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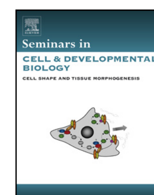

Review

Renal branching morphogenesis: Morphogenetic and signaling mechanisms

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ABSTRACT

The human kidney is composed of an arborized network of collecting ducts, calyces and urinary pelvis that facilitate urine excretion and regulate urine composition. The renal collecting system is formed in utero, completed by the 34th week of gestation in humans, and dictates final nephron complement. The renal collecting system arises from the ureteric bud, a derivative of the intermediate-mesoderm derived nephric duct that responds to inductive signals from adjacent tissues via a process termed ureteric induction. The ureteric bud subsequently undergoes a series of iterative branching and remodeling events in a process called renal branching morphogenesis. Altered signaling that disrupts patterning of the nephric duct, ureteric induction, or renal branching morphogenesis leads to varied malformations of the renal collecting system collectively known as congenital anomalies of the kidney and urinary tract (CAKUT) and is the most frequently detected congenital renal aberration in infants. Here, we describe critical morphogenetic and cellular events that govern nephric duct specification, ureteric bud induction, renal branching morphogenesis, and cessation of renal branching morphogenesis. We also highlight salient molecular signaling pathways that govern these processes, and the investigative techniques used to interrogate them.

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Abbreviations: CAKUT, congenital anomalies of the kidney and urinary tract; ND, nephric duct; UB, ureteric bud; MM, metanephric mesenchyme; GDNF, glial derived neurotrophic factor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; TGFβ, transforming growth factor β; OPT, optical projection tomography.

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1. Introduction

The capacity of the mammalian kidney to regulate water, sodium, potassium, and acid–base homeostasis is dependent on the collecting system, which is composed of collecting ducts, calyces, the renal pelvis, and ureter. In humans, an average of 785,000 nephrons are connected to ~60,000 collecting ducts [1,2]. Filtrate produced by nephrons aggregates in the collecting ducts which then converge at the renal pelvis to exit the kidney via the ureter. In humans, the collecting system is derived from the ureteric bud, a caudal epithelial derivative of the nephric duct that give rise to ureteric branches and daughter collecting ducts in a process termed branching morphogenesis. Remodeling of the initial ureteric branches results in formation of the calyces and pelvis. These morphogenetic events are completed by 34 weeks gestation in humans [3]. Defects in collecting system formation present with multiple or ectopic ureters, dilation of the collecting ducts, calyces and/or renal pelvis, and collecting duct and calyceal cysts. Since ureteric branch tips induce nephron formation, decreased branching morphogenesis also causes formation of a lower than normal number of nephrons and an overall decrease in kidney size, a condition called renal hypoplasia. This spectrum of developmental anomalies is classified as congenital abnormalities of the kidney and urinary tract (CAKUT) and is the most frequently detected congenital renal anomaly in infants [4]. Here we describe the developmental and cellular processes, and genetic mechanisms that control ureteric bud formation and renal branching morphogenesis (Table 1).

2. Overview of kidney development and renal branching morphogenesis

The kidney derives from reciprocal signaling interactions between distinct cellular lineages found within the intermediate mesoderm as early as embryonic day 8 (E8.0) [5–7]. At this stage, a population of cells within the intermediate mesoderm begins to epithelialize to form a simple epithelial nephric duct (ND, also known as the Wolffian duct, see Fig. 1) that will later give rise to the renal collecting system, ureter, and vas deferens in males [8]. At E10.5, a bud termed the ureteric bud (UB) forms on the caudal, ventral aspect of the ND in response to inductive signaling from the adjacent metanephric mesenchyme (see Fig. 2) (MM) [5,7]. Outgrowth of the nascent UB is marked by distinct and rapid changes in epithelial morphology. The initial bud, termed the ureteric ampulla, evaginates to form a 'T' shaped structure (see Fig. 3) that is subtended into genetically distinct tip and stalk domains by E11.5. The 'T' shape then undergoes a series of iterative branching events up until mouse post-natal day 3 (P3), forming an arborized epithelial collecting system in a process known as renal branching morphogenesis [9,10]. The correct positioning of the nascent bud site is dependent on the presence and position of the ND in relation to the inducing metanephric mesenchyme. Signaling cascades within the ND and adjacent metanephric mesenchyme dictate the sensitivity and position of the ND to inductive cues. When disrupted, aberrant signaling can inhibit kidney induction or conversely induce multiple ureteric buds to form resulting in renal aplasia or duplex/multiplex collecting systems, respectively. Thus nephric duct specification is an important determinant of ureteric induction and renal branching morphogenesis. The final structure of the kidney is divided into the renal cortex and renal medulla along a radial axis that is established at the onset of kidney induction. The inducing metanephric mesenchyme lays ventral to the nephric duct, and by position alone imparts a polarity to the responding ureteric bud. During development, the cortico-medullary axis of the kidney imparts distinct ureteric tip and stalk signaling domains necessary for normal patterning and development. The cortico-medullary axis also facilitates the urine-concentrating ability of the kidney by forcing filtrate to pass via collecting ducts through the hypertonic medullary interstitium. Perturbing cortico-medullary signaling domains in the ureteric bud itself or the adjacent mesenchyme and stromal populations alters branching morphogenesis by disrupting the UB branch pattern (dysplasia) or reducing the number of branches (hypoplasia). Similarly so, renal dysplasia and a loss of cortico-medullary patterning are associated with abnormal urine parameters.

3. Specification and patterning of the nephric duct

3.1. Specification of the nephric duct

The nephric duct (ND) is first detected at E8.0 with a distinct population of the intermediate mesoderm undergoing a mesenchyme-to-epithelial transition between somites 5 and 8 to form a simple epithelial tubule (schematized in Fig. 1) [11]. This tubule extends caudally toward the cloaca by E9.5. *Pax8*, a member of the Paired-box family of transcription factors, is detected in the nascent nephric duct at the 7-somite stage in mouse and is the earliest known genetic marker that distinguishes the nephric duct from the surrounding intermediate mesoderm [11–13]. Another member of the *Pax* family, *Pax2*, is detected by the 9-somite stage, shortly after *Pax8*, in the nephric duct [11,13,14]. Surprisingly, *Pax8*

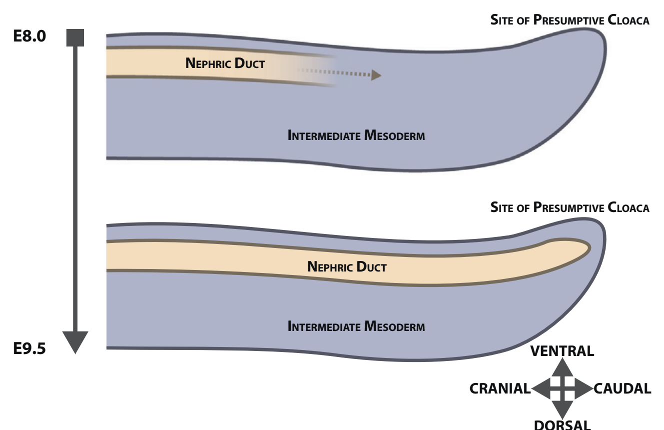

Fig. 1. The intermediate mesoderm exists as two bilateral sausage-shaped domains within the developing embryo and serves as the embryological primordium for the urogenital system. The nephric duct (ND) is specified at mouse embryonic day E8.0 and derives from cells within the intermediate mesoderm that begin to form a simple epithelial tubule. The tubule extends caudally to the cloaca by E9.5. Molecular signaling events that regulate these processes are discussed in the text.

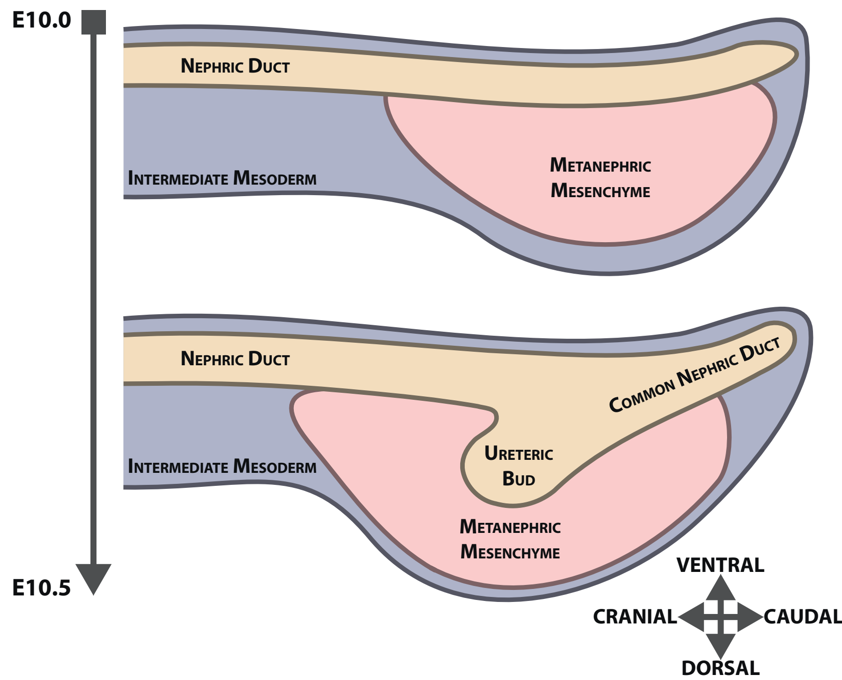

Fig. 2. The nephric duct (ND) lays adjacent to the newly specified metanephric mesenchyme by mouse embryonic day E10.0. The ND is composed up of cells that respond to inductive signals from the MM to form the nascent ureteric bud (UB). The sensitivity of these cells to inductive signals dictates the position and morphology of the bud site relative to adjacent tissues. Ureteric induction commences by E10.5 with a swelling and evagination of the ND into the adjacent MM to form the UB. Aberrant signaling within the ND or MM can lead to abnormal ureteric induction or malformations of the collecting system (see text for details).

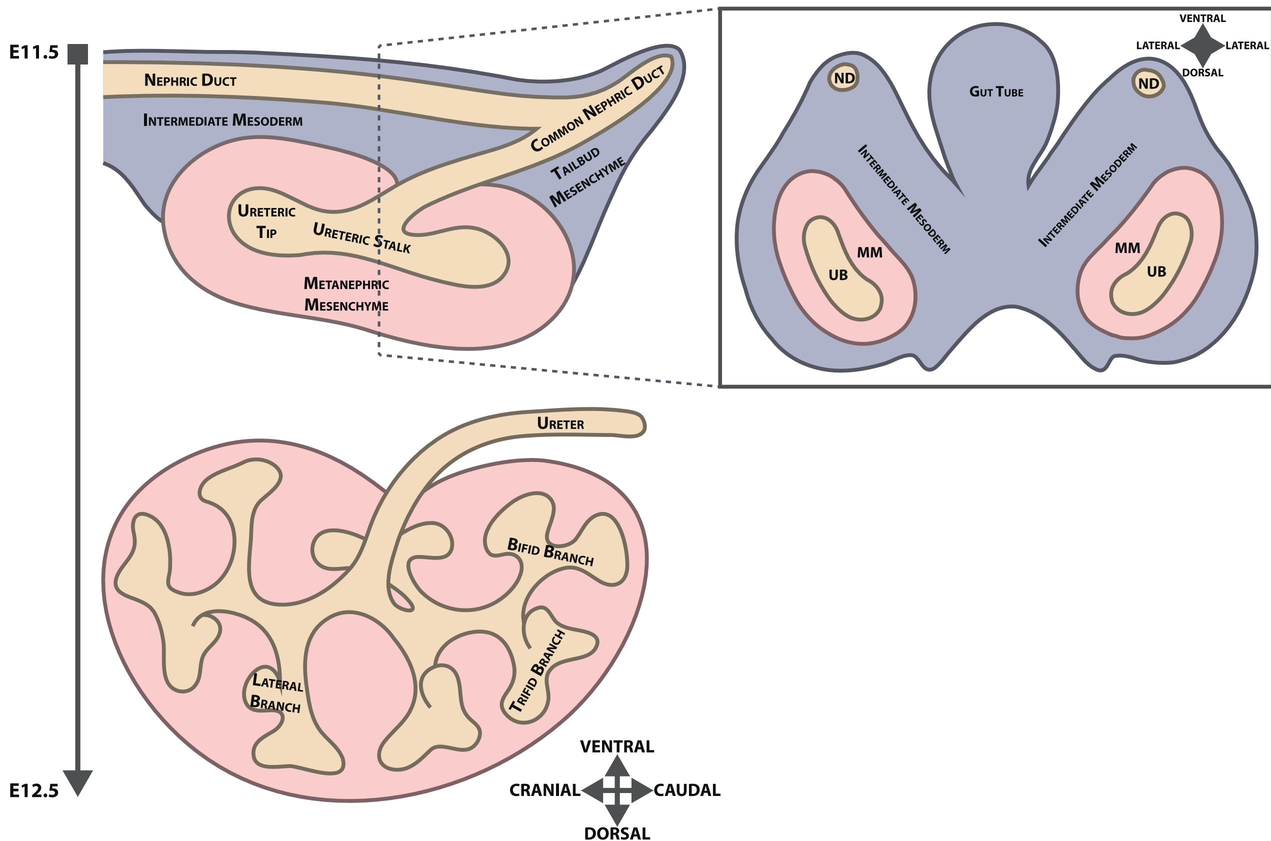

Fig. 3. By E11.5 the nascent UB has formed a T-shaped structure that is embedded within the metanephric mesenchyme (MM). At this time, genetically distinct ureteric tip and ureteric stalk domains exist within the UB. The distal ureteric stalk interfaces with the remaining nephric duct to form the common nephric duct. A cross-sectional view through the developing urogenital system reveals the medio-lateral and dorsal-ventral patterning of the nascent ND, UB, and MM. At this time, the T-shaped bud will continue to undergo a series of iterative branching events. By E12.5, stereotypical bifid branch events are observed, and less frequently, trifid and lateral branching events. The UB will undergo between 10 and 11 branch generations to form nearly 1600 ureteric branch tips in the mature kidney.

Table 1
Genetic mouse models of nephric duct specification, ureteric induction and renal branching morphogenesis.

Model	Phenotype	Reference
Pax8$^{-/-}$	No renal phenotype	Mansouri et al. [15], Bouchard et al. [13]
Pax2$^{-/-}$	Bilateral renal agenesis	Dressler et al. [6], Torres et al. [14], Bouchard et al. [13], Narlis et al. [12]
Pax2$^{+/-}$;*Pax8*$^{-/-}$	Bilateral renal agenesis	Narlis et al. [12]
Shh$^{-/-}$	Bilateral renal aplasia or a single ectopic dysplastic kidney	Hu et al. [19]
Lhx1$^{-/-}$	Nephric duct agenesis	Shawlot and Behringer [22], Tsang et al. [21]
Gata3$^{-/-}$	Nephric duct extension failure	Grote et al. [26]
Ret$^{-/-}$	Renal agenesis	Pachnis et al. [35], Schuchardt et al. [36]
Gdnf$^{-/-}$	Renal agenesis, renal hypoplasia	Vega et al. [38], Sainio et al. [37]
Foxc1$^{-/-}$, *Foxc2*$^{-/-}$	Renal hypodysplasia, ureteric duplication	Kume et al. [44]
Slit2$^{-/-}$, *Robo2*$^{-/-}$	Ureteric duplication	Grieshammer et al. [45]
Spry1$^{-/-}$	Multiple ectopic budding	Basson et al. [46], Basson et al. [47]
Fgfr2$^{Stroma-/-}$	Cranial localization of the bud site	Walker et al. [55]
Bmp4$^{+/-}$	Renal hypodysplasia, hydroureter, duplex collecting system	Miyazaki et al. [57]
Etv4$^{-/-}$;*Etv5*$^{+/-}$	Failure to bud	Lu Cebrian et al. [64]
Wnt11$^{-/-}$	Renal hypoplasia	Majumdar et al. [31]
Bmp2$^{+/-}$	Increased renal branching morphogenesis	Hartwig et al. [89]
Bmp4$^{+/-}$	Decreased renal branching morphogenesis	Miyazaki et al. [57]
Ilk$^{UB-/-}$	Renal hypoplasia	Smeeton et al. [90]
Rarα$^{-/-}$;*Rarβ2*$^{-/-}$	Renal hypoplasia	Mendelsohn et al. [97]
Fgfr2IIIb^{DN}	Renal hypoplasia	Celli et al. [104]
Cttnb1$^{UB-/-}$	Renal aplasia, renal hypodysplasia	Bridgewater et al. [110]
Wnt7b$^{UB-/-}$	Cyst formation	Yu et al. [72]
Wnt9b$^{Stalk-/-}$	Cyst formation	Karner et al. [74]

is dispensible for ND formation as *Pax8* deficient embryos die postnatally from thyroid deficiencies [13,15]. In *Pax2* deficient mouse embryos however, extension of the nephric duct arrests at the level of the mesonephros and fails to reach the cloaca [6,12–14]. In *Pax2/8* compound mutants, there is no evidence of nephric duct formation marked by an absence of *Lhx1* and *Ret* expression (discussed in detail in Section 3.3) [12]. These data suggest that *Pax2* or *Pax8* can serve redundant functions during initial ND epithelialization but only *Pax2* is necessary for the maintenance of the ND. The question then arises whether *Pax2* is sufficient to induce the nephric duct in the intermediate mesoderm. Indeed, misexpression of *Pax2* in early chick mid-streak tissue gave rise to ectopic nephric ducts two days later as indicated by *Lhx1* and *Ret* expression [13]. Taken together these data highlight a necessary and sufficient role for *Pax2* in initiating and maintaining the nephric duct. It has not been determined if other transcription factors are also sufficient to induced the ND in the intermediate mesoderm.

3.2. *Medio-lateral positioning of the ND*

The Hedgehog signaling pathway is implicated in the morphogenesis of many organs throughout the body including the lung [16], pancreas [17], and salivary gland [18]. Removal of Sonic Hedgehog (*Shh*) in the developing embryo causes renal aplasia or a single ectopic dysplastic kidney [19]. Examination of *Shh*-null embryos with renal aplasia revealed a loss of *Pax2* expression within the intermediate mesoderm and no evidence of nephric ducts. The genetic removal of the downstream transcriptional repressor GLI3R restored *Pax2* expression and permitted kidney development to occur (albeit with minor midline patterning defects) [19]. Consistent with these data, ablation of the notochord and neural floor-plate results in dorsalization and medialization of the intermediate mesoderm ultimately resulting in a horseshoe kidney [20]. Taken together these data suggest that Hedgehog signaling regulates positioning of the nephric duct within the intermediate mesoderm.

3.3. *Cranial–caudal extension of the nephric duct*

As the nephric duct will contribute to the ureteric tree, ureter, and male gonad, it is imperative that it extends caudally to the cloaca along the cranial–caudal axis. Here, we discuss the genetic and molecular signaling mechanisms that control ND elongation. A member of the LIM homeodomain containing transcription factors *Lhx1*, is expressed in the intermediate mesoderm as early as E7.5 and becomes uniformly restricted to the nephric duct by E9.5 [21]. The morphologic integrity of the nephric duct has not been clearly defined in *Lhx1* deficient embryos due to severe patterning defects [21,22]. Analysis of *Lhx1*-deficient ↔ WT chimeras demonstrated that *Lhx1*-deficient cells were unable to contribute to the nephric duct or its derivatives as early as E9.5 [23]. To better identify the role for *Lhx1* during early ND patterning, *Lhx1* was conditionally removed from the nephric duct in a *Pax2-cre* dependent manner. Analysis of so-generated mice revealed that the ND failed to extend caudally at E9.5, and did not reach the urogenital sinus by E10.5 [24]. In some conditional mutants, a hypoplastic ureteric bud formed, and expressed *Pax2*, *Emx2*, *Gata3*, and *Ret*, suggesting that *Lhx1* is not required for the specification of the nephric duct cell identity and acts downstream of *Pax2* [24]. Intriguingly, *Wnt9b* and *E-cadherin* expression were markedly reduced in the ND of these mutants at E10.5 indicating that Lhx1 is necessary for ND epithelial maintenance [24]. Additional studies found that the transcription factor *Emx2* lies upstream of *Lhx1* and *Pax2* [25]. Loss of *Emx2* abrogates *Lhx1* and *Pax2* expression in the nephric duct. Despite this finding, *Emx2* deficient embryos are phenotypically normal until E11.5 implying that *Emx2* may control an additional genetic program required for ND maintenance.

In an effort to better identify the downstream effectors of *Pax2/Pax8* signaling in the nephric duct that are responsible for ND extension, *Pax2*-deficient cells expressing GFP under the *Pax2* promoter were isolated and RNA expression data was generated to identify differentially regulated gene targets [26]. Analysis revealed that the transcription factor *Gata3* was significantly higher in WT versus *Pax2*-deficient cells. Indeed, closer examination of *Gata3* expression by RNA in situ hybridization revealed that *Gata3* is expressed in the nephric duct as early as E8.5, shortly after *Pax2* expression is detected [26]. Moreover, *Gata3* expression is markedly reduced in *Pax2*$^{-/-}$;*Pax8*$^{+/-}$ embryos indicating that *Gata3* expression is mediated by *Pax2/8*. Loss of *Gata3* expression in *Gata3*$^{GFP/GFP}$ embryos showed that the nephric duct failed to extend to the cloaca by E9.5 and was discontinuous [27]. Further analysis of *Gata3* deficient embryos is complicated by embryonic lethality at E11.0 necessitating conditional deletion of *Gata3* in a lineage specific manner. Hydroureter, hydronephrosis, and duplex

collecting systems were observed at E18.5 when *Gata3* was conditionally removed from the nephric duct lineage consistent with ureteric budding abnormalities. Examination of $Gata3^{ND-/-}$ urogenital ridges at E10.5 and E11.5 demonstrated ectopic budding along the entire length of the ND that could be rescued by attenuating *Gdnf-Ret* and *Fgf* signaling. Moreover the authors demonstrated that *Gata3* expression is genetically downstream of the *Wnt*/*β-catenin* signaling pathway as expression of canonical *β-catenin* targets remained unaffected in $Gata3^{ND-/-}$ mutants. This was further confirmed by manipulating *β-catenin* levels in a nephric-duct derived cell culture model and demonstrating an associated change in *Gata3* expression [27]. In a separate study, the role for *Gata3* was explored in tandem with a requirement for retinoic acid signaling mediated by *Raldh2* to facilitate the correct extension and insertion of the common nephric duct into the cloaca [28]. The authors discovered the presence of filpodia on the most distal cells of the nephric duct. These filopodia actively extend in order to promote nephric duct elongation. Each of *Ret*, *Gata3*, and *Raldh2* signaling are required for filopodia formation [28]. Although this study provides kinetic and cellular dynamic insight into the nephric duct elongation process, the precise *Gata3*-dependent intracellular mechanisms that mediate filopodia formation remain undefined.

4. Specification of the ureteric bud site and ureteric induction

4.1. Introduction

Ureteric bud site specification is a highly coordinated process that permits ureteric budding at one and only one site into the adjacent metanephric mesenchyme (schematized in Fig. 2). When this process is disrupted, a number of patterning defects can arise resulting in the CAKUT spectrum of congenital anomalies. A number of signaling molecules seem to be involved in this process, and many act by restricting or attenuating activity of the *Gdnf-Ret* signaling axis [29]. All evidence to date suggests that specificity of the ureteric bud site is dependent on the sensitivity of the ureteric epithelia to inductive signals [29,30]. Ureteric induction is also mediated by many of the same pathways as bud-site specification and requires the sustained outgrowth of the ureteric bud into the adjacent metanephric mesenchyme sufficient for UB derived signaling to maintain the metanephric mesenchyme [31]. In the absence of UB derived signals, the metanephric mesenchyme undergoes apoptosis and a kidney fails to form [32].

4.2. Determinants of the ureteric bud site

The receptor tyrosine kinase RET [33] and co-receptor GFRα1 [34] are expressed along the length of the nephric duct as early as E9.5 and binds glial-derived neurotrophic factor (GDNF) that is secreted by the adjacent metanephric mesenchyme. Ureteric induction fails to occur in *Ret* deficient embryos [35,36], and in over 50% of *Gdnf* deficient embryos [37,38]. Conversely, Gdnf soaked beads, when cultured next to the nephric duct, are sufficient to induce ectopic budding of the nephric duct suggesting that *Gdnf* is a major regulator of ureteric induction [37].

A number of distinct molecular pathways control the domain of *Gdnf* expression in the metanephric mesenchyme [30]. The genes *Pax2*, *Eya1* [39,40], *Gdf11* [41], and the *Hox11* paralogues [42,43] are known to promote *Gdnf* expression as gene-specific knockouts have reduced *Gdnf* signaling and phenocopy *Gdnf* knockout mice [40]. Conversely, the transcription factors *Foxc1* and *Foxc2* are expressed in the metanephric mesenchyme and when genetically removed, permit a cranial expansion of the *Gdnf* signaling domain [44]. This, in turn, leads to a duplication of the ureteric bud, hydroureter, and renal hypodysplasia, suggesting that *Foxc1/2* functions as negative regulators of *Gdnf* expression. Similarly, *Robo2* is expressed in the metanephric mesenchyme and signals via the nephric-duct expressed receptor *Slit2*. Loss of *Slit2/Robo2* signaling similarly leads to a cranial expansion of the *Gdnf* signaling domain [45], independent of *Foxc1/2*, and causes duplicate ureters [44].

At least two distinct molecular pathways have been identified that alter the downstream state of RET signaling in the ureteric epithelium. SPRY1, a receptor tyrosine kinase antagonist is expressed in the nephric duct at the onset of ureteric bud initiation and attenuates downstream RET signaling by inhibiting Erk/MAPK [46,47]. When *Spry1* is removed, supernumerary buds are detected along the length of the nephric duct and the primary bud itself swells, leading to grossly abnormal development of the ureteric stalk and distal urinary collecting system [46,47]. When maintained on a *Gdnf* heterozygous background, *Spry1*-null kidneys developed normally suggesting that *Spry1* controls the sensitivity of the nephric duct to *Gdnf* signaling from the adjacent metanephric mesenchyme [47].

The finding that receptor tyrosine kinase antagonist SPRY1 controls ureteric induction is important because it implies that other tyrosine-kinase dependent signaling pathways may also induce the ureteric bud. One candidate pathway includes the fibroblast family of growth factors (*Fgf*) and their ligand receptors (*Fgfr*). Surprisingly, a number of *Fgf*-specific knockouts have been generated in both ligand and receptor (*Fgf10*, *Fgf7*, *Fgfr1*, *Fgfr2*) and none appear to inhibit the actual nephric duct induction process itself [48–54]. One recent study demonstrated that the genetic elimination of *Fgfr2* in the peri-nephric duct mesenchyme caused a more cranial localization of the ureteric bud site relative to the common nephric duct [55]. As a primary mediator of ureteric induction, it is clear that *Fgf* signaling is not necessary. However in the context of a *Gdnf-Ret* deficient signaling environment, is *Fgf* signaling sufficient for ureteric induction? FGF7 supplementation of isolated nephric ducts induced nephric duct thickening without budding [51]. However when activin (a *Tgf-β* superfamily antagonist) signaling was inhibited by follistatin or anti-activin antibody, FGF7 was sufficient to induce budding from the nephric duct [56]. In a separate and more recent study, *Gdnf*-null urogenital ridges were cultured in the presence of FGF10, and multiple and ectopic buds formed along the nephric duct suggesting that FGF10 is capable of inducing ureteric budding [49]. Moreover in a *Spry1-null*;*Gdnf-null* background, the genetic deletion of just one allele of *Fgf10* causes ureteric budding to fail where it would otherwise occur [49]. These studies demonstrate that *Fgf* signaling in the absence of *Gdnf-Ret* signaling in the nephric duct is sufficient for ureteric bud induction and may serve to finely regulate the inductive process. Another recent study has demonstrated a *Gdnf* independent budding mechanism that is dependent upon AKT and JNK activation in the absence of RET signaling activity implying that other undescribed pathways, may also serve to fine-tune the inductive budding process [56].

Studies of the *Tgf-β* superfamily of signaling molecules have implicated *Bmp4* [57,58], its receptor *Alk3* [59], and the *Bmp4* antagonist *Gremlin1* [60] as important signaling molecules in regulating the ureteric induction process. *Bmp4* expression is restricted medially within the urogenital ridge at the time of ureteric induction and surrounds the nephric duct but is excluded from the metanephric mesenchyme [57,61,62]. $Bmp4^{+/-}$ mice exhibit hypodysplasia, hydroureter, or duplex collecting systems [57]. Further analysis revealed an abnormal positioning of the ureteric bud site relative to the common nephric duct, and increased stalk elongation at the expense of ureteric tip branching. Surprisingly, ureteric tip specific expression of *Wnt11* is lost in cultured ureteric buds adjacent to BMP4 coated beads suggesting that ureteric tip identity might be lost [57]. These data are further supported by studies in the *Bmp4*-antagonist *Gremlin1* model. *Gremlin1*-deficient

nephric ducts fail to form a ureteric bud [60]. When GREM1 is added exogenously in culture to *Gremlin1*-deficient nephric ducts, ureteric budding resumes and supernumerary buds form along more cranial portions of the nephric duct that express *Wnt11* [60]. Recent evidence places the transcription factor *Isl1* upstream of *Bmp4* [63]. The conditional removal of *Isl1* from the lateral plate mesoderm reduces *Bmp4* signaling and causes secondary budding from the nephric duct, renal hypoplasia, and a cranial localization of the bud site that later leads to obstruction of the ureter [63]. Surprisingly the addition of exogenous BMP4 to *Isl1* deficient urogenital ridges failed to rescue these phenotypes suggesting that *Isl1* may act independent of *Bmp4* signaling.

4.3. Determinants of ureteric induction

Recent work has begun to elucidate the downstream targets of *Gdnf-Ret* signaling in an effort to better define the cellular processes involved in ureteric induction, and by extension, branching morphogenesis. Denuded ureteric buds at E11.5 were cultured with or without *Gdnf*, and RNA microarray analysis was performed to determine the genes that were differentially regulated by *Gdnf-Ret* stimulation [64]. By selecting those genes unique to the ureteric lineage, the authors were able to define a number of classes of molecules that are differentially regulated by *Gdnf-Ret* signaling including the transcription factors *Etv4* and *Etv5* [64]. Functional redundancy of these two transcription factors required the generation of $Etv4^{-/-}$;$Etv5^{+/-}$ compound mutants in order to investigate the functional relationship that *Etv4/5* has during ureteric induction [64]. Indeed, compound mutants fail to produce a ureteric bud. In a separate study, the authors sought to better understand the developmental potential of *Etv4/5* deficient cells to the nephric duct and ureteric bud [65]. To do so, $Etv4^{-/-}$;$Etv5^{+/-}$ (mutant) chimeras were generated on a WT or *Ret*-deficient background and demonstrated that mutant cells were unable to contribute to the ureteric tip domain but were able to contribute to the ureteric stalk, nephric duct, and common nephric duct suggesting that *Etv4/5* specifies ureteric tip cell identity [65].

Similar chimeric analysis has afforded the ability to interrogate the cellular mechanism underlying *Ret*-dependent formation of the ureteric bud. In *Ret*-deficient ↔ Wildtype chimeras that have a nephric-duct specific membrane-bound fluorescent reporter [66], *Ret*-deficient cells are observed randomly throughout the nephric duct at E9.5 [67]. At the onset of ureteric induction, a population of wildtype cells are observed pooling together at the presumptive bud-site, and *Ret*-deficient cells are excluded from this domain. Simultaneously, the nephric duct swells by forming a pseudo-stratified epithelium in a *Ret*-independent manner. This process of pseudo-stratification is presumed to serve as a major repository of epithelial cells that will allow for the rapid outgrowth of the ureteric bud through cell-rearrangement in the absence of substantial nephric duct cell proliferation. Formal testing of this theory however has yet to be reported. Ureteric budding occurs subsequently with *Ret*-deficient cells being excluded from the nascent bud tip domain. Moreover, *Ret*-deficient cells were able to contribute to a ureteric stalk implying that the ureteric tip domain is vital to ureteric induction [67]. To better test whether bud site formation and the establishment of a tip domain is dependent on sensitivity to *Gdnf-Ret* signaling, the authors generated *Spry1*-deficient ↔ Wildtype chimeras. As hypothesized, *Spry1*-deficient cells were observed forming at the presumptive bud site, and in the nascent ureteric tips. Dp-ERK staining (a readout of RET-tyrosine kinase activity) in WT nephric ducts varied from cell to cell. This demonstrated that nephric duct epithelia have differing levels of sensitivity to *Ret* signaling that can be separated into distinct populations of *Ret*-high and *Ret*-low cells [67]. These data provide compelling evidence to support *Ret*-dependent arrangements of the nephric duct to establish the ureteric bud site, and generate unique insight into the cellular processes through which ureteric induction occurs. Despite these data, it remains unclear what cell-specific process mediates the restructuring of the nephric duct, and what distinguishes a ureteric tip cell from a nascent stalk cell.

A number of questions remain to be addressed in the context of ureteric induction and relate to the physical processes that nephric duct epithelia must undergo. What mechanism drives the aggregation of *Ret*-high and *Ret*-low cells? What strategy is used by the nephric duct epithelia to bud and elongate? Finally, is the *Gdnf-Ret* signaling pathway the only major determinant of ureteric budding? Indeed the technologies to address these questions, including live-culture microscopy and high throughput expression analysis, are surely being adopted within the field and ought to provide definitive answers to these questions.

5. Renal branching morphogenesis

5.1. Introduction

As is the case with many processes during embryogenesis, signaling pathways that control nephric duct patterning and ureteric induction are reused to control renal branching morphogenesis. Through reciprocal signaling interactions between multiple cell lineages within the kidney, the nascent ureteric bud undergoes a process of iterative branching that generates an arborized urinary collecting tree with as many as 1600 branch tips that interface with approximately 8000 nephrons in the newborn mouse kidney (schematized in Fig. 3) [10,68]. Renal branching morphogenesis does not follow a predefined program unlike what is observed in pulmonary branching morphogenesis [68–70]. Instead, between 10 and 11 branch generations are generated, with three different branching morphologies: tip-specific bifid branching, tip-specific trifid branching, and stalk-specific lateral branching [68,70]. Bifid branching is by far the most common branching mode, estimated to occur about 75% of the time in a mouse kidney explant model followed by trifid branching at 18%, and lateral branching at only 6% [70]. Branch tips are estimated to interface with at least 4–6 distal nephron segments in the newborn mouse kidney [70]. As a consequence, determinants of renal branching morphogenesis are directly related to the total number of nephrons, with major consequences for human health and disease. Throughout renal branching morphogenesis, the ureteric lineage can be segmented into ureteric tips and ureteric stalks based on regiospecific gene expression patterns and epithelial behaviors [69]. This segmentation strongly correlates with a cortico-medullary signaling axis that exists within the kidney, and that is described below. Moreover, at least three distinct cellular processes are known to contribute to the actively developing ureteric tree including ureteric cell proliferation [71], planar-cell-polarity dependent cellular reorganization [72–75], and a novel mitosis-dependent cellular delamination and reinsertion process [76]. Here we discuss a number of these cortico-medullary signaling mechanisms and distinct cellular processes that mediate ureteric branching morphogenesis.

5.2. Gdnf-Ret signaling activity and renal branching morphogenesis

By far the most intensely studied signaling pathway involved in renal branching morphogenesis is the previously described RET tyrosine kinase signaling pathway (reviewed in greater detail in [29,30,77]). *Ret* expression is detected in the ureteric bud tips throughout kidney development and must be maintained for ureteric branching to be sustained [35,36]. Specific point mutations on select RET intracellular tyrosine residues have provided insight

into the downstream pathways that are impacted by *Ret* signaling [78,79]. These include the PLC-gamma-PKC pathway, RAS-RAF-MEK-ERK pathway, and the AKT pathway [78,79]. The genetic ablation of *Mek1/2* that mediates ERK phosphorylation reduces the number of branch generations with little effect on ureteric bud elongation [80,81]. Moreover, epithelial cell adhesion markers were mislocalized in *Mek1/2* ureteric knockouts suggesting that *Mek1/2* regulates epithelial cell adhesion [81]. It remains however to be determined what role *Mek1/2* signaling has on promoting branch generation in a *Gdnf*-dependent context.

Gdnf is expressed in the metanephric mesenchyme surrounding the ureteric tips and is a potent inducer of the *Ret* signaling pathway [30,33,82]. The genetic removal of a single copy of *Gdnf* results in renal hypoplasia associated with decreased branching morphogenesis [37,38,82–84]. Similarly, genes that control *Gdnf* expression in the developing kidney include *Eya1* [39], *Hox11* paralogues [43], and *Gdf11* [41] cause renal hypoplasia when genetically removed. By manipulating the levels of *Gdnf* signaling in vitro, a number of *Ret*-dependent targets were detected including *Wnt11*, *Etv4/5*, and a number of other genes (*Cxcr4*, *Crlf*, *Dusp6*, *Myb*, and others) [64]. *Etv4/5* deficient embryos present with renal aplasia, and *Etv4*$^{-/-}$;*Etv5*$^{+/-}$ nephric duct cells are unable to contribute to the ureteric tip domain in a wildtype chimeric model[65]. Although mutant nephric duct cells had reduced levels of cell proliferation, it does not explain why they are preferentially excluded from the ureteric tip domain [65]. *Wnt11* is expressed in the ureteric tips and maintains *Gdnf* expression in the metanephric mesenchyme to mediate a feed-forward signaling mechanism [31]. The specific mechanism that underlies *Wnt11*-dependent Gdnf expression however is unclear. Consistent with a cortico-medullary signaling axis in the developing kidney, the transcriptional repressor GLI3R, controlled by Hedgehog signaling activity in the ureteric lineage has also been implicated in maintaining *Wnt11* expression in the ureteric tip [85].

The transcription factors *Sox8* and *Sox9* have also been shown to control the expression of genes that function downstream of *Ret* in a *Gdnf-Ret* independent manner [86]. Loss of *Sox8/9* leads to a marked decrease in branch tip number at E12.5, loss of expression of *Gdnf*-responsive genes: *Etv4*, *Cxcr4*, *Met*, *Spry1*, and *Dusp6*, and an expansion of stalk-specific *Wnt9b* into the ureteric tips [86]. Intriguingly, *Sox8/9* expression was unperturbed in *Etv4/5* mutant kidneys suggesting that *Sox8/9* acts in parallel with *Gdnf-Ret* signaling to mediate tip-specific gene identity [65].

5.3. Tgf-β signaling and RBM

The *TGF-β* signaling pathway is another important pathway that controls renal branching morphogenesis (reviewed in [62,87]). Components of the pathway include members of the BMP family of secreted ligands, *Tgf-β* superfamily receptors *Alk2*, *Alk3*, and *Alk6*, and the SMAD family of transcriptional effectors. Canonical BMP signaling mediates the phosphorylation of R-SMADs (SMAD1, 5, or 8) that then form a heterodimer with a coactivator SMAD4 before promoting target gene transcription. *Bmp2* expression is detected in mesenchyme adjacent to the ureteric tip [88]. The addition of exogenous *Bmp2* in culture inhibits renal branching morphogenesis [62,80]. Not surprisingly in *Bmp2* heterozygous mice, there is increased renal branching that is further exacerbated in a *Smad4* heterozygous background [89]. When the primary *Bmp2* receptor *Alk3* was genetically removed from the ureteric lineage, there was an increase in the number of first, second, and third branch generations, and a decrease in subsequent branch generations indicating that *Bmp2* signaling may regulate branch generations [59]. Paradoxically, BMP4-ligand, expressed in the tailbud mesenchyme adjacent to the ureteric bud site, promotes ureteric bud elongation and outgrowth and is expressed in the mesenchyme adjacent to the ureter [57]. *Bmp4* heterozygosity caused renal hypoplasia and was associated with fewer but broader ureteric tips. An additional role for *Bmp4* is to promote differentiation of distal ureteric segments [58]. Exogenous BMP4, when added to kidney explant cultures, induces ectopic expression UPKIII (a marker of the renal pelvis and ureter) in the ureteric tips [58]. Taken together these data illustrate a cortico-medullar axis of *Bmp* signaling that regulate renal branching morphogenesis. Non-canonical *Bmp* signaling in the kidney is mediated by p38-MAPK activity and facilitated, in part, by integrin-linked kinase (*Ilk*). Loss of *Ilk* in the ureteric lineage resulted in renal hypoplasia, a loss of *Wnt11* expression, and decreased p38-MAPK phosphorylation in the absence of altered pERK staining [90]. Taken together, these data highlight distinct roles for different BMP-ligand/receptor pairs, and downstream TGF-β signaling components in determining ureteric branch generation, branch diameter, and ureteric bud branching morphogenesis.

5.4. Retinoid signaling and RBM

The renal stroma plays an essential role within the developing and mature kidney, and its role in mediating renal branching morphogenesis is an active area of research [91]. The renal stroma secrete a large number of factors including bioactive retinoids and extracellular matrix [92,93]. Vitamin A deficiency is associated with renal hypoplasia implicating retinoid-dependent signaling in kidney development [94,95]. Components necessary for synthesizing bioactive retinoic acid from retinaldehyde are found throughout the cortical renal stroma (*Raldh2*) and ureteric bud (*Raldh3*) [96]. Retinoic acid signals via retinoic acid receptors *Rarα* and *Rarβ2*, and when genetically removed cause renal hypoplasia with reduced branching morphogenesis [97]. Strikingly forced expression of *Ret* in the ureteric lineage was able to restore branching morphogenesis implying that retinoic acid signaling is upstream of *Ret* expression [93]. The expression of a dominant negative *Rarα* receptor in only the ureteric lineage resulted in renal hypoplasia with reduced branching, and ureteric budding/induction defects similar to those seen in *Rarα*;*Rarβ2* double knockouts demonstrating that retinoic acid signaling in the ureteric lineage is essential for renal branching morphogenesis [98]. Other secreted stromal components shown to regulate branching morphogenesis include the recently described *Ecm1* [99]. It is very likely that the extracellular matrix and environment plays a vital yet relatively undescribed role during branching morphogenesis including mediating branch point decision and bud elongation.

5.5. FGF signaling and RBM

Another major source of tyrosine kinase signaling found in the developing kidney is mediated by the fibroblast-growth factor (*Fgf*) family of secreted ligands and receptors [54]. Components of the *Fgf* signaling pathway are detected in the ureteric bud and metanephric mesenchyme and interact through reciprocal signaling interactions. There are 24 described *Fgf* ligands in mammals that bind to 4 known receptors (that also have splice-specific isoforms). The complexity of ligand and receptor binding has necessitated the lineage specific removal of individual signaling components in order to interrogate their function throughout renal branching morphogenesis. To date, *Fgf* receptors 1-4 [100–102], and *Fgf* ligands *Fgf7* [51], *Fgf8* [103], and *Fgf10* [49,50] have been studied in the developing kidney. The loss of *Fgf7*, *Fgf10*, and *Fgfr2IIIb* receptor (the major *Fgf7/10* receptor) caused renal hypoplasia associated with decreased renal branching morphogenesis [50]. When the extracellular *Fgfr2IIIb* binding domain was expressed in a soluble fashion, kidney development was abrogated suggesting that additional and undescribed Fgf signaling might be occurring [104]. The ureteric specific knockout of *Fgfr2* resulted in increased branching lengths

and abnormal stromal patterning [105]. Compound mutants that abrogate function of *Frs2a* (mediating downstream PKC, AKT, and ERK signaling) and *Fgfr1a* in the metanephric mesenchyme led to fewer branches with thickened ureteric bud diameters [106]. Morphometric analysis identified abnormalities in the plane of ureteric cell division suggesting that non-autonomous *Fgf* signaling may control ureteric planar cell polarity.

5.6. Wnt signaling, planar cell polarity, and RBM

The WNT family of signaling ligands and receptors has been studied extensively during embryonic kidney development [107,108]. The *Wnt* signaling pathway operates through distinctly different canonical and non-canonical mechanisms [109]. During canonical *Wnt* signaling, ligand binds to *Fzd* receptors, inhibiting the degradation of β-catenin (*Ctnnb1*). CTNNB1 is then free to interact with the transcriptional cofactor TCF where it promotes expression of target genes. In the ureteric lineage, loss of *Ctnnb1* causes renal hypodysplasia, or agenesis [110]. Microarray analysis of $Ctnnb1^{UB-/-}$ kidneys identified *Lhx1* and *Emx2* as key ureteric-specific genes that are maintained by canonical *Wnt* signaling. It remains undetermined whether forced expression of *Lhx1* or *Emx2* in a *Ctnnb1* deficient background is sufficient to rescue patterning and branching of the ureteric tree.

Non-canonical *Wnt* signaling (Wnt/PCP signaling) does not lead to stabilization of CTNNB1 and instead alters Rho-RAC GTPase and JNK to modify actin cytoskeleton organization. Non-canonical signaling mediates a process known as planar-cell polarity that defines the axis and orientation of a cell within an epithelium. In the context of kidney development, PCP mediates the reorganization and interdigitation of ureteric epithelial cells promotes elongation of the ureteric bud at the expense of its diameter in a convergent-extension process. Of the 19 secreted WNT ligands in mouse, *Wnt7b* [72] and *Wnt9b* [74] have both been shown to mediate PCP specific effects in the ureteric lineage to control renal branching morphogenesis. *Wnt9b* is detected in the nascent ureteric stalk and excluded from the ureteric tips – an expression pattern that is consistent throughout kidney development [111]. Loss of ureteric *Wnt9b* expression leads to cystogenesis in postnatal kidneys in both the ureteric derived collecting ducts and the mesenchyme derived proximal tubules [74]. Normally, the angle of cell division within the ureteric lineage is random at E13.5 and E15.5 and only becomes longitudinally biased after P1 suggesting that oriented cell division does not contribute significantly to the arborized architecture of the ureteric tree. In contrast, a significant number of epithelia were found to be oriented in the radial axis of the ureteric tree at E15.5, a process that was disrupted when *Wnt9b* was lost in the ureteric stalk [74]. Conversely *Wnt7b*, expressed in the ureteric stalk but not the ureteric tips, has been found to control oriented cell division at E15.5 in the ureteric stalk [72]. When *Wnt7b* is removed from the ureteric epithelium, the renal pelvis fails to form, and the ureteric tree dilates at the expense of its length. Strikingly, the removal of *Cttnb1* in the adjacent renal stroma also abrogated renal pelvis formation, suggesting a non-autonomous, canonical Wnt signaling mechanism that controls ureteric stalk planar cell polarity. Although it is clear that both *Wnt9b* and *Wnt7b* contribute to planar cell polarity within the ureteric lineage at later stages of kidney development, it remains unclear if a planar cell polarity dependent process is required for the initial branch generations in a nascent kidney.

5.7. Cellular dynamics during renal branching morphogenesis

A detailed analysis of ureteric cell proliferation coupled to real-time confocal imaging of kidney explants has identified a novel process through which the ureteric lineage is patterned. In these experiments, single mitotic cells in the ureteric bud tips were indelibly labeled and their position within the ureteric epithelium was assessed over time [76]. Surprisingly, it was found that the mitotic cell produced a daughter cell that delaminated from the epithelial sheet into the lumen of the UB and reinserted itself nearby. Moreover, it was found that proliferating ureteric cells were more likely to be found in the lumen of ureteric tips whereas proliferating cells in the ureteric stalk were found to always exist within the epithelium, suggesting that a unique and tip-specific mechanism exists that maintains tip morphology or behaviors [76]. The authors conclude that a new and distinct cellular mechanism termed mitosis-associated cell dispersal is at play. A number of questions remain including what dictates the insertion site of daughter cells (if any), and whether or not disrupting mitosis-associated cell dispersal is pathogenic in the developing kidney. Such a process, if regulated, may serve as a novel alternative to traditional planar-cell polarity mechanisms that mediate epithelial tubule elongation.

6. Cessation of renal branching morphogenesis

The mechanisms that underlie the cessation of kidney development are largely undefined [9]. Arguably a detailed understanding of this process has the potential to be exploited in the context of regenerative medicine and cancer. It is easy to speculate that altering any combination of the signaling pathways that support kidney development could lead to an arrest of kidney development. Between P0 and P3, there is a progressive loss of the *Gdnf* expressing nephrogenic cap mesenchyme that leads to a ureteric tip-specific loss of *Wnt11* expression [9]. Surprisingly, P3 ureteric tips were still able to promote nephrogenesis when they were cultured with E11.5 metanephric mesenchyme implying that the ureteric bud retained its potential to branch. Moreover, there was a loss of embryonic stromal identity as indicated by *Foxd1* expression following the reduction in nephrogenic cap mesenchyme [9]. Although it was not determined, it remains possible that reduced *Gdnf* signaling from the cap mesenchyme coupled to a retinoic acid-dependent reduction in Ret expression abolishes tyrosine kinase activity within the ureteric tips. Consistent with studies in the nephrogenic mesenchyme, it seems the largest determinants of continued branching morphogenesis are due to reciprocal signaling from the adjacent mesenchyme and stromal populations. It would be interesting however to determine whether ureteric branch pattern is permanently altered or whether that too depends on the nephrogenic mesenchyme.

7. Future directions and conclusions

A vast body of research has contributed to our current understanding of the genetic, molecular, and cellular mechanisms that control nephric duct patterning, ureteric budding, and renal branching morphogenesis. Numerous transgenic mouse strains including those that mediate lineage-specific fluorescence [26,66] and/or cre-dependent [105,112–114] gene expression or deletion provide important tools to study renal branching morphogenesis. Recent advances in imaging techniques (ex. live culture microscopy) are affording researchers the ability to take advantage of these mouse models to image renal explants in real time in an effort to understand the physical behaviors of the nephric duct and ureteric bud [115]. When coupled to high throughput genomics and expression data, these imaging techniques can invaluable information regarding pathways that control specific processes. Furthermore, a high level of temporal and spatial resolution will help to better describe the nuances of nephric duct patterning, ureteric budding, and renal branching morphogenesis. Although

technically challenging, ex vivo live imaging of the murine nephric duct during its initial patterning and elongation could provide novel insight into specific cellular rather than phenotypic processes disrupted in a *Pax2/8* and *Lhx1* dependent manner. Live culture imaging however becomes difficult as the size of the explant is increased; the arborized tertiary structure becomes flattened in two dimensions. Some attempts have tried to recapitulate a 3-dimensional culture system with some success however detailed imaging is convoluted by adjacent tissue [70,116]. Additional technologies such as optical projection tomography (OPT) are well suited to imaging kidneys in situ and can provide for additional quantitative morphometric analyses [117,118]. Real-time analysis using OPT cannot however be performed due to the opacity of the tissue and intensity of the excitatory light source. Indeed further advances in imaging techniques will need to be achieved before later stages of kidney development can be imaged in real-time.

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