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Nephric duct insertion is a crucial step in urinary tract maturation that is regulated by a *Gata3-Raldh2-Ret* molecular network in mice

Ian Chia[1,*], David Grote[2,*], Michael Marcotte[2], Ekaterina Batourina[1], Cathy Mendelsohn[1,†] and Maxime Bouchard[2,†]

SUMMARY

Urinary tract development depends on a complex series of events in which the ureter moves from its initial branch point on the nephric duct (ND) to its final insertion site in the cloaca (the primitive bladder and urethra). Defects in this maturation process can result in malpositioned ureters and hydronephrosis, a common cause of renal disease in children. Here, we report that insertion of the ND into the cloaca is an unrecognized but crucial step that is required for proper positioning of the ureter and that depends on Ret signaling. Analysis of Ret mutant mice at birth reveals hydronephrosis and defective ureter maturation, abnormalities that our results suggest are caused, at least in part, by delayed insertion of the ND. We find a similar set of malformations in mutants lacking either Gata3 or Raldh2. We show that these factors act in parallel to regulate ND insertion via Ret. Morphological analysis of ND extension in wild-type embryos reveals elaborate cellular protrusions at ND tips that are not detected in *Ret*, *Gata3* or *Raldh2* mutant embryos, suggesting that these protrusions may normally be important for fusion with the cloaca. Together, our studies reveal a novel Ret-dependent event, ND insertion, that, when abnormal, can cause obstruction and hydronephrosis at birth; whether ND defects underlie similar types of urinary tract abnormalities in humans is an interesting possibility.

KEY WORDS: Gata3, Nephric duct, Ret-signaling, Retinoids, Hydronephrosis, Lower urinary tract, Mouse

INTRODUCTION

The mature urinary tract consists of kidneys that filter and regulate blood homeostasis, the bladder and urethra that store and excrete urine, and the ureters that conduct urine from the kidneys to the bladder. The upper (kidney and ureter) and lower (bladder and urethra) urinary tract compartments form independently, but must be connected during development to generate a functional outflow tract. Kidneys and ureters are derived predominantly from intermediate mesoderm (Grobstein, 1956; Saxen and Sariola, 1987), whereas the bladder and urethra are derived from cloacal endoderm and peri-cloacal mesenchyme (Haraguchi et al., 2007; Seifert et al., 2008).

Formation of the nephric duct (ND) which occurs on embryonic day (E)9.5 in the mouse, is one of the earliest events in urinary tract formation, establishing a primary connection between the upper and lower urinary tract (Obara-Ishihara et al., 1999; Schultheiss, 2003; Vize et al., 1997). During this process the ND extends along the rostrocaudal axis of the embryo, turns towards the midline at the level of the limb buds and inserts into the cloacal epithelium. Kidney development starts at E10.5, when the ureteric bud sprouts from the caudal ND. At this stage the distal ureter is joined to the ND and does not have a direct connection with the bladder. The ureter-bladder connection is established during ureter maturation, which occurs between E11.5 and E14.5, when the newly formed ureter moves to and inserts into the bladder primordium. During sexual differentiation, which begins at later stages, NDs regress in females, or differentiate into the seminal vesicles, vas deferens and epididymis in males.

Mackie and Stephens first hypothesized that the position at which the ureteric bud forms on the ND is an important determinant regulating the final position at which the ureter will join the bladder (Mackie and Stephens, 1975; Tanagho, 1976). According to this model, ureteric buds emerging posterior to the normal sprouting site would insert in the primitive bladder too early, which can lead to ectopically positioned distal ureters and vesico-ureteral reflux. Conversely, ureters emerging at an abnormally anterior site will fail to join the bladder or will join at a later stage than normal, a defect that can result in defective ureter maturation and obstruction (Hains et al., 2010; Kume et al., 2000; Miyazaki et al., 2000; Pope et al., 1999). Recent evidence supports this model and further shows that ureter maturation depends on apoptotic elimination of the common nephric duct (CND), the most caudal segment of the ND (Batourina et al., 2005; Uetani et al., 2009). This process brings the ureter into contact with the bladder epithelium, where the caudal ureter undergoes apoptosis forming a new ureteral orifice in the bladder primordium (Batourina et al., 2002; Batourina et al., 2005; Mendelsohn, 2009; Murawski and Gupta, 2008; Uetani et al., 2009; Uetani and Bouchard, 2009). Subsequent growth of the bladder moves the ureteral orifice to its final position in the bladder. Once the ureter is inserted in the bladder, the smooth muscle surrounding its caudal end will associate with bladder muscle to form the trigone, the site of the valve mechanism that prevents back-flow of urine from the bladder to the kidney, which can cause severe renal damage (Viana et al., 2007).

[1]Department of Urology, Columbia University, 1130 St. Nicholas Avenue, New York, NY 10032, USA. [2]Goodman Cancer Research Centre and Department of Biochemistry, McGill University,1160 Pine Avenue W., Montreal, QC H3A 1A3, Canada.

*These authors contributed equally to this work
†Authors for correspondence (clm20@columbia.edu; maxime.bouchard@mcgill.ca)

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Ret is a receptor tyrosine kinase that, in the urinary tract, is activated mainly through the Gdnf ligand and the GPI-linked co-receptor Gfrα1 (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Sanchez et al., 1996). In humans, mutations in the *RET* gene lead to Hirschsprung's disease, cancer, and renal abnormalities (Jain, 2009; Takahashi, 2001). Recent studies of families with Hirschsprung's disease indicate that *RET* mutations can also give rise to urinary tract defects known collectively as CAKUT (congenital abnormalities of the kidney and urinary tract), including renal malformations, vesico-ureteral reflux, duplicated kidneys and ureteral obstruction (Pini Prato et al., 2009). In mice, *Ret* gene mutations lead to similar defects as in humans, including enteric nervous system defects, renal agenesis, hypoplasia and ectopic ureter termination (de Graaff et al., 2001; Ishizaka et al., 1989; Jain et al., 2006; Schuchardt et al., 1996; Skinner et al., 2008; Takahashi et al., 1985).

Ret-Gdnf signaling activates downstream pathways, including PLCγ, MAPK and PI3K/AKT, which regulate cell survival, proliferation, chemotaxis, migration and adhesion (Fisher et al., 2001; Tang et al., 2002; Tang et al., 1998; Wong et al., 2005). In the urinary tract, *Ret* is crucial for outgrowth of the primary ureteric bud from the ND, for branching morphogenesis of the ureteric bud within the kidney (de Graaff et al., 2001; Jain et al., 2006; Schuchardt et al., 1994; Schuchardt et al., 1996; Shakya et al., 2005) and for ureter maturation (Batourina et al., 2005; Shakya et al., 2005; Uetani et al., 2009). Ret signaling mediates ureter formation and ureter maturation, at least in part, by inducing migration and cellular rearrangements (Natarajan et al., 2002; Young et al., 2001).

Ret expression in ND cells depends on Gata3 and retinoic acid (RA) signaling, both of which are potent transcriptional regulators. Gata3 is required for the development of several organs, including the kidney, mammary gland and hematopoietic system (Chou et al., 2010; Patient and McGhee, 2002; Uetani and Bouchard, 2009). In mice, *Gata3* inactivation leads to defects in nephric duct proliferation and guidance, and later prevents premature differentiation of nephric duct cells (Grote et al., 2008; Grote et al., 2006). In humans, *GATA3* loss-of-function mutations are associated with the HDR (or Barakat) syndrome, which includes hypoparathyroidism, deafness, renal dysplasia, renal agenesis and vesico-ureteral reflux (Grote et al., 2008; Van Esch and Devriendt, 2001; Van Esch et al., 2000). RA, the active form of vitamin A, activates transcription by binding to and activating RA receptors, which are members of the nuclear receptor superfamily (for a review, see Rochette-Egly and Germain, 2009). Studies in humans with mutations in *ALDH1A2* (hereafter referred to as *RALDH2*), the major RA-synthesizing enzyme, suggest that levels of maternal RA during late pregnancy may be important regulators of kidney size (El Kares et al., 2010; Rochette-Egly and Germain, 2009). In rodents, vitamin A deficiency or defective RA signaling can lead to urogenital malformations, including hydronephrosis, megaureter and ectopically terminating distal ureters (Batourina et al., 2002; Batourina et al., 2005; Duester, 2008; Kastner et al., 1997; Luo et al., 1996; Mendelsohn et al., 1994; Niederreither et al., 1999; Quadro et al., 2005; Wilson and Warkany, 1948).

We find here that Ret signaling is required for ND insertion prior to ureteric bud formation. This event establishes the primary connection between the upper and lower urinary tract and appears to depend on formation of cellular extensions commonly associated with migrating cells. A surprising observation from our studies is that delay or failure in ND insertion due to loss of *Ret* can cause ectopic ureters that join the bladder abnormally or remain fused to the NDs, malformations that have not been previously associated with defective ND morphogenesis. Importantly, mutations in the genes encoding the *Ret* regulators Gata3 or Raldh2 (Aldh1a2 – Mouse Genome Informatics), result in similar renal anomalies. Accordingly, these factors independently regulate *Ret* expression

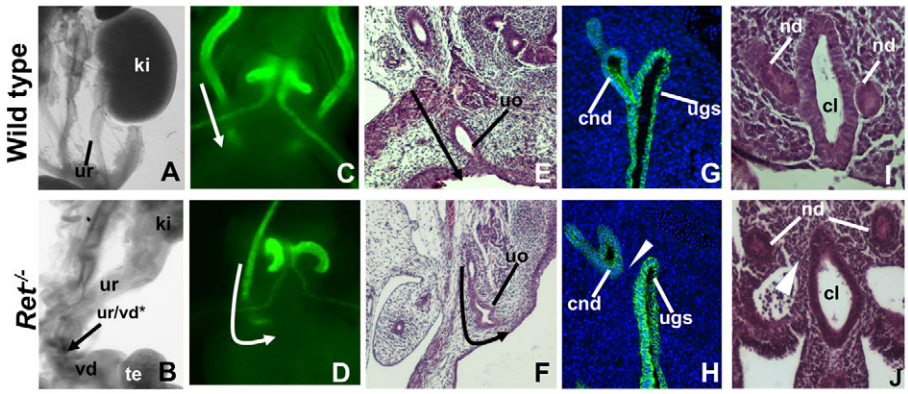

Fig. 1. Abnormal ureter insertion in *Ret*$^{-/-}$ mouse embryos. (**A**,**B**) Whole-mount urinary tract from wild-type (A) and *Ret* mutant (B) embryos at E18.5. The black arrow denotes the abnormal connection between the ureter and vas deferens, which are not separated and are dilated owing to obstruction (ur/vd*). (**C**,**D**) Whole-mount urinary tract from E17.5 control (C) and *Ret* mutant (D) embryos labeled with *Hoxb7-Gfp* transgene. The white arrow denotes the trajectory of the ureter as it passes through the bladder wall. Note the abnormal path of the intravesicular ureter as it crosses the bladder at a 90° angle (D), rather than a 45° angle as in controls (C). (**E**) Hematoxylin and Eosin (H&E) stained frontal section of the E17.5 wild-type embryo shown in C. Note the smooth trajectory of the intravesicular ureter as it passes through the bladder wall at a 45° angle and joins the lumen (black arrow). (**F**) H&E stained section from the sample shown in D. The black arrow traces the path of the mutant ureter that, in addition to its abnormal angle of entry, also appears to be kinked. (**G**,**H**) Sagittal section from wild-type (G) and *Ret* mutant (H) embryos labeled with *Hoxb7-Gfp* transgene. Note the separation between the common nephric duct and urogenital sinus in the *Ret* mutant embryo (white arrowhead). GFP is shown in green, DAPI staining in blue. (**I**,**J**) H&E stained sections through E9.5 wild-type (U) and *Ret* mutant (J) embryos. Note the distance between the nephric duct (ND) tips and cloaca (white arrowhead) in the *Ret* mutant embryo, whereas control NDs reach the cloaca at this stage. cl, cloaca; cnd, common nephric duct; ki, kidney; nd, nephric duct; te, testis; ugs, urogenital sinus; uo, ureter orifice; ur, ureter; vd, vas deferens. Magnifications: 2.5× in A,B; 10× in C-F; 20× in G,H.

in the ND. Together our studies suggest that ND insertion is crucial for proper ureter positioning and delays in this process can result in hydronephrosis and other urinary tract abnormalities.

MATERIALS AND METHODS

Mice and genotyping

Gata3$^{ND-/-}$ (*Hoxb7*^{cre}*; Gata3*$^{flox/flox}$), *Pax2*^{GFP}, *Raldh2*$^{-}$, *HoxB7GFP* and *Ret*$^{-}$ mice have been described previously (Grote et al., 2008; Grote et al., 2006; Niederreither et al., 1999; Pfeffer et al., 2002; Schuchardt et al., 1994; Srinivas et al., 1999). The *HoxB7Cre* (Yu et al., 2002) mice were purchased from Jackson laboratories.

To genotype *Raldh2* mutant animals, primers RALDH2KO-S (5′-GCCTGACCTATTGCATCTCCCG-3′) and RALDH2KO-AS (5′-GCCATGTAGTGTATTGACCGATTCC-3′) were used for PCR amplification of the mutant allele (annealing temperature 61°C; extension time 45 seconds), and RALDH2WT-S (5′-GAAGCAGACAAGGTGTGTATTGC-3′) and RALDH2WT-AS (5′-CCTTGTCTATATCCACCTGTTA-3′) detected the presence of the wild-type allele (annealing temperature 56°C; extension time 45 seconds). For genotyping of *Ret* mutant embryos, oligos P3 (5′-AGAGGCTATTCGGCTATGACTG-3′) and P4 (5′-CCTGATCGACAAGACCGGCTTC-3′) were used to amplify the mutant allele (annealing temperature 59°C; extension time 45 seconds), and primers P6 ext (5′-CTCTGGGAGAAGGCGAGTTTGGAAAAGTTGTC-3′) and P7ext (5′-CTCTTCAGGAACACTGGCTACCATGTCCATG-3′) detected the wild-type allele (annealing temperature 57°C; extension time 45 seconds).

The experimental design used in these studies was approved by the institutional animal care and use committees at Columbia and McGill Universities.

Retinoic acid rescue

For the generation of *Raldh2*$^{-/-}$ embryos, retinoic acid (RA) supplementation was performed as described previously (Batourina et al., 2005).

Histology, in situ hybridization and immunohistochemistry

Hematoxylin and Eosin staining was performed on 6 μm thick paraffin-embedded sections, according to standard procedures. In situ hybridization on tissue sections was performed as described previously (Pearse et al., 2007). The *Gata3* (George et al., 1994), *Ret* (Pachnis et al., 1993) and *Raldh2* (Batourina et al., 2001) in situ probes have been reported previously. Whole-mount in situ hybridization was performed as described previously (Henrique et al., 1995). Immunohistochemistry was carried out on 8 μm thick frozen sections using standard procedures (Bouchard et al., 2000). The following antibodies and detection reagents were used: rat-anti mouse E-cadherin antibody (Zymed Laboratories, 1:400), In Situ Cell Death Detection Kit (Roche, according to manufacturer's instructions), DAPI (50 μg/ml). Alexa Fluor 488-conjugated anti-rat antibody (Invitrogen, 1:200) was used for secondary detection. Slides were mounted with Slowfade Gold reagent (Invitrogen) and images were acquired on a Zeiss Axioplan2 microscope using Axiovision.

RESULTS

ND insertion is required for proper ureter positioning and depends on Ret signaling

Ret is expressed in a number of sites in the developing urinary tract, including ND epithelia, the CND, the nascent ureteric bud and the ureteric bud tips within the kidney. The majority of *Ret* mutant embryos display renal agenesis due to impaired ureteric bud formation (Schuchardt et al., 1994; Schuchardt et al., 1996); however, as previously reported, the remaining embryos in which the ureter forms display hydronephrosis (Batourina et al., 2002). Further analysis of *Ret*-deficient embryos that succeeded in forming a ureter revealed two types of abnormalities at birth: the first, which occurred in ~30% of embryos examined (4/13), was an abnormal junction between the sex ducts (vas deferens) and

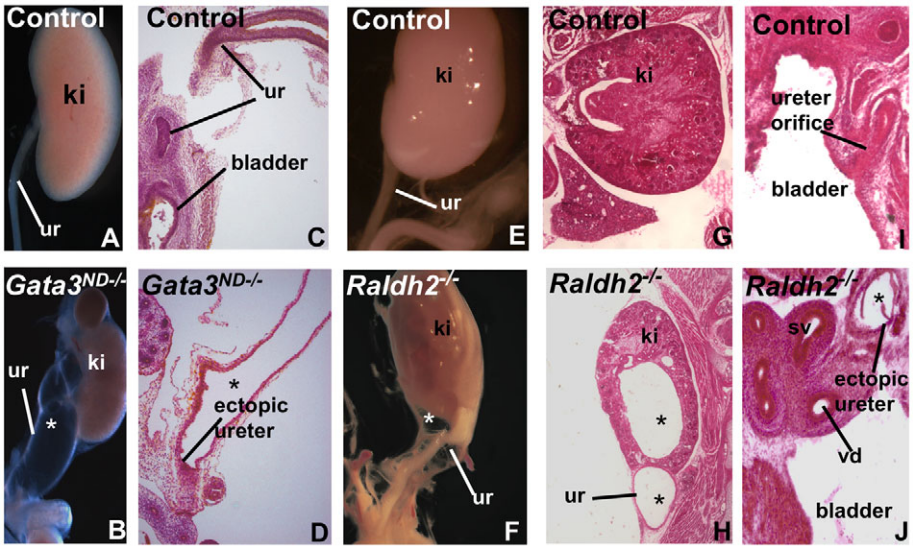

Fig. 2. Hydronephrosis and ectopic ureter termination in *Gata3* and *Raldh2* mutant mouse embryos resemble those present in *Ret* mutants. (**A**) Whole-mount urinary tract from a wild-type control E18.5 embryo showing the kidney and ureter. (**B**) Whole-mount E18.5 *Gata3*$^{ND-/-}$ embryo showing hydronephrosis and megaureter. The asterisk denotes the dilated mutant ureter. (**C**) Hematoxylin and Eosin (H&E) stained section through an E18.5 wild-type embryo at the position where the ureter enters the bladder. (**D**) *Gata3*$^{ND-/-}$ embryo showing an abnormal ureter that terminates bluntly rather than connecting to the bladder. The asterisk denotes the dilated mutant ureter. (**E**,**F**) Whole-mount urinary tracts from E18.5 wild-type (E) and *Raldh2*$^{-/-}$ (F) embryos. Note the hydronephrotic kidney and dilated ureter in F (asterisk). (**G**,**H**) H&E-stained section through E18.5 wild-type (G) and *Raldh2*$^{-/-}$ (H) kidneys. Note that the renal pelvis is not dilated in G, in contrast to the hydronephrosis and hydroureter observed in H (asterisks). (**I**) H&E-stained section through the bladder of an E18.5 wild-type embryo showing normal entry of the ureter. (**J**) Section through an E18.5 *Raldh2*$^{-/-}$ embryo at the level of the prostatic urethra, showing the ectopic ureteral connection in the vas deferens. The asterisk denotes the dilated distal ureter. ki, kidney; sv, seminal vesicle; ur, ureter; vd, vas deferens. Magnifications: 10× in A,B,E-H; 20× in C,D,I,J.

the ureter, without any connection to the urethra or bladder (compare Fig. 1A,B). In humans, such a malformation is called ectopic vas deferens (Delakas et al., 1996; Sukumar et al., 2010) and is likely to cause obstruction at birth. In the second class of *Ret* mutant embryos, which was more common, the sex duct was inserted in the urethra, but the ureters joined the bladder abnormally. Analysis of control and $Ret^{-/-}$ mice bred onto a *Hoxb7-Gfp* transgenic background (Srinivas et al., 1999) enabled us to clearly visualize the path of the intravesicular ureter. In controls, the ureter crossed through the detrusor muscle and submucosa at a 45° angle following a smooth path (Fig. 1C, white arrow). By contrast, $Ret^{-/-}$ ureters made a sharp turn and entered the bladder at an ectopic position compared with that in controls (Fig. 1D, white arrow). Histological analysis revealed that, in addition to an abnormal angle of entry, mutant ureters followed a jagged trajectory through the bladder wall (compare Fig. 1E,F, black arrows).

To elucidate the cause of ectopic ureters and vas deferens in *Ret* mutants at birth, we performed a developmental analysis comparing lower urinary tract formation at different stages. In E11.5 control embryos, the caudal ND segment (the common nephric duct, CND) was inserted in the primitive bladder, and the ureteric bud sprout had formed (Fig. 1G). In $Ret^{-/-}$ embryos, however, the CND was not joined to the primitive bladder (Fig. 1H). Analysis of embryos at later stages revealed that this phenotype persisted through E14.5 but had partially resolved in most embryos by E16.5 (Fig. 1F; data not shown). At this point in the majority of embryos, ureters and NDs were inserted; however, the position of the ureter was abnormal (Figs 1 and 3). Careful analysis of the cloaca and urogenital sinus in *Ret* mutant embryos failed to reveal any overt malformations (data not shown). Hence, the observation that the CND fails to join the primitive bladder in *Ret* mutant embryos points to an early defect in ND morphogenesis. To address this, we analyzed control and *Ret* knockout embryos at E9.5, when NDs normally fuse with the cloaca. Histological analysis of wild-type embryos revealed that, as expected, NDs joined the cloacal epithelium (Fig. 1I). However, in *Ret* mutant littermates, NDs extended close to the cloaca but failed to join (Fig. 1J). This phenotype was present bilaterally in all mutants examined (10/10) suggesting that ND defects are likely to underlie both ectopic vas deferens (Fig. 1B) and ectopic ureters (Fig. 1F). This is a surprising finding because ectopic ureters have not been associated previously with ND defects.

Ret regulators show similar ND insertion defects

Our previous studies reveal that *Ret* expression in the urinary tract depends on Gata3 and retinoid signaling, raising the possibility that these pathways might also be important for the regulation of *Ret* at the stage of ND insertion. To test this, we generated *Hoxb7Cre;Gata3*$^{f/f}$ (*Gata3*$^{ND-/-}$) mice, in which *Gata3* is selectively inactivated in the ND and its derivatives. To investigate whether RA signaling also regulates ND insertion via *Ret*, we analyzed urinary tract formation in embryos lacking *Raldh2*, which encodes the major RA-synthesizing enzyme (Duester, 2008; Niederreither et al., 1999). Analysis of *Gata3*$^{ND-/-}$ and *Raldh2*$^{-/-}$ embryos at birth revealed hydroureter and hydronephrosis (Fig. 2A,B,E,F). To better visualize the cause of hydronephrosis and hydroureter, we performed histological analysis of *Gata3*$^{ND-/-}$ and *Raldh2*$^{-/-}$ urinary tracts at E18.5. In wild-type controls, ureters were patent and joined the bladder (Fig. 2C,I). However, in *Gata3*$^{ND-/-}$ and *Raldh2*$^{-/-}$ embryos, ureters ended blindly or joined the urethra-bladder region at an ectopic location (Fig. 2D,H,J; data not shown). We have not observed ND defects in our previous studies of knockouts lacking both *Rara* and *Rarb2* (*Rarab2* mutants); however, ND-related defects were present in other classes of RA mutants that display more severe developmental abnormalities, consistent with the findings reported here (Batourina et al., 2002; Kastner et al., 1997; Mendelsohn et al., 1994).

To determine whether genitourinary tract anomalies in *Gata3*$^{ND-/-}$, *Raldh2*$^{-/-}$ and $Ret^{-/-}$ embryos were due to a common cause, we compared their respective phenotypes at different developmental stages. Histological staining at E14.5 revealed that in controls the ND was fully connected to the urogenital sinus at the level of the prospective urethra (Fig. 3A), whereas the ureter was inserted in the bladder region (the black arrowhead in Fig. 3A denotes the position of the ureter, which is out of plane). At this stage, the NDs of all three mutants reached the level of the

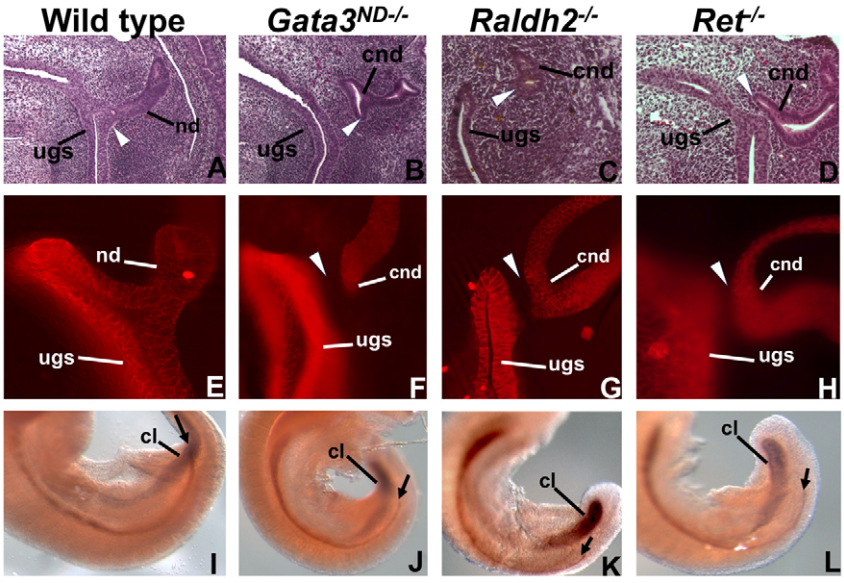

Fig. 3. Abnormal nephric duct (ND) insertion leads to disrupted ureter maturation in *Gata3*$^{ND-/-}$, *Raldh2*$^{-/-}$ and *Ret*$^{-/-}$ mouse embryos. (**A-D**) Sagittal Hematoxylin and Eosin (H&E)-stained sections through the urogenital sinus of E14.5 wild-type (A), *Gata3*$^{ND-/-}$ (B), *Raldh2*$^{-/-}$ (C) and *Ret*$^{-/-}$ (D) embryos. Note that in the three mutants, the NDs are not joined to the urogenital sinus (white arrowheads). Black arrowhead in A indicates ureter entry point. (**E-H**) E-cadherin-stained sagittal vibratome sections through E12.5 wild-type (E), *Gata3*$^{ND-/-}$ (F), *Raldh2*$^{-/-}$ (G) and *Ret*$^{-/-}$ (H) embryos. Unlike controls, the NDs in the three mutants are intact and have not inserted in the urogenital sinus (white arrowheads). (**I-L**) Whole-mount in situ hybridization showing *Pax2* expression in the cloaca and nephric ducts of E9.5 wild-type (I), *Gata3*$^{ND-/-}$ (J), *Raldh2*$^{-/-}$ (K) and *Ret*$^{-/-}$ (L) embryos. NDs in the control embryo terminate in the cloaca (arrow in I); however, mutant nephric ducts terminate prematurely and fail to join the cloaca (arrows in J-L). cl, cloaca; cnd, common nephric duct; nd, nephric duct; ugs, urogenital sinus. Magnifications: 20× in A-H, 10× in I-L.

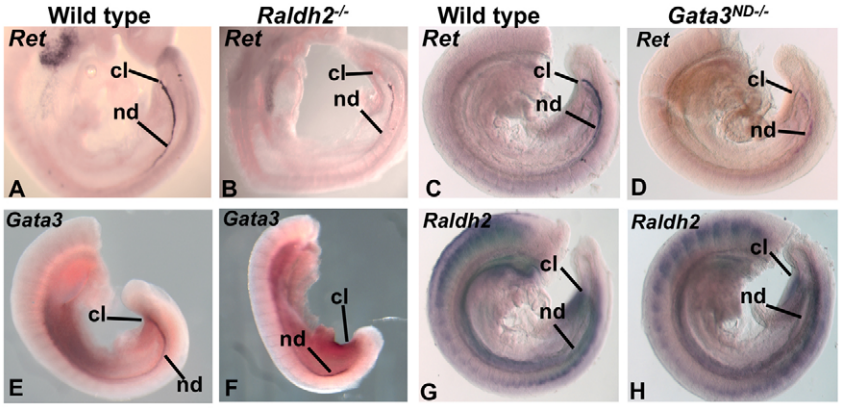

Fig. 4. Retinoids and *Gata3* act in parallel to regulate *Ret* expression in the mouse nephric duct (ND). (**A**) *Ret* expression in the nephric ducts of a whole-mount E9.5 wild-type embryo. (**B**) In situ hybridization analysis of an E9.5 *Raldh2*$^{-/-}$ mutant embryo showing downregulation of *Ret* in the ND, which has terminated prematurely. (**C**) Whole-mount in situ hybridization analysis of an E9.5 wild-type embryo hybridized with *Ret* probe. (**D**) Downregulation of *Ret* in an E9.5 whole-mount *Gata3*$^{ND-/-}$ mutant. (**E**) *Gata3* expression in a wild-type E9.5 embryo. (**F**) *Gata3* expression in an E9.5 *Raldh2*$^{-/-}$ embryo. (**G**,**H**) *Raldh2* expression in control (G) and in a *Gata3*$^{ND-/-}$ mutant embryo (H). cl, cloaca; nd, nephric duct. Magnifications: ×20 in all panels.

urogenital sinus but failed to join the epithelium and were separated by a layer of mesenchymal cells (Fig. 3B-D, white arrowheads). E-cadherin staining of vibratome sections of E12.5 urogenital tracts confirmed this result and further showed that the ureter remained connected to the ND in mutant embryos (Fig. 3F-H).

To determine whether, as in *Ret* mutant embryos, the primary defect leading to abnormal ureter insertion was delayed ND insertion, we performed whole-mount in situ hybridization analysis with *Pax2*, which is localized in both the ND and cloaca. These experiments revealed that the NDs of wild-type embryos had elongated to the posterior of the embryo and turned to fuse with the cloaca (Fig. 3I). By contrast, in all *Gata3*$^{ND-/-}$, *Raldh2*$^{-/-}$ and *Ret*$^{-/-}$ embryos examined, NDs extended successfully along the anteroposterior axis to a level below the limb bud but, in most cases, elongation was arrested and NDs failed to turn toward the midline and fuse with the cloaca (Fig. 3J-L). These results suggest a defect in ND elongation and guidance towards the cloaca as the primary cause of genitourinary tract defects in *Gata3*$^{ND-/-}$, *Raldh2*$^{-/-}$ and *Ret*$^{-/-}$ embryos.

Gata3 and Raldh2 act in parallel to regulate *Ret* expression

Our previous studies suggest that expression of *Ret* in the CND and ureteric bud depends on Gata3 and RA signaling. To determine whether *Ret* expression in the caudal ND also depends on these signaling pathways, we analyzed *Ret* expression in *Gata3* and *Raldh2* mutant embryos at the stage of ND insertion. In situ hybridization analysis of wild-type embryos at E9.5 revealed robust *Ret* expression in the ND extending to the cloaca (Fig. 4A), which was greatly reduced in NDs of *Raldh2*$^{-/-}$ and *Gata3*$^{ND-/-}$ embryos (Fig. 4B-D). Raldh2 and Gata3 might regulate *Ret* in a linear fashion or in parallel. To determine whether RA regulation of *Ret* was via Gata3, we analyzed *Raldh2* mutant and control embryos to determine whether *Gata3* expression was altered. We observed no significant change in *Gata3* expression in the ND of *Raldh2* mutants compared with wild-type embryos (Fig. 4E,F), suggesting that RA signaling is unlikely to be an upstream regulator of *Gata3* in ND cells. To determine whether Gata3 regulates *Ret* expression via the RA pathway, we compared expression of *Raldh2* and RA receptors in wild-type and *Gata3* mutant embryos. These experiments revealed little if any change in expression levels of any of these genes (Fig. 4G,H; data not shown), suggesting that Gata3 is unlikely to be an upstream regulator of RA signaling. Taken together, these experiments suggest that RA and Gata3 are important regulators of *Ret* expression in the ND, most likely acting in parallel pathways.

ND tip cells display cellular extensions that may be important for chemoattraction or migration during the final phase of ND morphogenesis

Ret signaling mediates a number of cellular behaviors including migration and chemoattraction, processes that have been shown to be important for ND formation in other species (Drawbridge et al., 2000). Analysis of whole-mount and sectioned *Hoxb7-Gfp* control embryos at E9.5 revealed the presence of thick

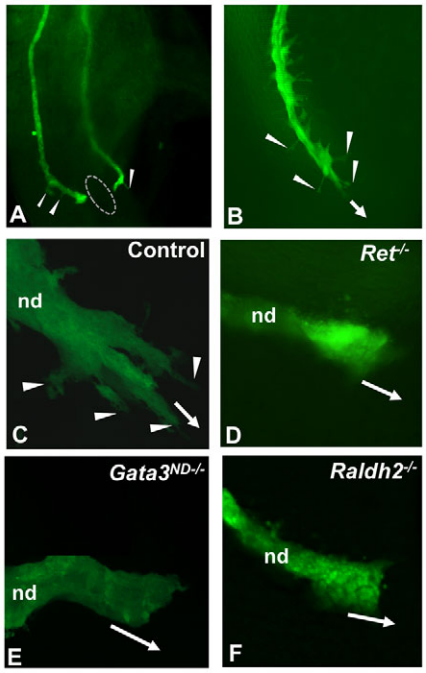

Fig. 5. Wild-type embryos display prominent extensions that are not visible in *Ret*, *Gata3* or *Raldh2* mutant embryos. (**A**) A whole-mount wild-type *Hoxb7-Gfp* embryo at E9.5 showing nephric ducts (NDs) that have extended and migrated to the cloaca (dashed oval). Cellular extensions are visible in the more posterior segment (arrowheads). (**B**) Higher magnification image showing posterior cellular extensions in a control *Hoxb7-Gfp* embryo (arrowheads). (**C**) High magnification image showing cellular extensions (arrowheads) at the tip of a E9.5 *Hoxb7-Gfp* control nephric duct. (**D**) Absence of visible ND extensions in *Hoxb7-Gfp*;*Ret* mutant at E9.5. (**E**) Absence of visible GFP-positive ND extensions in *Gata3*$^{ND-/-}$ mutant embryo at E9.5. (**F**) Absence of ND extensions in E9.5 *Raldh2* mutant embryo. White arrows in B-F denote the direction of growth. nd, nephric ducts. Magnifications: 10× in A; 12× in B; 40× in D-F.

extensions emanating from cells at the ND tip, which were more abundant in the caudal ND segment that has turned toward the midline (Fig. 5A,B). To investigate further whether these cellular processes might be important for ND morphogenesis, we analyzed the morphology of NDs in E9.5 *Ret*$^{-/-}$, *Gata3*$^{ND-/-}$ and *Raldh2*$^{-/-}$ embryos using GFP expression to visualize ND cells. These studies revealed few, if any, extensions in the posterior ND in the mutants compared with controls (Fig. 5C-F). The observation that ND tip cells display prominent cellular processes in wild-type embryos that are absent in all three mutant lines that also display delayed ND extension, suggests that these processes may normally be crucial for ND morphogenesis, and that their formation is regulated by the Gata3-Raldh2-Ret signaling network in ND cells.

DISCUSSION

Failure of nephric duct/cloaca fusion: a novel cause of lower urinary tract defects

The majority of the lower urinary tract anomalies identified to date originate from defects in the induction, growth or differentiation of the kidneys and ureters (Mackie and Stephens, 1975; Murawski and Gupta, 2008; Tanagho, 1976). ND insertion occurs prior to the onset of kidney development; hence, little attention has been paid to developmental defects at this early stage**.** Here, our studies suggest that a nephric duct extension or fusion defect may be a novel cause of urogenital system malformations. We show that in *Gata3*$^{ND-/-}$, *Raldh2*$^{-/-}$ and *Ret*$^{-/-}$ embryos, NDs fail to complete their caudal extension and, as a result, either fail to fuse with the cloacal epithelium or fuse later than normal. Consequently, ureters remain joined to the ND (ectopic vas-deferens), a likely cause of hydronephrosis at birth (Fig. 6).

Importantly, our results suggest that delayed ND insertion can also cause ectopic ureters and hydronephrosis by disrupting later stages of distal ureter maturation, the process by which apoptotic elimination of the CND and distal-most ureter positions the ureteral orifice in the bladder (Fig. 6). Examination of the migrating ND revealed the presence of cellular extensions at the ND tip that are regulated by the Gata3-Raldh2-Ret network and are likely to reflect the active process of ND guidance toward the cloaca. That abnormal ND insertion can lead to ectopically terminating ureters is in retrospect not surprising, as ND morphogenesis and ureter maturation occur at a stage when the bladder and urethra are undergoing dramatic growth and differentiation. Hence, developmental defects that occur prior to ureteric bud formation and that alter the timing of alignment or fusion of the ureters with the bladder, may be an important cause of ectopically terminating ureters and hydronephrosis (Fig. 6).

What factors contribute to ND morphogenesis?

Our studies suggest that there are at least four phases in ND morphogenesis: epithelialization of the ND from intermediate mesoderm, extension along the rostrocaudal axis, migration to the midline and fusion with the cloaca. *Pax2* and *Pax8* mediate one of the earliest events in ND formation. Deletion of these two transcription factors results in failure at the stage of specification of intermediate mesoderm, and failure of the ND to form (Bouchard et al., 2002). Studies in the chick and amphibians suggest that epithelializaton or elongation of the NDs may be regulated by *Bmp4* signals from the overlying ectoderm and laminin in extracellular matrix (Morris et al., 2003; Obara-Ishihara et al., 1999). In the present study, we find that NDs of *Ret* mutant embryos can extend successfully to the posterior of the embryo, but

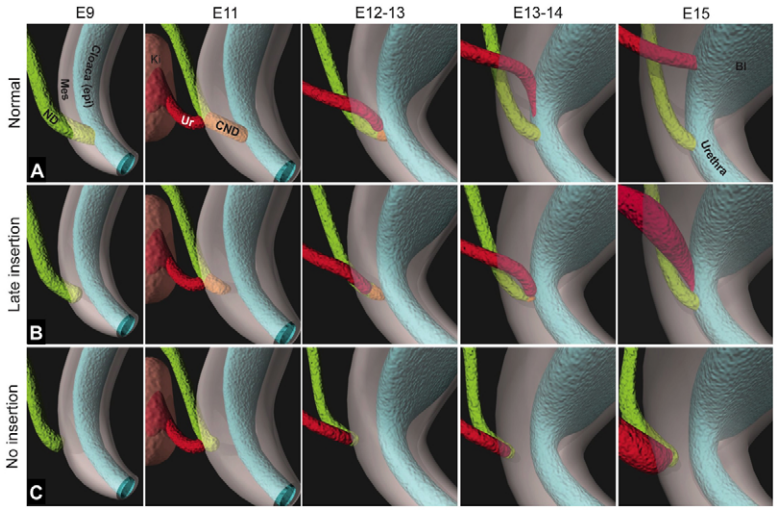

Fig. 6. Model showing how delayed or failed nephric duct (ND) insertion can influence the final position of the ureter orifice. (**A**) Nephric duct insertion and ureter maturation in wild-type embryos. NDs extend to the caudal region of the embryo and insert in the cloaca at E9.5. Ureteric bud formation begins at E10. By 11.5, ureters have elongated and reached the kidney blastema, but are still attached to the caudal ND segment (the CND). Between E12.5 and E13.5, the CND merges with the urogenital sinus and undergoes apoptosis, forming a patent connection between the kidneys and the bladder. Between E13.5 and E14.5, the ureter aligns with and fuses with the urogenital sinus epithelium; as this occurs, the caudal-most ureter segment undergoes apoptosis, generating a new ureter orifice in the bladder primordium. (**B**) Delayed ND insertion at E9.5 affects CND apoptosis and results in defects in alignment and insertion of the ureter into the bladder. As a consequence, distal ureters end up closer to the ND than in controls, which can cause obstruction once renal function begins (dilated ureters in right-most panel). (**C**) Complete failure in ND insertion at E9.5 results in ectopic vas deferens, where the ureter and vas deferens remain fused and fail to connect with the bladder and urethra, respectively, causing obstruction (dilated ureter in right-most panel). Bl, bladder; CND, common nephric duct; epi, cloacal epithelium; Ki, kidney; Mes, mesenchyme; ND, nephric duct; Ur, ureter.

fail to turn toward the midline, suggesting that there is a guidance mechanism that normally regulates late stages of ND extension. We find a similar phenotype in *Gata3*$^{ND-/-}$ and *Raldh2*$^{-/-}$ and we show that *Ret* expression is downregulated in the NDs of these mutants. These observations suggest that *Ret* is normally important in ND cells for the final phase of insertion, whereas Gata3 and RA are important upstream regulators of *Ret* expression in the ND. As *Ret* is also expressed in the cloaca, it is possible that ND defects could be non-cell autonomous, caused by a defect in cloacal morphogenesis. However, a careful examination of *Ret* mutant embryos has not revealed detectable defects in the cloaca, bladder or urethra, arguing against this possibility. In addition, chimeric embryos accumulate wild-type but not *Ret*$^{-/-}$ cells at the ND tip, suggesting a cell-autonomous function in ND tip cell guidance (Chi et al., 2009). Interestingly, we previously identified a ND guidance mechanism regulated by Gata3 at an earlier stage of extension. Germline inactivation of *Gata3* indeed results in a misguidance of the ND that ultimately fuses with the surface ectoderm (Grote et al., 2006), raising the idea of an early Ret-independent and a late Ret-dependent guidance mechanism, both of which would require Gata3 function.

The cellular protrusions extending from posterior ND cells are reminiscent of processes that form in migrating neural crest cells (Druckenbrod and Epstein, 2007), in protrusions at the tips of neuronal growth cones (Lowery and Van Vactor, 2009) and in sprouting vasculature (Gerhardt, 2008). In these systems, attractive and repulsive signals are regulating the directional migration of cells toward their targets. In this case, ND cells would normally respond to attractive signals from the cloaca (such as Gdnf) or repulsive signals from the lateral part of the embryo originating from limb buds or other tissues. Ret-Gdnf signaling regulates migration and cellular process formation in a number of different contexts. For example, Gdnf from gut mesenchyme guides Ret-expressing enteric nervous system precursors to their destination (Druckenbrod and Epstein, 2007; Natarajan et al., 2002), a process that might also be important in ND formation.

Studies in amphibians and fish suggest that Ret signaling acts in a cell-autonomous manner in ND cells, controlling ND migration by a chemotactic mechanism that can be regulated by exogenous Gdnf (Drawbridge et al., 2000). Consistent with this, in mouse embryos, *Gdnf* is localized in mesenchyme at the caudal-most portion of the ND adjacent to *Ret*-expressing ND cells (Hellmich et al., 1996; Pachnis et al., 1993), and its inactivation leads to arrested ND extension (Pichel et al., 1996), a defect similar to the one we observe in *Ret* mutant embryos. Hence, Gdnf might act as a chemoattractant during ND insertion in mammals, guiding the trajectory of ND tip cells toward the cloaca via a Ret-dependent mechanism. Interestingly, experiments in Axolotls suggest that NDs follow a gradient of Gdnf, enabling signaling over several cell diameters that guides the connection of NDs to the cloaca (Drawbridge et al., 2000). Ret-Gdnf signaling also plays an important role in migrating MDCK cells and other cell types, where Ret controls cytoskeletal arrangements that underlie process formation and polarity (Tang et al., 1998; Wang et al., 2010). It is unclear at present how protrusions of ND tip cells regulate migration to the cloaca. Further experiments will be needed to address this question. Together, our experiments suggest that ND insertion depends on Ret, and that *Ret* expression in ND cells depends on RA and Gata3. This novel developmental pathway performs multiple functions during kidney development and our findings suggest that the same is true in the lower urinary tract. Here, we find that Gata3 and RA regulation of *Ret* is required for ND insertion that establishes a primary connection between the upper and lower urinary tract. These signaling pathways are likely to be crucial again during ureter maturation, when the ureter makes final connections with the bladder. To our knowledge, this is the first report directly linking ND insertion to ureter positioning, opening up a new dimension in our thinking about the causes of obstruction and vesicoureteral reflux (VUR).

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Competing interests statement

The authors declare no competing financial interests.

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