Kidney development requires precise spatio-temporal coordination of numerous biomolecular interactions. Approaches geared toward elucidating the molecular and cellular basis of kidney development and branching morphogenesis have focused on the study of discrete molecules and signaling pathways by using genetic and in vitro approaches (reviewed in Refs. 11 and 71), more recently combined with microarray studies (66, 74, 75). To date, integration of this body of data at the protein, genetic, and cellular levels has proven difficult, and precise causal relationships mapped within a more general framework remain elusive. Moreover, apparent contradictions between in vitro and genetic experiments have so far limited our ability to assemble a complete mechanistic model.

A seemingly opposite yet broader tactic that is emerging in the postgenomic field is to use a system-level approach. Genome-scale protein-interaction maps have been constructed in numerous organisms. Indeed, high-throughput data collection techniques are being applied with increased frequency across many systems, including the kidney. This organization of data into a graphical arrangement of nodes and links, where nodes depict molecules (proteins, nucleic acids, proteoglycans, etc.) and links delineate the interactions between them, encompasses a network. Many classes of networks exist within a cell or an organism; these may include gene expression, protein-protein, metabolic, and signaling networks. It has been found that architectural features common to complex systems (which range from the Internet to societies to cells) result in a robust, relatively error-tolerant network (2, 4).

Obviously, construction of a topological and dynamic network of branching morphogenesis would require knowledge of all molecules involved in kidney development and an understanding of their intricate interactions. Although the kidney database of genes and proteins is far from complete, current in vitro and mutation data, albeit limited, support the presence of a network architecture that underlies the branching program (49). As our understanding of gene expression, protein-protein, and signaling pathways in branching morphogenesis progresses, organization of data into such a framework would enhance attempts to define, quantify, and model the structural and dynamic parameters that underlie branching morphogenesis. Combined with studies that aim to delineate the detailed roles played by specific molecules (Table 1), these two approaches may be able to provide complementary and converging viewpoints that would enable integration and a deeper understanding into the factors that control kidney development. Although branching during kidney development is the focus in this review, many of the issues are applicable to organogenesis in general.

Stages of Kidney Organogenesis

Branching morphogenesis is initiated with the invasion of the epithelial ureteric bud (UB) into a cluster of cells known as the metanephric mesenchyme (MM). The tip of the UB concomitantly induces the surrounding MM to condense and aggregate. The UB itself, under the direction of signals arising from the MM, undergoes a number of repetitive branching iterations. Continued growth and transformation of mesenchymal cell clusters results in epithelialization and tubulogenesis, ultimately forming the portion of the nephron comprising the glomerulus, proximal tubule, loop of Henle, and distal tubule. The MM and its epithelial derivatives continue...
to provide instructive and reciprocal signaling that leads to multiple iterations of UB branching and elongation. In this manner, the UB consequently forms the collecting system (FIGURE 1).

Development of the collecting system is often considered as a series of related events that occur in an iterative manner. Indeed, it has been shown that as kidney development progresses, global gene expression patterns shift in concert with specific developmental time points and morphological changes (74, 75). Furthermore, on the basis of in vitro models of development and characteristic patterns of kidney disease that correlate with time-specific mutations in knockout mice and in humans, branching morphogenesis can be represented by using a stage framework (10, 49, 68). These may comprise 1) initial UB outgrowth, 2) early branching, 3) late branching and maturation, and 4) branching termination and tubule maintenance (10, 68) (FIGURE 2). Finally, although branching does not occur after acute injury, a fifth regenerative stage should be considered. Cellular responses to injury often employ molecules that are expressed and thought to have morphogenetic roles in kidney development, including hepatocyte growth factor (HGF), transforming growth factor-β1 (TGF-β1), matrix metalloproteinases (MMP), fibroblast growth factors (FGF), and epidermal growth factor receptor (EGFR) ligands (7, 27, 29, 30). In addition, recovery may recapitulate several developmental processes, which include mitosis of surviving epithelial cells; recruitment, proliferation, and differentiation of progenitor cells; and basement membrane remodeling (78).

These stages are characterized by the balance of numerous stimulatory and inhibitory factors. UB outgrowth seems to be largely regulated by GDNF-dependent pathways, although other factors are

<table>
<thead>
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<th>Table 1. Genes that control kidney branching morphogenesis</th>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>Bone morphogenetic protein 4</td>
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<td>Bone morphogenetic protein 7</td>
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<tr>
<td>Endostatin</td>
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<tr>
<td>Epidermal growth factor receptor and ligands</td>
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<td>Eyes absent homolog 1</td>
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<td>Fibroblast growth factors</td>
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<tr>
<td>Glial cell line-derived growth factor</td>
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<td>GDNF family receptor-α1</td>
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<td>Glypican-3</td>
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<td>Hepatocyte growth factor</td>
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<td>Homeotic genes 11</td>
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<td>Integrin-α-6</td>
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<td>Lim homeodomain transcription factor</td>
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<td>Matrix metalloproteinases</td>
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<td>Paired-box gene 2 transcription factor</td>
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<td>Ret Tyrosine kinase</td>
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<td>Sal-like 1</td>
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<tr>
<td>Six family homeobox family</td>
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<tr>
<td>Tissue inhibitor of metalloproteinases</td>
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<td>Transforming growth factor-α</td>
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<td>Wilms tumor-suppressor</td>
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likely involved. Those factors that promote growth and branching are thought to be most highly active at stage II, during which UB branching is spurred on by multiple stimulatory factors, perhaps functioning as part of a feed-forward mechanism that predominates over feedback strategies (10, 68) (FIGURE 3A). Stage II is characterized by rapid arborization of the collecting system, and tissue-recombination studies support the view that the MM provides guidance cues that influence vectorial branching, elongation, and sculpting of the ureteric tree (10, 56, 72). At stage III, this growth and branching is slowed by increased preponderance of inhibitory molecules that provide negative feedback information compared with branch- and branching is slowed by increased preponderance of inhibitory molecules that provide negative feedback information compared with branch- and growth-inducing molecules (FIGURE 3A). Stage II is characterized by rapid arborization of the collecting system, and tissue-recombination studies support the view that the MM provides guidance cues that influence vectorial branching, elongation, and sculpting of the ureteric tree (10, 56, 72). At stage III, this growth and branching is slowed by increased preponderance of inhibitory molecules that provide negative feedback information compared with branch- and growth-inducing molecules (FIGURE 3A). For example, in undifferentiated MM, bone morphogenic protein 7 (BMP7) is expressed at low levels and activates branching; however, its expression is increased in differentiated epithelia, where its action at these higher concentrations actually suppresses branching (26). Finally, although clear “stop branching” signals have yet to be identified in the kidney, in vitro studies suggest that a shift in the balance from predominately stimulatory growth-promoting factors to increased expression of inhibitory molecules, such as the TGF-β superfamily members and endostatin, may act to terminate branching (10, 63, 74).

The properties of each one of these stages presumably are characterized by a distinct network structure of gene and protein interactions. Transitioning or “switching” between stages is mediated at the molecular level by changes in expression of MM- and UB-derived components. These include growth factors (e.g., glial cell line-derived neurotrophic factor [GDNF], FGFs, TGF–β superfamily members, Wnts, etc.) in the context of heparan sulfate proteoglycans (HSPGs), along with other extracellular molecules and MM cell-surface proteins. Depending on their combinations and configurations, these can positively or negatively impact each stage. Thus their balance, perhaps in combination with global and local gradients of growth factors, HSPGs, and extracellular molecules with the MM/stroma input, determines whether (and how) transitioning to the next stage of developmental occurs. Models of possible network configurations at each stage will be discussed below.

Network Models

“Genome-scale” models have been constructed for the protein-interaction maps of Drosophila melanogaster (24) and Saccharomyces cerevisiae (31, 81), and for the Haemophilus influenzae metabolic network (18). A common feature of their organization is a “scale-free” arrangement of nodes (proteins) and links (their interactions) (2). Such a scale-free organization differs from the arrangement of nodes and links found in random networks, which are characterized by the arbitrary assembly of nodes and their connectivities. In the latter, the number of connections remains close to a small, average value, so that the number of interactions between nodes is fairly homogeneous (i.e., each is characterized by a relatively constant number of connections). Conversely, nodes in scale-free networks are characterized by an uneven (or inhomogeneous) distribution of connectedness. Moreover, some of these nodes display a disproportionately greater number of links compared with those that are less connected but occur with greater frequency. The end result of this inhomogeneity in connectivity is that a small number of highly connected nodes act as hubs that shape the overall operation of the network (2). Because random disruption statistically is more likely to affect a node that, by number, outweighs the number of hubs, this type of scale-free architecture confers a resistance to system failure and tends to maintain overall network integrity (2).
It is important to note that disruption of one or more hubs may produce catastrophic effects and a “splintering” of the system into small, isolated node clusters.

Resilience to mutation has been demonstrated in a number of systems. For example, effects arising from deletion of one or more enzymatic reactions that comprise the metabolic network of *H. influenzae* can be circumvented by exploiting alternative pathways (18). It also has been found that ~40% of genes in *S. cerevisiae* do not yield an aberrant phenotype when ablated (86). Furthermore, simultaneous deletion of many *E. coli* genes is without substantial phenotypic effect (36, 89). Vulnerability to attack is demonstrated by the lethality that results upon mutation, however, in a smaller subset of genes: ~18.7% of *S. cerevisiae* and 14.4% of *Escherichia coli* gene deletions are lethal when individually deleted (22, 23, 86). The highly connected nature of hubs is demonstrated through deletion analysis in the *S. cerevisiae* protein-protein network. Only ~10% of proteins with fewer than five protein-protein interactions are essential. However, this fraction increases to >60% for proteins with >15 links (32) (such molecules would be classified as “hubs” given both their vulnerability to disruption and high degree of connectivity). This result supports the idea that the degree of connectivity plays an important role in the determination of the deletion phenotype (32). How these results might apply to organogenesis is just beginning to be explored.

**Parallels Between the Molecular Basis of Kidney Branching Morphogenesis and Scale-Free Networks**

The developmental stages of branching in kidney development enumerated above are important from several different perspectives. First, at the cellular level, they are characterized by recurring molecular events, the most elemental being growth factor binding to receptor and downstream signaling. Second, switching between stages appears to be dictated by growth factor/HEPG/extracellular matrix/MM interactions with the UB. Third, from the standpoint of disease, dysfunction at any given stage has the potential to result in certain pathological consequences, as recently reviewed (68). Finally, they each can be represented by specific time-dependent network assemblies. These arrangements may describe interacting genes that underlie development and that correlate with the phenotypic effects of gene disruption. It should be noted that network representations can model interactions on multiple levels. These may include protein-protein interaction as well as metabolic, signaling, and transcription-regulatory networks (5). For the sake of discussion, all references to kidney branching networks in this review refer to gene interactions.

**A Potential Hub in Stage I**

**Molecules Often Results in Renal Agenesis**

Factors that play key roles in UB outgrowth have been reviewed in detail (11, 54, 71). These include Gdnf, Wt1, Lim1, Paired-box gene 2 (Pax2), Eyes absent 1 (Eya1), Scl1, Sal-like 1 (Sall-1), the Hox11 homeobox gene paralogous group, Gdnf family receptor GFRα1, and Ret (12, 14, 37, 38, 42, 50, 59, 65, 69, 80). Gdnf has been found to play a crucial role in initial bud outgrowth (59). In mice, disruption of Gdnf, of molecules involved with Gdnf expression or signaling, or of members of the Gdnf receptor signaling complex most often result in phenotypes
ranging from renal agenesis to rudimentary UB systems and kidneys.

Given that hubs are defined as nodes comprised of numerous links and that their deletion results in catastrophic systemic effects, a potential stage I network configuration might be comprised of highly linked nodes and one or more hubs (FIGURE 4). This arrangement would be in accord with the nature of molecular interactions in stage I, whether they be physical associations (e.g., the ligand-receptor interaction between GDNF and Grf1/Ret) or whether they represent tightly linked upstream or downstream regulatory interactions between genes (e.g., Pax2, Foxc1, and Eya1 regulation of Gdnf expression). Furthermore, the fact that disruption of the preponderance of molecules active at stage I results in the absence of UB outgrowth and ultimately in renal agenesis is consistent with mutation-induced splintering or disastrous network effects. The fine structure of this potential hub needs to be fully worked out. Obviously, designation of a gene or molecule as a node or as a multilinked hub requires complete description of its phenotypic consequences and enumeration of all genes, molecules, and their interactions. Although this information currently is lacking, any of the above-mentioned molecules could conceivably act as hubs in UB outgrowth.

Decentralized Networks at Branching Stages II and III

Although the action of GDNF, Ret, and the numerous signaling and transcription factors linked to the GDNF-Ret axis are crucial to initial UB outgrowth, subsequent branching events (comprising stages II and III), although likewise requiring precise spatiotemporal molecular interactions, seem to exhibit a fair ability to withstand mutations, i.e., resilience. A number of gene-inactivation studies designed to target key developmental molecules for which there is strong in vitro evidence of participation have demonstrated a lack of resultant aberrant kidney phenotypes. For example, mouse studies demonstrate that disruption of molecules thought to be involved in early- and late-stage UB branching such as Wnt-1 (85), MMP-9 (3), integrin-β1 (21), and BMP5 (35) result in mutant animals with no apparent kidney defects. Compared with stage I, defects that result from disruption of molecules that have been implicated in UB outgrowth, phenotypic effects of knockout of factors that are expressed at these stages of branching morphogenesis and corticomedullary patterning have been noted to be much more subtle. For example, kidneys in Fgf7-null mice possess ~30% fewer nephrons compared with wild-type mice, yet they maintain normal branching architecture (57), and in mice, inactivation of Wnt11, a glycoprotein normally secreted by the UB, also results in a reduction in nephron number by 36% (46). Given the relative lack of severe phenotypic flaws in stages II and III, it is conceivable that the network structure is relatively decentralized and is able to safeguard against mutation to a much greater extent compared with stage I. Furthermore, other buffering mechanisms may be operative, such as molecular redundancy.

A

- Foxc1
- Pax2
- EyA1
- Sall1
- Sx1

Factors that regulate expression of GDNF.

GDNF interacts with the Ret-Gfr1 receptor complex to stimulate UB outgrowth

B

- Hgf
- Pleiotrophin
- TGF
- Endostatin
- Bmp2/4

Multiple ligands activate some receptors, possibly buffering mutation.

Stimulate branching

Inhibit branching

FIGURE 3. Molecules involved renal branching morphogenesis

A: several factors known to be involved in initial UB outgrowth. Disruption of GDNF, of molecules involved with GDNF expression or signaling, or of members of the GDNF-receptor signaling complex often result in phenotypes ranging from renal agenesis to rudimentary kidneys. B: several molecules involved in branching Stages II and III. Ligand-receptor interactions and their downstream pathways have been shown to be important in positive (green) and negative (red) regulation of branching. Various receptors may be activated by multiple ligands, possibly contributing to buffering against mutation. Stages II and III are likely characterized by differences in the preponderance of either positive or negative-acting branching factors. Branch-promoting factors favor a feed-forward iterative branching state; increasing expression of branching inhibitors slow down UB arborization via negative-feedback mechanisms (10).
Stage IV Branching Termination and Networks

Across all organ systems, relatively little is known regarding mechanisms involved in termination of epithelial branching, and in the kidney, specific stop signals remain to be identified. However, in vitro experiments have implicated the involvement of several molecules that act to slow down and/or terminate the branching program. For example, supplementation of endostatin to UB culture inhibits branching, and addition of endostatin-neutralizing antibodies enhances UB outgrowth and branching (34). Exogenous addition of tissue inhibitor of metalloproteinase 1 (TIMP1), the natural inhibitor of MMP9, has been found to inhibit UB branching (41), and TIMP2 also has been found to inhibit branching via its effects on MMPs (6). In the context of UB branching morphogenesis during kidney development, in vitro data suggest a model in which soluble factors produced by the MM, in the context of specific extracellular molecule components, modulate the expression of specific subsets of MMPs and TIMPs. As structures develop, this expression evolves and the matrix environment changes, suggesting distinct roles for different groups of MMPs and their inhibitors during each stage of branching morphogenesis and its cessation (55). Furthermore, TGF-β superfamily members, in concert with various effectors or matrix-inhibitory molecules, have been implicated in shifting the balance from a feed-forward branching mode to a negative feedback mechanism and, ultimately, to branching termination (10, 34, 60, 63).

There appears to be a correlation between developing structural features of kidney precursors and which is supported by in vitro studies (57), as will be discussed below. It is interesting to note that in both Fgf7 and Wnt11 knockout animals, the morphological defect correlates with what is effectively the loss of less than one branching generation. Thus compared with initial UB outgrowth, stages II and III are characterized by subtle or by a relative lack of mutant phenotypes in the setting of gene disruption. This robustness is consistent with a scale-free topology in which random failure affects mainly the numerous small-degree nodes, which do not have a major effect on the network’s integrity (FIGURE 4). Again, a complete description of molecules active in early and late branching stages is lacking, as well as their interactions, including the circuits that describe feed-forward and feedback information that regulate branching. Transitioning from stage II to stage III may require a shift from predominantly feed-forward mechanisms (i.e., signaling loops that sustain expression of branch-promoting factors) to feedback branching mechanisms, whereby negative regulators provide corrective information, which provides appropriate branch-slowing signals. Appropriate balance of positive and negative feedback loops has been shown to integrate growth and patterning in several model systems, including the developing Drosophila melanogaster respiratory appendage, vertebrate limb, and kidney (10, 20, 39, 76). With increasing availability of high-throughput and mutation data, it would be interesting not only to search for molecular function but, in addition, to determine if 1) the connectivities of more weakly interacting (or decentralized) nodes allow tolerance to multiple concurrent mutations and 2) whether the nature of these nodes limit the more far-reaching effects of local perturbations.

Stage IV Branching Termination and Networks

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molecular signaling. Cross-talk between the MM and UB must be coordinated in such a way that signals arising from each component appropriately terminate branching when respective MM- and UB-derived nephron segments have formed (10). Defects in stage IV stop signals and branching inhibitors, although not predisposing to excessive branching, tend to result in cystic phenotypes (68). Indeed, overexpression of several branch-promoting factors, including HGF, TGF-β, and EGFR, are associated with cystic phenotypes in mice (77), and knockout of inhibitory factors can also result in cystic dilation of tubules, as evidenced by heterozygous and null mutants of Bmp4 and Tgfβ2, respectively, in mice (17, 61, 68).

Together, these data suggest that mutation of genes important in branching termination, either via overexpression of branching-stimulatory or underexpression of branching-inhibitory molecules, results in cystic “overgrowth” phenotypes, characterized by rampant epithelial growth, although not necessarily by disturbance of the overall branching pattern. Human cystic disease also can result from a disruption of inhibitory molecules. These include genes that encode ciliary structural proteins such as PKD1 and PKD2, mutation of which can disrupt regulation of tubule epithelial cell proliferation and lead to autosomal-dominant polycystic kidney disease (ADPKD) (8, 88). Abnormal upregulation of the branch-promoting factors Tgfβ and Egfr also has been shown to be associated with ADPKD (40). In addition, mutation of the HSPG glypican-3 results in cystic phenotypes via disrupted binding to endostatin, a molecule that normally functions to modulate branching-inhibitory and -stimulatory growth factors (25, 53). In the aforementioned mouse and human studies, at one level it would seem that the network topology offers comparatively little buffering at this stage, as the disruption of factors that are mainly responsible for negative regulation of growth results in mutant phenotypes. This also may be due in part to the nature of inhibitory molecules: although their negative regulatory effects have been disrupted, the underlying developmental program is able to proceed, albeit in the setting of relatively unchecked proliferation, causing disordered yet continued growth. On the other hand, unchecked branching does not seem to occur, suggesting that at another level, inhibitory molecules continue to supply feedback control.

Toward Understanding Resilience and Vulnerability in the Branching Program

A finding that has emerged from the genome era is that a substantial number of genes do not yield an aberrant phenotype when disrupted. Consistent with this is the finding that many errors in kidney development do not result in overt renal phenotypes. We have explored the implications that graph and network theory convey to branching morphogenesis and kidney development. A fair amount of robustness has been attributed to the network organization of nodes, links, and hubs that underlie the functional organization of living systems (which also is likely a major source of phenotypic diversity). The error tolerance of such networks is not without cost, however. Removal of highly connected molecules can produce catastrophic systemic effects, as evidenced by the renal agenesis phenotype that is strongly associated with stage I-specific defects. It also must be noted that more subtle phenotypes may result from gene deletion and that some mutant phenotypes may only become evident at a later time, under specific environmental conditions, or only under more sophisticated means of detection. Again, this is consistent with the fact that, despite gene disruption, a complex network design allows for the use of alternative compensatory pathways to circumvent mutations.

Although a wealth of information has been obtained via reductionist approaches to analyzing kidney development, it is increasingly clear that an understanding of branching morphogenesis will require a detailed itemization of all genes and molecules that are involved in this process, as well as their interactions. Large-scale searches for branch-specific molecules are only just beginning to describe the genetic components of kidney development (66, 74, 75), and our understanding of the complex molecular signaling and events that underlie branching morphogenesis is far from complete. However, integration of molecular data into a more comprehensive framework will enhance our understanding of the cooperative action of genes and proteins and the effects of their mutation. Mapping of the underlying “complexity” in kidney development is critical, given that the effects of gene disruption have far-reaching and often unpredictable phenotypic consequences that lead to disease.

References


