compared with those of stalks and (at least in rabbits) do not express some of the cadherins that are expressed in the stalks.[29] This rather disorganised arrangement of the terminal epithelium may be an adaptation to allow rapid cell rearrangement during branching morphogenesis. Alternatively, or perhaps additionally, it may facilitate recruitment of extra cells from the surrounding mesenchyme.[30]

The process of branching alters the morphology of the tips cyclically, as emergence of new branches alternates with elongation. In mouse kidneys developing in organ culture, branching seems to take place mainly by simple bifurcation of the tips of the growing collecting duct tree. In microdissected human kidneys, though, the pattern seems to be more complicated, consisting of the emergence of a new tip just behind the old one followed by the bifurcation of this new tip only, resulting in a three-pointed structure consisting of the original tip and two new ones[31,32] (Fig. 4). The new tips elongate and, later, a new tip emerges just proximally to each and immediately bifurcates, the process repeating about 15 times in humans.[33] It is not clear whether the apparent difference between mouse culture data and human microdissection data results from a difference between species, a difference between behaviour in vivo and in vitro, or a difference between the early generations of branching seen in culture and the later generations that would have been represented in the human samples.

The morphogenetic mechanisms by which branching takes place are probably the least understood aspects of the ureteric bud tip. The kidney does not appear to use the clefting mechanism seen, for example, in the salivary gland (see Chapters 9, 12) in which bands of collagen divide an expanding ampulla into lobes that then extend, allowing the cycle to repeat.[34] Apart from the fact that there is no morphological evidence for clefting in the kidney, tissue inhibitors of metalloproteinases (TIMPs), which protect collagen from degradation, inhibit branching in the kidney[35,36] but do exactly the opposite in salivary glands.[37] This contrast suggests a fundamentally different mechanism may be at work. Rather than being generated by cleavage of an existing ampulla, new branch points seem to grow outwards from an existing ureteric bud tip, usually at a direction perpendicular to that of the original and 180 degrees away, at least initially (this may be seen from the excellent time-lapse sequences of the Costantini lab[38]). There are various possible mechanisms that might drive this emergence, but none have been investigated in any detail.

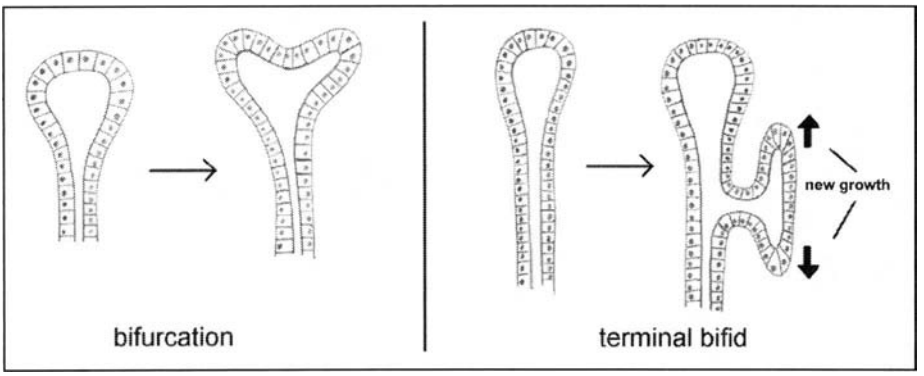

Figure 4. The two types of branching in the kidney: bifurcation, as seen in organ culture, and terminal bifid branching observed in later collecting duct development in humans. It is not yet clear whether the difference reflects developmental stage, species, or whether development is in vivo or in vitro.

One mechanism for branching that may be used in the kidney is epithelial folding driven by local contraction of apical actin, which would make cells wedge-shaped and therefore cause that part of the epithelium to curve outwards (Fig. 5). There are numerous bands of apical actin at the tip[39] and inhibitors of actin polymerisation and of myosin-mediated actin contraction inhibit branching in cultured mouse kidneys; these data support the mechanism, but the many roles of myosin makes such an experiment difficult to interpret. The relative lack of intercellular junctions in this area also makes the actin-contraction model less attractive, since the forces would have to be transmitted cell-to-cell by these junctions. This mechanism is used in other examples of epithelial development, though, such as invagination of the neural tube[40,41] or folding of the colon[42] so it should not be dismissed without further work.

Another possible way in which new tips are created, perhaps made more likely by the rather disorganized nature of the tip epithelium, is locomotion of epithelial cells. Fibroblast-like

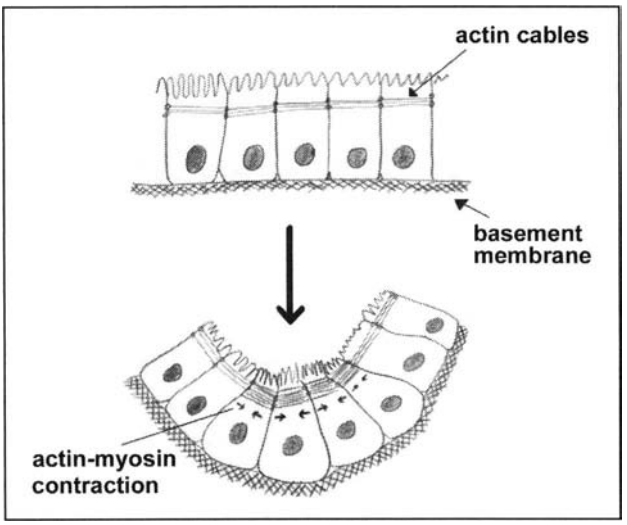

Figure 5. Bending of an epithelium by actin-myosin driven apical contraction.

locomotion of epithelia, using lamellipodia and filopodia, has been observed in wound healing in vertebrates,[43] in dorsal closure in *Drosophila*[44] and also in a variety of culture models. There has been no direct observation of lamellipodia and filopodia structures in ureteric bud cells, but when small amounts of cytochalasin are applied to kidneys growing in culture to compromise the integrity of the actin-adhaerens junction system, migratory cells do stream out specifically from the ureteric bud tips.[45] This suggests that these cells may be primed for migration, although it certainly does not prove the point. In most cells, the balance between actin being organised for contractile stress fibres or for lamellipodia and filopodia is controlled by competition between the small GTPases, Rac, Rho and cdc42. It would be very interesting to examine the activation states of these pathways during ureteric bud morphogenesis.

New tips might also perhaps be produced simply by localised cell proliferation and directional cytokinesis. Culture of mouse kidney rudiments in medium containing the thymidine analogue, bromodeoxyuridine (BrdU), reveals that there is much more cell proliferation in the tips than in the stalks as long as morphogenesis is taking place normally, but not when branching is inhibited by a variety of treatments.[39,46] It is not clear whether cell proliferation is a direct mechanism of morphogenesis or just a necessary accompaniment, although it is striking that a local increase in proliferation is seen in the nephric duct before any obvious morphological sign of ureteric bud emergence, suggesting that elevated proliferation is not simply a consequence of morphogenesis.[46] Proliferation in the tips is controlled, either directly or indirectly, by the MAP-kinase signalling pathway.[47]

Molecular Markers of 'Tip' Character

Until recently, it has not been obvious whether the special behaviour of tip cells reflects an unique state of gene expression in these cells or just different behaviours of otherwise identical cells being driven simply by the different shapes and stresses in the tissue. In recent years, however, evidence has been obtained for differential gene expression between tip and stalk, suggesting that tip cells are in a specific state of differentiation. The most striking marker for the tip cells is expression of *wnt11* gene, which encodes a signalling protein: *wnt11* is expressed only by the cells at the very tip and vanishes as soon as they leave for the stalk.[48-50] The as-yet uncharacterised glycoprotein that bears a ligand for the *Dolichos biflorus agglutinin* lectin has a reciprocal expression pattern, staining the stalks of the ureteric bud but not extending into the tip (Sweeney, Michael, Davies unpublished). Other genes, such as *ret* and *ros,* have been reported to be expressed in 'tips', but the regions described as 'tips' in these reports extend much further into the stalk than the region described as a 'tip' in this Chapter, and staining for the proteins confirms a less-restricted pattern.[39]

During normal growth, it is natural to assume that new stalk cells differentiate from tip cells that are 'left behind' by the advancing tip. In support of this view are the facts that most bud proliferation takes place in the tip, and that the ureteric bud begins as nothing but a 'tip' so it is difficult to see from where else stalk cells could come. The 'natural' direction of differentiation, from tip to stalk, may be reversible, though, and this might have important implications for the shape of the collecting duct tree. In a recent series of experiments, we have used microdissection to separate the tip and stalk regions of a once-branched ureteric bud (discarding the intermediate portion whose status may be ambiguous) and have cultured each in the presence of embryonic kidney mesenchyme. Isolated tips, which begin Wnt11-positive and DBA-negative, slowly generate a branched structure which includes DBA-positive stalks; this is not surprising given the 'normal' direction of differentiation. Isolated stalks, which begin DBA-positive and Wnt11-negative, seem to generate new tip regions which are Wnt11-positive (Sweeney, Michael and Davies, unpublished). This surprising result suggests that stalk cells can reverse their differentiation to become tip cells again, and may explain why cell-lines derived from mature collecting ducts are able to form branching structures when cultured in collagen gels.[51,52] Furthermore, the apparent ability of stalk to generate new tips when an existing tip has been removed suggests that the presence of a nearby tip normally inhibits stalk-to-tip differentiation; this may be an important mechanism for spacing the branches of the tree, and will be discussed in more detail below.

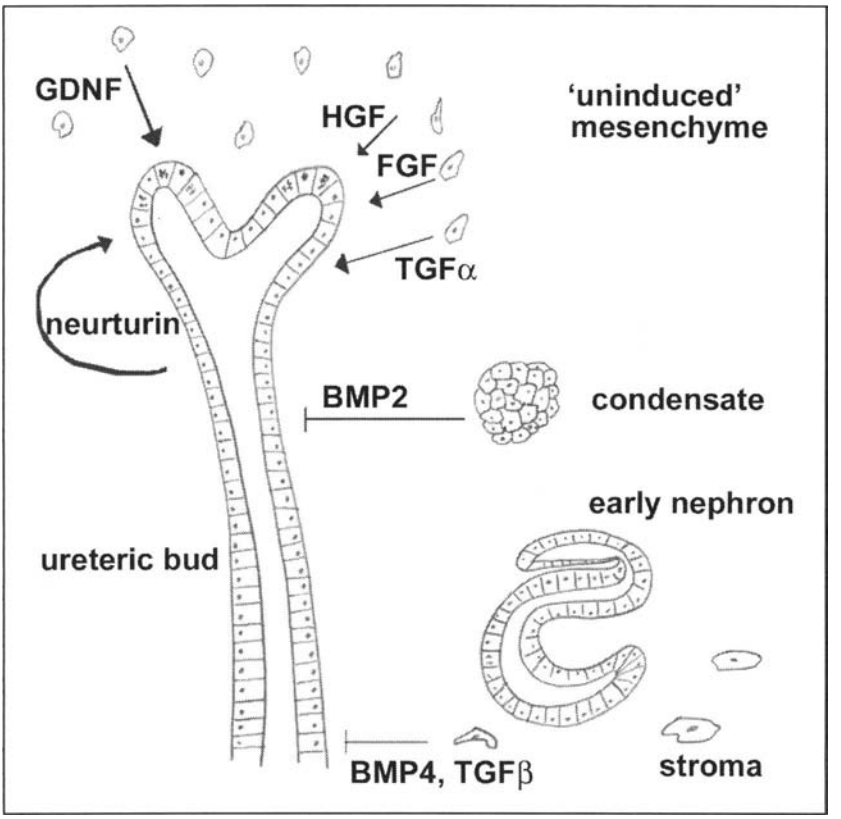

Figure 6. Regulatory inputs to the ureteric bud. Inputs that encourage branching morphogenesis are shown by ↓ and inputs that inhibit branching by ⊥.

Regulatory Inputs to the Bud Tips

Cells of the ureteric bud tip bear a large number of receptors for signalling molecules that are made by surrounding tissues (Fig. 6). One of the most important is the receptor system for glial cell-line derived neurotrophic factor (GDNF), which is made by the 'uninduced' mesenchymal cells into which the bud tips invade. The receptor complex for GDNF is composed of the Ret receptor tyrosine kinase, a GFRα1 co-receptor and 2-O-sulphated heparan sulphate glycosaminoglycan, which also seems to serve as a co-receptor.[53,54] Transgenic mice that are defective in any of these components, or cultured organs that have been subjected to treatments that remove or inhibit any of these components, cause failure of ureteric bud branching.[55-60] Conversely, provision of exogenous GDNF in culture increases the amount of branching, up to a point, and local sources of GDNF can cause the production of ectopic ureteric buds from the Wolffian duct.[61,62] GDNF therefore seems to serve an analogous role to that played by FGF7/10 signalling through FGFRIIIb in the branching epithelia of lungs, salivary glands, mammary glands, prostate and pancreas in that it seems to be the principal driver of branching. GDNF cannot be the only signal capable of inducing the emergence of the ureteric bud itself, though, because some *gdnf-/-* mice do manage to produce a ureteric bud although it does not then go on to branch.[63,64]

The Ret receptor tyrosine kinase also acts as a receptor for neurturin, produced by the bud itself, and persephin, both of which are members of the GDNF family and both of which

stimulate ureteric bud branching in organ culture. The ureteric bud also bears the Met receptor tyrosine kinase which is stimulated by hepatocyte growth factor (HGF) from the surrounding mesenchyme, the EGF receptor which is stimulated by TGFα from the bud itself, and FGF receptors which respond to mesenchyme-derived FGF: all of these stimulate bud growth and branching.[65,66,67,68] Different FGFs evoke different responses from ureteric buds in simple culture systems; FGFs1 and 10 induce long branches with ampullary tips, and FGFs 2 and 7 induce a more general and less organised proliferation.[69] Each of these receptor systems can signal intracellularly via the MAP-kinase and PI-3-kinase pathways, and there is evidence that implicates each of these pathways in the stimulation of ureteric bud branching.[39,70]

As well as having receptor tyrosine kinases, the bud expresses receptor serine/threonine kinases for BMPs, TGFβ and activin.[71-73] These receptors, which generally signal via Smad proteins, all tend to inhibit branching (although BMP7 is a little complicated, encouraging branching when applied at low concentrations but inhibiting it when applied at higher concentration, at least in complete cultured organs). The ability of TGFβ and BMPs to inhibit branching is a feature not just of kidneys, but also of lungs, pancreas and salivary, mammary and prostate glands.[74]

The ureteric bud bears also receptors for matrix components, particularly integrins. At least some of these, for example integrins containing the α8 chain, are necessary for normal morphogenesis.[75] Integrin α8 can associate with a variety of ligands (e.g., fibronectin, virtonectin, tensacin), but a kidney-specific matrix component called nephronectin seems to be a particularly important α8 ligand for development of the ureteric bud.[76] Connections within the matrix itself are important too, and inhibiting interactions between matrix components such as laminin and nidogen inhibits branching.[77] It is not clear whether the matrix provides an instructive or merely a permissive role in regulating bud morphogenesis.

Many of the receptors described above are not expressed exclusively in the tips, but also extend some way along the stalks. Some, such as Ret, extend back a relatively short distance while others, such as the serine/threonine kinases, are expressed along much of the length of the system.

Morphoregulatory Outputs from the Ureteric Bud Tips

As well as bearing receptors for signalling molecules coming from elsewhere, the ureteric bud is the origin of a number of important signals (Fig. 7). Some of these, such as neurturin, appear to act on the bud itself[78] but most act on the cells that surround it. At least three bud-derived signals, TGFα, FGF2 and TIMP2, act as survival factors and mitogens for cells of the mesenchymal blastema into which the ureteric bud grows.[79-84] Without these factors, the mesenchyme dies by apoptosis[85] but with them it proliferates enough to maintain growth of the organ rudiment as well as to contribute cells to various pathways of differentiation. It is interesting that some function of TIMP2 other than its well-known ability to inhibit metalloproteinase activity seems to be required in this context, because pharmacological inhibitors of the same metalloproteinases cannot subsititute for TIMP2.[86] The bud tips (and only the tips) produce Wnt11, a signalling protein that stimulates production of GDNF by the surrounding mesenchyme.[87-90]

The ureteric bud also makes an inductive signal, which may include the above growth factors and which probably includes as-yet unidentified components as well, that cause groups of mesenchyme cells to condense together and differentiate into nephrons. This inductive process is still not understood well—it is not even certain whether the mesenchyme is a homogenous population, the cells of which have the choice between remaining blastemal, differentiating into nephrons or into stroma, or whether the nephron and stroma lineages are distinct from the start. Once they have been induced to form nephrons, cells draw together into a tight cell condensate which then epithelializes to form a cyst. The cyst, the sequence of developmental events followed by these cells is reasonably well understood. They aggregate undergoes a stereotyped series of morphogenetic movements to produce a comma-shaped body, and S-shaped

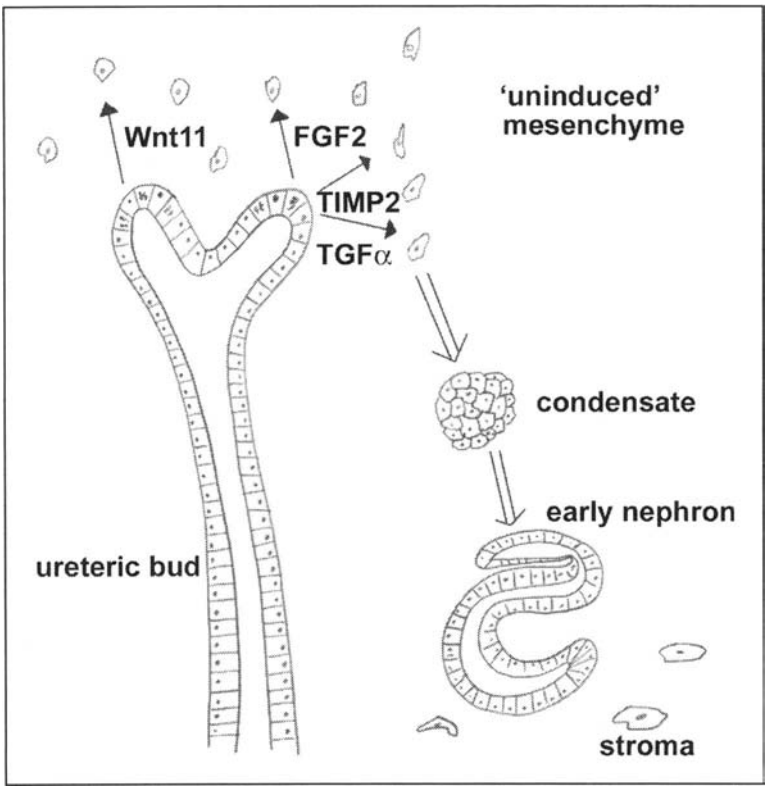

Figure 7. Regulatory outputs from the ureteric bud. The main outputs of the bud prevent mesenchymal apoptosis and drive the differentiation of mesenchyme to nephrons.

body and then the mature nephron. The changes in gene expression that take place during this process have been reviewed extensively elsewhere[9,91,92] so, because they are not really to do with branching, they will not be described in detail here. One aspect of them that is relevant to the branching of the ureteric bud is that cells at different stages of nephron and stroma differentiation produce different sets of growth factors, and these signal back to the ureteric bud (see the section on feedback, below).

Some 'outputs' of the ureteric bud may play a direct role in morphogenesis (rather than an indirect one by signalling to other tissues). One example is MT1-MMP, which is a membrane-bound metalloproteinase that cleaves and activates other metalloproteinases, that can in turn digest components of the extracellular matrix.[93] Ths digestion process may modulate signalling, by releasing matrix-bound growth factors, but it may also be important in simply clearing a path along which the ureteric bud can advance. In organ culture, metalloproteinases such as MMP9 are essential for ureteric bud branching.[94] This is also true for isolated ureteric buds in culture and for cell-line models.[95] The activation of metalloproteases is itself controlled by factors coming from the mesenchyme (/mesenchyme substitute), suggesting another level of feedback.[96]

Feedback Loops That Control Bud Branching

The simple descriptions of signalling to and from the ureteric bud tip presented above highlight two key facts; tip branching normally takes place only in the presence of the correct

mesenchyme-derived signals, and signals coming from the tip cause the mesenchyme to alter its expression of signalling proteins. These facts combine to create a feedback system that could potentially control the shape and extent of ureteric bud branching in the complete organ.

The main positive regulators of ureteric bud development, GDNF, HGF etc, are made by the mesenchymal blastema before it is invaded by the bud, and therefore before it is induced to differentiate into nephrons. Once the bud has begun to branch, the only parts of the kidney that are in this branch-promoting state would be those at its periphery, beyond the current reach of the bud. Branch-promoting factors would therefore be expected to form a gradient, increasing centrifugally and potentially guiding the ureteric bud tips outwards (although the presence of such a gradient has not yet been proven by direct measurement of protein concentrations). As soon as they begin to condense and to differentiate into nephrons, mesenchymal cells lose their expression of GDNF and HGF.[97,98] The processes of induction and mesenchymal differentiation take time (in culture, obvious condensation of mesenchyme takes place about 18 hours after contact with the inducing tissue[99]), so the mesenchymal cells that have just been reached by and induced by contact with the bud will still produce GDNF for a short while. By the time their expression of GDNF has been lost, the bud will have moved on a little way to invade fresh mesenchyme. The result of this is that, while the virgin mesenchyme that lies just ahead of the bud tip will be a rich source of GDNF, and that immediately around it will still have some, the mesenchyme that lies behind it will produce none.

The main negative regulators of ureteric bud branching, BMP2, BMP4 and TGFβ, are expressed only by (ex-)mesenchymal cells that are reaching more advanced states of differentiation. BMP2 is made by cells as they condense and become epithelial,[100] while TGFβ is expressed by the bud itself and by mesenchymal cells that have differentiated into mature stroma instead of nephrons, and BMP4 by both stroma and developing nephrons.[101,102] This pattern of expression probably reinforces the message given by the decline of GDNF expression in differentiating cells: the ureteric bud is provided with encouragement to grow and branch from ahead, but receives strong "keep out" signals from the zones that it has already induced into nephrogenesis (Fig. 8). This would be important, because invasion of groups of cells that are already forming nephrons by new bud branches would probably result in a tangled mess rather than orderly morphology.

Invasion and new branching are therefore confined mainly to the zone of virgin mesenchyme at the cortex of the developing organ and will take place proximally to that only if there happens to be a region of mesenchyme that has escaped earlier invasion by the bud and that therefore still expresses GDNF and does not yet express branch inhibitors. Even in the proximal zone, the subtle gradients of concentrations of branch activators and inhibitors may be used to direct branching so that a series of essentially two-dimensional branching events builds a tree that fills up three-dimensional space (the two-dimensional nature of organ culture makes this three-dimensional space-filling process difficult to observe by time-lapse photography).

It is perhaps surprising, in view of the potential feedback loops described above, that ureteric bud branching will still occur in the absence of normal mesenchymal differentiation. This can be seen to some extent in kidneys in which mesenchymal differentiation has been inhibited by specific treatments,[103] but is shown most dramatically by the ability of isolated ureteric buds to develop in three-dimensional matrices when provided with appropriate growth factors (the combination of matrix and growth factors acting as a mesenchyme-substitute.[104] Although the branching seen in such circumstances is not exactly of the form seen in real organs, the bud is clearly capable of organising itself into tips and stalks, and of spacing out branch points appropriately. This suggests that the bud has an intrinsic mechanism for suppressing the formation of tips (branches) that are too close together, and for avoiding collisions. The most obvious way in which this might occur would be for tips to suppress tip formation in nearby cells, and for both tips and stalks to secrete a repellant that prevents new branches from colliding with them. There is not yet any direct evidence for such inhibitory or repulsive mechanisms. The most promising hint is the presence in the ureteric bud of murine homologues of the *Droshopila* gene *sprouty*.[105] In *Drosophila*, *sprouty* suppresses the formation of tips in the branching

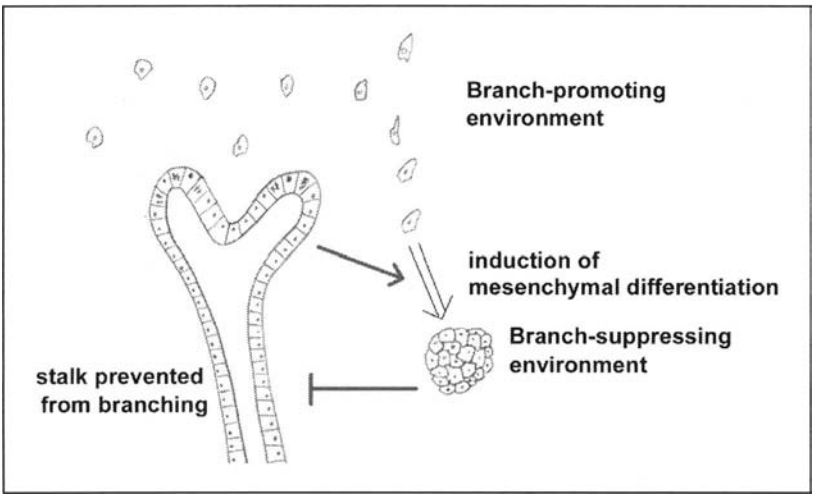

Figure 8. Hypothetical feedback loop that restricts branching activity to the tips.

tracheal system.[106] If *mSproutys* work in a similar manner in mice, this may be the basis of lateral inhibition by tip cells, and release from this inhibition may be the mechanism that allows regeneration of tips from stalks, described earlier.

As well as being driven by genetic mechanisms, the branching behaviour of the ureteric bud, whether in isolation or in the mesenchyme, may be controlled by the mechanical/ fluid dynamic forces of viscous fingering. These are discussed at length in Chapter 12 so will not be described here.

Branching of the Renal Blood System

The blood system of the mature kidney follows a branched arrangement. Arterial blood flows towards the kidney via the renal artery, which branches directly from the abdominal aorta and receives 20% of the cardiac output.[107] In humans, the renal artery divides into segmental arteries just before these arteries enter the kidney; in mice there is only one segment. Once in the kidney, the segmental arteries divide again to form interlobar arteries, which give rise to arcuate arteries that serve the nephrons themselves. Arterioles take blood from the arcuate arteries to a tight complex of specialized capillaries in the glomerulus, and a second arteriole conducts blood from the glomerulus to a second capillary network that surrounds other parts of the nephron; finally it drains, via venules and a vein network that follows the general arrangement of the arterial one, to the vena cava.

In general, two different processes can give rise to embryonic blood systems; angiogenesis produces blood vessels by branching from pre-existing vessels, in a manner morphologically similar to the branching of epithelia, and vasculogenesis produces them from mesenchyme-like precursor cells already present in the tissues. In kidneys, there is evidence for both processes. Grafting embryonic kidney rudiments to sites already rich in a blood supply, such as the chorioallantoic membrane of a bird egg, results in the formation of a blood system derived from the host tissue by angiogenesis.[108] Culture of isolated kidney rudiments in low oxygen, or in the presence of exogenous VEGF, causes the formation of primitive blood systems by vasculogenesis.[109-111]

Developing nephrons, especially the glomeruli, are strong sources of VEGF and attract endothelial precursors, this attraction being susceptible to being blocked by antibodies against VEGF.[112] The final arrangement of fine blood vessels within the glomerulus is complex, and

probably arises by intussusceptive division of vessels as described in more detail in Chapter 6. The fine capillaries of kidneys probably arise by vasculogenesis, while the largest vessels are more likely to arise by angiogenesis from the great vessels of the rest of the body. Unfortunately, very little is known about how these processes cooperate in renal development, or how the blood system aligns with that of the epithelia, although migration of vessels along the ureteric bud and its major branches is one obvious possibility.

Conclusions and Perspectives

Understanding the branching of the ureteric bud is, then, key to understanding the morphogenesis of the kidney as a whole. The last two decades have identified many signals are produced by or that converge on to the tips of the ureteric buds. While more of these signals no doubt remain to be discovered, it is perhaps more important to focus now on the largely uncharacterised mechanisms that couple these signals to morphological change. Some potential mechanisms have been implied by recent data, including roles for cell division, modulation of cell adhesion and cell locomotion: it would be useful to test these carefully, and to assess the extent to which known modulators of these processes, such as small GTPases, play a role in kidney development. Another very promising area of research is that of the feedback systems that seem to restrict 'tip' character to limited areas of the epithelium, and may be responsible for ensuring that the entire kidney is supplied with collecting ducts without any zones being over-supplied: it will be interesting to learn whether the same systems operate in all branching epithelia.

Understanding the ureteric bud is important for medical reasons as well: dysmorphologies of the bud produce clinically-devastating cystic diseases, and it is possible that the invasive behaviour of renal carcinomas results in part from inappropriate reactivation of the invasive mechanisms used in development. A deeper knowledge of these processes might allow them to be modulated medically, and offer the possibility or treatment, or at least amelioration, of debilitating and life-threatening medical conditions.

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