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REVIEW

Eph/ephrin signaling in the kidney and lower urinary tract

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Abstract Development and homeostasis of the highly specialized cell types and tissues that constitute the organs of the urinary system, the kidneys and ureters, the bladder, and the urethra, require the tightly regulated exchange of signals in and between these tissues. Eph/ephrin signaling is a bidirectional signaling pathway that has been functionally implicated in many developmental and homeostatic contexts, most prominently in the vascular and neural system. Expression and knockout analyses have now provided evidence that Eph/ephrin signaling is of crucial relevance for cell and tissue interactions in the urinary system as well. A clear requirement has emerged in the formation of the vesicoureteric junction, in urorectal septation and glomerulogenesis during embryonic development, in maintenance of medullary tubular cells and podocytes in homeostasis, and in podocyte and glomerular injury responses. Deregulation of Eph/ephrin signaling may also contribute to the formation and progression of tumors in the urinary system, most prominently bladder and renal cell carcinoma. While in the embryonic contexts Eph/ephrin signaling regulates adhesion of epithelial cells, in the adult setting, cell-shape changes and cell survival seem to be the primary cellular processes mediated by this signaling module. With progression of the genetic analyses of mice conditionally mutant for compound alleles of Eph receptor and ephrin ligand genes, additional essential functions are likely to arise in the urinary system.

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Introduction

The urinary system is a multi-component entity that primarily controls the water and ion balance of the blood by mediating the excretion of excess water, solutes, and waste products. In each of its constituting organs, the two kidneys and ureters, the bladder and the urethra, a large number of highly specialized cell types are organized in different functional tissues that are spatially integrated to fulfill specific sub-tasks in support of blood filtration and waste disposal. In the kidney, thousands (mouse) and millions (man) of nephrons constitute a large unit for filtration of blood and the subsequent modification of the primary urine. At the proximal end of these elaborated epithelial bodies, a double-layered Bowman's capsule is formed that harbors a capillary network, the glomerulus. Microfiltration of the blood through the endothelium, the underlying basal membrane, and the specialized cells of the inner leaflet of Bowman's capsule, the highly interdigitated podocytes, generates the primary urine. In the following tubular components of the nephron, the proximal and distal tubule, and the intermediary loop of Henle, selective resorption and secretion systems regain most of the water, proteins, and low molecular weight components but secrete waste products and ions.

The ureters, the bladder, and the urethra coordinately accomplish the expulsion of the urine to the outside. The ureters drain the urine by peristaltic contractions from the renal pelvis to the bladder where it can be transiently stored before being willingly released to the outside via the urethra. Given the common task of urinary drainage, it is not surprising that the components of this drainage unit share a common two-layered tissue design. On the inside, a highly specialized multi-layered

epithelium, the urothelium, seals the lumen with the urine from the interstitial space while providing flexibility to the changing urinary volume. An outer mesenchymal coating of fibroblasts and smooth muscle cells provides contractile activity (only the ureters), and additional flexibility and rigidity to the organs.

Although the contiguous and highly integrated nature of the components of the urinary system suggests a common developmental origin, it is in fact derived from different germinal tissues. The lower urinary tract (bladder and urethra) derives from an endodermal infolding and its surrounding mesenchyme, whereas the upper urinary system (the kidneys and ureters) stems from subregions of the intermediate mesoderm [1, 2].

Urinary tract development starts at approximately E8.5 in the mouse embryo, when an epithelial tube, the Wolffian or nephric duct (ND), emerges in the intermediate mesoderm at the forelimb level. The ND elongates within the intermediate mesoderm posteriorly until it reaches the epithelial infolding of the bladder primordium, the cloaca, at around E9.5, turns to the midline and fuses with it. At E10.5, an epithelial bud protrudes from the ND anterior to the cloaca at the hind limb level and invades the adjacent metanephric mesenchyme. Once this ureteric bud has entered this mesenchymal cell mass, it undergoes several rounds of dichotomous branching to give rise to an epithelial tree-like tubular structure that differentiates into the mature collecting duct system of the kidney. The mesenchyme next to the newly forming ureteric tips repeatedly aggregates to form renal vesicles that differentiate into the different regions of the nephron [3]. The initially simple epithelium of the distal ureters, the cloaca, and the proximal part of the urethra grows and thickens to differentiate into the urothelium. The surrounding mesenchyme differentiates in a radial fashion into the lamina propria, smooth muscle cells, and outer adventitial fibroblasts [4].

Considering the development of the urinary system from different cell lineages, it seems obvious that cell behaviors within and between the primordial tissues need to be highly coordinated. Embryological experiments first, and genetic experiments later, have demonstrated that this coordination is achieved by exchange of signals, often acting in a reciprocal fashion [1]. In the last years, the molecular nature of some of these paracrine signals has emerged from genetic experiments in mouse and man. Glial-derived neurotrophic factor (Gdnf) from the metanephric mesenchyme induces ureter budding and branching [5], Wnt9b from the ureteric tips induces nephron formation [6], and sonic hedgehog (Shh) from the epithelium of the developing ureter and bladder is required for induction of smooth muscle differentiation in the surrounding mesenchyme [7, 8], just to name some. One may state that signaling pathways that have emerged as essential players in cellular processes in the development of most other organs (e.g., Notch, fibroblast growth factor, Wnt, hedgehog) are also critically involved in development and homeostasis of the urinary system [3]. For some time it seemed that Eph/ephrin signaling, a pathway particularly well studied in neural and vascular development [9, 10], is a notable exception. Work in recent years, however, suggests that components of this signaling pathway are expressed in different tissues of the developing and mature urinary system and are of functional relevance at least for some of the numerous cell and tissue interactions therein (for a summary see Table 1).

Eph/ephrin signaling

The history of this signaling pathway can be traced back to 1987 when a search for new receptor tyrosine kinase genes involved in cancer pathogenesis and progression identified a cDNA encoding EphA1, a member of a novel subclass of this protein family that was named orphan due to the lack of its binding ligand at that time [11]. Subsequent work identified a large number of highly related proteins in vertebrates making Eph (erythropoietin-producing hepatocarcinoma cell) receptors the largest sub-family of receptor tyrosine kinases [12]. With the help of receptor affinity chromatography, the protein B61 (today referred to as ephrinA1) was subsequently discovered as the first binding partner of an Eph receptor (ECK, today referred to as EphA2) [13]. Eph receptors as well as their ligands, the ephrins are not only conserved in vertebrates but also occur in invertebrates, such as *Drosophila* and *Caenorhabditis elegans* and surprisingly also in sponges, pointing to the evolutionary conserved nature of this pathway [14]. In the mouse, 14 Eph receptors have been characterized that are divided into an A and B subfamilies based on sequence and function. The nine EphA receptors (EphA1-EphA8, EphA10) promiscuously bind to five ephrinA ligands, and the five EphB receptors (EphB1-4, EphB6) bind to three ephrinB ligands, with some exceptions (EphA4 and EphB2 can bind to ephrins of the other class) [12, 15, 16].

Ephs and ephrins have a complex modular design (see Fig. 1). The prototypical Eph receptor consists of an extracellular globular ephrin binding domain at the N-terminus followed by a cysteine-rich region and two fibronectin III repeats [17]. The Eph receptor is anchored in the cell with a transmembrane domain, which is followed by a short juxtamembrane region, which can be phosphorylated to activate the signaling cascade. Essential for signal transduction is the cytosolic tyrosine kinase domain, which represents the largest domain of the Eph receptor protein. The kinase domain is followed by a sterile alpha motif (SAM) protein–protein interaction domain and a PDZ domain, which epitomize binding sites for effector proteins [12, 18]. EphrinA ligands are tethered to the cell membrane via a GPI-(glycosylphosphatidylinositol) anchor, whereas ephrinB ligands are transmembrane proteins with a small cytoplasmic domain.

Table 1 Summary of Eph receptor and ephrin ligand expression and function in the kidney and urinary tract. Expression and function are separated for the embryonic and adult situation. In the latter, homeostatic function, injury responses, and tumors are distinguished. For ephrinA2, ephrinA3, ephrinA4, ephrinA5, EphA3, EphA5, EphA6, EphA8, EphA9, EphA10, and EphB5, no data on specific expression and function in the urinary system was reported. *ccRCC* clear cell renal cell carcinoma; *ND* nephric duct; *UUO* unilateral ureteral obstruction

Gene	Embryo		Adult			
	Expression	Function	Expression	Homeostatic function	Injury responses	Tumors
EphrinA1			Canine kidney cells [84]			ccRCC, bladder cancer, and urothelial carcinoma [68, 93, 94]
EphrinB1	Capillary loop of early stage glomerulus and in a mesangial pattern in mature glomerulus [66]		Mature glomeruli [66], podocyte and slit diaphragm [80], medullary straight tubules, proximal and distal convoluted tubules, loop of Henle [81]	Maintenance of tubular epithelial cells [81]	Reduced after induced nephropathy [80]	
EphrinB2	Epithelial cells of endodermal folds, mesoderm of Rathke's and Tourneux folds, urethral endoderm and sub-endodermal mesenchyme [38], bladder [39], nephric duct and cloacal epithelium [47], arterial capillary beds [54–57], podocyte- and nephron precursors [67]	Cloacal septation and tubularization of urethra [38], ND fusion with cloaca [47], endothelial sprouting, glomerulogenesis [54–57], attachment and spreading of mural cells on glomerular endothelium [65]	Pericytes and vascular smooth muscle cells [65], macrophages, pericytes, endothelial cells [82]	Angiogenic remodeling [54–57], maintenance of boundaries between arterial and venous capillary beds [61, 62]	Protective role after proximal tubule capillary destruction (rarefaction) and renal fibrosis after induced kidney injury by UUO [82]	Upregulated in arterial endothelium of kidney and bladder tumors [97]
EphrinB3	Bladder [39]					
EphA1			Kidney [68]			RCC [68], independent prognostic marker for survival endpoints [90]
EphA2			Rat medullary and cortical collecting ducts, renal papilla [78]	Adaptive response to medullary hypertonicity Refresh and urea stress, reorganization of cytoskeleton in MDCK and IMCD3 cells [78, 84, 85]	Upregulation in ischemia-reperfusion mouse model [77]	Deregulated in various tumors, e.g., ccRCC, bladder cancer and urothelial carcinoma [68, 85, 87, 88, 91, 92]
EphA4	Bladder [39], pericloacal and ureteric mesenchyme [47]	Nephric duct fusion with cloaca [47], ureter budding [48]			Loss leads to non-penetrant hydronephrosis, unilateral hydroureter, and salt-sensitive hypertension [48]	
EphA7	Bladder [39], pericloacal and ureteric mesenchyme [47]	Nephric duct fusion with cloaca [47]				
EphB1	Bladder [39], capillary loop of early stage glomerulus and in a mesangial pattern in mature glomerulus [66]		Rat glomerulus [80]			
EphB2	Cloacal epithelium, endoderm and sub-endodermal mesenchyme of urethra, mesoderm of Rathke's and Tourneux folds [38], bladder [39]	Cloacal septation and tubularization of urethra [38], regulation of cytoarchitecture of medullary tubules	Rat glomerulus [80], medullary tubules, loop of Henle, distal straight tubules [81], bladder urothelium [95]	Maintenance of tubular epithelial cells [81]		Transitional bladder cell carcinomas [95]
EphB3	Endoderm and sub-endodermal mesenchyme of urethra, mesoderm of Rathke's and Tourneux folds [38], bladder [39]	Cloacal septation and tubularization of urethra [38]				
EphB4	Bladder [39], venous capillary beds of glomerulus [61, 62]	Endothelial sprouting, glomerulogenesis [54–57]	Apical side of podocytes [79]	Podocyte survival (unclear), angiogenic remodeling [54–57]	Upregulated after Thy1.1 induced glomerulonephritis and involved in podocyte injury response [79]	Overexpressed in transitional bladder cell carcinomas and cancer cell lines [95, 96]
EphB6			Proximal and distal convoluted tubules, distal straight tubules [81]			

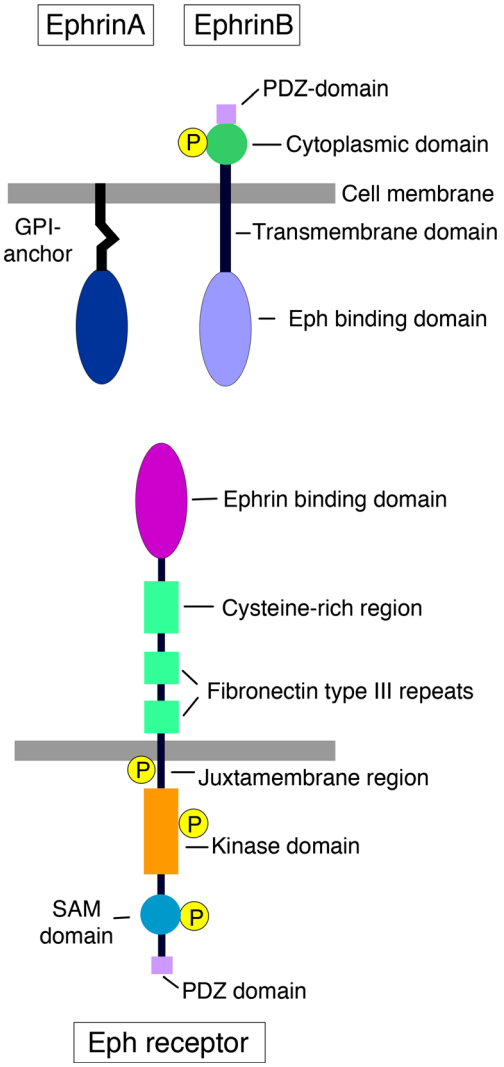

Fig. 1 Structure of Ephs and ephrins. Schematic portrayal of the structural and binding domains of ephrin ligands and Eph receptors. EphrinA ligands are tethered to the cell membrane with a glycosylphosphatidylinositol (GPI-) anchor. The globular extracellular part constitutes the Eph binding domain. EphrinB ligands are transmembrane proteins with a small intracellular cytoplasmic and a PDZ domain. The extracellular globular part constitutes the Eph-binding domain. The Eph receptor is comprised of an extracellular ephrin-binding domain, a cysteine-rich region and two fibronectin type III repeats which is followed by a transmembrane domain and a juxtamembrane region. The biggest part of the Eph receptor is represented by the catalytically active kinase domain, which is followed by a sterile alpha motif (SAM-) domain and a docking site for PDZ domain-containing proteins. The juxtamembrane region, the kinase domain, as well as the PDZ domain can be phosphorylated upon ligand binding

EphrinA ligands can be released from the cell surface to reach and activate Eph receptors at a distance [19].

Eph–ephrin interaction exclusively occurs upon direct contact of juxtaposed cells. Activation of Eph receptors is mediated by binding of membrane-clustered ligands (Fig. 2). The first downstream event after the binding interaction is receptor membrane clustering in dimers or oligomers followed by receptor autophosphorylation. Phosphorylation of conserved tyrosine residues in the juxtamembrane domain removes an inhibitory interaction with the kinase domain to allow efficient kinase activity [20]. The phospho-tyrosine residues of the kinase domain display docking sites for SH2 (Src homology 2) or SH3 (Src homology 3)-containing adaptor proteins including the non-receptor tyrosine kinase families like Src (Src kinase) and Abl (Abelson oncogene). These kinases then activate or inhibit downstream proteins such as the small GTPases RhoA, Rac, or Cdc42 to alter the actin cytoskeleton and ultimately resulting either in a cell-rounding or cell-spreading response [12, 16].

The Eph/ephrin signaling pathway constitutes a cellular communication module that harbors the ability to not only transduce signals in the receptor-bearing cell as "forward signaling", but also into the ephrin ligand-expressing cell referred to as "reverse signaling". Hereby, the cytosolic domain of the ephrin ligand is phosphorylated, and thereby acts as a "mini-receptor" to recruit signaling effector proteins such as Grb4 or Stat3 [21]. As a consequence, focal adhesion kinase gets activated, cytoskeletal changes occur, and/or transcriptional programs are initiated [22–24]. Phosphorylation-independent downstream signaling via the PDZ domain is also known [10]. This bidirectional signal transduction is unique and, although assumed for other pathways like e.g., Notch signaling [25], has not been demonstrated for any other signaling pathway to date. Eph–ephrin interaction cannot only occur in *trans*; it has been shown that receptor–ligand interaction in *cis* can alter or even inhibit signal transduction into the juxtaposed cell [19]. Termination of receptor–ligand adhesive interaction can be achieved by internalization of the receptor–ligand complex [26].

Eph/ephrin signaling is required in the embryo as well as in the adult to acquire and maintain organized tissue architecture. Ephs and ephrins enable cells to communicate contact-dependently to control cell morphology, adhesion, repulsion, and migration; emerging evidence suggests a role of this signaling module in mediating survival as well as apoptosis [27]. During embryonic development, Eph/ephrin signaling controls cell sorting, axon guidance, topographical mapping, synaptic plasticity, neural tube differentiation, blood vessel formation, as well as epithelial integrity [12, 16].

Here, we summarize the current information on expression and function of this signaling module in the kidney and urinary drainage system. We will first describe its role in three developmental contexts, the subdivision of the embryonic cloaca, the formation of a patent vesicoureteric junction (VUJ), and glomerulogenesis, before we review the literature with respect to its involvement in the mature urinary system, including conditions of injury and disease.

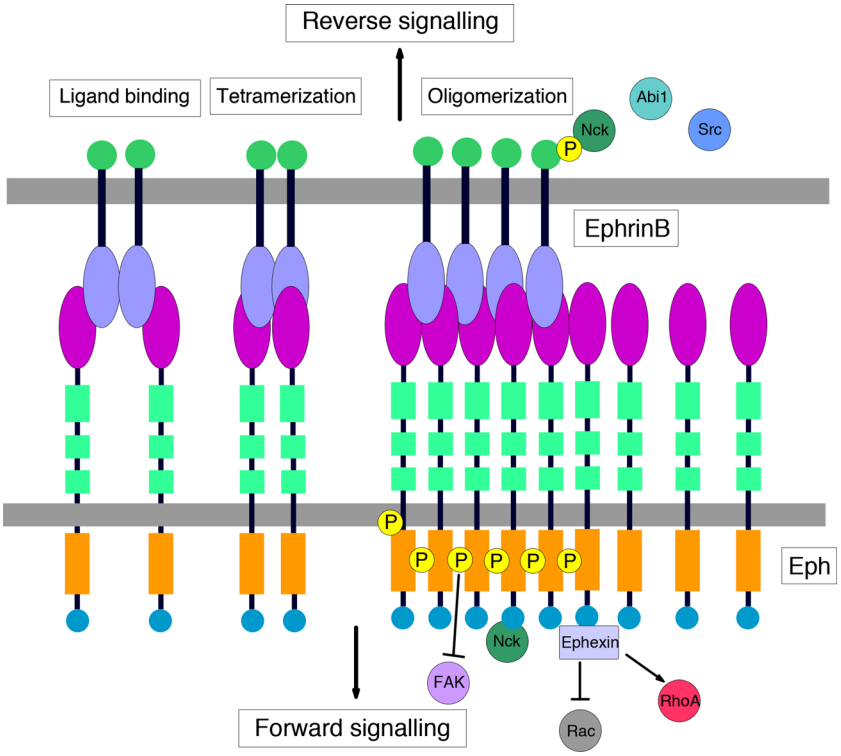

Fig. 2 Eph/ephrin signaling opportunities. Ephrin signaling is initiated by the binding of membrane-clustered ephrin ligands to Eph receptor dimers on the juxtaposed cell. Binding leads to the formation of a tetrameric ligand–receptor complex that initiates oligomerization of the Eph-ephrin cluster. Ephrin ligand binding that results in forward signaling leads to phosphorylation of the juxtamembrane region of the Eph receptor. This modification removes a steric hindrance at this region and leads to auto- and cross-phosphorylation of the kinase domain. Phosphorylation of the kinase domain recruits effector proteins like Nck, inhibits Fak and Rac activity, but activates RhoA kinase and its downstream effector Rock to mainly mediate repulsive responses. Reverse signaling solely transduced by ephrinB ligands leads to phosphorylation of the intracellular domain of the ephrinB ligand mainly by Src family kinases. This phosphorylation recruits effector proteins like Nck and Abelson family kinases or interacting proteins (Abi) that modify the cytoskeleton, resulting in mainly adhesive responses

Eph/ephrin signaling in urorectal septation

The endodermal infolding of the cloaca not only represents the exit point of the urinary system but is also the terminus of the genital and alimentary system in the early embryo (in the mouse E9.5 to 10.5). Starting from E10.5 to E13.5, the cloaca is partitioned into an anorectal canal dorsally (from which the rectum develops), and a urogenital sinus ventrally with the mesodermal urorectal septum lying between. This developmental program is mediated by ingrowth of three endodermally lined mesenchymal tissue folds, the Tourneux fold cranial-to-caudally and two Rathke's folds lateral-to-medially, and their fusion at the midline. In the male, the urogenital sinus subsequently elongates as a plate that then undergoes tubularization to become the urethra. In the female, the urogenital sinus contributes to the formation of a shorter urethra and the vagina [28–30]. Failure of movement and/or adhesion of epithelial cells in the endodermal Tourneux/Rathke's folds lead to incomplete or failed septation of the cloaca [31]. Anorectal malformations in human infants are rare (approximately 1 in 5000). They vary from mild anal stenosis to severe anorectal agenesis with an abnormal internal connection (fistula) of the rectum to the base of the bladder [32, 33]. Failures of urethral tubularization in the male are much more frequent, amounting to 1 in 125 newborns. They result in hypospadias, birth defects that involve the repositioning of the urethral orifice to the ventral side of the penis shaft [34, 35]. The molecular control of cloacal septation and urethral tubularization is poorly understood, but the fact that 14 % of patients with anorectal malformations also exhibit hypospadias point to shared cellular and/or molecular programs [36, 37]. Bidirectional ephrinB2-EphB2/EphB3 signaling was recently implicated in movements and adhesion of endodermal cell sheets that tubularize the urethra and septate the cloaca deciphering such a shared molecular module [38].

Knowing that ephrin ligands function as receptors and are capable of transducing signals, Dravis and colleagues generated an *EphrinB2* allele, in which the intracellular domain of ephrinB2 was replaced by a LacZ coding region [38]. The resulting protein acts as a dominant negative form that still allows Eph forward signaling but prevents reverse signaling. Twenty-eight percent of adult $EphrinB2^{LacZ/+}$ males displayed hypospadias. Female mutants showed similar but less obvious defects in the morphology of their external genitalia. Either

sex also failed to close the perineum at the midline. Surprisingly, mice homozygous for this allele survived embryogenesis and showed highly exaggerated phenotypes with a complete penetrance. In males, the rectum connected to the neck of the bladder by a fistula, and in females the rectum, vagina, and neck of the bladder similarly all converged into one tube. The external genitalia of all the mutants exhibited the typical severe hypospadias and un-tubularized external urethra (in males) or splayed clitoris (in females). To detect the onset and cellular causes of these phenotypic changes, mice were analyzed at early embryonic stages. At E11.5 control littermates had just initiated midline fusion of the cloaca, while the mutants showed a primitive cloaca with no obvious movement of epithelial cells towards the midline.

Notably, mice compound homozygous for null alleles of *EphB2* and *EphB3*, which encode binding partners of ephrinB2, exhibited the exact same phenotype of severe hypospadias and a reduced perineal distance in a quarter of the cases. To test whether EphB forward signaling contributes to the phenotype, the authors generated compound mutants of an *EphB3* null allele and an *EphB2* knock-in mutation that translates into a kinase-inactive C-terminally truncated EphB2-gal fusion protein. Roughly a third of the resulting males exhibited severe hypospadias and a reduced perineum, indicating that EphB2 forward signaling also plays a role in development of the caudal midline.

Expression analysis detected coexpression of ephrinB2 and EphB2 in the epithelial cells of the endodermal folds, which meet and adhere at the midline in the cloaca and in the urethral folds. Expression was strongly upregulated when the different epithelial sheets contacted each other. Additional ephrinB2 and EphB2 expression occurred in mesodermal cells of the Tourneux/Rathke's folds that migrate towards the midline underneath the septating cloaca to form the urorectal septum. The authors concluded from these detailed genetic analyses that both forward and reverse Eph/ephrin signals are transduced into cells that meet at the caudal-most midline of the embryo and that the outcome of this form of bidirectional signaling is cell-to-cell adhesion.

In a microarray screen for genes enriched in E13.5 versus E18.5 bladders, it was found that not only *EphrinB2*, *EphB2*, and *EphB3* but also *EphB1*, *EphB4*, *EphA4*, *EphA7*, and *EphrinB3* were upregulated at E13.5 [39]. As an expression analysis has not been performed at the level of individual mRNA or protein, it remains open whether these factors act redundantly with ephrinB2-EphB2/EphB3 signaling or are part of other cellular programs in early bladder development.

Eph/ephrin signaling in ND insertion

We described above that the bladder and the ureters derive from different precursor tissues, namely the endodermal cloaca and the mesodermal ND. As a consequence, formation of a functional VUJ requires the interaction and finally the fusion of these two tissues. Analyses of mutant mice has revealed that this is just one of a number of cellular processes that are critical for achieving the contiguity of the upper and lower urinary system [40]. First, the ND has to elongate properly and contact the cloacal epithelium in a spatially and temporally defined manner. Distal ND elongation is controlled by a number of transcription factors including Pax2, Lhx1, and Gata3 that converge on transcriptional activation of *Ret*, a gene encoding a receptor tyrosine kinase [41]. After the initial fusion event, the common nephric duct (CND), the piece of ND distal to the ureteric bud, is removed by apoptosis. This step depends on retinoic acid signals from the peri-cloacal mesenchyme that act on *Ret* expression in the CND [42]. In a further step, the distal ureter lies down onto the bladder to be again removed by apoptosis, a process mediated by LAR family receptor protein kinases [43]. This finally allows integration of the distal ureter in the bladder and the separation from the ND. Failure in any of these cellular programs leads to ectopically or blind-ending ureters, causing a severe or complete physical obstruction of urinary drainage into the bladder [44]. Considering the complexity of these events, it may not come as a surprise that congenital defects of the VUJ represent a large subgroup of congenital anomalies of the kidney and the urinary tract (CAKUT) that are frequently found in human newborns [45, 46].

Recently published work characterized Eph/ephrin signaling as an additional crucial molecular module in establishing the VUJ [47]. Analyzing the expression of all *Eph* receptor genes in the ureteric mesenchyme, it was found that only the genes encoding the two closely related EphA receptors *EphA4* and *EphA7* are specifically expressed in this tissue from E11.5 to E14.5. From E10.5 to E14.5, expression was additionally found in the peri-cloacal mesenchyme, i.e., in mesenchyme surrounding the distal ND and the bladder primordium, and later in the urethral mesenchyme. To address the functional significance of these expression domains, single mutants for null alleles of *EphA4* and *EphA7* were analyzed. Neither of them showed obvious morphological changes in the urogenital system at newborn stages, suggesting functional redundancy between the two genes. In fact, roughly half of $EphA4^{LacZ/LacZ};EphA7^{-/-}$ (DKO) and 20 % of $EphA4^{LacZ/LacZ};EphA7^{+/-}$ mutants displayed a spectrum of CAKUT-like phenotypes including ureterocele, blind-, or ectopically ending ureters, megaureter, and hydronephrosis at birth. Hydroureter and megaureter formation was not caused by a functional obstruction, as the smooth muscle cell differentiation program initiated normally prior to onset of urine production at E15.5. Analysis of VUJ patency by ink injection and histological analysis proved a physical barrier to urinary drainage as the cause of the observed CAKUT-like phenotypes.

Analysis of earlier stages detected survival of the CND, which was due to a late or absent fusion of the ND with the cloacal epithelium. Live cell imaging of dorsal trunk cultures as well as confocal microscopy with markers for epithelial integrity revealed that the tip of the ND of DKO embryos lost its integrity around the fusion event. Expression of *Lhx1*, *Gata3*, and *Ret*, genes crucial for ND elongation and guidance, was specifically down-regulated in the caudal part (but not in the mesonephric part) of the ND, which can explain the late or lacking fusion of the two epithelial compartments. An expression analysis in search for a possible ligand of the two Eph receptors, revealed *ephrinB2* expression in the ND epithelium. In DKO embryos, ephrinB phosphorylation at Y316, a residue specifically phosphorylated by Src-kinases in response to Eph binding, was dramatically decreased in the ND and the cloaca, supporting the model of reverse signal transduction in this tissue context. Conditional deletion of *EphrinB2* from the ND epithelium resulted in similar defects in the UGS, including megaureter and ureter ectopia as well as downregulation of the same set of ND marker genes as in the DKO situation. These findings suggest that EphA4 and EphA7 from the peri-cloacal mesenchyme activate ephrinB2 reverse signaling in the ND to mediate ND fusion with the cloaca, most likely by maintaining the adhesive integrity of the distal ND and/or mediating adhesion between ND and cloacal epithelium (Fig. 3). How the activation of ephrinB2 is intracellularly transmitted to trigger a cell adhesion program, whether EphA4/EphA7 signaling interacts with Ret signaling, and what the cellular consequences of potential EphA4/EphA7 forward signaling is, remains to be seen. Additional Eph receptors and ephrin ligands may participate in these processes to account for the partial penetrance of the observed phenotypic changes.

Interestingly, it was previously reported that mice homozygous for a GFP knock-in allele of *EphA4* display variable degrees of pelvic dilation at 3–10 months after birth [48]. Bilateral hydronephrosis was found in 17 % of all animals, whereas unilateral hydroureter was evident in half of all animals. Hydronephrotic $EphA4^{GFP/GFP}$ mice developed variable and salt-sensitive hypertension, reduced urine concentrating ability and renal plasma flow, lower glomerular filtration rate, increased renal fibrosis, inflammation, and glomerular and tubular changes similar to physiological and structural defects observed in experimentally induced hydronephrosis by unilateral ureteral obstruction (UUO). Analysis at E15.5 revealed duplicated ureters that became associated with hydroureter and hydronephrosis after birth. Duplex kidneys/ureters are caused by ectopic ureter budding rather than by a defect in ND fusion [49]. Therefore, it remains to be seen whether the nature of the mutant allele and the different genetic background used in this study (C57Bl6 rather than outbred) has influenced the phenotypic outcome of the urinary tract phenotype as well as its penetrance.

Eph/ephrins in congenital anomalies of the human kidney and urinary tract

Considering the important role of Eph/ephrin signaling in different subprograms of urinary tract development in the mouse, it is obvious to speculate that mutations in Ephs and ephrins are causative for congenital anomalies of this organ system in humans. Efforts to identify disease-causing mutations in Ephs and ephrins in congenital anomalies of the kidney and the urinary tract, focused on *EphrinB2*, the crucial mediator of cloacal subdivision and VUJ formation in mice [38, 47]. Although an excellent candidate gene for human anorectal malformations, a comprehensive mutation analysis of the *EphrinB2* gene in 331 patients with isolated anorectal malformations did not identify any disease-causing mutation [50, 51]. A smaller study on 20 patients with a persistent cloaca also failed to detect mutations in *EphrinB2* [50]. Finally, polymorphisms in the *EphrinB2* gene do not appear to contribute in a substantial way to non-diabetic, diabetic, or all-cause end-stage renal disease in African Americans [52].

Eph/ephrin signaling in glomerulogenesis

Blood vessels form a highly branched hierarchical network of arteries, capillary beds, and veins in the vertebrate body to ensure that all tissues and organs are sufficiently supplied with nutrients and oxygen. In the kidney, the association of the vascular system with the kidney parenchyma is particularly tight, as the primary task of this organ is to filter the blood through a largely increased interface between capillary loops of the numerous glomeruli and the adjacent layer of podocytes as well as the intervening basal membrane. Glomerulogenesis follows a poorly understood program that, however, is likely to engage the same processes by which blood vessels develop throughout the body during development. The initial glomerular plexus is formed by vasculogenesis, i.e., from endothelial progenitors (angioblasts) that immigrate into the narrow slit between the two leaflets of the proximal end of the developing nephron. Angiogenesis subsequently expands and remodels the existing plexus by growth and sprouting [53].

Global and endothelial-specific deletion experiments in the mouse have implicated *EphB4* and *EphrinB2* in angio-genetic remodeling in general, and thus, most likely in glomerulogenesis as well [54–57]. The phenotype of *EphB4*-null or *ephrinB2*-null mice was indistinguishable, suggesting that forward signaling, reverse signaling, or a combination of forward and reverse signaling is necessary for normal vascular development. More recent studies have deciphered the cellular role of reverse ephrinB2 signaling in sprouting angiogenesis, particularly in the cells at the tip of the sprouts. It seems that reverse ephrinB2 signaling promotes the formation of filopodial extension in these tip cells (as shown in the retina)

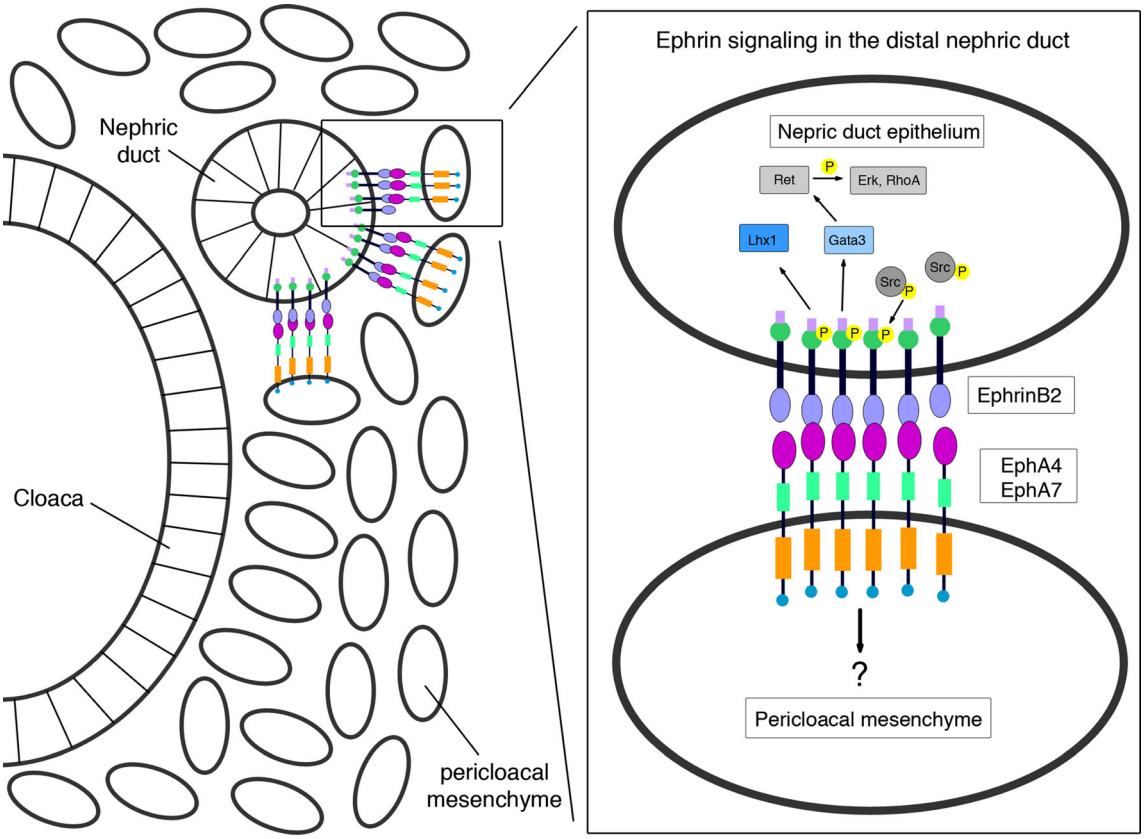

Fig. 3 Eph/ephrin signaling in the distal nephric duct. The ligand ephrinB2 is expressed on the surface of the distal nephric duct epithelium, whereas the Eph receptors EphA4 and EphA7 are present on juxtaposed cells of the pericloacal mesenchyme. Binding of ephrinB2 to EphA4 and EphA7 leads to activation of reverse signaling by the phosphorylation of the intracellular domain mediated by Src family kinases. The phosphorylated form of ephrinB2 activates the transcription of the nephric duct (ND)-specific marker genes *Lhx1* and *Gata3* (by a yet unknown mechanism), which in turn activates the expression of the *Ret* receptor. Ret activity in the distal ND leads to phosphorylation of Erk and (potentially) RhoA kinases. Activation of these kinases leads to an adhesive response of the ND epithelium to maintain its cellular integrity

[58, 59]. Filopodia from adjacent tip cells then connect to each other, leading to the development of a lumen that permits blood flow in the new vessel [60]. The role of EphB forward signaling in endothelial cells has remained less clear. Two studies have concluded that EphB4 forward signaling in endothelial cells represses endothelial cell migration, adhesion, and proliferation in vitro, and may serve to repel endothelial cells from each other and maintain boundaries between arterial (ephrinB2-positive) and venous (EphB4-positive) capillary beds [61, 62]. However, another study suggested that EphB4 forward signaling in endothelial cells promotes some degree of endothelial cell proliferation and angiogenesis [63].

Once an initial vascular bed has formed by spouting angiogenesis, changing mechanical forces and the altered chemical environment lead to the regression of some of the newly formed vessels whereas others mature through the establishment of a basal membrane and the recruitment of pericytes/smooth muscle cells that stabilize the vessel wall and regulate endothelial cell survival, growth, and permeability [64]. EphrinB2 is expressed and signals in mural cells of nascent vessels arguing for another independent cellular function in vessel development. In fact, conditional deletion of *EphrinB2* in pericytes and smooth muscle cells caused perinatal lethality associated with developmental defects in small-diameter blood vessels of the skin, lung, gastrointestinal tract, and interesting for this review in kidney glomeruli, which were not properly covered with smooth muscle cells or pericytes [65]. Further in vitro experiments showed that *EphrinB2*-deficient smooth muscle cells are defective in spreading, focal-adhesion formation and polarized migration, and show increased motility [65].

Daniel and coworkers reported that EphB1 (originally named ELK) and ephrinB1 (originally named Lerk-2) are observed as an endothelial pattern along capillary loop in early stage glomeruli. They also reported that the EphB1 was observed as a mesangial pattern in matured glomeruli, and that the glomerular expression of ephrinB1 was weak in matured glomeruli [66]. Takahashi and coworkers identified ephrinB2 expression in presumptive podocytes in glomeruli of comma-shaped nephron precursors, and noted that the staining in podocytes disappeared in matured glomeruli. They also analyzed the expression of EphB4, a binding partner of ephrinB2,

but failed to detect EphB4 signals in matured glomeruli [67]. Together, these expression analyses indicate that Eph/ephrin signaling is also implicated in the development of different non-vascular cell populations of the glomerulus and Bowman's capsule.

Eph/Eph signaling in the mature kidney and urinary tract

Eph/ephrin signaling has been well studied in embryonic development; its role in healthy adult tissues and in human disease, however, has been much less well defined. Using an RT-PCR approach, it was shown that the family of Eph receptors and ephrin ligands are widely expressed in adult tissues with organ-specific patterns [68]. Expression of many family members was also found in kidney and bladder, confirming a series of earlier reports [69–76] that, however, failed to provide a meaningful resolution with respect to renal and urinary physiology.

Evidence for a functional involvement in kidney homeostasis and injury has been gained by non-genetic methods for ephrinB1 in podocyte architecture, EphB4 in podocyte injury response, EphB2 in cell-shape changes of medullary tubule cells, and for EphA2 as a sensor for osmolarity, urea, and mechanical stress [77–81], while genetic experiments have provided convincing evidence for a protective role for ephrinB2 reverse signaling in capillary destruction and fibrosis after kidney injury [82].

Hashimoto and colleagues focused on the question whether Eph/ephrins may participate in the permeability function of the slit diaphragm [80]. The slit diaphragm is an extracellular protein network that connects the highly interdigitated foot processes of the podocytes. It is composed of specialized proteins like Nephrin (Nphs1) that connect via adaptor proteins such as Cd2ap to a complex actin cytoskeletal network [83]. Hashimoto and colleagues identified mRNA expression of *EphB1*, *EphB2, EphrinB1*, and *EphrinB2* in rat glomeruli. After experimental induction of nephropathy only *EphrinB1* was reduced correlating it but not the other family members with the glomerular permeability barrier. EphrinB1 was subsequently localized to the slit diaphragm in association with Nphs1 and Cd2ap. RNA silencing of *EphrinB1* in cultured podocytes resulted in dis-localization of Cd2ap, suggesting that ephrinB1 maintains the slit diaphragm structure, possibly by proper arrangement of the Cd2ap adaptor.

Wnuk and colleagues confirmed the expression of ephrinBs at the basal side of podocytes but found additional weak expression of EphB4 at the apical side of these cells [79]. Remarkably, expression of EphB4 was dramatically upregulated 9 days after Thy1.1 induced glomerulonephritis. In this procedure, an antibody against the membrane protein Thy1.1 leads to lysis of mesangial cells, which affects the capillary network in the glomerulus. A critical role in glomerular disease recovery has been attributed to podocytes that may help to preserve glomerular architecture until mesangial cells have recovered. To analyze the contribution of EphB4 signaling to this process, a small molecular inhibitor of EphB4 phosphorylation was administered to rats. Thy1.1 nephritic, but not control rats, showed inhibition of capillary repair, podocyte damage and loss, and albuminuria after prolonged periods of time. This argues for a protective role of EphB4 signaling in podocytes after mesangiolysis. How EphB4 signaling is activated and how EphB4 signaling acts to mediate podocyte survival remains unclear.

Insight into a function of Eph/ephrinB signaling in medullary tubule cells was provided by a study from Ogawa and colleagues [81]. Using immunofluorescence analysis they showed that the different regions of the nephron express different sets of Ephs and ephrins. The proximal and distal convoluted tubes express ephrinB1 and EphB6, the loop of Henle coexpresses ephrinB1 and EphB2, and the distal straight tubules coexpress ephrinB1, EphB2, and EphB6. EphB2 was found to be tyrosine-phosphorylated, indicating active signaling in the kidney. In primary cultures of medullary tubule cells, soluble ephrinB1-ligand induced tyrosine phosphorylation of EphB2. EphrinB1 stimulated cell retraction by remodeling of focal adhesions. This was mediated by activation of RhoA and inactivation of Rac1, resulting in stress fiber formation and loss of lamellipodia. Together, Eph/ephrin signaling in tubular cells may regulate cytoarchitecture and spatial organization of tubular cells by affecting focal adhesion signaling.

EphA2 has been implicated in cell–cell and cell–matrix interactions of many epithelial cell types [84, 85]. *EphA2* mRNA and EphA2 protein was detected at low levels in rat renal cortex but at high levels in the collecting ducts of the renal medulla and papilla [78]. EphA2 expression in the renal papilla was induced by water deprivation, whereas dietary supplementation with 20 % urea increased EphA2 expression in the outer medulla, implicating EphA2 expression as an adaptive response to medullary hypertonicity or urea exposure. In another study it was found that expression of EphA2 was strongly upregulated throughout tubules in the corticomedullary junction in a mouse model of ischemia-reperfusion injury as well as in cultured renal tubular epithelial cells and in MDCK and mIMCD3 cells following administration of hydrogen peroxide, and to a lesser extent after mechanical wounding [77]. Although these changes are only correlative in nature, one may speculate that EphA2 participates in cytoskeletal reorganization after stress induction in epithelial cells in a more general fashion [85].

Based on the functional involvement of bidirectional ephrinB2/EphB4 signaling in embryonic angiogenesis, Kida and colleagues addressed the role of ephrinB signaling under conditions of capillary injury and fibrosis [82]. They found that ephrinB2 reverse signaling was activated in the kidney

only after injury in the UUO model. The major population of phosphorylated ephrinB-positive cells were macrophages, whereas smaller populations were microvascular endothelial cells and pericytes. In mice lacking the PDZ intracellular signaling domain of ephrinB2 (ephrinB2 DV), angiogenesis was impaired and kidney injury led to increased destruction of proximal tubule capillaries (rare-fraction) and fibrosis. EphrinB2 DV primary kidney pericytes migrated more than wild-type pericytes and were less able to stabilize capillary tubes in three-dimensional culture and to stimulate synthesis of capillary basement membrane. EphrinB2 DV primary kidney microvascular endothelial cells migrated and proliferated less than wild-type microvascular endothelial cells in response to vascular endothelial growth factor (Vegf)A and showed less internalization and activation of Vegf receptor-2. The authors concluded that that PDZ domain-dependent ephrinB2 reverse signaling protects against destruction of proximal tubule capillaries by regulating angiogenesis and vascular stability during kidney injury. Furthermore, this signaling protects against pericyte-to-myofibroblast transition and myofibroblast activation, thereby limiting fibrogenesis.

Deregulation of Eph/ephrin signaling in tumors of the kidney and bladder

Eph/ephrin signaling is a critical mediator of angiogenesis, and furthermore, involved in the regulation of cell morphology, growth, migration, adhesion, and survival in tissue homeostasis [16]. Given these important functions—and reminiscing the discovery of the first Eph receptor in a liver cancer cell line—it may not come as a surprise that Eph receptors and Ephrin ligands are differentially expressed in a variety of human malignant tumors, and an imbalance in the receptor–ligand ratio or an impaired receptor–ligand interaction can affect the cellular behavior of cancer cells in vitro and in vivo. Depending on the tumor type and context, Eph/Ephrin signaling can suppress tumor progression or promote cancer growth (for a comprehensive review see [15]).

Analysis of Eph/Ephrin deregulation in tissues of the urinary system has primarily focused on renal cell carcinoma and bladder cancer, the most relevant tumor entities in this system. Given that EphA2 maintains the epithelial phenotype that it can mediate ligand-dependent inhibition and ligand-independent stimulation of cell migration and invasion in other contexts [86] and is deregulated together with EphA2 and EphrinA1 in a variety of tumors [85, 87, 88], these three proteins also became the focus of analyses in renal cell carcinoma (RCC, mostly of the common clear cell (cc) subtype). In a very small sample setting, mRNA of *EphA1*, *EphA2*, and their ligand *EphrinA1* was detected in normal and malignant kidney tissues [68]. In a small RCC cohort with mixed histological subtypes including 30 ccRCC and four non-ccRCC EphA2 protein levels inversely correlated with progression-free interval and overall survival period [89]. In a more extensive study expression and prognostic relevance of EphA1, EphA2 and EphrinA1 was studied in a large cohort of 241 ccRCC patients. Gene and protein expression of all three factors was altered in tumor specimens with EphA1 and EphA2 being generally diminished in tumors compared to normal renal tissue, whereas EphrinA1 was commonly elevated. A positive EphA1 and EphA2 protein staining as well as a low EphrinA1 protein level were significantly linked to more aggressive tumor features, but only a positive EphA1 immunoreactivity was significantly associated with poor survival. In subgroup analyses, EphA1 and EphA2 protein levels were significantly higher in metastatic than in primary lesions. Patients with EphA1/EphA2-positive tumors or with tumors with positive EphA1 and low EphrinA1 immunoreactivity had the shortest survival rates compared to the respective other combinations. In a multivariate model, EphA1 was an independent prognostic marker for different survival endpoints [90]. In a series of 62 RCC samples it was also found that high EphA2 protein expression in renal cell carcinoma is associated with a poor disease outcome [91]. Interestingly, EphA2 expression is inhibited by miR-141 that is significantly down-regulated in RCC [92]. In conclusion, altered EphA1/A2-EphrinA1 signaling may significantly contribute to the pathogenesis and progression of ccRCCs.

Increased expression of EphA2 was also detected in bladder cancer cell lines as well as in advancing stages of urothelial carcinoma. Similarly, the staining intensity of ephrinA1 was low in normal tissues and high in cancerous tissues, but similar across the various stages of urothelial carcinoma. Intriguingly, adenovirus delivery of ephrinA1 inhibited proliferation of bladder cancer cells, suggesting ephrinA1 as a potential therapeutic target [93, 94].

Li and colleagues described that EphB2 protein is expressed in the urothelium of a normal bladder. In transitional cell carcinomas of the bladder, EphB2 is down-regulated, whereas EphB4 was strongly upregulated, possibly acting as a cell survival factor [95]. Furthermore, high expression of arterial ephrinB2 and venous EphB4 was observed in kidney and bladder tumors, and was suggested to contribute to their involvement in the progression of tumor angiogenesis [96]. Overexpression of EphB4 in bladder cancer cells and cell lines was confirmed and correlated with a survival advantage of bladder cancer cells [97].

All of these findings suggest that deregulated EphA/ephrinA expression and signaling contributes to tumor initiation, or more likely tumor progression and metastasis. EphrinB2/EphB4 signaling may be critical in tumor angiogenesis. Given the complexity of combinatorial or parallel Eph/ephrin expression and signaling in tumor cells, the tumor microvasculature and additional surrounding cells development of Eph/ephrin-based anticancer drugs clearly represents a formidable challenge [93].

Outlook

Eph receptor tyrosine kinases and their ephrin ligands represent an important signaling system with widespread roles in cell physiology and disease. The analysis of the Eph/ephrin signaling module in the kidney and lower urinary tract has been relatively slow probably due to redundant factors and the magnitude of signaling opportunities of this pathway. Nonetheless, genetic experiments have now characterized a role for this pathway in mediating cell-adhesion programs crucial for cloacal subdivision, fusion of the ND with the cloaca, and glomerulogenesis while non-genetic methods implicated Eph/ephrin signaling in different aspects of cytoarchitectural adaptations in normal and diseased podocytes and tubular cells (Table 1). Further analysis should aim to further characterize the cellular and molecular programs acting downstream of both receptors and ligands in all of these contexts and decipher additional requirements of this module in the kidney and urinary tract preferably by unambiguous genetic methods. Additional efforts should be directed towards understanding the significance of deregulated Eph/ephrin signaling in tumors of the kidney and the bladder. The progress will be slow but the insights gained will be rewarding.

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Conflict of Interest The authors declare that they have no conflict of interest

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