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Non-cell-autonomous retinoid signaling is crucial for renal development

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SUMMARY

In humans and mice, mutations in the *Ret* gene result in Hirschsprung's disease and renal defects. In the embryonic kidney, binding of Ret to its ligand, Gdnf, induces a program of epithelial cell remodeling that controls primary branch formation and branching morphogenesis within the kidney. Our previous studies showed that transcription factors belonging to the retinoic acid (RA) receptor family are crucial for controlling *Ret* expression in the ureteric bud; however, the mechanism by which retinoid-signaling acts has remained unclear. In the current study, we show that expression of a dominant-negative RA receptor in mouse ureteric bud cells abolishes *Ret* expression and *Ret*-dependent functions including ureteric bud formation and branching morphogenesis, indicating that RA-receptor signaling in ureteric bud cells is crucial for renal development. Conversely, we find that RA-receptor signaling in ureteric bud cells depends mainly on RA generated in nearby stromal cells by retinaldehyde dehydrogenase 2, an enzyme required for most fetal RA synthesis. Together, these studies suggest that renal development depends on paracrine RA signaling between stromal mesenchyme and ureteric bud cells that regulates Ret expression both during ureteric bud formation and within the developing collecting duct system.

KEY WORDS: Ret expression, Kidney development, Retinoic acid, Mouse

INTRODUCTION

Kidney development depends on reciprocal signaling between metanephric mesenchyme that differentiates into the nephron, the ureteric bud epithelium that forms the collecting duct system, and stromal mesenchyme that differentiates into the renal interstitium. As the fetal kidney grows, nephron induction and branching morphogenesis occur continuously in the nephrogenic zone in the outer cortex, while further differentiation occurs in deeper regions of the kidney. In the nephrogenic zone, ureteric bud tips generate signals important for survival and maintenance of nephron progenitors while, at the same time, signals from metanephric mesenchyme, which trans-differentiates into the epithelial components of the nephron, induce ureteric bud tips to grow and branch (Grobstein, 1953). Surrounding ureteric bud branches and nephron progenitors, cortical stromal cells generate signals required for nephron differentiation and branching morphogenesis (Hatini et al., 1996; Levinson et al., 2005).

Retinoic acid (RA), the active form of vitamin A, is crucial for formation of most organs and tissues, including the kidney. In rodents, maternal vitamin A deficiency results in renal hypoplasia, the severity of which depends on the extent of vitamin A deprivation (Lelievre-Pegorier et al., 1998; Quadro et al., 2005; Wilson, 1953). RA is synthesized from retinol, an inactive precursor, by enzymes belonging to the retinol dehydrogenase and retinaldehyde dehydrogenase (Aldh1a, hereafter referred to as Raldh) families. Retinol dehydrogenases mediate the first step in RA synthesis that converts retinol to retinaldehyde and, with the exception of Rdh10, are widely expressed (Duester et al., 2003). However, enzymes belonging to the Raldh family that convert retinaldehyde to retinoic acid are selectively expressed in the embryo at many sites of active RA signaling, where they are thought to be crucial for regulating the availability of RA (Duester, 2008; Duester et al., 2003; Lin et al., 2003; Marlier and Gilbert, 2004; Niederreither and Dolle, 2008). Gene targeting and studies with RA reporter mice suggest that *Raldh2* accounts for most RA synthesis in the embryo (Mic et al., 2002; Niederreither et al., 1999; Zhao et al., 2009). In the embryonic kidney, *Raldh2* is localized in stromal mesenchyme of the outer cortex and *Raldh3* is expressed in the ureteric bud (Batourina et al., 2001; Mic et al., 2002; Niederreither et al., 2002). It is unknown, however, whether one or both of these enzymes are important for kidney organogenesis.

Once RA becomes available in cells, it binds to and activates retinoic acid receptors (Rars), a family of transcription factors that control gene expression via retinoid response elements in regulatory sequences of target genes. The RA receptor family includes 8 members encoded by 3 genes, *Rara*, *Rarb* and *Rarg*, that are widely expressed during development (Dolle et al., 1990; Leid et al., 1992; Ruberte et al., 1990). Inactivation of individual *Rars* results in few embryonic abnormalities (Lohnes et al., 1993; Lufkin et al., 1993; Luo et al., 1995; Mendelsohn et al., 1994c). However, compound mutants lacking multiple Rar family members display a set of malformations that mirror those induced by maternal vitamin A deficiency (Ghyselinck et al., 1998; Kastner et al., 1995; Lohnes et al., 1994; Luo et al., 1996; Mendelsohn et al., 1994b). Hence, RA signaling in the embryo is either

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functionally redundant, requiring a sufficient level of RA receptor expression in a given cell type, or depends on the concerted activity of more than one receptor species.

Our previous studies of *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants revealed abnormalities in many tissues, including the kidney. At birth, the nephrogenic zone, which in wild-type animals contains nephron progenitors, stromal cells and ureteric bud tips, was absent. (Mendelsohn et al., 1999; Mendelsohn et al., 1994b). *Ret*, a gene required for formation of the ureteric bud and its branching in the kidney (Schuchardt et al., 1996), was downregulated in mutant kidneys and branching morphogenesis was impaired (Mendelsohn et al., 1999). The observation that all of these phenotypes could be rescued by forced expression of *Ret* in ureteric bud cells of *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants suggests that a major function of RA during renal development is in regulating *Ret*, while *Ret* signaling acts downstream of RA, regulating branching and stromal cell patterning (Batourina et al., 2001). The observation that renal hypoplasia and abnormal stromal cell patterning were also observed in mutants lacking *Ret9*, one of two *Ret* isoforms (de Graaff et al., 2001), supports the conclusion that branching and stromal cell abnormalities are linked to impaired *Ret* signaling in the ureteric bud.

Despite what we have learned, a number of questions remain. For example, it is unclear whether branching morphogenesis and *Ret* expression depend on RA generated by Raldh3 in ureteric bud cells, by Raldh2 in stromal cells or both, and it is unclear whether RA receptor signaling regulates *Ret* via an autocrine pathway in ureteric bud cells or by a paracrine mechanism via mesenchyme. The observation previously, that inactivation of *Rara* and *Rarb2* together, but not separately, resulted in renal abnormalities, led us to suggest that their concerted function was required in a given renal cell type for regulating branching morphogenesis and *Ret* expression. In situ hybridization studies revealed that *Rara* and *Rarb2* were colocalized in stromal cells, but not in other cell types, suggesting that stromal cells were the prime mediators of RA signaling (Mendelsohn et al., 1999; Dolle et al., 1990; Visel et al., 2004). Based on these findings, we proposed that RA receptor signaling in stromal cells was important for generating secreted signals that control *Ret* expression and branching in ureteric bud cells (Mendelsohn et al., 1999). More recently however, microarray studies reveal that *Rara* and *Rarb2* are also colocalized in the ureteric bud (Schmidt-Ott et al., 2005) (GUDMAP, http://www.gudmap.org/). Hence, *Rara* and *Rarb2* regulation of *Ret* could occur either via ureteric bud cells or stromal cells.

To begin to address these questions in the current study, we analyzed mutants lacking Raldh2 and Raldh3 to test the requirement for RA-synthesis in the stroma and in ureteric bud cells, respectively. We find that inactivation of Raldh3 has little, if any, effect on kidney development, whereas inactivation of Raldh2 results in phenotypes that are similar to, but slightly less severe than, those in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants. Deletion of both Raldh2 and Raldh3 together increased the severity of malformations, suggesting that Raldh2 is the major source of RA required for kidney development, but that, in its absence, Raldh3 can generate sufficient levels of RA to partially rescue renal morphogenesis.

The observation here that RA can induce branching and *Ret* expression in ureteric buds cultured without mesenchyme, suggests that RA, but not other stromal cell signals, is normally important for regulating branching morphogenesis. We find that in the absence of either Gdnf or RA, *Ret* expression was undetectable and branching was inhibited. Hence, RA and Gdnf are likely to be required independently: RA for inducing expression of *Ret* in ureteric bud cells and Gdnf for activating *Ret* RTK (receptor tyrosine kinase) signaling that controls ureteric bud remodeling. Finally, using a dominant-negative mouse model, we show that blocking RA receptor signaling in ureteric bud cells results in renal hypoplasia and renal agenesis, supporting the idea that RA receptors are required in ureteric bud cells rather than in stroma. Together, these studies reveal a novel stromal-ureteric bud signaling pathway that is required for formation of the renal collecting duct system and in which RA secreted from cortical stroma induces the expression of *Ret* in ureteric bud cells by activating RA receptor signaling. This pathway is likely to be conserved and utilized at other sites in the developing embryo.

MATERIALS AND METHODS

Mouse strains and genotyping

All matings were with Swiss-Webster mice (Taconic). Embryonic day 0.5 (E0.5) was considered to be noon on the day when a vaginal plug was observed. Littermates were used for all experiments in which normal and mutant embryos were compared. The *Hoxb7-Cre* line was generated in the McMahon laboratory (Yu et al., 2002). *RARE-hsp68-lacZ* mice were a generous gift from the Rossant laboratory (Rossant et al., 1991). Genotyping was done by PCR of the tail or yolk sac. PCR genotyping of the *HoxB7-Cre* mice was performed using primers 5′-TGATGAGGTTCG-CAAGAACC-3′ and 5′-CCATGAGTGAACGAACCTGG-3′, generating a 400 bp product. *RARE-hsp68-lacZ* mice were genotyped using primers 5′-CGTCGTCCCCTCAAACTGGCAGATGC-3′ and 5′-TTCGGCG-CTCCACAGTTTCGGGTTTTC-3′ generating a 570 bp product. *RARaDN* mice were genotyped using primers 5′-ATGGTGTACACGT-GTCACC-3′ and 5′-CACCTTCTCAATGAGCTCC-3′. For the wild-type allele we used the primers 5′-TGGCTCGTGTCAAAGAACTG-3′ and 5′-TGGTCGGTAGAAAGGCAGAG-3′. The mutant and wild-type bands were 210 bp and 426 bp, respectively. All PCR protocols were performed using a DNA Thermal Cycler PTC-100 (BIO-RAD, Hercules, CA, USA) with 40 cycles of 94°C for 30 seconds, 53.5°C for 30 seconds and 72°C for 40 seconds, except for the *RARaDN* mutant, where we performed 45 cycles of 94°C for 30 seconds, 54.5°C for 30 seconds and 72°C for 40 seconds.

RA rescue

For the generation of *Raldh2*$^{-/-}$ and *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ embryos, retinoic acid (RA) supplementation was performed as previously described (Batourina et al., 2005). The experimental design used in these studies was approved by the institutional animal care and use committee at Columbia University.

Immunochemistry, histology and non-radioactive in situ hybridization

For histology and in situ analysis, embryos were dissected into ice cold PBS and then transferred to 4% paraformaldehyde (PFA) and fixed overnight at 4°C. Following fixation, embryos were transferred to 30% sucrose overnight at 4°C and then embedded in OCT. Sagittal cryosections (14 mm) were cut, dried and processed directly or stored at –80°C. Histology was performed using Hematoxylin and Eosin staining. Non-radioactive in situ hybridizations and *lacZ* histochemistry were performed as previously described (Mendelsohn et al., 1999).

Quantitation of branching morphogenesis

Hoxb7-GFP;Raldh2$^{-/-}$ and control kidneys were cultured for 3 days to allow explants to flatten, then were fixed in 4% PFA. Images were generated from each sample at low magnification and ureteric bud tips were counted by dividing each sample into four parts. The Student's t-test was used for statistical analysis.

Organ culture

Whole kidneys were cultured under conditions previously described (Mendelsohn et al., 1999). To isolate ureteric buds, kidneys from E11 embryos were placed in DMEM supplemented with collagenase and incubated at 37°C for 15 minutes to dissociate the metanephric mesenchyme from the ureteric bud then transferred to medium supplemented with DNAse

and mechanically separated from the surrounding mesenchyme with fine needles. Isolated ureteric buds were then transferred to growth-reduced Matrigel (BD)-coated Transwell (Costar) filters and embedded in growth-reduced Matrigel. Cultures were in DMEM with or without 125 ng/ml Gdnf and 200 nM all-trans-RA and 9-cis RA.

RT-PCR

Total RNA was isolated from isolated E11 ureteric buds cultured for 32 hours under the conditions described above. RNA was extracted with Trizol reagent (Invitrogen) according to manufacturer's protocol. The quality and concentration of isolated RNA was assessed using an RNA600 Pico Assay Kit (Agilent Technologies). Each RNA dilution (10 ng to 1 mg) was used as a template for first-strand cDNA synthesis using oligo (dT) as a primer, in the presence of Superscript III reverse transcriptase (Invitrogen). One microliter of cDNA was used as a template for the PCR reaction using Platinum Blue PCR Super Mix (Invitrogen). Each experiment was performed in triplicate using the following primers to detect expression: *Foxd1*, 5′-CTCCTCCGTGTCCTCGTCCG-3′ and 5′-AGTTTAGCTCAGAGGGTCCA-3′ generating a product of 286 bp; *Raldh2*, 5′-TGGTGGAACGGGACAGGGCA-3′ and 5′-AGCGATTG-CTGCCCCTGCTG-3′ generating a 418 bp product; *Gdnf*, 5′-TACGGA-GACCGGATCCGAGG-3′ and 5′-CAGGCATATTGGAGTCACTGG-3′ generating a 207 bp product; *Ret*, 5′-GCTATGCCCAGATCGGGAAAG-3′ and 5′-TCAGTAATGGATGTCCCCTCC-3′ generating a 280 bp product; and actin, 5′-CTAAGGCCAACCGTGAAAAG-3′ and 5′-TCT-CAGCTGTGGTGGTGAAG-3′ generating a 282 bp product. β-actin-specific primers were included in all PCR reactions as an internal control. PCR conditions were 35 cycles of: denaturation at 94°C for 2 minutes, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. Amplified products were separated on a 1.2% agarose gel using a 1 kb DNA ladder (GIBCO-BRL) for size comparison.

Generation of a *Rosa26-RARa403* dominant-negative mouse

The human *Rara* truncation mutant (*RARaT403*) (Damm et al., 1993) was isolated from the *pCAGGS* expression vector (Novitch et al., 2003) and subcloned into the multiple cloning site of the *pBIGT* vector (Srinivas et al., 2001) using *Sal*I and *Apa*I enzymes to generate *pBIGT-RARaT403*. Next, the *pBIGT-RARaT403* vector was digested with *Pac*I and *Asc*I to release the floxed *neo-tpa* and *RARaT403* cDNA insert, and the resulting fragment was cloned into the *pROSA26PA* plasmid, which contained the *ROSA26* genomic targeting arms, to generate the final targeting vector, *pROSA26PA-BIGT-RARaT403*. This plasmid was then linearized with *Kpn*I and electroporated into 129/SvEv embryonic stem (ES) cells. Following selection with G418 for 10 days, 96 colonies were picked, expanded and their ES cell genomic DNA screened by genomic Southern blot hybridization after digestion with *Eco*RV. A 5′ probe (Srinivas et al., 2001) was used for Southern blot hybridizations and correctly targeted clones were identified by the presence of an 11 kb wild-type band and a 3.8 kb targeted band, which reflects the targeted allele and includes an additional *Eco*RV site. One clone, E9, was expanded and used to generate the *RaraDN* mouse line.

RESULTS

To begin to study the mechanism by which RA signaling controls renal development, we mapped the distribution of *Raldh2* and *Raldh3*, RA-synthesizing enzymes which account for most of the RA in the embryo. *Raldh2* expression was strong at E11 in mesenchyme and at later stages became restricted to cortical stroma in the nephrogenic zone (Fig. 1A,B). *Raldh2* was also present in comma shaped bodies, then in the differentiating glomerulus in the visceral layer and in podocytes, a pattern similar to that reported in the rat (Marlier and Gilbert, 2004) (data not shown). Our analysis revealed an additional site of *Raldh2* expression in the Loop of Henle: in the hairpin turn that is thought to be a site of tubule elongation (Fig. 1C-E).

Raldh3 expression was observed exclusively in epithelial cells beginning at E11 in the ureteric bud tips and trunk (Mic et al., 2002; Niederreither et al., 2002) (Fig. 1F,G). As the papilla began to form,

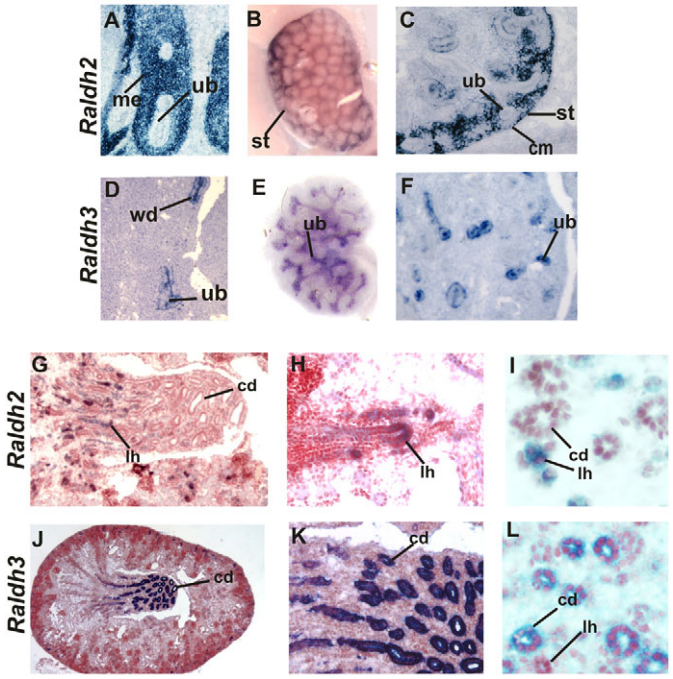

Fig. 1. *Raldh2* and *Raldh3* are selectively expressed in cortical stroma and in the ureteric bud during kidney development. (**A-C**,**G-I**) *Raldh2* expression in a section from an E11 wild-type embryo (A), a wholemount E14 wild-type kidney (B), a section of an E14 wild-type embryonic kidney (C), a section of an E18 wild-type kidney (G), a higher magnification of G (H), and a section through an E18 wild-type embryonic kidney (I). (**D-F**,**J-L**) *Raldh3* expression in a section of an E11 wild-type embryonic kidney (D), a wholemount E14 wild-type embryonic kidney (E), a section of an E14 wild-type embryonic kidney (F), a section of an E18 wild-type embryonic kidney (J), a higher magnification J (K), and a section of an E18 wild-type embryo (L). cd, collecting duct; cm, cap mesenchyme; lh, Loop of Henle; me, mesenchyme; st, stroma; ub, ureteric bud; wd, Wolffian duct. Magnifications: 40× in I,L; 20× in A,C,D,F,H,K; 10× in G; 5× in B,E,J.

Raldh3 expression became particularly intense in the large collecting ducts adjacent to the Loop of Henle where *Raldh2* was localized (Fig. 1H-J). The localized expression of RA-synthesizing enzymes in the Loops of Henle and large collecting ducts suggests that in addition to a role in branching morphogenesis, they might also control RA-dependent functions required for medullary patterning.

Branching morphogenesis depends mainly on RA synthesized in cortical stroma

The distribution of RA-synthesizing enzymes in the embryonic kidney suggests that *Ret* expression and branching depend on *Raldh2* in cortical stromal cells, *Raldh3* in ureteric bud cells, or both. To address this question, we analyzed renal development in mutant mice lacking either Raldh2 or Raldh3. To bypass lethality due to heart defects, *Raldh2* mutants were rescued by adding a small amount of RA to the maternal diet between E7 and E9 as described (Niederreither et al., 1999). Histological analysis at E12.5 revealed that branching had progressed to a similar extent in *Raldh2* mutants compared with wild-type controls (Fig. 2A,B). By E14.5 however, mutant kidneys were greatly reduced in size compared with controls but displayed relatively normal architecture (Fig. 2C,D). By contrast, kidney development was apparently normal in mutants lacking Raldh3 (Fig. 2C,E).

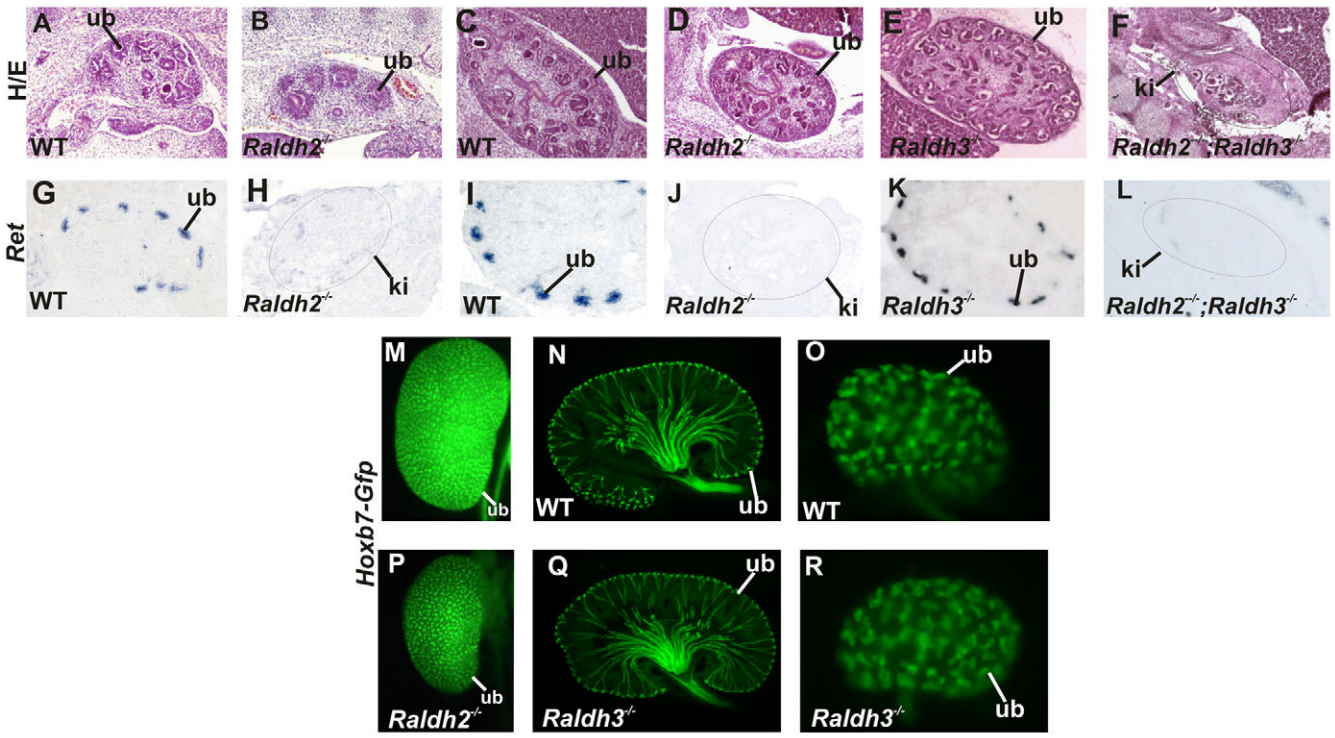

Fig. 2. Renal development depends mainly on *Raldh2*. (**A-F**) A Hematoxylin and Eosin (H/E)-stained section through an E12 kidney from a wild-type embryo (A), an E12 *Raldh2* mutant embryonic kidney (B), an E14 wild-type kidney (C), an E14 *Raldh2* mutant kidney (D), an E14 *Raldh3* mutant kidney (E), and an E14 kidney from a *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ mutant (F). (**G-L**) In situ hybridization showing *Ret* expression in an E12 wild-type kidney (G), an E12 *Raldh2* mutant kidney (H), an E14 wild-type kidney (I), an E14 *Raldh2* mutant kidney (J), an E14 *Raldh3* mutant kidney (K), and an E14 *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ compound mutant kidney (L). (**M**) A wholemount E18 kidney from a wild-type *Hoxb7-Gfp* transgenic embryo. (**N**) A vibratome section through an E18 *Hoxb7-Gfp* wild-type embryo. (**O**) A wholemount E14 control *Hoxb7-Gfp* embryonic kidney. (**P**) A wholemount E18 *Hoxb7-Gfp*;*Raldh2*$^{-/-}$ kidney. (**Q**) A vibratome section through an E18 *Hoxb7-Gfp*;*Raldh3*$^{-/-}$ embryonic kidney. (**R**) A wholemount *Hoxb7-Gfp;Raldh3*$^{-/-}$ E14 kidney. ki, kidney; ub, ureteric bud. Magnifications: 10× in A-L,O,R; 5× in M,N,P,Q.

The observation from our previous studies that forced expression of *Ret* in the ureteric bud could rescue renal development in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants suggests that RA signaling is likely to be crucial for *Ret* expression. To determine whether RA-synthesized by Raldh2 is also important for expression of *Ret*, we performed in situ hybridization analysis to determine whether *Ret* expression was altered in *Raldh2* mutant kidneys. Analysis of embryonic kidneys from E12.5 wild-type and *Raldh2* mutants revealed robust expression of *Ret* in the ureteric buds of wild-type embryos, which was nearly undetectable in *Raldh2* mutants (Fig. 2G,H). At E14, *Ret* expression was greatly reduced in the ureteric buds from *Raldh2* mutant kidneys compared with controls (Fig. 2I,J). Analysis of *Raldh3* mutants revealed wild-type levels of *Ret* expression (Fig. 2K), consistent with the apparently normal renal development observed in this line.

Loss of *Ret* expression is likely to result in reduced levels of branching morphogenesis. To determine whether this was the case, *Raldh2* mutants were crossed with *Hoxb7-Gfp* mice, a transgenic line expressing *Gfp* throughout the renal collecting duct system (Srinivas et al., 1999). Analysis of *Hoxb7-Gfp*;*Raldh2*$^{-/-}$ mutant kidneys at E18 and earlier stages revealed that branch numbers were reduced by 50% compared with wild type littermates (Fig. 2M,P; n=5; data not shown); an 80% reduction in branch numbers was observed in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants (Mendelsohn et al., 1999). Conversely, analysis of *Raldh3*$^{-/-}$;*Hoxb7-Gfp*$^{+/-}$ mutant kidneys revealed branch numbers and ureteric bud patterning that were comparable to controls and consistent with normal histology and *Ret* expression in this line (Fig. 2N,O,Q,R).

One explanation for the relatively mild phenotype in *Raldh2* mutants compared with RA-receptor knockouts could be that Raldh3, which is expressed in the ureteric bud, might provide sufficient RA to partially rescue renal development. To assess whether this was the case, we analyzed compound mutants lacking both *Raldh2* and *Raldh3* to determine whether removing *Raldh3* increases the severity of renal phenotypes in *Raldh2* mutants. Histological analysis of *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ mutant kidneys indeed revealed a more severe pattern of abnormalities reminiscent of those in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ knockouts. *Raldh2*$^{-/-}$;*Raldh3*$^{-/-}$ mutant kidneys were greatly reduced in size compared with mutants lacking *Raldh2* alone, contained few ureteric bud branches or developing nephrons and *Ret* expression in mutant ureteric buds was essentially undetectable (Fig. 2F,L). Hence, *Raldh3* is unlikely to be required normally for renal morphogenesis. However, the observation that deletion of *Raldh3* together with *Raldh2* increases the severity of renal malformations compared with those in single *Raldh2* knockouts, suggests that RA generated by Raldh3 can marginally support development.

One possible explanation for the apparent minimal requirement for Raldh3 during kidney formation is that there might be less RA synthesis in ureteric bud cells or that RA synthesized in ureteric bud

cells is less stable than RA synthesized in stroma. To address this question, we examined whether isolated ureteric bud explants and mesenchymes were capable of synthesizing RA. To detect RA, we performed co-culture experiments, plating isolated ureteric buds and mesenchymes on a lawn of *F9-RARE-lacZ* cells, a cell line containing a stably integrated RA-response element fused to the *lacZ* gene that is activated by nanomolar quantities of RA (Wagner et al., 1992). After 16 hours of culture, *lacZ* activity was undetectable in *F9-RARE-lacz* cells maintained in serum-free medium without added RA (Fig. 3A), indicating that reporter activity was RA-dependent. Addition of 10 nM RA to the culture medium resulted in robust *lacZ* expression (Fig. 3B); however, there was no reporter activity detected in cultures with 10^{-7} M retinol, an inactive RA precursor (Fig. 3C). Hence, reporter activity in *F9-RARE-lacZ* cells is RA-dependent and is not activated by retinol at a concentration of 10^{-7} M.

To determine whether isolated ureteric buds and mesenchymes secrete RA, we cultured explants on lawns of *F9-RARE-lacZ* cells in serum-free medium then assayed co-cultures for *lacZ* expression. After 16 hours, *lacZ* staining was present in a ring of cells surrounding both mesenchyme and ureteric bud explants (Fig. 3D,F), indicating that both tissues secrete at least nanomolar amounts of RA that are sufficient to activate reporter expression. To determine whether ureteric buds and mesenchyme contain active RA-synthesizing enzymes, we investigated whether explants could convert retinol, an inactive RA precursor, to RA at sufficient levels to induce reporter activity. Explants were plated on lawns of *F9-RARE-lacZ* cells with 10^{-7} M retinol then stained for *lacZ* activity. We found that retinol stimulated *lacZ* expression in both ureteric bud and mesenchymal explants (Fig. 3F,G). Although both ureteric bud and mesenchymal compartments synthesize and secrete RA, it is nonetheless possible that stromal cells secrete higher levels of RA compared with ureteric bud cells. As *F9-RARE-lacZ* cells are extremely sensitive to RA, ureteric bud cells might generate RA at concentrations that activate RA-reporter-driven *lacZ* expression that is too low to efficiently activate RA-dependent transcription of *Ret* in ureteric bud cells.

RA, but not other stromal cell-derived signals, is likely to be crucial for inducing *Ret* expression and ureteric bud branching

The observations from microarray analyses that *Rara* and *Rarb2* are co-localized both in the ureteric bud and in stromal cells, suggests that RA-receptor activity could be important in either cell type. To begin to address this question, we investigated whether stromal cell-derived signals were necessary for *Ret* expression and branching by culturing ureteric buds in the absence of mesenchyme. Isolated ureteric buds from E11 embryos were plated in Matrigel in serum-free medium with added Gdnf (Meyer et al., 2004) for 32 hours either with or without added RA. In control experiments, RT-PCR analysis of explants did not reveal detectable expression of *Raldh2* or *Foxd1*, which are localized in the cortical stroma, or *Gdnf*, which is localized in metanephric mesenchyme (see Fig. S1 in the supplementary material), suggesting that ureteric bud cultures were not significantly contaminated with mesenchyme.

In cultures containing both RA and Gdnf, ureteric buds underwent extensive branching and *Ret* expression was upregulated (Fig. 4; see Fig. S1 in the supplementary material). However, in cultures lacking either RA or Gdnf, branching was blocked and *Ret* was undetectable (Fig. 4; see Fig. S1 in the supplementary material). These observations suggest that branching and *Ret* expression depend on RA and Gdnf and do not require additional signals from the stromal mesenchyme. The observations that RA and Gdnf can activate branching and *Ret* expression together, but not separately, suggests that these two signaling pathways act independently in ureteric bud cells, with RA inducing *Ret* expression in ureteric bud cells and Gdnf activating tyrosine-kinase signaling via the *Ret* receptor.

RA-receptor-mediated signaling is required in the ureteric bud for maintaining *Ret* expression and branching

The observation that *Ret* can be induced in isolated ureteric buds in the absence of stromal mesenchyme suggests that, in contrast to our original model, RA receptors are likely to be required for *Ret* expression and branching in ureteric bud cells rather than in stroma.

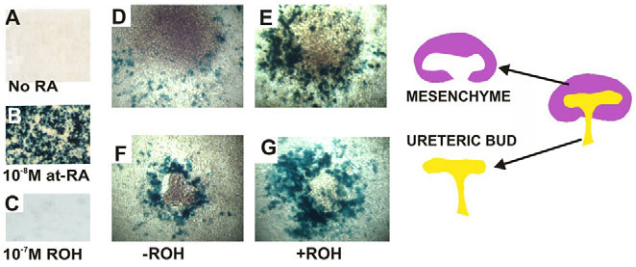

Fig. 3. RA is synthesized by both the ureteric bud and mesenchyme in the embryonic kidney. (**A-C**) *LacZ* expression in *F9-RARE;lacZ* reporter cells cultured for 16 hours in medium without added retinoic acid (RA) (A), with 10^{-8} M at 9-cis RA (B) and with 10^{-7} M retinol (C), an inactive RA precursor. (**D-G**) *LacZ* expression in explants cultured for 16 hours on lawns of *F9-RARE;lacZ* reporter cells: E11 mesenchyme without a source of vitamin A (retinol) (D), E11 mesenchyme with 10^{-7} M retinol (E), E11 ureteric buds plated without retinol (F) and E11 ureteric buds with 10^{-7} M retinol (G). The schematic on the right shows separated metanephric mesenchyme (purple, assayed for *lacZ* activity in 3D,E) and ureteric bud explants (yellow, assayed for *lacZ* activity in 3F,G). Magnification: 20× in A-G.

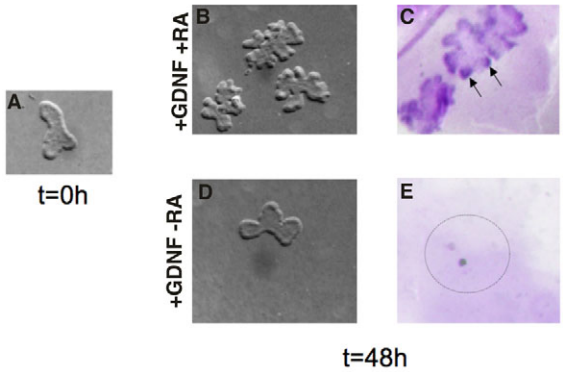

Fig. 4. RA is sufficient for maintaining *Ret* expression and branching of isolated ureteric buds in the absence of mesenchyme. (**A**) A wholemount E11 ureteric bud prior to culture. (**B**) E11 ureteric buds cultured in serum-free medium including Gdnf, with added retinoic acid (RA). (**C**) In situ hybridization showing *Ret* expression in ureteric buds cultured for 48 hours in medium containing Gdnf and RA. (**D**) A wholemount ureteric bud cultured for 48 hours in medium with Gdnf and without added RA. (**E**) Undetectable *Ret* expression in an E11 ureteric bud cultured for 48 hours in medium with Gdnf and without RA. Magnification: 20× in A-E.

To directly address this question, we tested whether inhibition of RA-receptor signaling in the renal collecting duct system would lead to abnormalities similar to those seen in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants.

RaraT403 is a truncated form of human *RARa* that lacks the carboxyl terminal sequence that is normally required for RA-dependent transcriptional activation. Numerous studies show that this mutant receptor inhibits endogenous RA-mediated transcription of target genes in a dose-dependent manner (Blumberg et al., 1997; Damm et al., 1993; Rajaii et al., 2008). Inhibition of RA-receptor signaling would be likely to generate lethal phenotypes, hence we generated a line of mice carrying a dormant *RaraT403* transgene (hereafter called *RaraDN*) inserted into the *ROSA26R* locus (Soriano, 1999). The transgene is preceded by a floxed transcriptional and translational *STOP* sequence that is excised in cells expressing the *Cre* recombinase, activating the expression of the mutant RA receptor (see Fig. S2 in the supplementary material).

To ensure that the *RaraDN* protein was synthesized and actively suppressed RA signaling in the ureteric bud, we first tested whether its expression could efficiently block *RARE-lacZ* expression in RA-reporter mice (Giguere et al., 1990), a line containing a stably integrated RARE-driven *lacZ* transgene, whose expression in the ureteric bud is RA-dependent (see Fig. S3 in the supplementary material). Analysis of E14 control embryos revealed abundant *lacZ* expression in the ureteric bud tree (Fig. 5A). In E14 *Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/+}$;*RARE-lacZ* embryos expressing a single allele of the *RaraDN* transgene, *lacZ* expression in the ureteric bud was greatly reduced but still detectable (Fig. 5B), indicating that RA-signaling was not completely blocked. However, in kidneys from mice expressing both alleles of the *RaraDN* transgene (*Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/flox}$;*RARE-lacZ* animals), *lacZ* activity was undetectable, indicating that, as seen by other groups, this RA dominant-negative mutant can block endogenous RA-receptor-dependent signaling in a dose-dependent manner.

Histological analysis revealed that expression of both alleles of the *RaraDN* mutant also resulted in a phenotype very similar to that in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants; *Hoxb7-Cre*;*RaraDN*$^{flox/flox}$ embryonic kidneys were greatly reduced in size compared with controls, had few ureteric bud branches or nephrons and displayed severe patterning abnormalities (Fig. 5D,E). To determine whether, as in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ and *Raldh2* mutants, these phenotypes were due to loss of *Ret* expression in ureteric bud cells, we analyzed kidneys from dominant-negative mutants to determine whether *Ret* was downregulated. This analysis revealed abundant expression of *Ret* in the ureteric bud tips of controls, which was downregulated in most ureteric bud tips, but still detectable in others (Fig. 5F,G). Consistent with the observation that the *RaraDN* mutant functions in a dose-dependent manner, expression of two alleles of the *RaraDN* transgene in ureteric bud cells resulted in undetectable *Ret* expression (Fig. 5H).

To determine whether branching defects were more severe in kidneys from embryos expressing two alleles of the *RaraDN* transgene compared with those expressing a single allele, *Hoxb7-Cre;RaraDN* mice were intercrossed with *Hoxb7-Gfp* mice to enable us to clearly visualize branching morphogenesis. Analysis of kidneys from at E14 *Hoxb7-Cre;RaraDN*$^{flox/+}$;*Hoxb7-Gfp* embryos expressing one allele of the *RaraDN* receptor revealed renal hypoplasia with a 50-80% reduction in branch numbers (n=11; Fig. 5I,J), whereas in embryos expressing two alleles of the RA receptor mutant (*Hoxb7-Cre;RaraDN*$^{flox/flox}$;*Hoxb7-Gfp* mutants), we observed fewer branch numbers and renal agenesis (n=5/6; Fig. 5L).

Renal agenesis was not observed previously in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants but is the most common phenotype (second to renal hypoplasia) in *Ret* mutants (Schuchardt et al., 1996), indicating that

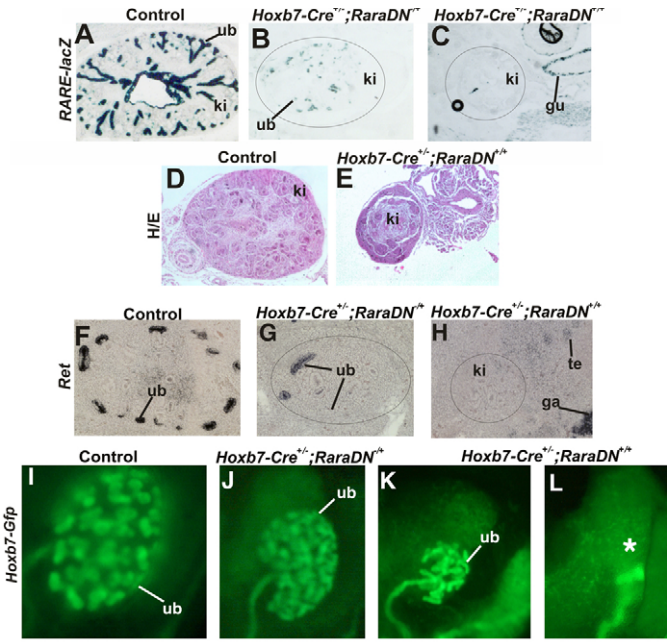

Fig. 5. Expression of *RaraDN* in ureteric bud cells inhibits *Ret* expression and branching in a dose-dependent manner. (**A**) A *lacZ*-stained section from a control (*Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/flox}$;*RARE-lacZ*) embryo. (**B**) A *lacZ*-stained section of an embryo expressing one allele (*Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/+}$;*RARE-lacZ*) of the *RaraDN* transgene. (**C**) Undetectable *lacZ* expression in kidneys in an embryo expressing two alleles (*Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/flox}$;*RARE-lacZ*) of the *RaraDN* transgene. (**D**) A Hematoxylin and Eosin (H/E)-stained section from an E14 *Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/flox}$ control embryonic kidney. (**E**) An H/E-stained section through a kidney from an *Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/flox}$ embryo. (**F**) In situ hybridization analysis of a control (*Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/+}$) embryonic kidney. (**G**) In situ hybridization analysis of an embryo expressing one allele (*Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/+}$) of the *RaraDN* transgene. (**H**) Undetectable *Ret* expression in a sectioned kidney from an E14 (*Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/flox}$ embryo) expressing two alleles of the *RaraDN* transgene. (**I**) *Hoxb7-Gfp* expression in a control (*Hoxb7-Cre*$^{-/-}$;*RaraDN*$^{flox/flox}$) E14 embryonic kidney (**J**) Reduced branching morphogenesis in a *Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/+}$;*Hoxb7-Gfp* E14 kidney from an embryo expressing one allele of the dominant-negative transgene. (**K**,**L**) Renal hypoplasia and renal agenesis, respectively, in *Hoxb7-Cre*$^{-/+}$;*RaraDN*$^{flox/flox}$;*Hoxb7-Gfp* E14 embryonic kidneys from animals expressing two alleles of dominant-negative *RaraDN* transgene. The asterisk in (L) denotes renal agenesis. gu, gut; ki, kidney; te, testes; ub, ureteric bud. Magnification: 10× in A-L.

both ureteric bud formation and branching are *Ret*-dependent events. Hence, as in the kidney, RA signaling might normally be important for maintaining *Ret* expression during ureteric bud outgrowth, and loss of *Ret* expression in the emerging ureteric bud of *Hoxb7-Cre;RaraDN*$^{flox/flox}$ mutants might be the underlying cause of renal agenesis. Ureteric buds sprout from the caudal-most portion of the Wolffian duct between E10.5 and E11, then invade kidney mesenchyme and begin to branch. To determine whether renal agenesis in *Hoxb7-Cre;RaraDN*$^{flox/flox}$ was linked to defective ureteric bud outgrowth, we analyzed *Hoxb7-Cre;RaraDN*$^{flox/flox}$;*Hoxb7-Gfp* mutants at E10 and E11, to determine whether ureteric bud formation was impaired. At E10 in control embryos, the ureteric bud had begun to extend from the Wolffian duct, whereas 1 day later, it had invaded kidney mesenchyme and undergone 1 to 2 generations of branching (Fig. 6A,C). In *Hoxb7-Cre*$^{+/-}$;*RaraDN*$^{flox/flox}$;*Hoxb7-Gfp*

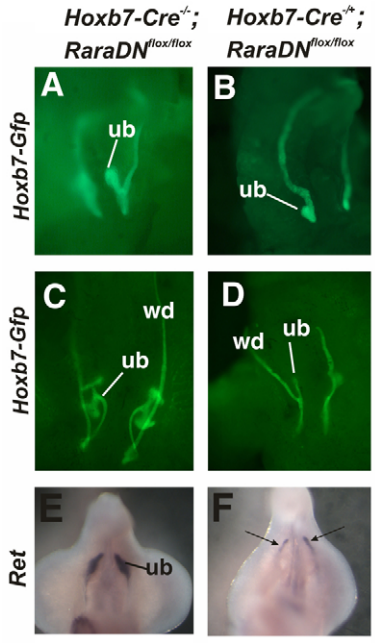

Fig. 6. RA signaling is important for primary ureteric bud formation as well as for branching morphogenesis within the kidney. (**A**,**B**) Ureteric bud formation in a wholemount E10.5 control (*Hoxb7-Cre*$^{-/-}$*;RaraDN*$^{flox/flox}$*;Hoxb7-Gfp*) embryo (A) and an E10.5 *Hoxb7-Cre*$^{-/+}$*;RaraDN*$^{flox/flox}$*;Hoxb7-Gfp* embryo expressing two alleles of the *RaraDN* transgene (B). (**C**,**D**) *Gfp* expression in an E11.5 control embryo (C) and an E11.5 *Hoxb7-Cre*$^{-/-}$*;RaraDN*$^{flox/flox}$*;Hoxb7-Gfp* embryo (D). (**E**,**F**) Wholemount in situ hybridization showing *Ret* expression in the emerging ureteric bud of a control (*Hoxb7-Cre*$^{-/-}$*;RaraDN*$^{flox/flox}$) E10.5 embryo (E) and an E10.5 *Hoxb7-Cre*$^{-/+}$*;RaraDN*$^{flox/flox}$*;Hoxb7-Gfp* embryo (F). The black arrows point to the caudal Wolffian ducts. Magnification: 20× in A-F.

embryos, the ureteric bud was smaller at E10.5 than in control embryos, and at E11 it had barely begun to branch, suggesting that primary ureteric bud formation was indeed abnormal. To determine whether this phenotype was linked to downregulation of *Ret*, we analyzed E10.5 *Hoxb7-Cre;RaraDN*$^{flox/flox}$ embryos and controls for *Ret* expression by wholemount in situ hybridization. In controls not expressing *RaraDN* (*Hoxb7-Cre*$^{-/-}$*;RaraDN*$^{flox/flox}$ embryos), *Ret* was abundant in the Wolffian ducts and in the emerging ureteric bud (Fig. 6E). However, ureteric bud formation was delayed and *Ret* expression was greatly reduced in the Wolffian ducts of *Hoxb7-Cre*$^{+/-}$*;RaraDN*$^{flox/flox}$ mutants. These results suggest that RA signaling is likely to be important at the onset of kidney formation for primary ureteric bud outgrowth and also within the embryonic kidney for branching morphogenesis, in both cases acting as a crucial regulator of *Ret* expression.

DISCUSSION

Studies performed in rodents demonstrate that maternal vitamin A is required for formation of most organs and tissues including the kidney (Lelievre-Pegorier et al., 1998; Quadro et al., 2005; Wilson, 1948). Our previous analysis of animals lacking two RA receptor family members (*Rara*$^{-/-}$*;Rarb2*$^{-/-}$ mice) revealed a similar pattern of defects as that reported in vitamin A deficiency studies (Batourina et al., 2001; Mendelsohn et al., 1994a). The mutant kidney displayed reduced branch numbers, abnormal patterning and downregulation of *Ret*, a gene that is important both for ureteric bud formation and for branching morphogenesis within the developing kidney (Schuchardt et al., 1996). That forced expression of *Ret* in the ureteric bud cells of *Rara*$^{-/-}$*;Rarb2*$^{-/-}$ mutants rescued these kidney defects suggests that a major role of RA-receptor signaling during renal development is to regulate *Ret* expression in the ureteric bud (Batourina et al., 2001).

RA-dependent transcription is regulated via a switch-like mechanism; in the absence of RA, *Rars* can bind to enhancers in regulatory sequences of target genes where they repress basal transcription, but when bound by RA, receptors undergo a conformational change and become potent transactivators. Recent studies suggest that in many cases, RA signaling occurs via a paracrine mechanism where RA receptors expressed in one cell type are activated by RA that is generated in a neighboring cell type (Duester, 2008). Our studies suggest that renal development also depends on paracrine signaling, in this case between stromal cells that synthesize RA and ureteric bud cells that respond to RA. This pathway in turn regulates ureteric bud formation and branching by controlling the expression of *Ret*.

Raldh2 in cortical stroma generates a crucial source of RA that maintains renal development.

Among the Raldh family members, only *Raldh2* and *Raldh3* are expressed at robust levels in the mouse embryonic kidney, where they are localized in cortical stroma and ureteric bud cells respectively (Fig. 1) (Niederreither et al., 2002). Paradoxically, we find that deletion of *Raldh3* has little, if any, effect on renal development, whereas deletion of *Raldh2* results in defects that are similar to, but less severe than, those in RA receptor knockout mice, including reduced kidney size, impaired branching and downregulation of *Ret* in the ureteric bud. The decreased severity of renal defects in *Raldh2* mutants compared with those in RA receptor knockouts suggests that *Raldh3* provides a source of RA that can partially rescue renal development. This suggestion is supported by the observation here that removal of *Raldh3* on a *Raldh2* mutant background increased the severity of kidney defects to the level of those found in *Rara*$^{-/-}$*;Rarb2*$^{-/-}$ knockout mice.

Our findings are in general agreement with those of other groups; *Raldh2* mutants display a spectrum of defects in organogenesis that mirror those observed in vitamin A deficiency (Duester, 2008; Niederreither and Dolle, 2008; Wilson, 1953). However, despite its robust and widespread expression in the embryo, malformations in *Raldh3* mutants are limited to the nasal passage, eyes and brain (Dupe et al., 2003; Halilagic et al., 2007; Molotkova et al., 2007). The observation from our studies and from those of others that deletion of *Raldh2* and *Raldh3* together can increase the severity of embryonic phenotypes compared with those in mutants lacking either enzyme alone, together with studies in RA reporter mice (Molotkov et al., 2006), demonstrates that *Raldh3* is a bona fide RA-synthesizing enzyme. One explanation for the disparity between its expression and function could be that although *Raldh3* is able to synthesize sufficient quantities of RA to activate the expression of sensitive RA reporters, RA synthesis might be at levels that are insufficient to fully activate transcriptional programs in the developing embryo. Whether *Raldh3* or other RA-synthesizing enzymes play a role in post-natal renal development is an interesting question that we will further pursue.

What renal cell types are important for mediating RA-dependent signaling?

We found previously that inactivation of *Rara* and *Rarb2* together, but not separately, generated renal malformations including defective branching and downregulation of *Ret* in ureteric bud

cells (Mendelsohn et al., 1999). In situ hybridization analysis revealed that *Rarb2* and *Rara* were colocalized in stromal cells but not in other renal cell types, suggesting that their concerted function was required in the stromal cell compartment for inducing expression of secreted signals that control *Ret* expression in ureteric bud cells (Mendelsohn et al., 1999). However, more recent microarray studies reveal *Rarb* expression both in stroma and in ureteric bud cells (Schmidt-Ott et al., 2005) (GUDMAP, http://www.gudmap.org/); therefore, RA-receptor signaling could be via ureteric bud cells or via stromal cells. The finding that without stromal mesenchyme, ureteric buds can be induced by RA to express *Ret* and branch, suggests that RA, but not other stromal-cell signals, regulates *Ret* expression by activating RA-receptor-dependent transcription in ureteric bud cells (Fig. 7). Direct evidence for this model comes from experiments in which we demonstrate that blocking RA-dependent transcriptional activity in ureteric bud cells by expressing a dominant-negative RA receptor results in phenotypes resembling those in *Rara*$^{-/-}$;*Rarb2*$^{-/-}$ mutants and in *Ret* mutants (Fig. 5).

How does RA regulate *Ret* in ureteric bud cells?

RA regulates *Ret* in the embryonic kidneys of several species, including rodents (Gilbert, 2002) and amphibians (Osafune et al., 2002). Withdrawal of RA or impaired RA signaling results in downregulation of *Ret* in the ureteric bud (Mendelsohn et al., 1999); however, even at high concentrations, RA is unable to expand the domain of *Ret* expression or induce ectopic expression of *Ret* in embryonic kidneys (Batourina et al., 2001). These findings suggest that RA is likely to be required for maintaining *Ret*, but does not normally activate de novo *Ret* expression in ureteric bud cells. The question of whether RA receptors act by binding to enhancers in *Ret* regulatory sequences or by inducing expression of an intermediary transcription factor in ureteric bud cells is unclear. Several candidate RA response elements have been identified in the *Ret* promoter; however, none of these has been shown to drive RA-dependent expression (Grice et al., 2005; Patrone et al., 1997), suggesting that RA might control *Ret* indirectly. Potential direct activators of *Ret* include *Pax2* (Brophy et al., 2001), Hox genes (Lui et al., 2008), *Ttf1* (Garcia-Barcelo et al., 2007), *Nkx2.1* (Garcia-Barcelo et al., 2008) and *Nurr1* (*Nr4a2* – Mouse Genome Informatics) (Wallen et al., 2001), all of which are localized in the developing kidney.

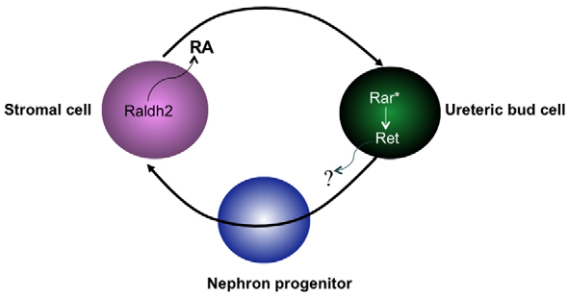

Fig. 7. Model showing *Ret* regulation in ureteric bud cells by paracrine RA signaling. Secreted retinoic acid (RA) synthesized by *Raldh2* in cortical stroma binds to RA receptors in ureteric bud cells and activates *Rar*-dependent signaling (Rar*). RA-dependent transcription induces *Ret* expression in ureteric bud cells, either directly or indirectly.

RA signaling regulates formation of the primary ureteric bud from the Wolffian duct and its branching morphogenesis within the developing kidney via *Ret*

Ret is a gene that, when mutated in humans, can cause renal defects, Hirschsprung's disease and cancer (Dressler, 2008; Moore and Zaahl, 2008; Runeberg-Roos and Saarma, 2007). The majority of *Ret* mutant mice display renal agenesis due to defects at the first stage of branch formation, whereas a minority display renal hypoplasia due to impaired ureteric bud branching within the kidney (Schuchardt et al., 1996). The expression of the *RaraDN* mutant receptor in ureteric bud cells produces a set of renal phenotypes that overlap with those observed in *Ret* knockouts, whose severity depends on the gene dosage. Mutants expressing one *RaraDN* allele display renal hypoplasia, reduced branching and reduced *Ret* expression in the embryonic kidney compared with mutants expressing two *RaraDN* alleles that display renal agenesis or severe hypoplasia and downregulation of *Ret* both at early stages in the emerging ureteric bud and at later stages within the kidney (Fig. 5; Fig. 6). The observation that RA and *Ret* act in a similar manner in the lower urinary tract, regulating ureter insertion via remodeling of the common nephric duct (the portion of the Wolffian duct below the ureteric bud) (Batourina et al., 2005), suggests that this conserved signaling pathway controls epithelial cell remodeling at multiple stages of urinary tract formation: during primary ureteric bud outgrowth from the Wolffian duct, during ureteric bud branching within the kidney and during ureter insertion when common nephric duct remodeling is required for proper insertion of ureters in the bladder.

Studies in rodents suggest that even mild vitamin A deficiency can lead to impaired branching and a reduction in the number of nephrons (Lelievre-Pegorier et al., 1998; Moreau et al., 1998). In this case, renal size and architecture appeared normal but fewer nephrons formed, a phenotype which is probably owing to impaired branching morphogenesis and partial loss of *Ret* expression in the ureteric bud (Moreau et al., 1998). In humans, nephron deficits are associated with increased risk of hypertension (Brenner and Mackenzie, 1997). That mild (sub-clinical) vitamin A deficiency, which is widely found in the human population, might also lead to nephron deficits is a real possibility (Bhat and Manolescu, 2008).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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