Branching morphogenesis and kidney disease

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Summary

Branching morphogenesis in the kidney is a tightly regulated, complex process and its disruption potentially can lead to a broad spectrum of diseases, ranging from rare hereditary syndromes to common conditions such as hypertension and chronic kidney failure. This review synthesizes data on branching during kidney development derived from in vitro and in vivo rodent studies and to apply them to human diseases. It discusses how the broad organization of molecular interactions during kidney development might provide a mechanistic framework for understanding disorders related to aberrant branching.

Introduction

Formation of a tree-like structure via the ramification of epithelial tubules during embryogenesis is known as branching morphogenesis. This process is fundamental to the development of a number of organ systems that share similar tissue architecture, such as the kidney, lung, breast and salivary gland. In the mammalian kidney, branching morphogenesis leads to the formation of the urinary collecting system, and a number of key developmental pathways have now been identified through the study of model systems. These have provided important insights into the mechanisms of disease that result from alteration of the branching program. Defective branching may result in clinical phenotypes that range from complete renal agenesis to diseases, such as hypertension, that exist in the setting of grossly normal appearing kidneys.

This review focuses on the intersection between human kidney disease with in vitro model systems and in vivo mutation data with the aim of correlating established molecular events in kidney development, particularly in the context of branching morphogenesis, with final renal architecture, function and pathology. These events are reviewed in the context of the morphological steps that occur during kidney development, from ureteric bud outgrowth to the cessation of branching.

Kidney organogenesis

The mammalian kidney, or metanephros, arises at approximately day 35 of human gestation. The kidney originates from two mesenchymally derived components: the Wolffian duct and the metanephric mesenchyme (MM). Directed by inductive signals emanating from the MM, the Wolffian duct forms an epithelial out-pouching called the ureteric bud (UB), which invades the adjacent MM cell cluster. A remarkable series of events follow; reciprocal induction between the UB and MM leads to multiple iterations of branching and elongation of the UB to form the collecting system (where final production of a concentrated urine occurs), while the mesenchyme is induced to condense and epithelialize around the branched tips. Known as the mesenchymal-to-epithelial transformation, these pre-tubular mesenchymal aggregates then proceed through several morphological stages, including comma- and S-shaped bodies, forming metanephric tubules that eventually mature into the parts of the nephron responsible for regulation of ion and organic molecule transport (proximal and distal tubules), as well as the epithelial glomerular (filtering) components (Fig. 1A).

Iterative branching of the UB occurs ~15 times during human development, normally generating between 300,000 and 1 million nephrons per human kidney (Nyengaard and Bendtsen, 1992). The formation of lateral branches (i.e. branches that grow out from a main stem) is followed by terminal bifid branching (bifurcation of one stem into two daughter branches). As each ureteric tip connects to a nephron, it effectively is removed effectively from further branching events. This type of branching appears to induce the majority of nephrons formed initially. The remaining fraction develops through the poorly understood process of arcade formation and late-phase branching (al-Awqati and Goldberg, 1998; Bush et. al, 2004b). The formation of the renal calyces and renal pelvis – the part of the urinary collecting system that leads to the ureter – occurs through the growth and dilation of early branching segments (Fig. 1B). There is great variability in the number of nephrons per kidney between animal species and currently it is unknown whether similar modes of branching occur across species (Moritz et al., 2003).

Ureteric bud outgrowth

In vitro studies have shown that glial cell-derived neurotrophic factor (GDNF), produced in the MM, is a primary inducer of ureteric budding (Sainio et al., 1997). GDNF interacts with a number of different molecules to play a crucial role in the initiation of downstream branching events. In mice, knockout of GDNF, or factors that affect GDNF expression or signaling, can result in phenotypes ranging from renal agenesis to rudimentary UB systems and kidneys. These factors have been comprehensively reviewed (Steer et al., 2003; Bush et al., 2004b) and include Wilms tumor-suppressor gene 1 (*Wt1*), *Lim1*, Paired-box gene 2 (*Pax2*), Eyes absent *1* (*Eya1*), *Six1*,

A
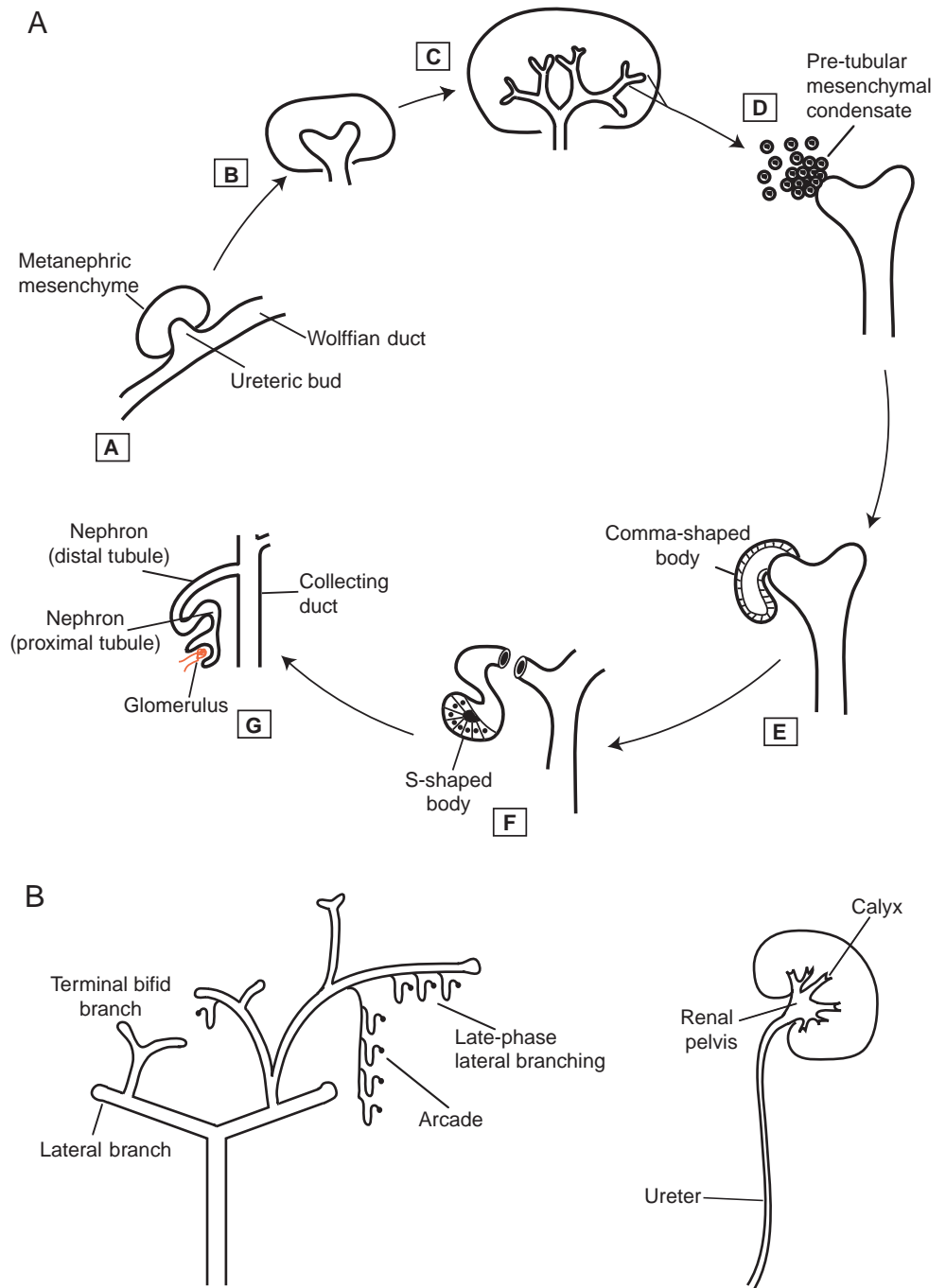
Fig. 1. (A) Stages of renal branching morphogenesis and nephron formation. Ureteric bud (UB) outgrowth from the Wolffian duct is induced by signals from the metanephric mesenchyme (MM) (A). (B,C) Invasion of the MM by the UB is followed by iterative branching of the UB and elongation of UB stalks. (D) At the tips of the branches, the epithelium induces the mesenchyme to form pre-tubular aggregates, which are stimulated to undergo mesenchymal to epithelial transformation (E,F) through the formation of comma-shaped (E) and S-shaped (F) bodies to form components of the nephron (G): renal tubules (proximal and distal) and the epithelial component of the glomerulus. (B) Nephron endowment is thought to be largely determined through branching of the UB. (Left) The UB adopts a strategy of lateral branching followed by bifurcation of a stem into two daughter branches (terminal bifid branching) to form the collecting system of the kidney. Nephrons are induced at UB tips but are also formed around the stem of elongating branches during the later branching iterations (arcades) and late-phase lateral branching. (Right) The segments of the collecting system proximal to the ureter (the renal pelvis and calyces) are formed from early branching segments of the UB that have dilated.

B

Sal-like 1 (*Sall1*) and the *Hox11* homeobox gene paralogous group (Davis et al., 1995; Kreidberg et al., 1993; Laclef et al., 2003; Nishinakamura et al., 2001; Shawlot and Behringer, 1995; Torres et al., 1995; Xu et al., 1999) (see Table 1 for details). In addition, knockout of GFRα1 and *Ret*, members of the GDNF receptor signaling complex, can also lead to the failure of UB outgrowth and result in renal agenesis (Cacalano et al., 1998; Schuchardt et al., 1996). In many of these knockouts, penetrance is variable; a certain percentage of mutants have rudimentary ureteric bud systems and kidneys (Table 2), indicating that there are compensatory mechanisms that allow kidney development to proceed despite disruption of the GDNF pathway. Conversely, the depletion of factors that limit the expression of GDNF in the MM, such as Foxc1 (Mf1), or that antagonize inductive signals from the MM, such as BMP4, leads to supernumerary budding and duplication of the collecting system (Kume et al., 2000; Miyazaki et al., 2000).

GDNF and BMP4 both require heparan sulfate proteoglycans (HSPG) to promote and suppress, respectively, UB outgrowth along the Wolffian duct (Barnett et al., 2002; Paine-Saunders et al., 2000). HSPGs are glycoproteins that are found on the cell surface and within the extracellular matrix. They are molecules with two distinct components: a core protein and a covalently linked glycosaminoglycan (GAG) side

Table 1. Known expression patterns of genes expressed in kidney development, and corresponding branching defects in human disease

Gene	Location of expression	Mutant mouse phenotype	Human (naturally occurring mutation)
Ureteric bud outgrowth			
Wt1	MM	Bilateral agenesis (Kreidberg et al., 1993)	
Pax2	MM, UB	Bilateral agenesis (Torres et al., 1995)	Renal coloboma syndrome (renal agenesis/hypoplasia, optic nerve coloboma) (Sanyanusin et al., 1995)
Sall1	MM	Agenesis (32%); single hypoplastic kidney (29%); bilateral remnant kidneys (39%) (Nishinakamura et al., 2001)	Townes-Brock syndrome (anal, renal, limb, ear anomalies) (Kohlhase et al., 1998)
Eya1	MM	Bilateral agenesis (100%) (Xu et al., 1999)	Brancho-oto-renal syndrome (branchial fistulae, deafness, renal agenesis) (Abdelhak et al., 1997)
Six1		Bilateral agenesis (Xu et al., 2003)	
Hoxa11/*Hoxd11*	MM	Variable severity: agenesis to dysplasia. Abnormal branching with elongated primary branches (Davis et al., 1995)	
Lim1	UB	Bilateral agenesis (Shawlot and Behringer, 1995)	
Gdnf	MM	Agenesis caused by lack of UB (Pichel et al., 1996)	
Ret	UB	Bilateral agenesis (58%); unilateral agenesis (31%); decreased branching and undifferentiated MM (11%) (Schuchardt et al., 1996)	
Gfra1	MM, UB	Bilateral agenesis (76%); unilateral rudiment (23%) (Cacalano et al., 1998)	
Foxc1 (*Mf1*)	MM	Duplicated collecting system (Kume et al., 2000)	
Bmp4	MM	Homozygotes are lethal at E6.5; no mesoderm formation. (Winnier et al., 1995). Heterozygotes have a duplicated collecting system (Dunn et al., 1997)	
Unknown			Duplicated collecting system
Early branching			
Gdnf heterozygotes	MM	Dysplasia; 30% fewer nephrons (Cullen-McEwen et al., 2001)	
Fgf7	S	Thirty percent fewer nephrons (Qiao et al., 1999b)	
Fgf10	MM	Hypoplasia (Ohuchi et al., 2000)	
Emx2	MM, UB	Bilateral agenesis secondary to failure of UB branching. Outgrowth normal (Miyamoto et al., 1997)	
Foxd1 (BF2)	S	Hypoplasia (Hatini et al., 1996)	
Rara/*Rarb2*	S	Fourfold decrease in the number of UB tips; 4-5 versus 8-9 branching iterations when compared with wild type (Mendelsohn et al., 1999)	
Pod1	MM	Hypoplasia and 61% decreased branching (Quaggin et al., 1999)	
Wnt4	MM	Small, dysgenic kidneys; undifferentiated MM interspersed with UB branches (Stark et al., 1994)	
Wnt11	UB	Thirty-six percent fewer nephrons (Majumdar et al., 2003)	
Itga8 (integrin α8)	MM	Bilateral dysgenesis (Muller et al., 1997)	
Kama5 (laminin α5)	MM	Dysgenesis; decreased UB branching (Miner and Li, 2000)	
Heparan sulfate 2-sulfotransferase	MM, UB	Bilateral agenesis (Bullock et al., 1998)	
Unknown			Possibly hypertension
Late branching and maturation			
Tgfb1	MM, UB	Perinatal lethal; kidneys normal (Kulkarni et al., 1993)	
Activin	MM	Perinatal lethal; kidneys normal (Jhaveri et al., 1998)	
Bmp2	MM	Embryonic lethal (Zhang and Bradley, 1996)	
Unknown			Possibly hypertension
Branching termination and tubule maintenance			
Hgf	MM	Overexpression leads to cysts (Takayama et al., 1997)	
Tgfa	MM	Overexpression leads to cysts (Jhappan et al., 1990)	
Egfr	MM	Overexpression leads to cysts (Orellana et al., 1995), knockout leads to dilated collecting ducts (Threadgill et al., 1995)	
Tgfb2	MM	Cysts (Sanford et al., 1997)	
Gpc3 (glypican 3)	MM, UB	Enhanced branching, medulla degenerates by E15.5; cysts (Cano-Gauci et al., 1999)	Simpson-Golabi-Behmel syndrome (cystic overgrowth) (Pilia et al., 1996)
Pkd1, *Pkd2*	UB (cilia)		ADPKD (Wu and Somlo, 2000)
Invs (inversin)	UB (cilia)	Cysts (Morgan et al., 2002)	
Kif3a (kinesin)	UB (cilia)	Conditional knockout leads to cysts (Lin et al., 2003)	
Polaris	UB (cilia)	Cysts (Yoder et al., 2002)	
Regeneration			
			Acute tubular necrosis
			Acquired cystic kidney disease

UB, ureteric bud; MM, metanephric mesenchyme; S, stroma.

Table 2. Knockout phenotypes of early branching molecules*

Gene	Bilateral agenesis	Rudimentary UB system	Unilateral agenesis/ hypoplasia	References
Gdnf	73.0	27.0		Moore et al., 1996
Gfra1	76.0	24.0		Cacalano et al., 1998
Ret	66.0	24.0		Schuchardt et al., 1996
Wt1	100.0			Kreidberg et al., 1993
Lim1	Bilateral agenesis (not quantified)			Shawlot et al., 1995
Pax2	100.0			Torres et al., 1995
Six1	97.5	2.5		Xu et al., 2003
Sall1	32.0	39.0	29.0	Nishinakamura et al., 2001
Eya1	100.0			Xu et al., 1999
Hs2st	100.0			Bullock et al., 1998

*Values are the percentage displaying the phenotype.

chain, heparan sulfate (HS). Many growth factors bind to the HS side chains of HSPGs, and the specificity of these interactions is often based on the location and clustering of sulfate modifications along segments of the HS chain (e.g. the degree of 2-O-sulfation versus 6-O-sulfation) and the core protein family (e.g. glypican versus syndecan) (Box 1). For example, GDNF activity is dependent on its binding to HS (Barnett et al., 2002). BMP4 activity, instead, is dependent upon its binding to the cell surface HSPG glypican 3 (Paine-Saunders et al., 2000). These studies indicate that HSPGs influence budding via the specificity of their interactions with morphogenic molecules, a theme that recurs throughout kidney branching morphogenesis.

Defects during initial ureteric bud outgrowth

Renal agenesis is a relatively frequent congenital defect in humans. An estimate of congenital absence of the kidney is 0.48 to 0.58 per 1000 live births (unilateral agenesis occurs in 1/200 births and bilateral agenesis in 1/5000-1/10000 births) (Pohl et al., 2002). Current data indicate that ureteric budding requires the presence of GDNF as a primary inducer. In humans, renal agenesis arises from mutation of single genes known to affect GDNF expression or signaling, for example, in diseases such as Townes-Brock syndrome (*SALL1*), Renal-coloboma syndrome (*PAX2*) or Branchio-Oto-Renal syndrome (*EYA1*) (Abdelhak et al., 1997; Kohlhase et al., 1998; Sanyanusin et al., 1995) (Table 1). These genes are known to interact with GDNF expression or signaling in the mouse, and the clinical phenotype of these gene mutations supports the view that regulation of GDNF expression is a key event in ureteric outgrowth in humans. Collecting system duplication (resulting in bifid ureter and duplex kidney) is another common renal malformation; however, the gene mutations leading to ectopic budding in humans have not yet been identified. A better understanding of congenital renal abnormalities in humans will, in part, depend on the creation of detailed large-scale databases of kidney and lower urinary tract developmental disorders.

Early ureteric bud branching

Initial UB outgrowth is followed by iterative branching of the UB, and this depends upon reciprocal epithelial-mesenchymal interactions (Fig. 1A). Gradients of soluble factors from the MM are likely to be essential for appropriate spatiotemporal regulation of the arborization pattern (Santos and Nigam, 1993). In vitro, both in UB cell and isolated bud culture, conditions can be optimized to sustain iterative branching in the absence of direct cell-cell contact between epithelia and mesenchyme (Qiao et al., 1999a; Sakurai et al., 1997a). Soluble factors derived from the mesenchyme have been found to support branching and growth so robust that structures eventually occupy much of the matrix, and a major limitation seems to be one of cell nutrition. Whether the reciprocal epithelial-mesenchymal interactions between the UB and the MM are analogous to the positive feedback loops that coordinate growth and patterning in vertebrate limb (Laufer et al., 1994; Sun et al., 2000) needs to be determined.

Multiple factors regulate early branching

In vitro data indicates that GDNF continues to play a prominent role during early branching (Qiao et al., 1999a; Vega et al., 1996). Transcriptional modulators of GDNF expression also influence ureteric branching. For example, *Emx2*, a homeobox gene expressed in UB epithelia during early branching, maintains expression of GDNF in the MM. Murine *Emx2* knockouts exhibit normal UB budding, but the absence of subsequent branching events leads to rapid mesenchymal apoptosis and renal agenesis (Miyamoto et al., 1997). Similarly, maintenance of localized Ret expression in the ureteric bud tips via expression of RARα, RARβ2, Foxd1 and Pod1 appears to be crucial for proper branching morphogenesis (Hatini et al., 1996; Mendelsohn et al., 1999; Quaggin et al., 1999). Homozygous null mutation of *Rara*, *Rarb2*, *Foxd1* and *Pod1* results in decreased ureteric branching within the embryonic kidney (Table 1).

Although GDNF plays an important role in continued branching morphogenesis, in vitro studies indicate that, while necessary, it is not sufficient to induce robust branching in an isolated UB culture model; other soluble factors such as pleiotrophin are also important (Sakurai, 2001). Cell growth continues to be prominent during this stage; however, sculpting of the UB also occurs. Some members of the fibroblast growth factor (FGF) family have been implicated in the early patterning of the UB. FGF1, FGF2, FGF7 and FGF10 all have different effects on branching morphogenesis of the isolated UB (Qiao et al., 2001). Other factors may also operate during early branching. In vitro, hepatocyte growth factor (HGF) induces branched tubule formation in cells embedded within a 3D extracellular matrix (Montesano et al., 1991; Santos and Nigam, 1993), as do the epidermal growth factor (EGF) receptor ligands EGF, TGFα and heparin binding EGF (HB-EGF), as well as amphiregulin (Barros et al., 1995; Sakurai et al., 1997b). Thus, a plethora of soluble mesenchymally derived factors induce the isolated UB or mature collecting system cells to branch. A single key factor has yet to be identified and current studies suggest that multiple factors, perhaps acting in combination, are required during early branching in vivo (Perantoni et al., 1995; Sakurai et al., 1997a; Bush et al., 2004a).

HSPGs again feature prominently during early branching morphogenesis. GDNF, pleiotrophin, HGF and various FGFs all bind to HS side chains of HSPGs, and HSPGs appear to differentially modulate the activity of these growth factors (Box 1) (Barnett et al., 2002; Iseki et al., 2002; Rickard et al.,

Box 1. Heparan sulfate proteoglycans: multifunctional molecules

A

B
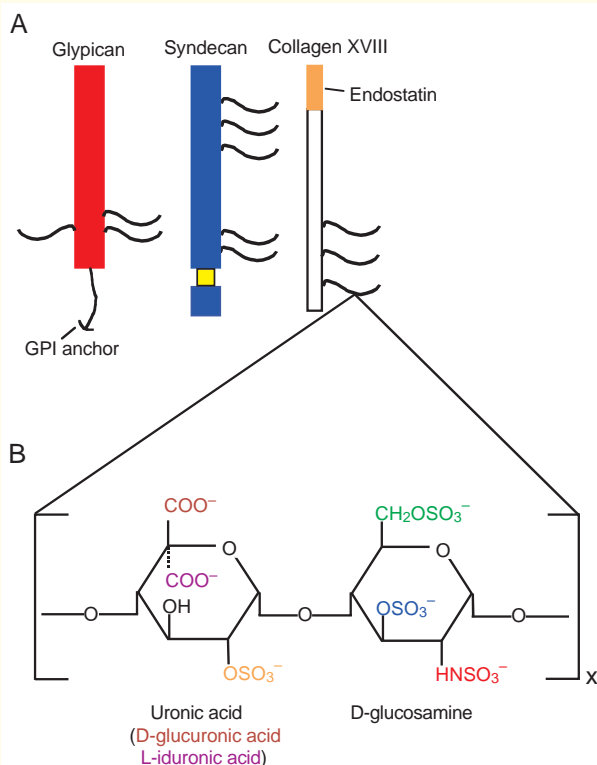
Table I. Morphogen and heparan sulfate proteoglycan interactions

Factor	Characteristics of factor-HS interaction
GDNF	2-O-sulfation required for binding to GFRα1 (Rickard et al., 2003)
BMP4	Activity modulated by glypican 3 (Paine-Saunders et al., 2000) BMP4 antagonist, Noggin, binds HS (Paine-Saunders et al., 2002)
BMP2	Activity modulated by glypican 3 (Grisaru et al., 2001)
WNT	Activity simulated by desulfation at 6-O position (Ai et al., 2003)
Pleiotrophin	Binds to syndecan (Mitsiadis et al., 1995)
FGF1	2-O- and 6-O-sulfation required for binding (Pye et al., 1998) Cell-bound HS mediates receptor activity (Wu et al., 2003) Highly sulfated HS potentiates mitogenesis (Lundin et al., 2000)
FGF2	Pentasaccharide with N-sulfation and 2-O-sulfation sufficient for binding (Pye et al., 1998) 6-O-sulfation required for activation of FGFR1 (Pye et al., 1998)
FGF7	Heparin abrogates activation of FGFR2IIIb (Ye et al., 2001)
HGF	6-O-sulfation important for binding (Lyon et al., 1994)
TGFβ1	HS potentiates dimerization Requires high level of N-sulfation for binding (Lyon et al., 1997)
HB-EGF	Cell-surface HSPG optimizes activity (Higashiyama et al., 1993)
Endostatin	Proteolytic cleavage product of collagen XVIII Requires interaction with glypican 3 for activity (Karihaloo et al., 2001)

HSPGs are modular structures composed of a glycosaminoglycan side chain – heparan sulfate (HS) – covalently linked to a specific core protein. Two major families of plasma membrane-bound HSPGs are known as glypicans (A, red), of which there are six isoforms, and syndecans (A, blue), of which there are four isoforms. Each of the core protein families has distinct functions and tissue specific expression. Minor members of the HSPG superfamily include collagen XVIII (A, white), perlecan and agrin.

Heparan sulfate chain synthesis occurs in the Golgi compartment and is mediated by a variety of biosynthetic enzymes. HS chains are composed of alternating uronic acid and D-glucosamine residues (B) of varying lengths. Following chain polymerization, a series of enzymes modify the HS chain through variable sulfation and C-5 epimerization (of D-glucuronic acid to L-iduronic acid). Variable sulfation can occur at the HNAc- residue (red residue in B) (mediated by the N-deacetylase/N-sulfotransferase enzymes), or at the 6-O (green), 2-O (orange) or 3-O (blue) positions (mediated by the respective O-sulfotransferase enzymes) (Esko and Lindahl, 2001). In addition, cells can alter the structure of the HS chain in response to the environment through the action of sulfatases (Ai et al., 2003). Thus, HSPGs are highly diverse and are capable of multifunctional regulation.

HSPGs play multiple roles by interacting with growth factors: they bind growth factors in proximity to high affinity receptors, act as co-receptors in the growth-factor-receptor complex and protect growth factors from proteolytic degradation (reviewed by Bernfield et al., 1999). A feature of many of the factors involved in kidney branching morphogenesis is their interaction with HSPGs (Table I). The interaction of a growth factor with an HSPG is very specific, based on the length of the glycosaminoglycan side chain (Turnbull et al., 1992) and the fine sulfation pattern (Nakato and Kimata, 2002).

2003; Rubin et al., 2001; Pye et al., 1998). In addition, mice with a gene trap mutation for heparan sulfate 2-O-sulfotransferase (*Hs2st*), an HS biosynthetic enzyme, die perinatally because of renal agenesis (Bullock et al., 1998). Mice with this mutation appear to have normal UB outgrowth, but mesenchymal condensation and subsequent UB branching is absent. *Hs2st*$^{-/-}$ mice display a very consistent phenotype, indicating that 2-O-sulfated HS plays an important role during early branching (Table 2). It is important to note that *Hs2st*, may also function at later stages of kidney development, but termination of development at the early ureteric bud branching stage precludes analysis of the role of *Hs2st* during late branching morphogenesis.

The binding of growth factors to their receptors ultimately induces cellular proliferation, shape changes and migration, all of which are required for branching to occur. In UB branching, the ECM may serve several purposes in this regard: providing a substrate for cell migration (Pohl et al., 2000b; Santos and Nigam, 1993), acting as a reservoir for morphogenetic molecules (which exist in gradients) (Pohl et al., 2000a) and transmitting environmental information to the cell via adhesion molecules such as integrins (Kanwar et al., 1997; Zent et al., 2001). Many of the ECM molecules that carry out these functions have been shown to regulate branching morphogenesis in vitro (Pohl et al., 2000a). For example, an appropriate balance of matrix metalloproteinases (MMPs) and their inhibitors – the tissue inhibitors of matrix proteinases (TIMPs) – at tips and leading edges may result in
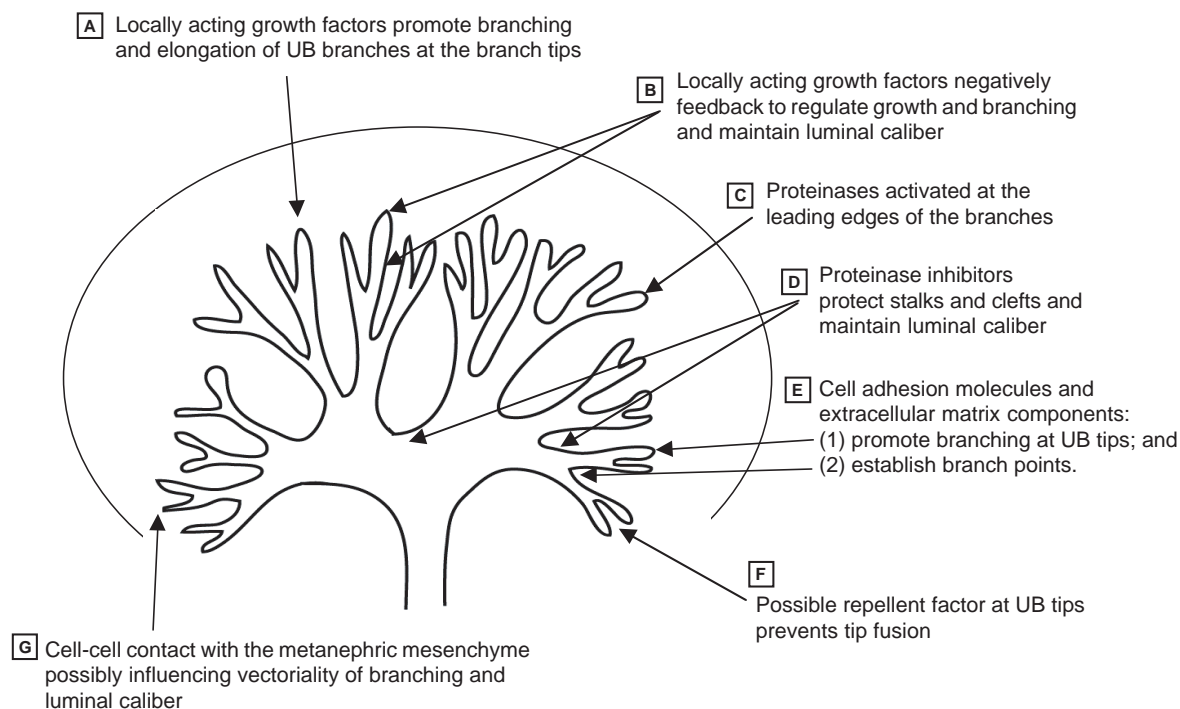
Fig. 2. The interactions between soluble morphogens, metanephric mesenchyme and matrix molecules during the branching process of the ureteric tree. (A) Local and possibly global gradients of soluble branch promoting growth factors, such as pleiotrophin and fibroblast growth factors, may promote vectorial branching and elongation of the ureteric bud (UB) stalks (Qiao et al., 2001; Sakurai et al., 2001). (B) Inhibitory growth factors such as transforming growth factor β and the bone morphogenetic proteins may negatively feed back to regulate growth and branching and may also function to maintain luminal caliber (Santos and Nigam, 1993; Dunn et al., 1997; Bush et al., 2004a). These stimulatory and inhibitory branching morphogens may govern the process of vectorial branching by altering expression of distal effector molecules at tips, branches and stalks, which are known to be important in branching morphogenesis, such as (C) proteinases [e.g. matrix metalloproteinases (Lelongt et al., 1997; Pohl et al., 2000a)], (D) inhibitors of proteinases (Sakurai and Nigam, 1997), (E) extracellular matrix proteins (e.g. proteoglycans) (Pohl et al., 2000b), and cell-adhesion molecules (e.g. integrins) (Qiao et al., 2001; Zent et al., 2001). Cell-cell contact with the metanephric mesenchyme may also be necessary for proper patterning (vectorial branching and stalk elongation) of the ureteric tree (Qiao et al., 1999a) (G). Adapted, with permission, from Stuart and Nigam (Stuart and Nigam, 1995).

matrix degradation that promotes both branching and elongation (Stuart et al., 1995; Sakurai and Nigam, 1998) (Fig. 2).

Defects during early branching

Aside from complete renal agenesis, very few knockout mutations of genes that operate during early branching result in moderate to severe branching defects (e.g. of the order of 5-10 branching generations) (Table 1). Instead, these phenotypes tend to be slight or undetectable. *Fgf7*-null mice are characterized by mildly affected phenotypes, having only 30% fewer nephrons than wild type (equivalent to loss of less than one branching generation), although the overall branching architecture is maintained (Qiao et al., 1999b). *Wnt11*-null mice have a similar defect; these kidneys are characterized by 36% fewer nephrons than wild type with no apparent abnormality in branching pattern (Majumdar et al., 2003).

The lack of moderate to severe branching defects may arise from a requirement for a crucial number of nephrons for organ survival. For example, defects in early branching processes can result in malformed (dysplastic) kidneys that can involute eventually (Hiraoka et al., 2002). In addition, mechanisms such as redundancy may allow for activation of compensatory processes that are able to buffer the effects of genetic mutations (Melton, 1994; Wagner, 2000). For example, although

individual disruption of *Hoxa11* or *Hoxd11* produces no apparent kidney defects (Small and Potter, 1993; Davis and Capecchi, 1994; Davis et al., 1995), double mutants (*Hoxa11/Hoxd11*) demonstrate variable kidney hypoplasia (Davis et al., 1995; Patterson et al., 2001) and removal of the last *Hox11* paralogous member, *Hoxc11*, results in renal agenesis (Wellik et al., 2002). Similarly, null mutations of a number of Fgf genes (e.g. *Fgf1*, *Fgf2*, *Fgf3*, *Fgf5*, *Fgf6*, *Fgf9*), another set of growth factors important for in vitro UB branching, result in kidneys that, with respect to growth and branching, appear normal, raising the possibility that substitution of function occurs between these proteins (Kanwar et al., 1997; Mahmood et al., 1995; Okada-Ban et al., 2000; Qiao et al., 2001). Moreover, in vitro data indicate that certain combinations of growth factors may also result in phenotypically equivalent branching; the addition of TGFβ and FGF7 to UB culture results in a tree that appears similar to one cultured in the presence of FGF1 alone (Bush et al., 2004a).

Finally, gene dose may also play a role in the lack of moderate to severe branching defects. In other words, expression of the gene product must reach a certain threshold for normal branching to occur; below the threshold abnormalities in renal branching may result. For example, although *Gdnf* null mice lack kidneys and die, heterozygotes show an array of renal phenotypes ranging from small kidneys

bilaterally (with approximately a 30% decrease in nephron number) to unilateral agenesis with no intermediate phenotypes (Cullen-McEwen et al., 2001). A similar requirement for adequate gene dose may be operative in human urinary tract development, as highlighted in distal 10q monosomy. In this condition, individuals carrying a single copy of the genes present on the distal 10q chromosome display urinary anomalies such as small under-developed kidneys and defects in the posterior urethral valves. The gene(s) responsible for the observed phenotypes have not yet been identified. However, in some of the cases, haploinsufficiency was not associated with the urinary abnormality, perhaps owing to variable penetrance and/or a dose-dependent gene-phenotype relationship (Ogata et al., 2000). In total, these studies indicate that functional redundancy and/or dose effects exist during early branching.

Late branching and maturation

At some point during development, branching slows down, presumably owing to negative feedback (i.e. corrective information) that serves to dampen the intrinsic branching signals of the UB (slowing growth and proliferation) and promote nephron patterning. There is evidence for these types of signals in other model systems. For example, during the development of respiratory appendages in the *Drosophila* embryo, the integration of positive and negative feedback systems allows for proper spatiotemporal expression of signals, and hence appropriate patterning (Freeman, 2000). A similar mechanism may operate when differentially expressed modulatory molecules, such as TGFβ and BMPs, act as branching inhibitors and sculptors in the developing kidney (Bush et al., 2004a).

Factors involved in negative feedback

Data obtained from whole organ culture suggest that members of the TGFβ superfamily (BMP2, BMP4, activin and TGFβ1) have direct inhibitory effects on the overall growth and development of the embryonic kidney and cell lines, particularly on UB-derived components (Rogers et al., 1993; Piscione et al., 1997; Raatikainen-Ahokas et al., 2000; Sakurai and Nigam, 1997; Bush et al., 2004a). The in vivo role of these factors has more been difficult to elucidate, as knockout of these genes results in phenotypes that range from early embryonic lethality (*Bmp2*, *Bmp4*) (Zhang and Bradley, 1996) to no overt renal phenotype (*TGFβ1*, activin) (Jhaveri et al., 1998; Kulkarni et al., 1993). The generation of conditional knockouts will help to further clarify these functions.

Negative feedback signals may arise from the MM, perhaps timed to a specific point in MM-derived tubule formation. Fusion between a lateral ureteric branch and metanephric tubule (Fig. 1A, stage F) effectively removes the ureteric branch from further divisions (Oliver, 1968). The 'stop signal' may even be present earlier during MM-derived tubule formation, occurring in the earliest branching generations and/or as soon as comma- or S-shaped bodies appear (Fig. 1A) to prevent the intertwining and fusion of branches. The observed shift in expression of the branch-inhibitory factors, BMP2 and BMP7 (Dudley and Robertson, 1997) and the slit proteins (which function in axon repulsive guidance) (Piper et al., 2000) from the condensed mesenchyme to the comma- and S-shaped bodies is consistent with this hypothesis.

Patterning during kidney development also requires structural remodeling by the ECM scaffolding (Fig. 2). Specific ECM proteins can either enhance or inhibit branching in cultured cells (Santos and Nigam, 1993; Sakurai and Nigam, 1997). In addition, differential expression of MMPs and TIMPs at stalks and/or non-branching areas may provide feedback information that prevents further branching and growth (Nigam, 1995; Ota et al., 1998; Pohl et al., 2000a).

As in previous stages, regulatory molecules during late branching and maturation require HSPGs for receptor binding and signaling. Signaling by BMP4 and BMP2 is modulated by glypican 3 (Paine-Saunders et al., 2000; Takada et al., 2003), while the activity of TGFβ appears to be modulated by its binding to HSPGs with N-sulfated HS chains (Lyon et al., 1997). HSPGs may also function to establish the formation of growth factor gradients that modulate patterning of the nephric tree. HSPGs regulate the cellular distribution of Noggin, a BMP antagonist, and may therefore regulate cellular responsiveness to BMPs in vivo through the establishment of a BMP activity gradient (Paine-Saunders et al., 2002).

Derivatives of HSPGs can also influence UB branching directly. For example, endostatin, a proteolytic cleavage product of the HSPG collagen XVIII, inhibits branching in the isolated UB and in cell culture (Karihaloo et al., 2001). Although collagen XVIII is initially present throughout the ureteric epithelium, as development progresses, its expression is lost at the ureteric tips and becomes restricted to the stalk (Lin et al., 2001). Endostatin may suppress further branching specifically at the stalk as a result of this differential spatial expression, which is evident as early as embryonic day 11.5 in the mouse.

Disorders of reduced nephron number

Defects during early or late branching may predispose to disorders of reduced nephron number. The extent of UB branching is thought to reflect nephron number (Oliver, 1968). A widely discussed hypothesis (Brenner et al., 1988) regarding the pathogenesis of certain forms of hypertension is based on the argument that inadequate nephron number (which correlates with reduced filtration surface area) results in increased glomerular volume and increased mean arterial pressure (MAP), compensatory mechanisms that have evolved to maintain adequate renal blood flow and function. Indeed, kidney cross-transplantation experiments demonstrate that hypertension can be 'transplanted' from rat to rat and that in humans, antihypertensive drug requirements of kidney donors and recipients become similar (Rettig et al., 1996). Long-term follow-up of GDNF heterozygous mice demonstrates that aged mice (analogous to late-middle age humans) maintain normal kidney function and blood flow despite reduced nephron number, but that MAP is significantly elevated (Cullen-McEwen et al., 2003). Similarly, rats that are growth restricted in utero are found to have lower nephron number and higher MAP as compared with controls (Lisle et al., 2003). Finally, a recent study demonstrates a strong correlation between low nephron number and hypertension among matched individuals involved in fatal accidents (Keller et al., 2003). All of these examples raise the possibility that some forms of essential hypertension may be disorders of branching morphogenesis. Similarly, the progression of chronic kidney failure also may be inversely proportional to nephron number (Hughson et al.,

2003). Thus, it is conceivable that defects during early and late branching processes may lead to the development of two very common clinical syndromes: hypertension and progressive renal failure. Importantly, reduced nephron number reflects only one of many possible pathogenic mechanisms that may lead to these diseases.

The number of factors involved in the determination of nephron number is likely to be even more complex than those listed here. It is conceivable that genetic variants (the existence of two or more alleles at significant frequencies in a population) that interact differently with modifiers, suppressors and enhancers can cause subtle changes in nephron number that eventually lead to disease. This could also explain the apparently continuous distribution of nephron number in humans, ranging from approximately 230,000 to 1,800,000 (Nyengaard and Bendtsen, 1992). Variant alleles, identified by single-nucleotide polymorphisms (SNPs), are a source of genetic diversity and are expected to be neutral (or nearly so) with respect to fitness (Fay et al., 2001). During early and late branching, SNPs present in genes governing the branching process may not affect the viability or gross architecture of the kidney, but might predispose to hypertension or chronic kidney disease later in life. This idea may be supported by the recent identification of a SNP in the MMP9 gene, which appears to increase end-stage renal disease susceptibility in humans (Hirakawa et al., 2003). During branching, SNPs might be phenotypically subtle or even silent, consistent with the observation that the branching program seems relatively resistant to single mutations. Because of this resilience, it can be postulated that multiple adverse SNPs in branching genes would be required for a clinically significant decrease in nephron number. Identifying the factors that are involved in these stages of branching may lead to candidate genes for hypertension research and may even be useful in devising therapies.

Branching termination and tubule maintenance

Branching termination remains one of the biggest unresolved problems in epithelial organogenesis. Developmental mechanisms must be 'turned off' when the structure reaches a certain size and maturity. In the developing kidney, branching must cease and the caliber of the tubule lumen must be regulated. Although clear stop signals have not yet been identified, in vitro studies have suggested several possible candidates. During later stages of branching, increased expression levels of negative regulators (e.g. TGFβ) may overwhelm positive growth-promoting factors (FGFs) and thereby act as stop signals (Santos and Nigam, 1993; Stuart et al., 2003; Bush et al., 2004a). For example, branching of the murine inner medullary collecting duct (mIMCD) cells is inhibited by the addition of TGFβ, leading to the formation of straight, non-branched tubular structures (Sakurai and Nigam, 1997).

Stop/maturation signals also may be correlated with the differentiation of the MM. Recombination assays between cultured UB and freshly isolated MM indicate that the MM regulates tubule lumen caliber and provide signals that induce UB branch elongation (Qiao et al., 1999a; Steer et al., 2003). However, branching does not extend beyond the confines of the MM, which may provide specific stop signals during nephron formation and maturation. Such stop signals could be short-range secreted molecules or cell-surface proteins (Pohl et al., 2000c). In other systems, membrane-bound ephrins act as stop and maturation signals of epithelial morphogenesis. In *C. elegans*, ephrins are thought to act as a repulsive signal that prevents the migration of epidermal cells (George et al., 1998). Interestingly, media conditioned by embryonic murine MM cells upregulates expression of ephrin A5 and ephrin B2 in UB cells (Pavlova et al., 1999) suggesting a role for these proteins in UB branching morphogenesis.

Finally, sulfated proteoglycans may also function as stop signals of branching, similar to their roles in other developmental systems. For example, chondroitin sulfate proteoglycans (CSPGs) inhibit axon growth in vitro – perhaps forming a protective 'jacket' that masks the target stimulatory molecule(s) (Bradbury et al., 2002; Morgenstern et al., 2002). It also is possible that changes in the proteoglycan environment lead to either enhanced binding of inhibitory factors or diminished activity of stimulatory factors; in effect creating gradients of activity. This appears to be the case in *Drosophila* wing development, where the expression of the HSPG *dally* regulates both the cellular response to Dpp (the *Drosophila* TGFβ/BMP protein homolog) and the distribution of Dpp morphogen in tissues (Fujise et al., 2003).

Disorders of branching termination

As yet, a clear clinical manifestation of disordered stop signals for branching, e.g. a kidney with 4 million nephrons, has not been reported. In fact, clinical studies that have measured gross kidney parameters have failed to find large variation in normal kidney size (kidneys tend to be between 11-12 cm in length in the general population (Brenner and Rector, 2000)). It is possible that a high level of functional redundancy exists among the stop signals. It also is feasible that nutritional or external factors (retroperitoneal space constraints and/or restriction by the kidney capsule) prohibit the generation of significantly over-branched organs.

It is plausible that molecules involved in stop mechanisms also play a role in tubule maintenance. Aberrations in these signals then may result in generation of cysts (thin-walled cavities arising from a single epithelial cell) with corresponding clinical manifestations ranging from asymptomatic (simple cysts) to end-stage kidney disease (polycystic kidney disease). For example, Simpson-Golabi-Behmel (SGB) syndrome is a human disease characterized by cystic renal overgrowth. The genetic basis for this disease is a mutation in the glypican 3 gene (*Gpc3*) (Pilia et al., 1996), an HSPG that is a low-affinity receptor for the inhibitory molecule endostatin (see above) and which modulates inhibitory and stimulatory growth factors involved in branching (Grisaru et al., 2001). *Gpc3*-null mice and individuals with SGB have similar cystic phenotypes. Evaluation of these mice early in embryogenesis demonstrates an increased number of UB branches when compared with controls; thus, *Gpc3* may modulate a branching stop signal(s). Furthermore, in these mice, epithelial cysts progressively form as nephrogenesis proceeds; therefore, it is possible that GPC3 also functions as a maintenance molecule (Cano-Gauci et al., 1999). Culture of *Gpc3*-deficient kidneys demonstrates that glypican 3 modulates the effects of FGF7, BMP7 and BMP2 during renal branching morphogenesis, thereby mediating the activity (facilitatory or repressive) of these growth factors (Grisaru et

al., 2001). In *Gpc3*-deficient mice, there is altered regulation of FGF7, BMP7 and BMP2 that then correlates with increased epithelial cell turnover (i.e. upregulated proliferation and apoptosis), analogous to cyst-forming mechanisms found in human disease (Lanoix et al., 1996).

Mechanisms of cyst formation

Gradients of stimulatory and inhibitory growth factors and other secreted morphogenetic molecules are thought to be normally responsible for maintaining tubule architecture (Pohl et al., 2000c). Gradient disruption and/or alteration of regulatory information presumably leads to an imbalance in the relative concentration of growth and survival signals received by cells (Sakurai and Nigam, 1998), and may play a role in cyst development. Overexpression or upregulation of several branch-promoting factors including HGF, TGFα and EGFR are associated with cystic phenotypes in mice (Takayama et al., 1997). A similar outcome is seen following the disruption of factors that inhibit branching. For example, cystic dilation of tubules occurs in heterozygous mutants of *Bmp4* (Dunn et al., 1997) and null mutants of *Tgfb2* (Sanford et al., 1997). Similar mechanisms may contribute to human cystic disease as well; autosomal dominant polycystic kidney disease (ADPKD), the most common form of human polycystic kidney disease, is associated with abnormal upregulation of TGFα and EGFR (Lee et al., 1998).

The ECM is another component involved in tubule maintenance and cyst formation. Its influence on epithelial cell behavior is highlighted by cell culture models in which differences in ECM composition can result in phenotypes ranging from branching structures to cysts. For example, immortalized UB cells grown in a matrix of 80% type I collagen/20% Matrigel (an ECM extracted from mouse EHS sarcoma composed of laminin, collagen IV, and HSPGs) form branched tubules; however, when grown in 100% Matrigel matrix, these cells form cysts, even in the presence of tubulogenic growth factors (Sakurai et al., 1997a). Likewise, when Madin-Darby canine kidney (MDCK) cells, which have the properties of the kidney distal tubule and collecting duct cells, are grown in type I collagen alone, they form branched tubules in the presence of HGF, a potent tubulogenic factor for this cell line. However, MDCK cells grown in collagen mixed with Matrigel form cysts even with the addition of HGF (Santos and Nigam, 1993). ECM remodeling by MMPs and their inhibitors (TIMPs) is also important in the formation and maintenance of tubule structure (Pohl et al., 2000a). In the rat model of ADPKD, upregulation of MMP RNA occurs predominantly in cyst lining epithelia. Treatment with the MMP inhibitor called batimastat results in a significant reduction of cyst number (Obermuller et al., 2001). This mechanism may also apply to Von-Hippel-Lindau (VHL) disease, an autosomal dominant tumor syndrome associated with cystic kidney disease. In cell culture, *VHL* inactivation leads to decreased TIMP and increased MMP levels, suggesting abnormal regulation by these cells (Koochekpour et al., 1999). This re-emphasizes the idea that the relative balance between MMPs and TIMPs is important in proliferation versus stopping and, in this case, cyst versus tubule formation.

Cilia

Although an appropriate balance of stimulatory and inhibitory growth factors and ECM constraints normally maintains a steady state of proliferation and apoptosis, another cellular feature is emerging as a prominent regulator of tubular architecture: cilia. Cilia are organelles present on the apical surface of tubule-lining epithelial cells that are thought to function as environmental sensors, evident by the presence of many chemo- or mechano-sensing receptors (Calvet, 2003). Loss of ciliary structure and function appears to induce overgrowth of the epithelial cells, thereby leading to cyst formation, although the mechanism for this remains undefined (Bhunia et al., 2002; Lin et al., 2003; Morgan et al., 2002; Hou et al., 2002; Yoder et al., 2002). In humans, cilia gene mutations disrupt regulation of tubule epithelial cell proliferation, thereby resulting in cyst formation. Mutation of *PKD1* and *PKD2*, genes that code for ciliary structural proteins, underlie most forms of ADPKD, whereas mutations in *OFD1*, a gene involved in cilia generation and structure, and are associated with X-linked dominant polycystic kidney disease (Romio et al., 2003). These studies underscore the importance of cilia function in the regulation of tubule lumen diameter. However, the interactions between cilia, growth factors and ECM proteins in the regulation of branching morphogenesis remain undefined.

Injury/regeneration

Although branching does not occur following acute tubule injury, regeneration employs a combination of mechanisms that overlap with those that are involved in branching morphogenesis (Nigam and Lieberthal, 2000). Unlike glomerular or vascular injury, damage to the nephron tubule can be repaired through regeneration. The most common injury response is acute tubular necrosis (ATN), a sloughing of a large population of epithelial cells occurring in response to oxygen deprivation or chemical insult. Although the proximal tubule is the primary site of injury, more distal injury occurs as well. Recovery from ATN is possible if the adjacent blood supply and basement membrane remain intact, thereby guiding reconstitution of the polarized epithelia. Several mechanisms of cellular repair are activated in the recovery process, including mitosis of neighboring cells, recruitment, differentiation and proliferation of stem cells, and basement membrane remodeling.

Tubule regeneration processes are in part often mediated by molecules that are usually highly expressed during development such as HGF, MMPs, FGFs and EGFR ligands (among others), and their putative roles in the recovery process are listed in Table 3. During regeneration, however, cellular responses to these factors may be different. It is possible that regenerating cells can adopt radically different strategies to repair tubule breaches using the same fundamental tools that lead to tube formation in vitro or during development. The process of tubule regeneration is poorly understood, but studies in other developmental systems demonstrate that considerable plasticity exists within the transcriptional state of polarized epithelial cells (Lin et al., 2001). It is conceivable that, in order to replenish tubule components, surviving epithelial cells exhibit very different behavior to that seen during organogenesis. This may occur, for example, via a less differentiated phenotype, reminiscent of the invasive cellular processes (so-called 'invadopodia') characteristic of MDCK or mIMCD cells undergoing tubulogenesis in three-dimensional

Table 3. Growth factors in development and renal recovery

Growth factor	Expression following acute renal injury	Role in recovery from acute renal injury in animals	Branching/ tubulogenic activity	References
GDNF	Undetermined	Undetermined	Stimulatory*,†,‡	Vega et al., 1996; Qaio et al., 1999
FGF2	Undetermined	Undetermined	Stimulatory*,‡	Sakurai et al., 1997; Qaio et al., 2001
FGF7	Increased	Undetermined	Stimulatory‡	Sakurai et al., 1997a; Ichimura et al., 1996
HGF	Increased	Enhanced recovery	Stimulatory*	Igawa et al., 1993; Kawaida et al., 1994; Santos and Nigam, 1994
EGF	Unclear	Enhanced recovery	Stimulatory*	Humes et al., 1989; Sakurai et al., 1997b
TGFα	Increased	Enhanced recovery	Stimulatory*	Sakurai et al., 1997b; Hise et al., 2001; Safirstein et al., 1990
TGFβ	Increased	Undetermined	Inhibitory*,†,‡	Basile et al., 1996; Sakurai et al., 1997c

*Activity in cell culture (MDCF, mIMCD or UB cells).
†Activity in whole organ culture.
‡Activity in isolated UB culture.
Adapted, with permission, from Bush et al. (Bush et al., 1999).

culture (Stuart et al., 1995). Cells also may adopt a strategy similar to the one that occurs during *Drosophila* tracheobronchial development, whereby, after proliferation, extreme cell shape changes primarily account for tube formation (Samakovlis et al., 1996). The end result is a restored, intact epithelial tubule without scar formation.

The process of tubule repair, although ultimately beneficial in the setting of injury, may also go awry and lead to pathology such as in acquired cystic kidney disease (ACKD). ACKD, which is found in adults with end-stage renal disease, is characterized by a bilateral cystic change occurs in response to profound tissue loss. ACKD has been associated with overexpression of EGFR ligands and MMP abnormalities (Concolino et al., 1993; Ito et al., 2000).

Summary/future directions

We have reviewed the molecular basis of kidney epithelial branching; molecules thought to provide key points of control have been parsed within the framework of stages that describe branching morphogenesis from ureteric bud outgrowth to branching termination (Stuart et al., 2003; Nigam, 2003; Bush et al., 2004a). Applied to in vitro systems developed to date, this description may enable characterization of the independent morphogenetic mechanisms functioning during stages of kidney development and shed insight on disease.

Whereas secreted peptides, ECM molecules, growth and transcription factors comprise the mainstay of signals operative during branching, HSPGs have been found to interact with the majority of growth factors at each stage. As seen in other developmental systems, HSPGs may be instrumental in establishing growth factor gradients, as well as acting as morphogenetic modulators via their ability to regulate 'positive' and 'negative' growth factor activity. Further studies are needed to in order to understand how the integration of tremendous HSPG diversity with growth factor pathways at each branching step drive and/or regulate UB morphogenesis. Such studies may provide key information into the mechanisms underlying branching defects and disease.

As we have discussed, disruption at key points throughout kidney development may lead to disease, including renal agenesis. However, mutagenesis and gene deletion in animal models, including many genes thought to be important in branching based on in vitro studies, often results in kidneys that appear normal. Thus, as judged by these reports, relatively few phenotypes lie between these two extremes. A decentralized network of molecular interactions that comprise the developmental program, with regulation at many levels, may account for this distribution (Nigam, 2003). Loss of a centrally acting molecule that interacts with or is connected to multiple other molecules (e.g. GDNF) can produce irreparable downstream effects on kidney development. However, the phenotypic consequences of knocking out other genes (e.g. *Hgf*) may be buffered by the inhomogeneous wiring of complex systems. In certain cases, the plasticity of the system allows development to proceed via a bypass route and/or through alternative molecular mechanisms that are phenotypically equivalent (Barabási et al., 2002; Venter et al., 2001; Nigam, 2003). Thus, a major task of future studies will be to unravel this complex network of molecular interactions, in order to decipher and predict the pathological consequences of developmental errors. Application of microarray analysis to kidney development and model systems is likely to provide essential information about the (non)hierarchical structure of gene networks and key points of regulation (Stuart et al., 2001; Stuart et al., 2003; Schwab et al., 2003).

This review is dedicated to the memory of Robert Oden Stuart II, MD (1962-2004), who made major contributions to the field of kidney development and epithelial biology.

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