Epithelial Tubulogenesis Through Branching Morphogenesis: Relevance to Collecting System Development


ABSTRACT

Branching tubulogenesis of the ureteric bud, which gives rise to the urinary collecting system, is of considerable clinical interest because this process plays a major role in determining nephron number in the kidney. Data from in vitro model systems, including organ culture of the embryonic kidney and renal epithelial cells cultured in three-dimensional collagen matrices, indicate that growth factors, extracellular matrix composition, matrix-remodeling proteinases, and integrin expression are important factors in tubulogenesis and branching in the renal epithelium. One possibility is that gradients of soluble factors in the interstitial milieu of the embryonic kidney regulate the directionality of tubulogenesis and the degree of branching. Growth factors that enhance branching also appear to up-regulate matrix-remodeling extracellular proteinases. Redundancy in the ability of growth factors to induce tubulogenesis and branching may explain the apparent lack of a renal phenotype observed in targeted growth factor gene deletion experiments in mice. At the same time, differential effects in the efficiency of growth factors in inducing branching and/or changes in local matrix composition may be important in regulating the degree of arborization of the developing collecting system.

Key Words: Tubulogenesis, branching kidney development, collecting system

The process of epithelial branching morphogenesis, which gives rise to the urinary collecting system, is of considerable basic and clinical interest; 5 to 10% of the population appears to have some developmental abnormality of the kidney, and, though often clinically silent, many of these affect the collecting system (1-4). Current evidence indicates that tubular portions of the nephron are derived from two distinct embryologic sources: the metanephric mesenchyme and the ureteric bud (5). Cells from the proximal tubule through the distal tubule are derivatives of the mesenchyme, whereas the entire collecting system down to the trigone of the bladder is derived from the ureteric bud (6). The two embryonic tissues participate in a mutual induction in which the advancing ureteric bud induces the condensation and epithelialization of previously mesenchymal cells, whereas the mesenchyme induces branching behavior in the ureteric bud (6). The result of this mutual interaction is that branches of the ureteric bud recruit renal vesicles (which eventually develop into nephrons) from the surrounding mesenchyme. Thus, the ultimate number of nephrons (~1 million in humans) appears to be a function of two processes: branching morphogenesis in the ureteric bud and the ability of terminal branches of the ureteric bud tree to induce renal vesicles.

Osathanondh and Potter divided human collecting system development into four stages (7,8). The first stage is characterized by multiple iterations of symmetric and asymmetric dichotomous branching and recruitment of an initial population of nascent nephrons. Dilation of the first several generations of ureteric bud branches gives rise to the renal pelvis and calyces; the initial nephrons connected to these segments either degenerate or move with the advancing ureteric bud tip. During the second stage, so-called terminal branches of the ureteric bud elongate and induce a series of several renal vesicles as they elongate. Although these nephrons are initially each connected directly to the collecting duct, the points of attachment of the more proximal nephrons shift upward and onto the connecting segment of the next most recently formed nephron. This process results in the formation of “arcades” where several nephrons join in a common connecting segment (Figure 1). The third stage of collecting system development consists of elongation of the terminal branch of the ureteric bud past the arcade and the induction of a set of terminal nephrons that join the collecting duct directly (Figure 1). In the last stage, the collecting duct stops advancing and nephron formation ceases (7,8).

The exact relationship of branching events and the induction of terminal nephrons to the final number of nephrons is ill defined; however, even a small decrease in dichotomous branching efficiency iterated ~20 times would result in a large deficit in terminal collecting system branches (5,9). The efficiency of branching may have important clinical consequences...
Epithelial Tubulogenesis

Figure 1. Urinary collecting system development through branching morphogenesis of the ureteric bud. (a) Formation of the metanephric (permanent) kidney in vertebrates begins with the penetration of the initially unbranched epithelial tube (the ureteric bud) into the metanephric blastema. Mutual inductive events between the advancing ureteric bud and cells of the metanephric mesenchyme lead to the condensation and epithelialization of mesenchymal cells (not shown) and to branching behavior in the ureteric bud. (b to d) The ureteric bud experiences many iterations of dichotomous branching (four generations shown). The renal pelvis and major and minor calyces are thought to result from the dilation and coalescence of the first 6 to 12 generations of ureteric bud branches. Subsequent generations give rise to a set of terminal branches (e) that elongate dramatically and are capable of inducing several nephrons per branch. Osathanondh and Potter's second stage of nephrogenesis results in the formation of arcades where several nephrons join the collecting duct through a common connecting segment (7, 8). It is thought that the arcade nephrons originally are induced by and join directly to the nascent collecting duct, but that later "sliding" of their points of attachment results in arcades (e, inset). During the third stage, extension of the terminal branch of the ureteric bud beyond the connection site of the arcades results in the induction of a final set of nephrons that join the collecting duct directly (e, inset). Heavy lines, ureteric bud derivatives; fine lines, connecting segments.

In that nephron number has been suggested to affect the risk of, and response to, renal disease (10–12). In addition, 30 to 40% of all childhood cases of renal failure may be the result of developmental abnormalities of the kidneys; many involve the collecting system (3, 4). Thus, bifid ureter (associated with back and forth reflux and stagnation between the Y-arms) is ultimately a consequence of premature branching of the ureteric bud before reaching the mesenchyme. Similarly, duplication of the ureters (associated with ectopic ureters ending variously in the seminal vesicle, uterus, or vagina) follows from the creation of two separate buds from the mesonephric duct (1). In addition, a variety of hypoplastic diseases of the kidney such as segmental atrophy (Ask-Upmark syndrome) and renal cystic disease may ultimately be due to abnormal development of the collecting system (2).

Branching morphogenesis is a characteristic of most, if not all, epithelial tissues as well as endothelium. In tissues as diverse as the developing lung, salivary gland, mammary gland, capillary endothelium, and the ureteric bud, growth factors and the extracellular matrix (ECM) have emerged as critical determinants of branching morphogenesis (13–15). Growth factors, the ECM, integrin receptors for the ECM, and matrix metalloproteinases appear to participate in exceedingly complex interactions in these developing epithelial tissues. Fortunately, several models have been developed that allow the dissection of the process of branching at various levels of detail. Branching events can be followed in the embryonic kidney in culture (16), and the isolated ureteric bud itself can be maintained in organ culture (17). Thus, growth factors and neutralizing antisera, as well as antisense oligonucleotides, may be used to analyze the function of various molecular targets and the effect on branching observed in the embryonic kidney. Nevertheless, a multiplicity of cell types, lack of synchronization, and questions of the tissue penetration of reagents make conclusions about specific function difficult.

Fortunately, the process of branching in epithelial cells can be observed in isolation by observing renal epithelial cells in three-dimensional collagen matrix gels. At least two cell lines, Madin-Darby canine kidney (MDCK) cells and murine inner medullary collecting duct (mIMCD3) cells, undergo branching morphogenesis in response to certain growth factors. MDCK cells cultured in collagen matrix gels in the presence of hepatocyte growth factor (HGF) develop multicellular branching tubular structures (18) with distinct apical-basolateral polarity (19). In the absence of HGF, the cells form round cystic structures without any branching. On the other hand, the mIMCD3 cells form branching tubular structures in serum alone (20) and in response to transforming growth factor (TGF)-α and epidermal growth factor (EGF) under serum-free conditions, albeit to a lesser degree than HGF (21). In mIMCD3 cells, the ability of the growth factors to induce branching, both in terms of the number of branches and the complexity of arborization observed in the branches, appears to be as follows: HGF > TGF-α > EGF. With these models, it is possible to investigate the functional roles of ECM components and growth factors as well as cell signaling processes in a very simple, defined system that may have relevance to collecting system development.

GROWTH FACTORS

Many growth factors have been implicated in renal development on the basis of expression in the embryonic kidney and by antisense oligonucleotide and antiserum experiments in the organ culture model; however, the results are not always concordant with knockout experiments (Table 1). However, despite results from targeted gene disruption experiments, considerable evidence in both the organ culture and cell/
**TABLE 1. Knockout mouse and organ culture experiments and the developing kidney**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Antisera/Antisense Inhibition</th>
<th>Targeted Gene Disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-α</td>
<td>Decreased branching (22)</td>
<td>None apparent (24)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Increased branching (23)</td>
<td>None apparent (27)</td>
</tr>
<tr>
<td>NGFR (low affinity)</td>
<td>Decreased branching (25,26)</td>
<td>Proportionately small for body size (29)</td>
</tr>
<tr>
<td>ILGF-1</td>
<td>Uniformly small (28)</td>
<td>None apparent, though fetal death before nephrogenesis is complete (32,33)</td>
</tr>
<tr>
<td>HGF</td>
<td>Small with decreased branching (30,31)</td>
<td>Renal failure in certain strains with collecting system dilatation (34)</td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
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*Numbers in parentheses indicate reference numbers.*

culture models suggests a role for growth factors in the branching of the ureteric bud. Antiserum directed against TGF-α, for instance, appears to inhibit the branching of the ureteric bud in organ culture, whereas antisera against TGF-β appears to promote branching (22,23). These results are consistent with recent findings in mIMCD3 cells where TGF-α promotes branching in serum-free culture (21), whereas TGF-β inhibits branching in response to HGF in MDCK cells (see Reference 46).

The MDCK and mIMCD3 cell-branching response to HGF appears to be relevant to the situation in vivo. HGF and HGF-receptor, cMET, are present in the developing kidney in appropriate locations for a role in ureteric bud branching (HGF being expressed in the mesenchyme and cMET being present in both the ureteric bud and the mesenchyme; Figure 2) (20,30,31). In addition, the embryonic kidney appears to elaborate several soluble factors that induce branching in the MDCK and IMCD cells. The branching response of the MDCK cells in coculture with the metanephric mesenchyme is nearly abolished by anti-HGF antiserum, indicating that the primary "branching-morphogenetic" factor elaborated by the mesenchyme for the MDCK cells is HGF (30). However, anti-HGF antisera does not affect the branching of the mIMCD3 cells in coculture with the mesenchyme, indicating that at least in these cells (and perhaps the ureteric bud), there are other factors that may induce branching morphogenesis (21). These factors include TGF-α, EGF, and possibly others. Thus, the activation of at least two separate signal-transduction pathways (HGF-cMET and EGF/TGF-α-EGF-receptor) are capable of inducing branching morphogenesis in cells of renal epithelial origin. Tubule formation by EGF and TGF-α has also been described in baby mouse kidney cells maintained in Matrigel deprived of growth factors, although the origin of the cells within the nephron is not clear (35). The recent finding that mice in which the EGF receptor gene has been disrupted have renal dysfunction and collecting system dilatation supports an important role for EGFR ligands in nephrogenesis in general and collecting system development in particular. Indirect evidence suggests that downstream effectors may include protein kinase C, cAMP-dependent protein kinase, and phosphoinositol-3-kinase (19,36). These growth factors may also modulate gene expression for ECM proteins and ECM-modifying enzymes.

**ECM**

ECM components acting through integrins and other cellular receptors are capable of affecting gene expression in a wide variety of systems (37). Thus, it is not surprising that ECM components are capable of modulating the process of branching in a number of epithelial and endothelial tissues. It has long been known that tissues such as salivary gland maintained in organ culture require a "clot" of some ECM substance such as collagen in order to develop branching structures (38). In addition, antiserum to various ECM components has been shown to inhibit the process of branching. For instance, antibodies to laminin...
B-chain have been observed to reduce the degree of branching in developing mouse lung explants maintained in organ culture (39). Furthermore, it has been shown that β-p-xyloloside, which inhibits the production of chondroitin sulfate proteoglycan, inhibits branching morphogenesis in a variety of epithelial tissues (including ureteric bud) in organ culture (40,41).

There is very little direct evidence implicating a role for other ECM components in branching morphogenesis of the ureteric bud. It is known for instance that mice deficient in collagen-1 (α1(I)-chain) do not have a renal phenotype (42). Monospecific laminin A-chain antibodies, which have been argued to affect the polarization of mesenchymally derived cells, do not appear to have any effect on the branching morphogenesis of the ureteric bud (43). In fact, large-scale developmental changes in renal ECM composition occur mostly in the mesenchyme and not in the ureteric bud, (through the matrix context for ureteric-bud branching may be supplied by the mesenchymally derived cells), although it appears that laminin A-chain is expressed transiently in the ureteric bud at early times when branching is occurring (44). Many questions remain, however, because the number of known isoforms of ECM molecules is increasing rapidly and the specificity of reagents used in previous studies is not generally known (45).

In contrast to the embryonic kidney itself, the MDCK cell models have provided functional data with respect to the possible role of the ECM in epithelial branching morphogenesis. Although HGF induces branching tubulogenesis in MDCK cells maintained in collagen-1 matrix, the cells do not branch when cultured in Matrigel, a crude basement membrane extract, suggesting that some component(s) of Matrigel prevent the effect (46). Analysis of individual components revealed that collagen-I, laminin, fibronectin, and entactin (nidogen) facilitated branching in these cells, whereas collagen-IV and heparan sulfate proteoglycan HSP (as well as TGF-β) inhibited branching.

In a very general sense, it is those ECM molecules that are expressed in the interstitial matrix of the metanephric mesenchyme that facilitate branching morphogenesis, whereas some of those that are characteristic of mature basement membranes inhibit branching. Nevertheless, the situation is not clear cut in that some mature basement membrane components (laminin, entactin) do support branching morphogenesis *in vitro*. Another approximate view is that those ECM molecules that permit branching in MDCK cells are expressed earlier in nephrogenesis, whereas those that prevent branching (*in vitro*) are expressed later. The presence of a very insoluble, cross-linked array of basement membrane proteins (some of which inhibit branching *in vitro*) in intimate contact with the ureteric bud probably presents a critical barrier to both elongation and branching. Therefore, it is likely that ECM-remodeling enzymes are critical for generating the branching pattern of the collecting system.

### EXTRACELLULAR PROTEASES

Proteinases have been implicated in the development of several epithelial tissues, and several appear to be regulated by the very growth factors that promote tubulation in the MDCK/IMCD model. There are at least four classes of neutral proteinases that potentially act in matrix remodeling (47). The best characterized are the serine proteinases, which include plasmin, urokinase type plasminogen activator (uPA), and tissue plasminogen activator. These proteinases are situated at the top of a proteolytic cascade in which uPA cleaves plasminogen and the resulting plasmin activates various matrix metalloproteinases (MMP) that have varying specificity against ECM components (48,49). Moreover, extracellular proteolytic activity can be localized to cell surfaces and perhaps only restricted domains of the cell surface through the use of cell surface receptors such as the uPA receptor (50). In this way, a localized zone of plasmin and activated MMP can be generated in order to allow the vectorial migration of cells.

In other tissues, most notably capillary endothelium, the action of extracellular proteolytic activity has been shown to be critical for the formation of new branches (13,51,52). Endothelial branching begins with the extension of a cellular process across the basement membrane (53). This action depends on the ability of the endothelial cell to express localized proteolytic activity in order to degrade the basement membrane. Subsequent streaming of the cellular contents to fill the process, followed by proliferation, results in a new branch. The fusion of autolytic vacuoles in adjacent cells results in lumen formation (52).

Proteinase activity also directly correlates with branch formation in mammary epithelium. Mammary epithelial tissue from female mice inappropriately expressing active stromelysin-1 (an MMP) develop roughly twice the number of branch sites (54).

Conversely, it has been proposed that ECM components may act as localized barriers to an advancing epithelial structure and thereby create a cleft at the sites of branching. Some evidence for this has been presented in salivary gland and in the developing lung (55,56). For instance, focal areas of collagen-III deposition have been described at cleft sites in the developing salivary gland in mouse (56). Whether such structures are important for the generation or maintenance of branches remains to be determined. Presumably, if the ECM is important in cleft formation, proteinase activity is negatively regulated at such sites.

In addition, enhanced proteinase (Gelatinase-A) expression has been described in the developing lung in response to exogenous TGF-α and EGF, which appeared to inhibit branching (55). If branching events in the lung are mediated by ECM-dependent cleft formation, then up-regulated (or dysregulated) MMP expression might be expected to disrupt branching. Thus, on the one hand, proteinase expression at the leading edges of branching epithelial structures...
may facilitate branching, whereas on the other, there may be a requirement to decrease proteolytic activity at cleft sites and in areas that do not branch.

Although extracellular proteolytic activity has been implicated in the morphogenesis of other epithelial tissues, surprisingly little is known regarding their role in the embryonic kidney. Although it is known that some of the relevant molecules are expressed in the embryonic kidney, such as uPA and Gelatinase-A, there is not enough detailed information available regarding their precise spatiotemporal patterns of expression to draw unambiguous conclusions about their role in renal development (57–59). There are only minimal data that enable the dissection of the potential roles of ECM and proteinases in the developing kidney.

On the other hand, it has been shown that exogenous serine protease inhibitors inhibit branching tubulogenesis in the MDCK cell model (60). Moreover, HGF (an inducer of branching morphogenesis in these cells) has been shown to induce a fivefold increase in uPA activity and uPA receptor (uPAR) mRNA in MDCK cell monolayers, suggesting that HGF is capable not only of inducing extracellular proteolytic activity but in a manner by which to localize it to the cell surface in these cells through inducing a protease receptor (61). Other MMP also appear to be induced (Figure 3). Our recent work with the IMCD3 cell line (which forms branching tubular structures in response to HGF, TGF-α, and EGF) indicates that all three of these growth factors induce the expression of uPA in collagen matrix culture (data shown for HGF; Figure 3).

On the basis of the clues provided by the MDCK and IMCD cell models, it is likely that effectors implicated in branching in various epithelial and endothelial tissues are also mediators of branching in the developing kidney. Thus, a model explaining how the interaction of growth factors, the ECM, integrins, and ECM remodeling proteinases might participate in post-inductive vectorial branching of the ureteric bud as it develops into the mature collecting system has recently been proposed (9). In this model, it has been argued that the focal expression, or activation, of matrix-degrading proteinases at the leading edges of the advancing bud plays an essential role in ureteric bud morphogenesis. In addition, proteinase activity might be modulated by the differential expression of tissue inhibitors of MMP between branching and non-branching segments. It is also possible that matrix molecules serve a barrier function in forming initial clefts and conversely as facilitatory scaffolding for cell migration at the leading edges of the advancing tip (Figure 4).

In its details, a model of branching morphogenesis that depends on local matrix degradation by metalloproteinases is reminiscent of the mechanism by which tumor cells invade beyond the bounds of the basement membrane. Tumor cell invasion is characterized by the development of so-called "invadopodia" that cross the basement membrane by means of localized proteolysis and cell movement. It has been shown that the invadopodia represent specialized regions of the plasma membrane to which cell surface receptors for proteinases localize (50). With some modification, such a scheme could help to account for the ECM dependence of branching morphogenesis, as observed in the MDCK and IMCD models, in which the ECM modulates branching despite being separated from the cells by a basement membrane. In the MDCK and IMCD cell models, there is morphologic evidence for the existence of "invadopodia" (18,19,60). Specifically, in order for the cells to be able to crawl forward into the surrounding matrix (collagen-I, fibronectin), it would be anticipated that they secrete basement membrane-degrading proteinases and their activators. In addition, it would be expected that they would express integrin receptors for collagen-I (or another
facilitatory ECM component) in order to generate the traction necessary to pull forward. The model also might explain the inhibitory influence of collagen IV or HSP in that integrin-mediated signaling through receptors for these molecules might suppress epithelial process formation (Figure 5). Thus, in the presence of facilitating ECM versus inhibitory ECM, the cellular signaling "state" might be very different; this is also suggested by the involvement of multiple signaling pathways in tubulogenesis and branching (19). A similar cellular signaling "state" promoting tubulogenesis and branching could be activated by multiple factors (HGF and collagen-I or TGF-α and laminin) and vice versa for inhibitory conditions. Of course, these considerations remain speculative, especially regarding the embryonic kidney itself, but represent a testable hypothesis.

COORDINATION AND TERMINAL DIFFERENTIATION

The development of the precise geometric pattern and array of terminally differentiated cell types in the urinary collecting system would appear to require a great deal of spatiotemporal coordination of gene expression. Factors that potentially guide gene expression during ureteric bud morphogenesis include morphogenetic gradients of growth factors (or other soluble factors), the ECM, and perhaps cell adhesion molecules that ultimately exert their effects through the action of nuclear proteins on specific promoter and enhancer elements in DNA. There exists the potential for a great deal of complexity in the functional relationships of these effectors. For instance, a given growth factor may up-regulate the expression of an ECM component that itself subsequently influences gene expression by way of integrin-mediated signaling.

Many cascades of cause and effect involving the above effectors are likely to be active in collecting system development; however, it remains unclear how events are coordinated. On the basis of data from the MDCK and IMCD models, it has been proposed that, apart from cell-cell interactions with cells derived from the mesenchyme, morphogenetic gradients of soluble growth factors (presumably established by cells of mesenchymal origin) exist in the developing kidney that exert differential effects on the expression of the effectors of branching morphogenesis (9). In this model, HGF-like activity (and/or TGF-α-like activity, both of which promote branching), acting at the leading edges of the branching tubules, and TGF-β-like activity (inhibits branching), acting in nonbranching areas, might explain vectorial branching morphogenesis (Figure 4). TGF-β-like activity may be especially important during Osathanondh and Potter's third stage of nephrogenesis, in which there is a considerable amount of collecting duct elongation without branching (7,8).

In other systems, expression patterns in the ECM (and of integrin ECM receptors) are influenced by growth factors (62–66). Thus, morphogenetic gradients of growth factors may influence tubulogenic and
branch-inducing gene expression both directly and indirectly through changes in the ECM. Much attention has been given recently to ECM-response elements associated with genes active in the fully differentiated phenotype of several epithelial tissues. In mammary epithelium, for instance, the expression of differentiated phenotype of several epithelial tissues. In mammary epithelium, for instance, the expression of milk protein genes representative of the fully differentiated phenotype depends on cellular interaction with laminin through integrin receptors (67). It is not known to what degree full differentiation of the ureteric bud derivatives (such as the expression of collecting ducts and blind-ending branches of the ureteric bud without nephrons are not observed in the mature kidney. Although there is evidence for selective cell dropout (apoptosis) of mesenchymal cells that fail to match with a branch of the ureteric bud (68), little is known about the possible role of apoptosis in collecting system development. It is possible that the appearance of the extremely precise regulation of branching observed in the collecting system is actually the result of somewhat random branching and the subsequent rescue from apoptosis of only those branches that succeed in inducing nephrons.

In summary, the basis of findings in other organ systems and in vitro models of branching morphogenesis, it seems likely that growth factors, the ECM, ECM-remodeling proteinases, and perhaps apoptosis are critical in the morphogenesis and differentiation of the urinary collecting system. Yet there are obvious pitfalls in extrapolating from other organ systems and from reliance on immortalized cell culture systems in which the context is radically simplified and the cells are already quite differentiated. The hypotheses must be tested in the developing kidney itself. The relative rarity of direct data in this field is somewhat surprising, especially given the possibility that branching morphogenesis influences eventual nephron count and, presumably, susceptibility to renal disease. Key questions remain concerning the identity and spatial distribution of soluble morphogenetic factors, their effect on gene expression including ECM proteinases, and integrins, and the feedback effects of the ECM on gene expression. Finally, defects in any of these factors (ECM molecules, growth factors, integrins, proteases) could conceivably lead to inefficient branching and, therefore, decrease nephron numbers; likewise, defective tubular morphogenesis may occur, manifested by cystic dilatation of the collecting system (since in vitro, in the absence of factors promoting tubulogenesis, cyst formation results).

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REFERENCES


"The methods which I have been describing have not yet been made adaptable to mammalian study, hence the answer will be less direct. I think, however, that it is nonetheless certain that the mammalian glomerulus is a filter and that the chief function of the mammalian tubule in the elaboration of normal urine is reabsorption... The normal work of the kidney itself consists in very large part in salvaging to the organism those constituents of the glomerular filtrate whose loss would entail irretrievable damage."