Development 126, 1139-1148 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV6389

Stromal cells mediate retinoid-dependent functions essential for renal development

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Accepted 5 January; published on WWW 15 February 1999

SUMMARY

The essential role of vitamin A and its metabolites, retinoids, in kidney development has been demonstrated in vitamin A deficiency and gene targeting studies. Retinoids signal via nuclear transcription factors belonging to the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families. Inactivation of RARα and RARβ2 receptors together, but not singly, resulted in renal malformations, suggesting that within a given renal cell type, their concerted function is required for renal morphogenesis. At birth, RARαβ2$^-$ mutants displayed small kidneys, containing few ureteric bud branches, reduced numbers of nephrons and lacking the nephrogenic zone where new nephrons are continuously added. These observations have prompted us to investigate the role of RARα and RARβ2 in renal development in detail. We have found that within the embryonic kidney, *RARα* and *RARβ2* are colocalized in stromal cells, but not in other renal cell types, suggesting that stromal cells mediate retinoid-dependent functions essential for renal development. Analysis of RARαβ2$^-$ mutant kidneys at embryonic stages revealed that nephrons were formed and revealed no changes in the intensity or distribution of molecular markers specific for different metanephric mesenchymal cell types. In contrast the development of the collecting duct system was greatly impaired in RARαβ2$^-$ mutant kidneys. Fewer ureteric bud branches were present, and ureteric bud ends were positioned abnormally, at a distance from the renal capsule. Analysis of genes important for ureteric bud morphogenesis revealed that the proto-oncogene *c-ret* was downregulated. Our results suggest that RARα and RARβ2 are required for generating stromal cell signals that maintain *c-ret* expression in the embryonic kidney. Since *c-ret* signaling is required for ureteric bud morphogenesis, loss of *c-ret* expression is a likely cause of impaired ureteric bud branching in RARαβ2$^-$ mutants.

Key words: Retinoid, Renal development, Stromal cell, c-ret

INTRODUCTION

Kidney development is initiated when metanephric mesenchyme is invaded by an outgrowth of the Wolffian duct, the ureteric bud. Nephrons, functional units of the kidney, are formed via reciprocal inductive interactions between metanephric mesenchyme, which differentiates into the epithelial components of the nephron, and the ureteric bud, which gives rise to the renal collecting duct system (Saxen, 1987). The ureteric bud generates signals inducing metanephric mesenchyme to proliferate, aggregate, epithelialize and differentiate into nephrons (Grobstein, 1953, 1955), while metanephric mesenchymal cells generate signals required for growth and arborization of the ureteric bud (Erickson, 1968; Ekblom, 1992; Grobstein, 1953, 1955). Metanephric mesenchyme also gives rise to other cell types, including stromal cells, which do not differentiate into nephrons or the collecting duct system (Aufderheide et al., 1987; Herzlinger et al., 1992). Although it was initially thought that signaling between the ureteric bud and metanephric mesenchyme was sufficient for nephron induction and ureteric bud arborization, recent studies indicate that stromal cells are also likely to generate important signals required for renal morphogenesis (Hatini et al., 1996; reviewed in: Bard, 1996; Lechner and Dressler, 1997), however, these stromal cell signals have not yet been identified.

Renal morphogenesis depends on retinoids, biologically active metabolites of vitamin A. Vitamin A deficiency results in severe renal malformations (Wilson and Warkany, 1948), while in vitro, retinoids modulate renal morphogenesis. Exogenous retinoids stimulate ureteric bud branching and nephron formation in rat embryonic kidneys in culture (Vilar et al., 1996) and lead to upregulation of expression of *c-ret* (Moreau et al., 1998), a receptor tyrosine kinase required for ureteric bud outgrowth and branching (Schuchardt et al., 1994, 1996). This modulation of *c-ret* expression is reversible;

without addition of exogenous retinoids, expression declines, but is reinduced after retinoids are added to the culture medium (Moreau et al., 1998). Thus, in vitro, retinoid stimulation of renal morphogenesis may be mediated in part via c-ret.

The retinoid signal is transduced by transcription factors belonging to the retinoic acid receptor (RAR) and retinoid X receptor (RXRs) families. RAR/RXR heterodimers bind to enhancer elements located in regulatory regions of retinoid-responsive genes, where they activate transcription only in the presence of retinoids (for reviews, see Chambon, 1996; Mangelsdorf and Evans, 1995). The RAR family contains eight major isoforms (RARα1 and α2, RARβ1, β2, β3 and β4, and RARγ1 and γ2), which are differentially expressed during embryonic development and display evolutionary conservation (Dolle et al., 1990; Giguere et al., 1990; Krust et al., 1989; Leroy et al., 1991; Zelent et al., 1989, 1991). Nonetheless, few developmental defects were observed in mouse mutants lacking individual RAR isoforms (Ghyselinck et al., 1997; Li et al., 1993; Lohnes et al., 1994; Lufkin et al., 1993; Mendelsohn et al., 1994b). Compound mutant mice lacking multiple RAR isoforms died before or shortly after birth, however, and displayed severe malformations of many organs and tissues, including the kidney (Lohnes et al., 1994; Mendelsohn et al., 1994a; Luo et al., 1996; Kastner et al., 1997). These findings suggest that retinoid receptors can mediate overlapping functions required for renal morphogenesis.

We have previously shown that the kidneys of RARαβ2^{-} mutants, which lack RARα1, RARα2 and RARβ2, are small at birth and contain reduced numbers of nephrons and ureteric bud branches. In addition, RARαβ2^{-} mutant kidneys lack the nephrogenic zone, the outer cortical region where new nephrons are continuously added until the first week of post-natal life (Mendelsohn et al., 1994a). This led us to examine in detail the role of RARα and RARβ2 in renal development.

We show here that in normal embryonic kidneys, *RARα* and *RARβ2* are coexpressed in stromal cells, but not in other renal cell types, suggesting that stromal cells mediate retinoid-dependent functions required during renal development. We found that nephron differentiation and morphology appeared normal in RARαβ2^{-} mutants at early embryonic stages, but growth and branching of the ureteric bud was impaired. At E12, *c-ret* expression was almost undetectable in the mutant ureteric bud, and expression of *wnt-11*, a molecule whose transcription is modulated by GDNF-c-ret signaling in embryonic kidneys (Pepicelli et al., 1997) was also reduced. These findings suggest that retinoid-dependent stromal cell signals are normally required to maintain *c-ret* expression in the embryonic kidney, and that in their absence, c-ret signaling and ureteric bud morphogenesis are impaired. These stromal cell-derived signals may regulate *c-ret* expression by acting on the ureteric bud, or because of the reciprocal nature of renal induction, may be required in metanephric mesenchyme for maintaining *c-ret* expression in the ureteric bud.

MATERIALS AND METHODS

Generation and genotyping RARαβ2^{-} mice

RARαβ2^{-} mice were generated by intercrossing RARα$^{+/-}$/RARβ2$^{-/-}$ males with RARα$^{+/-}$/RARβ2$^{+/-}$ females (Mendelsohn et al., 1994a). Offspring were genotyped using the polymerase chain reaction. To detect the *RARα* mutant allele, three primers were used: a 5′ *RARα* primer, 5′-TGTGCCCTTCCCTCCATCTTCCTTA-3′, a 3′ *RARα* primer, 5′-TCCGACTTGCGACTCCCTCTACTCA-3′, and a 3′ Neomycin primer, 5′-GCCTTCTATCGCCTTCTTGACGAGTTC-TTC-3′. Both *RARα* primers were derived from exon 9 of the *RARα* gene. The amplified wild-type and mutant products were 580 bp and 365 bp, respectively. To detect the *RARβ2* mutant allele, two *RARβ2* primers were used, both derived from exon 1 of the *RARβ* gene: a 5′ primer, 5′-GATTCTGGGCTGGGAAAAAG-3′, and a 3′ primer, 5′-CGGTGTAGAAATCCAGGATC-3′. The 3′ Neomycin primer was the same as above. The RARβ2 wild-type and mutant products were 165 bp and 300 bp, respectively. The PCR run for both the *RARα* and *RARβ2* genes included 30 cycles of 30 seconds at 94°C, 30 seconds at 62°C and 30 seconds at 72°C.

Histology

Dissected embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3 overnight at 4°C, washed in PBS (0.1 M PB, pH 7.3 and 0.15 M NaCl), then immersed in 30% sucrose in 0.1 M PB, pH 7.3, overnight at 4°C and embedded in OCT compound (Miles). Frozen sections (10-15 μm) were cut, dried and stained with Gill's Hematoxylin and Eosin.

In situ hybridization

In situ hybridization analysis was performed with digoxigenin-labeled riboprobes. cDNAs were linearized and riboprobes were generated as previously described (Pfaff et al., 1996). *BF-2* was linearized with *Eco*RI and a 0.7 kb antisense RNA transcript was synthesized with T7 polymerase (Hatini et al., 1996); *BMP-7* was linearized with *Xba*I and a 0.3 kb RNA transcript was generated with Sp6 polymerase (Lyons et al., 1995); *c-ret* was linearized with *Sac*II and a 3.3 kb transcript was generated with T3 polymerase (Pachnis et al., 1993); *GDNF* was linearized with *Hin*dIII and a 0.7 kb antisense transcript was generated with SP6 polymerase (Hellmich et al., 1996); *GDNFRα* was linearized with *Hin*dIII and a 1.4 kb antisense transcript was generated with T7 polymerase (S. Srinivas and F. Costantini); *Pax-2* (Dressler et al., 1990) was linearized with *Bam*HI and a 0.6 kb transcript was generated with T3 polymerase; *WT-1* (Kriedberg et al., 1993) was linearized with *Not*1 and a 1.4 kb transcript was synthesized with T3 polymerase. *Wnt-4* (Stark et al., 1994) was linearized with *Bam*HI and a 0.4 kb transcript was generated with T7 polymerase. *Wnt-11* was linearized with *Xho*I and a 2.1 kb transcript was generated with T3 polymerase (Kispert et al., 1996). The *RARα* cDNA was linearized with *Eco*RV and a 1.6 kb transcript was generated with T3 polymerase (Dolle et al., 1990). This probe detects expression of both *RARα1* and *RARα2* mRNAs. The *RARβ2* cDNA was linearized with *Hin*dIII and a 1.7 kb antisense transcript was generated with T7 polymerase (Dolle et al., 1990). Note that although the *RARβ2* riboprobe contains 3′ sequences conserved among all *RARβ* isoforms, and can cross-hybridize with *RARβ1/β3* mRNAs, the hybridization seen represents *RARβ2* expression; in situ hybridization with riboprobes that distinguish between *RARβ* isoforms revealed *RARβ2* but not *RARβ1/β3* transcripts in the embryonic kidney, while *RARβ1/β3*-specific riboprobes detected *RARβ1/β3* transcripts at extra-renal sites, including the ventral neural tube (data not shown).

Embryos were collected in ice-cold PBS and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer and stored either in 100% methanol at –20°C or processed directly for embedding in OCT as above. Sections were cut, air-dried for 1-3 hours, then fixed in 4% PFA for 10 minutes at room temperature. After washing 3× with PBS, sections were treated with proteinase K (1 μg/ml for 3 minutes at room temperature), then washed 3× with PBS, acetylated for 10 minutes at room temperature and washed again with PBS. Prehybridization was for 2 hours or overnight at room temperature in a humidified chamber. Hybridizations were performed at 68-72°C overnight in a humidified chamber. Prehybridization and hybridization solution was 50% formamide, 5× SSC, 5× Denhardts,

250 μg/ml baker's yeast RNA (Sigma), 500 μg/ml herring sperm DNA (Sigma). Washes were at 72°C in 5× SSC for 5-10 minutes, then at 72°C in 0.2× SSC for 1 hour. Sections were stained overnight with anti-digoxigenin antibody (Boehringer-Mannheim), at a 1:5000 dilution in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl with 1% heat-inactivated goat serum. After staining overnight at 4°C in a humidified chamber, slides were washed in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl. Alkaline phosphatase activity was detected by developing slides in BCIP, NBT (Boehringer-Mannheim) and 0.25 mg/ml levamisole in a humidified chamber for 1-4 days in the dark. Sections were dehydrated and mounted in Permount (Fisher Scientific).

Quantification of ureteric bud branches and visualization of nephrons

Embryonic kidneys were dissected at E14 and grown overnight at 37°C in a humidified incubator on Transwell-Clear polyester membranes (Costar) floating in DMEM/F12 50/50 mix (Cellgro) in the absence of serum or growth factors, which allows the embryonic kidneys to flatten on the filter, but prevents further development in vitro. Embryonic kidneys were then fixed with 2% paraformaldehyde and processed for lectin histochemistry as already described (Gilbert et al., 1994). Embryonic kidneys were stained with rhodamine-coupled peanut agglutinin (PNA), which labels glomeruli, and fluorescein-coupled dolichous biflorus agglutinin (DBA), which labels the ureteric bud epithelium. Briefly, following fixation, kidneys were washed in PBS and permeabilized with saponin and neuraminidase, then incubated with Tritc-PNA to label glomeruli. To visualize the ureteric bud, following culture, embryonic kidneys were stained with fluorescein coupled-DBA, and the number of ureteric bud ends at the periphery of the flattened kidneys were counted.

RESULTS

RARα and *RARβ2* are co-expressed in stromal cells but not in other renal cell types

The observation that renal morphogenesis was impaired in animals lacking RARα and RARβ2 (RARαβ2^{-} mutants), but not in animals lacking either RARα or RARβ2, suggested that they can function redundantly in renal development. To identify cells expressing *RARα* and *RARβ2* transcripts in normal embryonic kidneys, in situ hybridization analysis was performed. *RARα* transcripts were expressed at low levels throughout the embryonic kidney, in the ureteric bud, metanephric mesenchyme and stromal cells (Fig. 1A,B). *RARβ2* expression, however, was restricted to the stromal compartment (Fig. 1C,D). *RARβ2* was seen as early as E11, after invasion of the metanephric mesenchyme by the ureteric bud, in a ring of cells at the edge of the metanephric blastema, which may contain presumptive stromal cells (data not shown).

RARβ2 expression was then compared with that of *BF-2*, a transcription factor whose expression within the embryonic kidney is confined to cells of the presumptive stromal lineage (Hatini et al., 1996). At E12, *RARβ2* and *BF-2* transcripts were excluded from the ureteric bud and metanephric mesenchyme, but were found in the renal capsule and in the stromal cells surrounding the ureteric bud branches and nephrogenic mesenchyme (Fig. 1C,E). *RARβ2* transcripts were also present in stromal cells surrounding the ureter (star in Fig. 1C), where *BF-2* expression was not detected. By E14, the embryonic

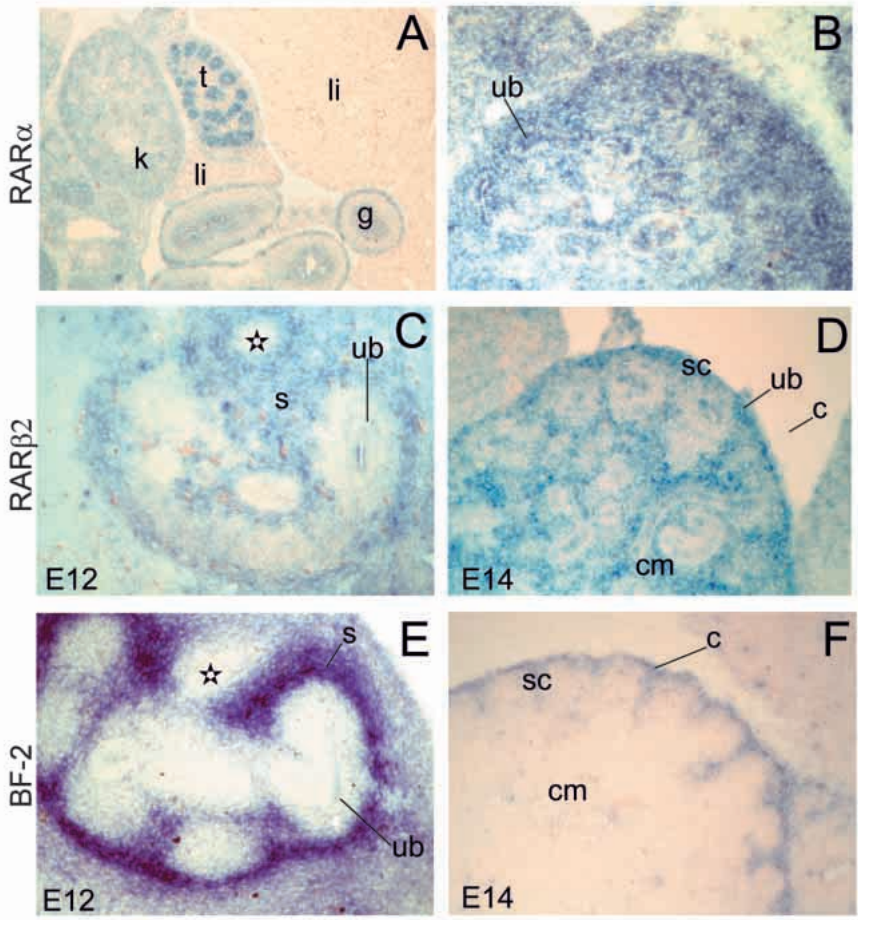

Fig. 1. *RARα* and *RARβ2* are colocalized in stromal cells in the wild-type embryonic kidney. In situ hybridization with digoxigenin-labelled riboprobes. (A) Transverse section of an E14 wild-type embryo hybridized with an *RARα*-specific riboprobe that recognizes both *RARα1* and *RARα2*. Note the absence of *RARα* transcripts in the liver, and the robust expression in seminiferous tubules of the testes and in the mesenchyme of the gut. (B) Higher magnification of A, showing *RARα* expression in the embryonic kidney, in stromal cells, metanephric mesenchymal cells and in the ureteric bud. (C-F) Comparison of *RARβ2* (C,D) and *BF-2* (E,F) expression in E12 (C,E) and E14 (D,F) embryonic kidneys. (C) In E12 embryos, *RARβ2* transcripts are present in presumptive stromal cells surrounding the ureteric bud tips and metanephric mesenchyme, and in the presumptive stromal cells surrounding the ureter (star). (D) At E14, *RARβ2* expression is observed in the renal capsule and subcapsular stromal cells and in the cortico-medullary stromal cells of the embryonic kidney. (E) In E12 wild-type embryos, *BF-2* transcripts are present in presumptive stromal cells, which surround the ureteric bud and metanephric mesenchyme, but not in the cells which surround the ureter (star). (F) At E14, *BF-2* expression is localized in the renal capsule and in the subcapsular stromal cells. *BF-2* transcripts are undetectable in cortico-medullary stromal cells, which express *RARβ2* transcripts. c, renal capsule; cm, cortico-medullary region; sc, subcapsular region; k, kidney; li, liver; g, gut; s, stromal cells; t, testes; ub, ureteric bud. (A) ×50; (B-F) ×200.

kidney is subdivided into cortical and medullary regions. At this stage and at subsequent stages, *RARβ2* was expressed in both cortico-medullary and in subcapsular stroma, while *BF-2* expression was maintained in subcapsular stroma, but barely detectable in cortico-medullary stroma (Fig. 1D,F). The findings that both *RARβ*2 and *BF-2* are expressed in the capsule and subcapsular stroma, but only *RARβ2* expression is maintained in cortico-medullary stroma, suggest that the subcapsular stroma and cortico-medullary stroma are distinct sub-populations which may also perform distinct functions.

Ureteric bud branching is impaired in RARαβ2⁻ embryonic kidneys

We have previously shown that, at birth, RARαβ2⁻ kidneys are severely malformed (Mendelsohn et al., 1994a). We first analyzed mutants at earlier embryonic stages to assess the extent of ureteric bud branching. Embryonic kidneys were dissected from wild-type embryos and RARαβ2⁻ mutants, and stained with dolichous biflorus, which labels the ureter epithelium. In mutants, the number of ureteric bud tips was reduced fourfold compared with wild-type embryos (Fig. 2A,B; 15.0±1.4 ureteric bud branches were counted for mutants (n=4) versus 61.8±6.6 for wild-type embryos (n=8), P=<0.01). At this early stage of renal morphogenesis, only 4-5 generations of branches were observed in mutants compared to 8-9 in wild-type embryos (Fig. 2A,B). Histological analysis performed on serial sections of RARαβ2⁻ mutant embryos confirmed the reduction in ureteric bud ends and revealed additional abnormalities. At E14, wild-type embryonic kidneys displayed numerous ureteric bud tips in the subcapsular region (Fig. 2C,E). The corresponding region in mutant embryonic kidneys lacked ureteric bud termini and instead contained a dense ring of mesenchymal cells (Fig. 2D,F). Thus in the absence of RARα and RARβ2 in stromal cells, ureteric bud branching is impaired, and the subcapsular region appears histologically abnormal, as a dense layer of mesenchymal cells lacking ureteric bud ends.

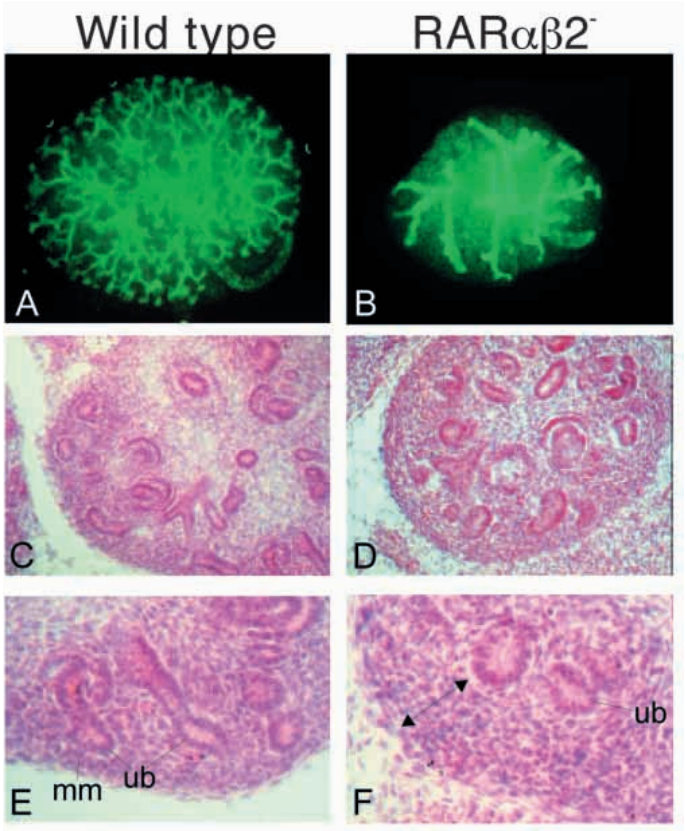

Fig. 2. Ureteric bud branching is impaired in RARαβ2⁻ mutants. Comparison of the pattern of DBA staining in mutant and wild-type embryonic kidneys. (A) Wild-type kidney at E14 showing numerous ureteric bud branches. (B) Mutant E14 embryonic kidney. The number of ureteric bud ends is reduced about fourfold in mutants compared to wild-type embryos. Transverse sections of embryonic kidneys from E14 wild-type (C,E) and mutant (D,F) embryos stained with Hematoxylin and Eosin. Note that mutant embryonic kidneys are much smaller than wild type. (C) Ureteric bud termini are present in the subcapsular domain of wild-type embryonic kidneys. (D) Ureteric bud ends of RARαβ2⁻ mutant embryonic kidneys are located in a recessed position away from the subcapsular region. (E) Higher magnification of C, showing ureteric bud ends in the subcapsular region and adjacent induced metanephric mesenchymal cells of a wild-type embryonic kidney. (F) High magnification of D. The double-headed arrow denotes the subcapsular domain, which does not contain ureteric bud ends, but instead contains a thick layer of mesenchymal cells. ub, ureteric bud; mm, metanephric mesenchyme. (A-D) ×100; (E,F) ×400.

c-ret is downregulated in the ureteric bud of RARαβ2⁻ mutants

The reduction in the number of ureteric bud branches in RARαβ2⁻ mutant embryonic kidneys suggests that retinoid-regulated signals are required either by the ureteric bud or the metanephric mesenchyme for normal branching morphogenesis. To determine whether ureteric bud morphogenesis was abnormal, embryonic kidneys from RARαβ2⁻ mutants were analyzed for ureteric bud-specific expression of genes that are likely to be important for branching morphogenesis. c-ret is required for ureteric bud outgrowth and branching (Schuchardt et al., 1994, 1996), GDNFRα facilitates c-ret signaling by forming a complex between GDNF and c-ret (Treanor et al., 1996; Sanicola et al., 1997), and wnt-11 is a signaling molecule whose expression in embryonic kidney is upregulated by exogenous GDNF (Pepicelli et al., 1997). At E11, RARαβ2⁻ mutant and wild-type embryonic kidneys appeared similar in size and morphology. At this stage, *c-ret* was expressed in the ureteric bud of wild type embryonic kidneys (Fig. 3A), and in extra-renal sites in RARαβ2⁻ mutants (data not shown); however, expression was reduced in the ureteric bud of mutant embryos (n=3) (Fig. 3B). By E12, *c-ret* expression was restricted to the ureteric bud tips of wild-type mutants, but was almost undetectable in the ureteric bud tips of RARαβ2⁻ mutants (n=4) (Fig. 3C,D). This downregulation of *c-ret* expression was seen at later stages in mutant embryonic kidneys (n=5) (Fig. 3E,F), although transcripts were present at extra-renal sites, including the gut, ventral neural tube and dorsal root ganglia (data not shown).

Analysis of RARαβ2⁻ mutants at E12 revealed that, although *c-ret* transcripts were almost undetectectable (Fig. 3C,D), *GDNFRα* expression was maintained in the ureteric bud at levels comparable to wild-type embryos (n=2) (Fig. 4A,B). *Wnt-11* was also expressed in the ureteric bud of RARαβ2⁻ mutants; however, overall levels of expression were somewhat reduced compared to wild-type embryos (n=2) (Fig.

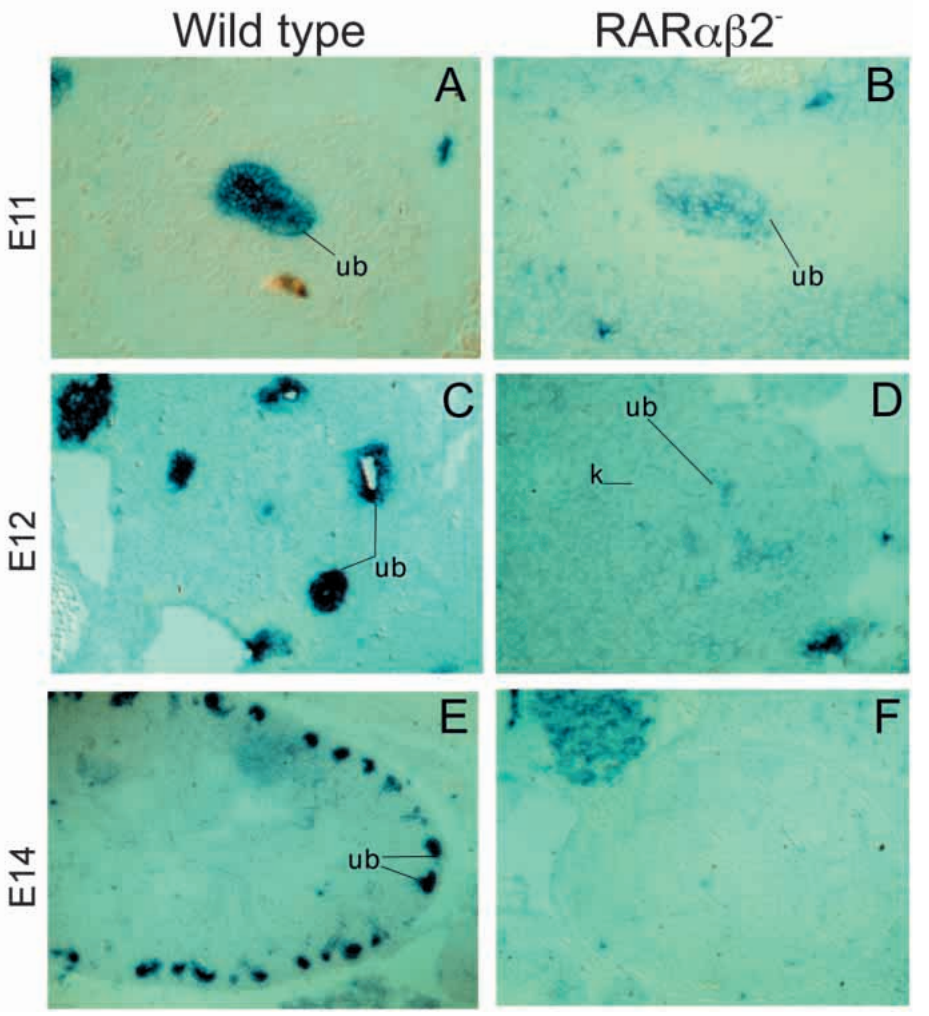

Fig. 3. *c-ret* expression is downregulated in the ureteric bud of RARαβ2⁻ mutant embryonic kidneys. In situ hybridization of wild type (A,C,E) and RARαβ2⁻ mutant (B,D,F) embryonic kidneys with *c-ret* riboprobe. (A) *c-ret* is expressed in the ureteric bud in wild-type embryonic kidneys at E11. (B) *c-ret* expression is greatly reduced in the ureteric bud of E11 RARαβ2⁻ mutants. (C) Expression of *c-ret* in the embryonic kidneys of E12 wild-type embryos. (D) Although ureteric bud ends are present, *c-ret* expression is almost undetectable in RARαβ2⁻ mutant embryonic kidneys at E12. (E) *c-ret* expression in the ureteric bud ends of wild-type embryonic kidneys at E14. (F) *c-ret* expression is undetectable in the ureteric bud of RARαβ2⁻ mutants at E14. The white circles in D and F designate the outline of the mutant embryonic kidneys. k, kidney; ub, ureteric bud. (A,B,E,F) ×200; (C,D) ×100.

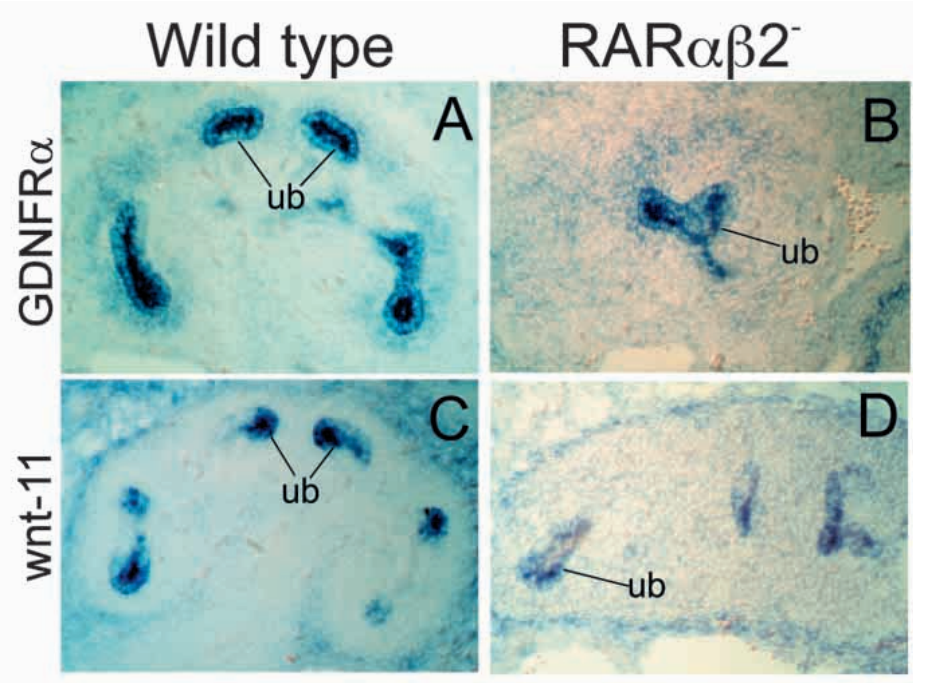

Fig. 4. *Wnt-11* and *GDNFRα* are maintained in the ureteric bud of RARαβ2⁻ mutants. (A) Expression of *GDNFRα* in wild-type E12 embryonic kidneys. Note strong expression in the ureteric bud ends. (B) Expression of *GDNFRα* is maintained in the ureteric bud of E12 mutant kidneys. (C) Expression of *wnt-11* in the ureteric bud of wild-type embryonic kidneys at E12. Transcript expression is localized in ureteric bud tips. (D) *Wnt-11* expression is maintained in ureteric bud tips of RARαβ2⁻ mutants, but overall levels are slightly reduced compared to wild-type embryos. ub, ureteric bud. ×200.

4C,D). Expression of GDNFRα and *wnt-11* in the mutant ureteric bud at a time when *c-ret* is almost undetectable suggests that loss of *c-ret* is likely to be an underlying cause of impaired ureteric bud morphogenesis in RARαβ2⁻ mutants, rather than a result of a general defect in ureteric development.

Metanephric mesenchymal cell differentiation appears normal in RARαβ2⁻ mutants at E12

Since renal morphogenesis involves reciprocal signaling between the ureteric bud and metanephric mesenchyme, loss of *c-ret* expression in the ureteric bud of RARαβ2⁻ mutants may result from defects in the metanephric mesenchyme. We therefore examined mutant embryonic kidneys at E12 with three molecular markers required for renal development that label metanephric mesenchymal cell types at different stages of differentiation: *WT-1* (Kriedberg et al., 1993), *GDNF* (Hellmich et al., 1996; Moore et al., 1996; Pichel et al, 1996) and *Pax-2* (Dressler et al., 1990; Rothenpieler and Dressler, 1993). *WT-1* was expressed at similar levels in both wild-type and RARαβ2⁻ mutant embryonic kidneys in peripheral and condensing metanephric mesenchyme surrounding the ureteric bud tips (Fig. 5A,B). *GDNF* expression in condensing metanephric mesenchyme surrounding the ureteric bud tips, was equivalent in wild type and RARαβ2⁻ mutants (Fig. 5C,D), while *Pax-2* expression was maintained at similar levels in wild type and RARαβ2⁻ mutants in induced metanephric mesenchyme surrounding the ureteric bud (Fig. 5E,F). Thus, at E12, metanephric mesenchymal cells in RARαβ2⁻ mutant embryonic kidneys expressed early differentiation markers, despite the absence of *c-ret* in the ureteric bud tips.

The distribution of stromal cells in the subcapsular region is abnormal in RARαβ2⁻ mutants

We examined mutant embryonic kidneys to determine whether survival or differentiation of stromal cells was affected in the absence of RARα and RARβ2. In normal embryos, *BF-2* labels subcapsular stromal cells and *RARβ2* is localized in both subcapsular and cortico-medullary stroma. Note that we have previously shown that *RARβ2* transcript expression is maintained in RARβ2 knockout mice, although no protein is made (Mendelsohn et al., 1994b). Expression of both *RARβ2* and *BF-2* were maintained in RARαβ2⁻ embryonic kidneys at E14, but their expression pattern differed from that in wild-type embryos, and was expanded to include a thick ring of cells encircling the mutant embryonic kidneys (Fig. 6A-D). Thus, stromal cells survive in RARαβ2⁻ mutants, but their distribution at the periphery of the embryonic kidney appears to be abnormal.

To determine whether a defect in the stromal cell compartment led to a secondary defect in the metanephric mesenchyme, mutant embryonic kidneys were examined to determine whether differentiation or survival of metanephric mesenchymal cell types was affected. At E14, *GDNF* expression was maintained in RARαβ2⁻ mutants in condensing cells surrounding the ureteric bud (Fig. 6E,F). *WT-1* transcripts were expressed throughout the thickened layer of putative stromal cells at the periphery of the mutant embryonic kidneys, in condensing mesenchyme adjacent to the ureteric bud and in the podocyte precursors in S-shaped bodies and glomeruli (Fig. 6G,H). Consistent with *WT-1* expression in differentiating glomeruli, PNA staining of mutant embryonic kidneys independently confirmed that morphologically normal nephrons were formed in mutant embryonic kidneys (data not shown). *Pax-2* and *BMP-7* transcripts were present in both mutant and wild-type embryonic kidneys, in condensing mesenchyme adjacent to the ureteric bud, in pretubular aggregates and in the ureteric

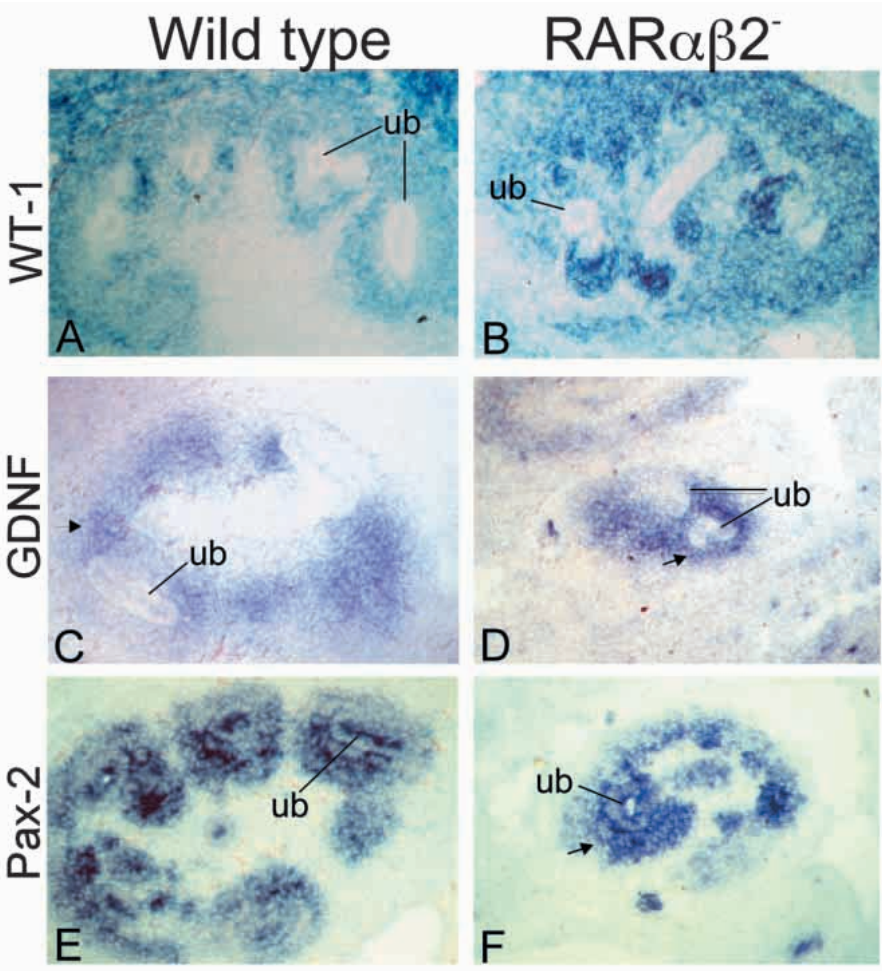

Fig. 5. Expression of early markers of induced metanephric mesenchyme in RARαβ2⁻ mutant embryonic kidneys. In situ hybridization of transverse sections of wild-type (A) and mutant (B) E12 embryos showing expression of *WT-1* in peripheral and condensing metanephric mesenchyme surrounding the ureteric bud tips. Expression of *GDNF* is maintained in condensing metanephric mesenchyme surrounding the ureteric bud tips in wild-type (C) and mutant (D) embryonic kidneys at E12. *Pax-2* expression in metanephric mesenchymal condensates adjacent to the ureteric bud in wild-type (E) and mutant (F) embryonic kidneys at E12. ub, ureteric bud. ×200.

bud (Fig. 6I-L). The presence of metanephric mesenchymal cells expressing *GDNF*, *Pax-2* and *BMP-7* and *WT-1*, inside the putative stromal layer surrounding the recessed ends of the ureteric bud, suggests that loss of retinoid signaling does not affect metanephric mesenchymal cell survival or differentiation. Rather, retinoids are likely to be required for regulating stromal cell signaling or differentiation.

In summary, metanephric mesenchymal cell differentiation appears normal and nephrons are formed despite the absence of RARα and RARβ2 in stromal cells. However, ureteric bud

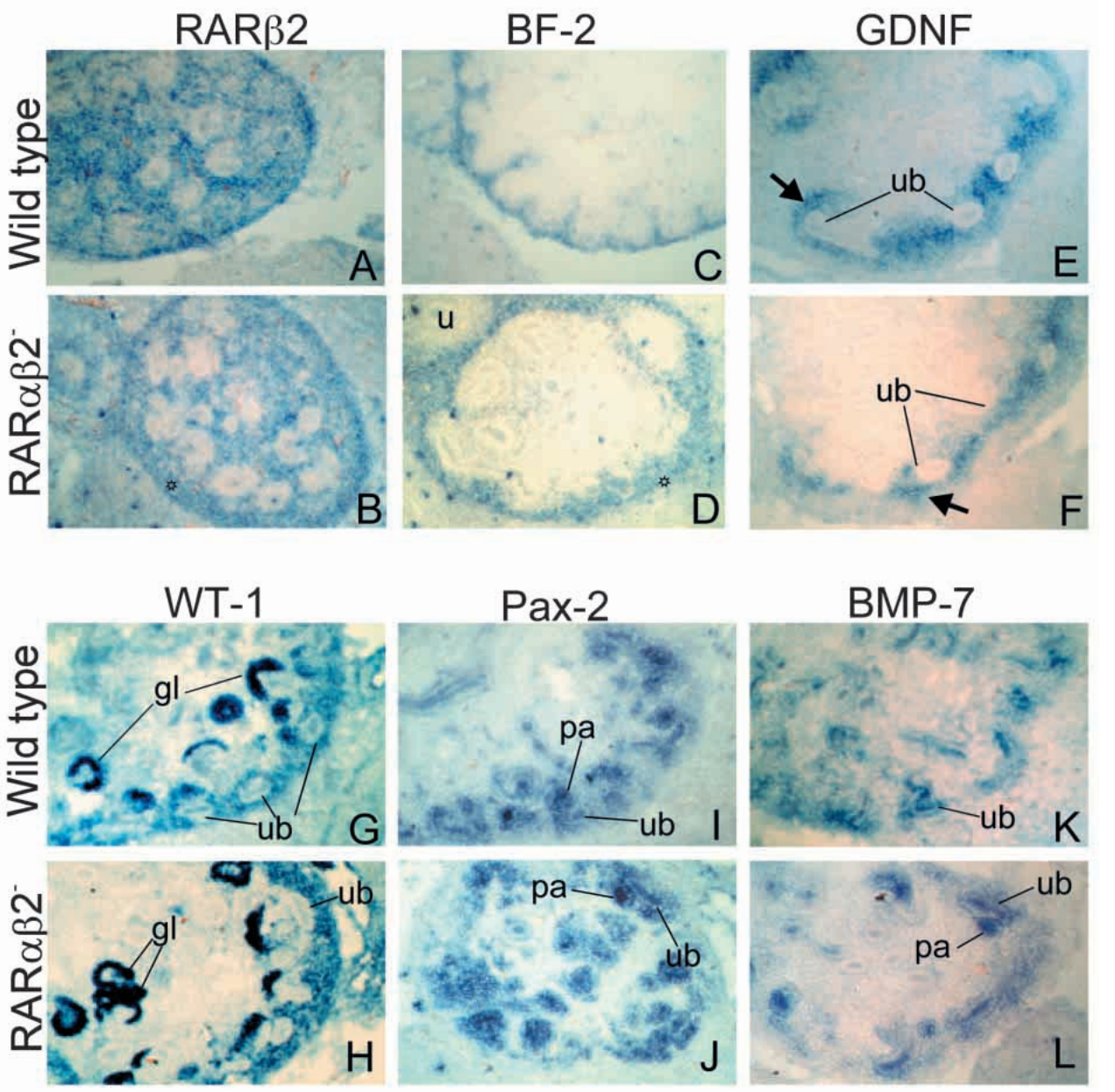

Fig. 6. Stromal cells survive but are distributed abnormally in RARαβ2⁻ mutant embryonic kidneys. Transverse sections of E14 embryonic kidneys hybridized with digoxigenin-labelled riboprobes. (A) Expression of *RARβ2* in the stromal cells of wild-type embryos at E14. (B) *RARβ2* expression is maintained in stromal cells in RARαβ2⁻ mutants. Note that *RARβ2* transcripts are generated in RARβ2⁻ knockout mice although the protein is not made. (C) *BF-2* expression in subcapsular stromal cells of wild-type E14 embryonic kidneys. (D) *BF-2* expression is maintained in the subcapsular stroma of RARαβ2⁻ mutants. Note the increased thickness of the peripheral stromal layer in the mutant embryos (star). (E-L) Metanephric mesenchymal cell differentiation appears normal in RARαβ2⁻ mutant embryonic kidneys at E14. *GDNF* expression in condensing metanephric mesenchymal cells surrounding the ureteric bud (arrow) in wild type (E) and mutant (F) embryonic kidneys. *WT-1* expression in wild-type (G) and mutant (H) embryonic kidneys. Note *WT-1* expression in the mutants, in metanephric mesenchyme surrounding the ureteric bud and in podocyte precursors in S-shaped bodies. Expression of *Pax-2* in pretubular aggregates, condensing metanephric mesenchyme adjacent to the ureteric bud and in the ureteric bud of wild-type (I) and RARαβ2⁻ mutant embryonic kidneys (J). *BMP-7* expression is maintained at E14 in pretubular aggregates, condensing metanephric mesenchyme and in the ureteric bud of wild-type (K) and RARαβ2⁻ mutant embryonic kidneys (L). gl, glomeruli; pa, pretubular aggregates; ub, ureteric bud. ×200.

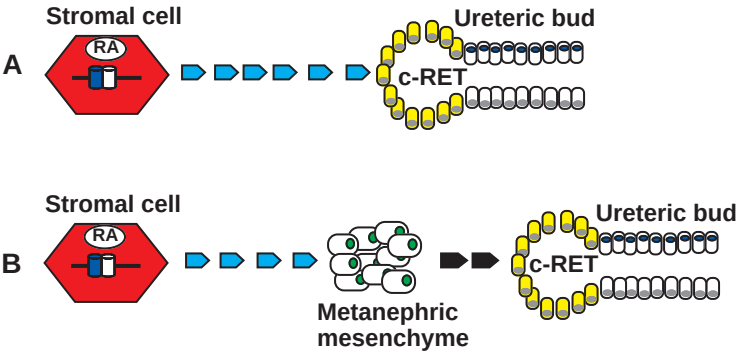

Fig. 7. A model for the role of retinoid signaling mediated by stromal cells in the embryonic kidney. Retinoid-dependent transcription of stromal cell genes leads to the production of signals required for renal morphogenesis. (A) Retinoid-dependent stromal cell signals act on the ureteric bud controlling *c-ret* expression. (B) Retinoid-dependent stromal cell signals act first on metanephric mesenchyme. In response to stromal cell signals, metanephric mesenchymal cells generate signals regulating *c-ret* expression in the ureteric bud. Red hexagons represent stromal cells containing retinoid receptors and RA. Blue arrowheads represent retinoid-dependent stromal cell signals. Cells in the ureteric bud tips expressing *c-ret* are shown in yellow and cells in the ureteric bud trunk are shown in white.

branching is impaired, and *c-ret* expression is downregulated in the mutant ureteric bud.

DISCUSSION

Retinoids are essential for renal development

Depletion of vitamin A or inactivation of retinoic acid receptors during embryonic development generates renal malformations. The most severe phenotype observed is renal agenesis, in which the ureteric bud fails to form, probably due to agenesis of the caudal portions of the Wolffian duct (Mendelsohn et al., 1994a). Renal hypoplasia is present in both vitamin A-deficient fetal rats and in mutants lacking multiple retinoid receptors (Wilson and Warkany, 1948; Mendelsohn et al, 1994a; Luo et al., 1996; Kastner et al., 1997). At birth, RARαβ2^{-} mutant kidneys contained fewer ureteric bud branches and nephrons, and lacked the nephrogenic zone (Mendelsohn et al., 1994a). These initial observations have led us to a more detailed investigation of kidney development in RARαβ2^{-} mutants. We have shown that stromal cells, but not other renal cell types, co-express *RARα* and *RARβ2* and are therefore likely mediators of retinoid-dependent functions essential for renal development. Early in renal development, metanephric mesenchymal cell differentiation appears normal, but ureteric bud branching is impaired, and *c-ret* is downregulated in the ureteric bud of RARαβ2^{-} mutants. Consistent with disruption of the c-ret signaling pathway, expression of *wnt-11*, a downstream target of c-ret-GDNF signaling (Pepicelli et al., 1997), is also reduced in the ureteric bud of RARαβ2^{-} mutants. These findings suggest that RARα and RARβ2 are required for generating stromal cell-derived signals that maintain *c-ret* expression in the ureteric bud (Fig. 7), and that in the absence of these signals, *c-ret* expression is downregulated, and branching morphogenesis is impaired.

Stromal cells produce multiple signals required for renal development

BF-2^{-} and RARαβ2^{-} knockout mice both display impaired stromal cell function, but their respective phenotypes differ, suggesting that BF-2 and retinoids activate distinct stromal cell signaling pathways. Stromal cells survived in both BF-2^{-} and RARαβ2^{-} mutants; however, in BF-2^{-} mutants, the stromal cell compartment was morphologically normal (Hatini et al., 1996), while in RARαβ2^{-} mutants, stromal cells were abnormally distributed, forming a thick peripheral layer devoid of ureteric bud ends. Nephrogenic mesenchymal cells and ureteric bud ends were localized inside the peripheral layer of putatative stromal cells, rather than in their normal position beneath the renal capsule. This thickening of the stromal cell layer in RARαβ2^{-} mutants may reflect intrinsic abnormalities in stromal cell differentiation or proliferation resulting from loss of retinoid signaling, or alternatively, may be a secondary effect of impaired ureteric bud signaling, or metanephric mesenchymal differentiation.

Nephron formation was reduced in both BF-2^{-} and RARαβ2^{-} mutant kidneys; however, in BF-2^{-} mutants, nephrogenesis was blocked at the level of epithelialization, leading to a dramatic reduction in nephron formation (Hatini et al., 1996), while in RARαβ2^{-} mutants, metanephric mesenchymal cell differentiation was apparently normal, and nephrons were formed. In both BF-2^{-} and RARαβ2^{-} embryonic kidneys, the extent of ureteric bud branching was reduced. However, examination of *c-ret* expression suggests that in these two cases the underlying causes of impaired ureteric bud function differ. In wild-type embryos, *c-ret* is initially expressed throughout the ureteric bud; however, once the ureteric bud begins to branch, expression is downregulated in the ureteric bud trunk, becoming restricted to the ureteric bud tips in the subcapsular region (Pachnis et al, 1993). In BF-2^{-} mutant embryonic kidneys, *c-ret* expression was inappropriately maintained in both the ureteric bud trunk and tips, perhaps a reflection of impaired ureteric bud differentiation (Hatini et al., 1996), whereas in RARαβ2^{-} mutant embryonic kidneys, *c-ret* expression was downregulated throughout the ureteric bud. Thus, both BF-2 and retinoids are required for stromal cell function; however, they appear to activate different signaling pathways. Retinoid-dependent signals maintain *c-ret* expression in the ureteric bud, and BF-2-dependent signals are required for epithelialization of nephrogenic mesenchyme and ureteric bud differentiation.

Retinoid regulation of *c-ret*

Ureteric bud branching was impaired in RARαβ2^{-} mutants, and *c-ret* expression was downregulated in the embryonic kidney. Consistent with loss of *c-ret* expression, *wnt-11*, a downstream target of c-ret-GDNF signaling (Pepicelli et al., 1997), was also reduced in the ureteric bud of RARαβ2^{-} mutants. These findings suggest that retinoid-dependent signals normally maintain *c-ret* expression, and that loss of c-ret signaling may underly renal malformations in RARαβ2^{-} mutants. Results of in vitro studies support this suggestion. In serum-free cultures of rat embryonic kidneys, exogenous retinoids stimulate nephron induction via enhanced ureteric

bud morphogenesis (Vilar et al., 1996), and this is closely correlated with an increase in expression of *c-ret*. In the absence of retinoids, *c-ret* expression was lost in embryonic kidneys after 2 days in culture, but could be reinduced after addition of RA (Moreau et al., 1998). These findings indicate that retinoid-dependent signals propagated within the embryonic kidney can modulate *c-ret* expression, and suggest that these signals may normally be required for maintaining its expression. Inactivation of *RARα* and *RARβ2* in stromal cells could result in downregulation of *c-ret* by disrupting stromal cell signaling. These stromal cell signals may normally maintain *c-ret* expression by acting either on the metanephric mesenchyme or ureteric bud (Fig. 7).

Mouse mutants in which *c-ret* function is inactivated by gene targeting, display severe renal malformations (Schuchardt et al., 1994). In most cases, the ureteric bud failed to branch from the Wolffian duct, or never reached the metanephric mesenchyme (Schuchardt et al., 1996). In RARαβ2$^-$ mutants, the ureteric bud was always present, but its growth and branching were impaired later on. *c-ret* expression was greatly reduced at E11 and almost undetectable by E12, suggesting that a small amount of *c-ret* transcript or protein may persist in mutant embryonic kidneys. The presence of low levels of *c-ret* in RARαβ2$^-$ mutants at E11 could be sufficient initially to support ureteric bud outgrowth and branching, but insufficient to support continued growth and branching of the ureteric bud. Clearly *c-ret* expression does not depend solely on RARα and RARβ2, since in RARαβ2$^-$ mutant embryos, *c-ret* mRNA was present in several extrarenal locations, suggesting that at these sites, expression depends on other RAR family members or is retinoid-independent.

Our results suggest a model where retinoid-dependent signals produced by stromal cells play an essential role in renal development, at least in part, by regulating *c-ret* expression in the ureteric bud (Fig. 7). In this model, activation of the retinoid signaling pathway induces expression of RA-responsive stromal cell genes, including molecules important for maintaining-*ret* expression. These stromal cell signals may act first on the ureteric bud (Fig. 7A), or may act first on metanephric mesenchyme, which in turn generates signals regulating *c-ret* expression in the ureteric bud (Fig. 7B). Thus, retinoids are likely mediators of a new stromal cell signaling pathway required for renal development.

We would like to thank Qais Al-Awqati, Frank Costantini, Jonathan Barasch, Jill Heemskirk, Doris Herzlinger, Vivette D'Agati and Janet van Adelsberg for helpful discussions and critical reading of the manuscript, and Frank Costantini, Eseng Lai, Gregory Dressler, Jordan Kreidberg, Andy McMahon and Pierre Chambon for probes. We would also like to thank Pierre Chambon for the RARαβ2$^-$ mice. This work was supported by NIH grant NS 30532 to J.D. and NIH grant DK 54057 to C.M.

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