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Renal agenesis and hypodysplasia in *ret-k⁻* mutant mice result from defects in ureteric bud development

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SUMMARY

The *c-ret* gene encodes a receptor tyrosine kinase that is expressed in the Wolffian duct and ureteric bud of the developing excretory system. Newborn mice homozygous for a mutation in *c-ret* displayed renal agenesis or severe hypodysplasia, suggesting a critical role for this gene in metanephric kidney development. To investigate the embryological basis of these defects, we characterized the early development of the excretory system in mutant homozygotes, and observed a range of defects in the formation, growth and branching of the ureteric bud, which account for the spectrum of renal defects seen at birth. Co-culture of isolated ureteric buds and metanephric mesenchyme show that the primary defect is intrinsic to the ureteric bud. While the mutant bud failed to respond to induction by wild-type mesenchyme, mutant mesenchyme was competent to induce the growth and branching of the wild-type bud. Furthermore, the mutant metanephric mesenchyme displayed a normal capacity to differentiate into nephric tubules when co-cultured with embryonic spinal cord. These findings suggest a model in which *c-ret* encodes the receptor for a (yet to be identified) factor produced by the metanephric mesenchyme, which mediates the inductive effects of this tissue upon the ureteric bud. This factor appears to stimulate the initial evagination of the ureteric bud from the Wolffian duct, as well as its subsequent growth and branching.

Key words: kidney development, renal agenesis, ureteric bud, metanephric mesenchyme, receptor tyrosine kinase, induction, *c-ret* gene, mouse

INTRODUCTION

Development of the mammalian excretory system is characterized by the successive formation of pronephric, mesonephric and metanephric kidneys (Saxen, 1987). The pronephric and mesonephric kidneys are transient organs, which consist of the nephric, or Wolffian, duct and a series of tubules and glomeruli which form in the adjacent nephrogenic cord. The formation of the metanephric, or permanent, kidney is initiated when a small epithelial outgrowth, the ureteric bud, emerges from the Wolffian duct and grows caudally into an adjacent region of mesenchyme, the metanephric blastema. The subsequent organogenesis of the kidney is believed to be controlled by a series of reciprocal inductive interactions between the ureteric bud and the metanephric mesenchyme (reviewed by (Bard, 1992; Ekblom, 1992; Hardman et al., 1994; Saxen, 1987). The mesenchymal cells induce growth and repeated branching of the ureteric bud, which eventually gives rise to the renal collecting system (Erickson, 1968; Grobstein, 1953a, 1955). At the same time, the tips of the branching ureteric bud induce the surrounding mesenchymal cells to condense into epithelial vesicles, which ultimately differentiate into the various segments of the nephron (glomeruli, proximal and distal tubules, and Henle's loops) (Grobstein, 1955, 1956; Saxen, 1970).

Despite intensive study, very little is currently known about the signals and receptors that mediate these inductive events. One gene that was recently shown to play an important role in metanephric kidney development is the *c-ret* proto-oncogene (Takahashi et al., 1988; Takahashi and Cooper, 1987), which encodes a member of the receptor tyrosine kinase (RTK) superfamily (Hanks, 1991). Like other RTKs, the RET protein contains an intracellular kinase domain, a membrane-spanning segment, and an extracellular domain that is believed to bind a ligand (whose identity remains unknown). During murine embryogenesis, *c-ret* is expressed in the developing excretory system, as well as in several sites in the developing peripheral and central nervous systems (Abantaggiato et al., 1994; Pachnis et al., 1993; Tsuzuki et al., 1995). Within the excretory system, *c-ret* mRNA is first detected in the nephric duct of the pronephros and mesonephros at E8.5-E10.5, with the highest levels at the caudal end of the duct, from which the ureteric bud will later evaginate. At E11.5, when the ureteric bud has first branched within the metanephric mesenchyme, *c-ret* mRNA is observed throughout the bud. The gene is apparently not expressed in the uninduced or induced metanephric mes-

enchyme, or in mesenchymally derived nephric elements at later stages. As kidney development progresses, *c-ret* expression becomes restricted to the growing tips of the ureteric bud within the peripheral nephrogenic zone, where bud elongation and branching take place, and it is no longer detected in the kidneys of adult mice.

The importance of the *c-ret* gene for the development of both the metanephric kidney and the peripheral nervous system was directly demonstrated by the production of mice carrying a mutant allele, *ret-k⁻*, which was designed to abolish RET kinase activity (Schuchardt et al., 1994). All mice homozygous for the *ret-k⁻* mutation died within 24 hours of birth, and displayed severe renal defects, in addition to an absence of neurons and glia of the enteric nervous system and the superior cervical sympathetic ganglia. The excretory systems of the newborn *ret-k⁻* homozygotes exhibited a spectrum of abnormalities ranging in severity from bilateral or unilateral renal agenesis (the absence of both ureter and kidney), to blind-ending ureters with no renal tissue, to small, dysplastic kidney rudiments. Histological analysis of the kidney rudiments revealed that the extent of ureter branching was minimal and that, while all elements of the nephron were present, they were greatly decreased in number and severely disorganized.

The effects of the *ret-k⁻* mutation on the newborn kidney, together with the gene's normal pattern of expression during excretory system development, and the predicted biochemical nature of the RET protein, suggested that RET might serve as a receptor for an inductive factor involved in the development of ureteric bud derivatives in the kidney (Schuchardt et al., 1994). To further test and refine this hypothesis, we performed the studies reported in this paper. We first characterized the early development of the excretory system in *ret-k⁻* homozygotes, to trace the embryological basis of the defects observed in newborn mutant mice. We then studied the ability of mutant embryonic kidney primordia to develop in organ culture, and the capacity of isolated mutant ureteric bud and metanephric mesenchyme to participate in inductive interactions following co-culture with wild-type tissues. The results confirm the importance of the RET protein for the development of the ureteric bud lineage, and suggest a more detailed model for the role of RET in the development of the metanephric kidney.

MATERIALS AND METHODS

Mice

The heterozygous *ret-k⁻* mice that were intercrossed to obtain homozygous embryos were on a mixed genetic background derived from strains MF1 and 129/SvEv. The observed variability in ureteric/renal development was due only in part to this mixed genetic background, as the variability was only modestly reduced in *ret-k⁻* mice on an inbred 129/SvEv background (unpublished data). Gestational age was estimated from the time of mating, with noon of the day of plug detection defined as day E0.5. The gestational age was further defined according to Theiler by assaying crown-to-rump length and hand, foot, eye and branchial arch development (Theiler, 1989). Embryos were genotyped using a polymerase chain reaction assay.

Polymerase Chain Reaction (PCR) Assay

A portion of the embryo measuring less than 0.2 cm was lysed by overnight incubation at 55°C in 200 μl of a buffer containing Proteinase K and non-ionic detergents (Perry et al., 1995), and boiled for 10 minutes. 5 μl of the lysate was added to a buffer containing 39 pmoles each of primers p3 and p4, 6.5 pmoles each of primers p1 and p2, 0.1 mM dNTP, 0.5 units of Taq polymerase (Boehringer Mannheim) and 1/10 volume of Taq polymerase incubation buffer. The PCR was performed at 94°C, 1 minute; 65°C, 2 minutes; 72°C, 3 minutes for thirty cycles and the amplification products were separated on a 2% agarose gel. The sequences of the primers used in the reactions were: P1, 5′-tgggagaaggcgagtttggaaa-3′; P2, 5′-ttcaggaacactggctaccatg-3′; P3, 5′-agaggctattcggctatgactg-3′; P4, 5′-cctgatcgacaagaccggcttc-3′.

Histological analysis of embryos

Embryos were fixed overnight in 10% formalin, washed overnight in 1× PBS containing 0.25 M sucrose and 0.2 M glycine, and subsequently dehydrated and embedded in paraffin. 6 μm sections were cut and stained with hematoxylin and eosin.

Organ cultures

Metanephric rudiments were dissected from E11.5 to E12.0 embryos in Leibovitz's L15 medium (Specialty Media, Inc.) and the Wolffian duct was removed. Although mutant rudiments could be reliably distinguished from wild-type and heterozygous ones based on their phenotypes, the genotypes of all embryos were confirmed using the PCR. If necessary, the epithelial and mesenchymal components of the rudiments were separated by a combination of enzymatic and mechanical treatments (Qiao et al., 1995). The rudiments were first incubated for 15 minutes at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mg/ml collagenase (Gibco 17018), and 50 u/ml DNase I (Boerhinger Mannheim). Following three washes in DMEM plus 10% FBS, the ureteric bud and metanephric mesenchyme were teased apart with tungsten needles. Spinal cord was obtained from the hindbrain and cervical region of E11.5-E12.0 embryos and dissected free from spinal ganglia and surrounding tissues. Spinal ganglia were also dissected from embryos of the same stage. In cultures which involved induction with spinal cord, the mesenchyme from one metanephric rudiment was placed adjacent to the dorsal edge of a piece of spinal cord. In other cultures, one spinal ganglion was placed adjacent to the mesenchyme from one metanephric rudiment. In co-cultures between isolated ureteric buds and metanephric mesenchymes, one ureteric bud was sandwiched between the mesenchyme obtained from the left and right metanephroi of one embryo. Although preliminary experiments indicated that development was improved when two ureteric buds were combined with two mesenchymes, as previously observed (Gluecksohn-Waelsch and Rota, 1963), the number of mutant ureteric buds that could be obtained on a given day was insufficient to allow this experimental design.

All wild-type ureteric buds used in co-culture experiments had contacted the metanephric mesenchyme and initiated branching. Mutant ureteric buds were at various stages of development (Fig. 1A, phenotypes *ii-v*). Mutant metanephric mesenchyme came both from blastemas containing a ureteric bud and those lacking a ureteric bud. To reduce any effects of genetic background, mutant and wild-type tissues were obtained from littermates.

Cultures were placed on Transwell-Clear filters of 0.4 μm pore size (Costar) and cultivated in a transwell system containing DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10^{-5} M glutamine in the bottom chamber. Cultures were grown at 37°C in 5% CO_2 for 6-8 days, with a change of medium every 3 days, and were evaluated daily under a dissecting and/or phase-contrast microscope. On alternate days, photographs were taken and the cultures were evaluated by an independent investigator who was blind to the genotypes of the culture components and the conditions used.

X-gal staining of whole-mount organ cultures

Organ cultures still attached to the filter were fixed in the Transwell dishes in 2% formaldehyde, 0.2% glutaraldehyde at room temperature for 1 hour. Following three 20 minute detergent washes, they were incubated with X-gal for 48 hours at 37°C, as described (Beddington et al., 1989).

Histological analysis of organ cultures

Fixation and embedding were carried out in the Transwell dishes with the cultures still attached to the filters. Cultures were fixed for 1 hour at room temperature in 10% buffered formalin, dehydrated in a graded series of ethanols, stained with 0.01% toluidine blue to permit visualization of the culture, cleared in Histoclear (National Diagnostics HS-200) and embedded in paraffin. 6 μm sections were cut and stained with hematoxylin and eosin.

For identification of proximal tubule or collecting duct segments, 4 μm sections were stained with the biotinylated lectins Tetragonolobus Lotus or Dolichos Biflorus, respectively, using chromagen diaminobenzidine and hematoxylin counterstain, as described (D'Agati and Trudel, 1992).

RESULTS

To trace the embryological origin of the renal defects in newborn *ret-k*⁻ homozygotes, the early stages of kidney development were investigated by histological analysis of serially sectioned embryos and by visual inspection of dissected metanephric kidney rudiments. At all stages examined, development of the excretory system in *ret-k*⁻ heterozygous and wild-type embryos was indistinguishable. We will therefore refer to both of these classes as 'wild type', and to *ret-k*⁻ homozygotes as 'mutant', throughout this paper.

Mesonephric kidney development in mutant embryos

Although *c-ret* is normally expressed in the nephric duct during the development of the pronephros and mesonephros (Pachnis et al., 1993), no obvious morphological defects in the development of the mesonephros could be detected in E11.5 mutant embryos (data not shown). However, there was a slight but significant difference in the numbers of mesonephric tubules seen in comparable sections of mutant and wild-type mesonephroi (averages of 8.5 versus 10.7 tubules per section, respectively; $P<0.005$).

Metanephric kidney development in mutant embryos: defects in the ureteric bud

At E10.5, the mutant and wild-type excretory systems appeared identical. All embryos had a Wolffian duct, which extended into the cloacal region, no evidence of a ureteric bud and a small metanephric mesenchymal blastema. By E11.0, however, development of the metanephroi in mutant embryos clearly differed from that in their wild-type littermates. In E11.0 wild-type embryos, all ureteric buds had entered the metanephric mesenchyme and had initiated the first branching event (Figs 1A, 2A). The first branching was generally completed by E11.5-E12.0, and more than half of the ureteric buds had begun to branch for the second time by E12.0 (Fig.

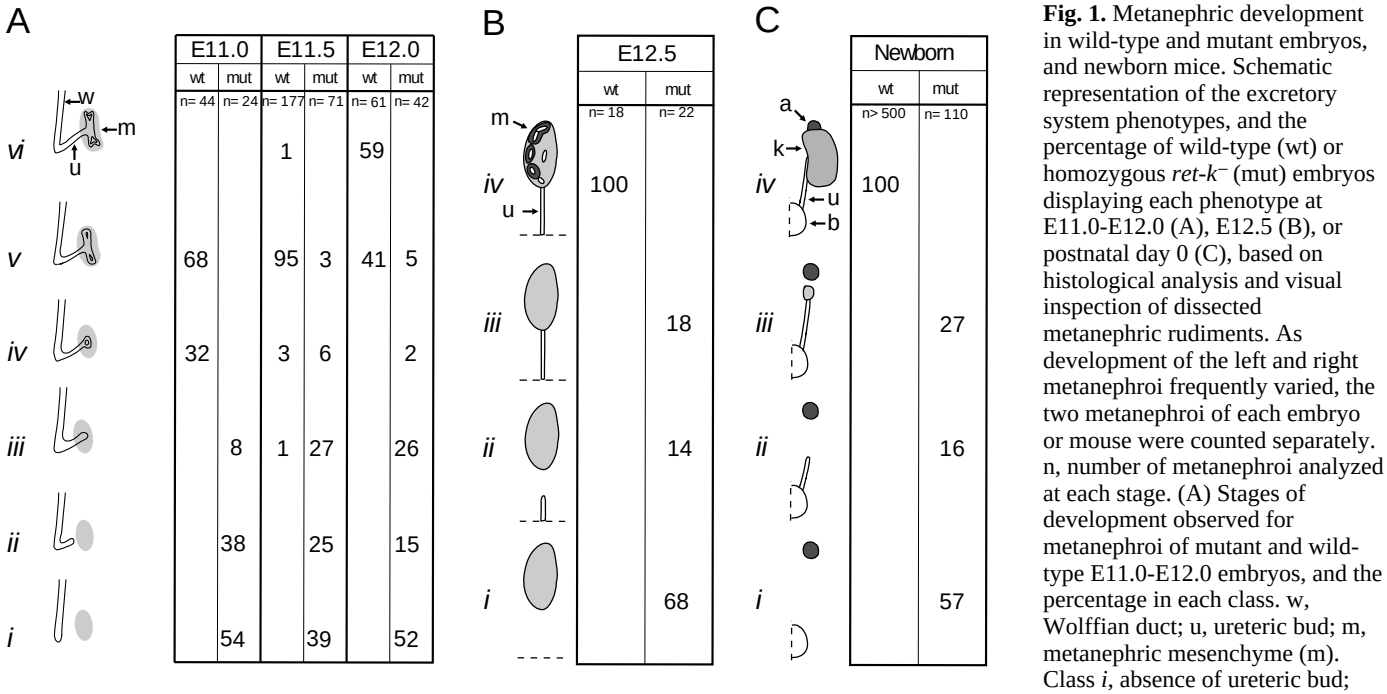


Fig. 1. Metanephric development in wild-type and mutant embryos, and newborn mice. Schematic representation of the excretory system phenotypes, and the percentage of wild-type (wt) or homozygous *ret-k*⁻ (mut) embryos displaying each phenotype at E11.0-E12.0 (A), E12.5 (B), or postnatal day 0 (C), based on histological analysis and visual inspection of dissected metanephric rudiments. As development of the left and right metanephroi frequently varied, the two metanephroi of each embryo or mouse were counted separately. n, number of metanephroi analyzed at each stage. (A) Stages of development observed for metanephroi of mutant and wild-type E11.0-E12.0 embryos, and the percentage in each class. w, Wolffian duct; u, ureteric bud; m, metanephric mesenchyme (m). Class *i*, absence of ureteric bud; class *ii*, ureteric bud not contacting mesenchyme; class *iii*, unbranched ureteric bud within mesenchyme; class *iv*, ureteric bud initiating first branch; class *v*, completion of first branching; class *vi*, second branching event beginning. (B) The excretory system at E12.5 is depicted as if sectioned through the metanephric mesenchyme to reveal the ureteric bud branches (hollow ovals), and the condensed (dark gray) and uncondensed (light gray) mesenchyme. The phenotypes depicted are: class *i*, absence of a ureteric bud; class *ii*, a ureteric bud that has failed to reach the mesenchyme; class *iii*, a ureteric bud that has reached the metanephric mesenchyme but not branched; and class *iv*, a wild-type metanephros, with ureteric bud branching and mesenchymal condensations. (C) Representation of the excretory system phenotypes in mutant (*i-iii*) and wild-type (*iv*) newborn mice. Mutant phenotypes include renal agenesis (*i*), blind-ending ureter (*ii*) and ureter with kidney rudiment (*iii*). a, adrenal gland; b, bladder; u, ureter; k, kidney.

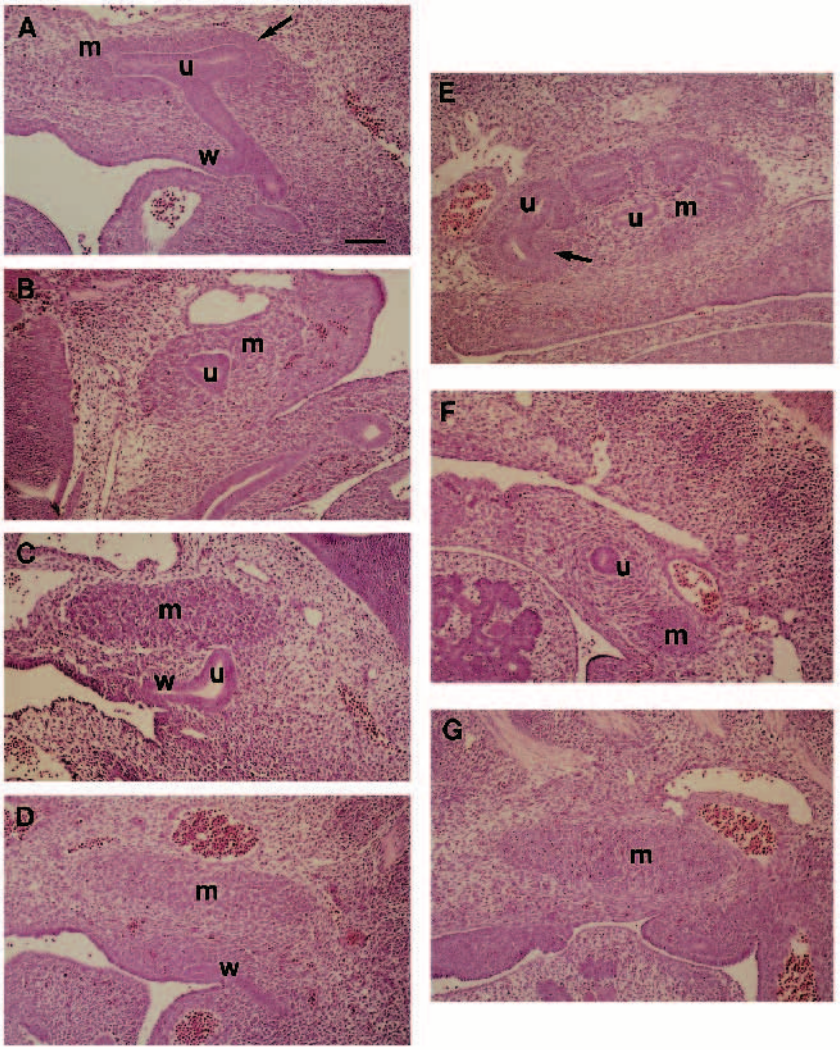


Fig. 2. Histological analysis of metanephroi at E11.5 and E12.5. Sagittal sections through metanephric rudiments from wild-type embryos at E11.5 (A) or E12.5 (E), and *ret-k*$^{-}$ homozygous embryos at E11.5 (B-D) or E12.5 (F,G). In the wild type at E11.5 (A), the ureteric bud (u) has grown from the Wolffian duct (w), entered the metanephric mesenchyme (m) and branched once, and mesenchymal condensation (arrow) is seen around the ureteric bud tips. In the specimen in B, one of the better developed mutant metanephroi observed at E11.5, ureteric bud branching is retarded and no mesenchymal condensation is observed. (C) Exemplifies a metanephric rudiment in which the ureteric bud has formed, but has not entered the metanephric mesenchyme, and (D) a rudiment in which no ureteric bud has formed, although the Wolffian duct and metanephric mesenchyme are present. At E12.5, the wild-type metanephros (E) displays multiple branches of the ureteric bud surrounded by condensed metanephric mesenchyme (arrow). Two sections (F,G) through a mutant E12.5 metanephros reveal that although the ureteric bud has entered the mesenchyme, it has not branched or induced mesenchymal condensation. Scale bar in A = 100 μm. H and E stain.

1A). By E12.5, multiple branches of the ureteric bud could be visualized in histological sections (Figs 1B, 2E).

In contrast, the mutant embryos between E11.0 and E12.5 displayed a range of metanephric phenotypes, which were generally much less developed than the age-matched wild-type population (Figs 1A,B 2B-D,F,G). Throughout this period of embryogenesis, approximately half of the mutant metanephroi contained a mesenchymal blastema and a Wolffian duct, but no sign of ureteric bud evagination (class *i* in Figs 1A,B, 2D). The frequency of this phenotype was approximately equal to the frequency of renal agenesis in the newborn *ret-k*$^{-}$ homozygous mice (Fig. 1C), suggesting that renal agenesis results from failure of the ureteric bud to form.

In cases where a ureteric bud was observed in the mutant embryos, its growth was retarded and it was delayed in entering the metanephric mesenchyme, or never contacted the mesenchyme during the stages examined (class *ii* in Figs 1A,B, 2C). While all wild-type ureteric buds had entered the mesenchyme by E11.0, only 8% of mutant buds had done so (class *iii* in Fig. 1A). The fraction of mutant ureteric bud that had failed to contact the mesenchyme by E12.0-E12.5 (14-15%) corresponded well to the frequency of blind-ending ureters in newborn mutants (16%), suggesting a lineal relationship between these embryonic and newborn phenotypes.

In those cases where the mutant ureteric bud eventually contacted the metanephric mesenchyme, its growth and branching was severely retarded and often abnormal. This class of metanephroi appears to develop into the hypodysplastic kidney rudiments seen in some newborn mutant mice. Few of the mutant ureteric buds that reached the metanephric mesenchyme showed any indication of branching by E11.5 or E12.0 (class *iii*, Fig. 1A). Even by E12.5, no branching was observed in any of the ureteric buds that had reached the metanephric mesenchyme (Fig. 1B, class *iii*, and Fig. 2F,G), although fewer rudiments were analyzed at this stage.

In the rare cases where the initiation of ureteric bud branching was observed in mutant metanephroi (6/71 cases at E11.5 and 3/42 at E12.0), it was often abnormal. Four of these nine ureteric buds had branched asymmetrically, with one branch longer than the other (e.g., Fig. 3C,F), while in wild-type embryos the initial branching event was always symmetrical (Fig. 3A,E). Furthermore, three mutant buds with no apparent branches showed an abnormal curvature (Fig. 3B).

Metanephric kidney development in mutant embryos: defects in the mesenchyme

Development of the metanephric mesenchyme was also affected in *ret-k*$^{-}$ homozygous embryos. In wild-type

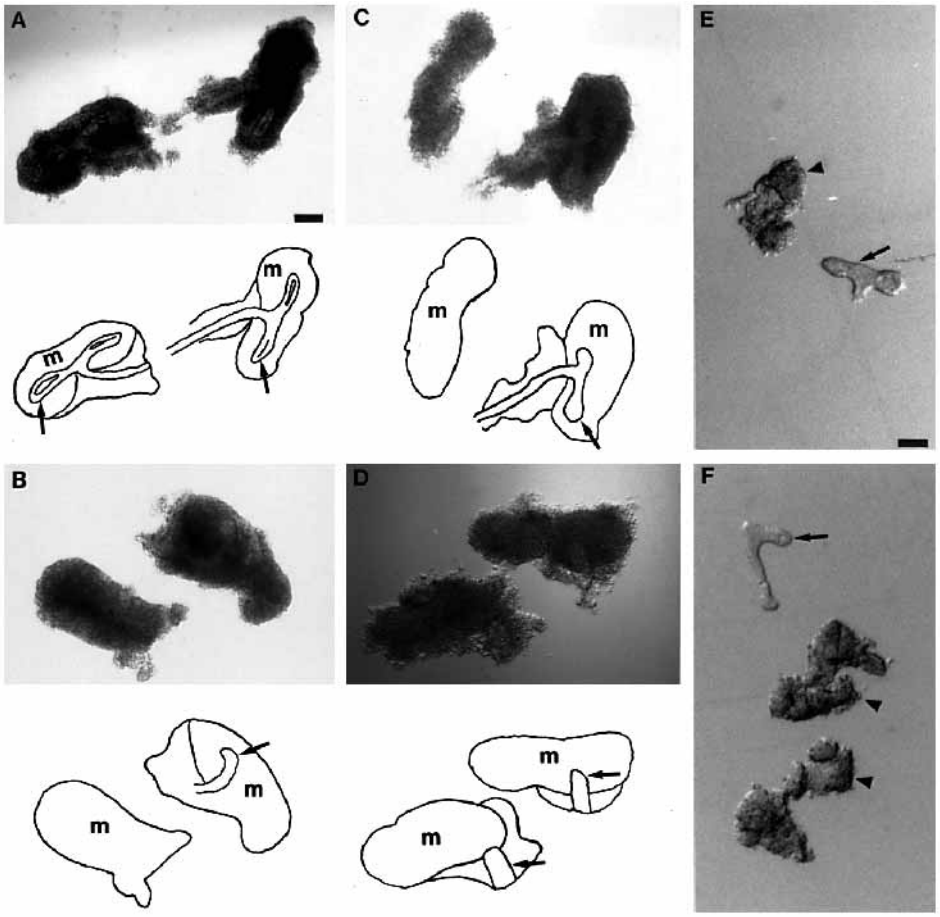


Fig. 3. Dissection of mutant E11.5 metanephric rudiments reveals asymmetric branching of the ureteric bud. (A-D) Whole metanephroi dissected from wild-type (A) and *ret-k*⁻ homozygous (B-D) E12.0 embryos. Below each photograph, a tracing of the rudiment depicts the shape of the ureteric bud (arrow) and its position with respect to the metanephric mesenchyme (m). The mutant phenotypes included: a curved ureteric bud (B, right); an asymmetrically branched ureteric bud (C, right); an unbranched ureteric bud that enters the metanephric mesenchyme (D, right); and a ureteric bud that barely contacts the metanephric mesenchyme (D, left). Two rudiments (B, left and C, left) lack a ureteric bud. (40×, bar = 200 μm). (E,F) Ureteric buds (arrows), which have been enzymatically separated from the metanephric mesenchyme (arrowheads), display more clearly the symmetrically branched wild-type (E) and the asymmetrically branched mutant (F) phenotypes. Scale bars, 200 μm.

metanephroi, condensation of the mesenchyme around the ureteric bud could be seen as early as E11.0 (not shown) and was readily apparent by E11.5 (Fig. 2A). These morphological changes, which correspond to the initial stages of nephron formation, are thought to result from induction by the ureteric bud (Ekblom, 1992; Saxen, 1987). In sections of seven E11.0 or E12.0 mutant metanephroi in which the ureteric bud had entered the mesenchyme, no condensation was observed (e.g., Fig. 2B). Whether the lack of condensation of the metanephric mesenchyme was due to the delay in contact with the ureteric bud, or whether it represented an independent effect of the *ret-k*⁻ mutation, could not be determined from this analysis.

Wild-type metanephric mesenchyme that fails to receive an inductive stimulus undergoes programmed cell death, or apoptosis (Koseki, 1993; Koseki et al., 1992). As expected, uninduced metanephric mesenchyme from *ret-k*⁻ homozygous embryos underwent apoptosis at E12.5 and disappeared by E13.5 (data not shown).

In vitro development of intact metanephric rudiments

The *ret-k*⁻ mutation appeared to affect the development of both the ureteric bud and the metanephric mesenchyme in vivo. However, it was unclear whether both components were directly affected by the mutation, or whether the defects in one component were secondary. This question could be addressed by isolating mutant ureteric bud and metanephric mesenchyme, and evaluating the ability of each component to develop in vitro when induced by an appropriate wild-type tissue. However, it was first necessary to determine whether intact mutant metanephroi displayed defects when cultured in vitro.

All 41 metanephric rudiments isolated from wild type E11.5-E12.0 embryos developed to varying extents when cultured in vitro, with visible ureteric bud branching and mesenchymal differentiation in nearly every case (Fig. 4A,B and data not shown). In contrast, few of the 24 metanephric rudiments cultured from *mutant* embryos showed any development in vitro and, when development did occur, it was much less extensive (Fig. 4C-J). As expected, none of the 14 mutant rudiments lacking a ureteric bud displayed any signs of mesenchymal differentiation during seven days of culture (Fig. 4I,J). In two rudiments in which the ureteric bud tip had not entered the metanephric mesenchyme when dissected and in three rudiments where the bud had entered the mesenchyme, no branching of the bud or differentiation of the mesenchyme occurred in vitro, although the buds persisted throughout the culture period (Fig. 4G,H). Four mutant rudiments with a ureteric bud inside the mesenchyme underwent a limited amount of mesenchymal tubulogenesis, but no ureteric bud branching (Fig. 4E,F). Only one mutant rudiment displayed ureteric bud branching in vitro (Fig. 4C,D). The bud in this rudiment had not branched when dissected, although its tip had entered the mesenchyme, and it branched in vitro in an asymmetric pattern (Fig. 4C,D) similar to that seen in some of the mutant embryos in vivo. After 8 days, this culture was much smaller than most wild-type cultures, and histological analyses

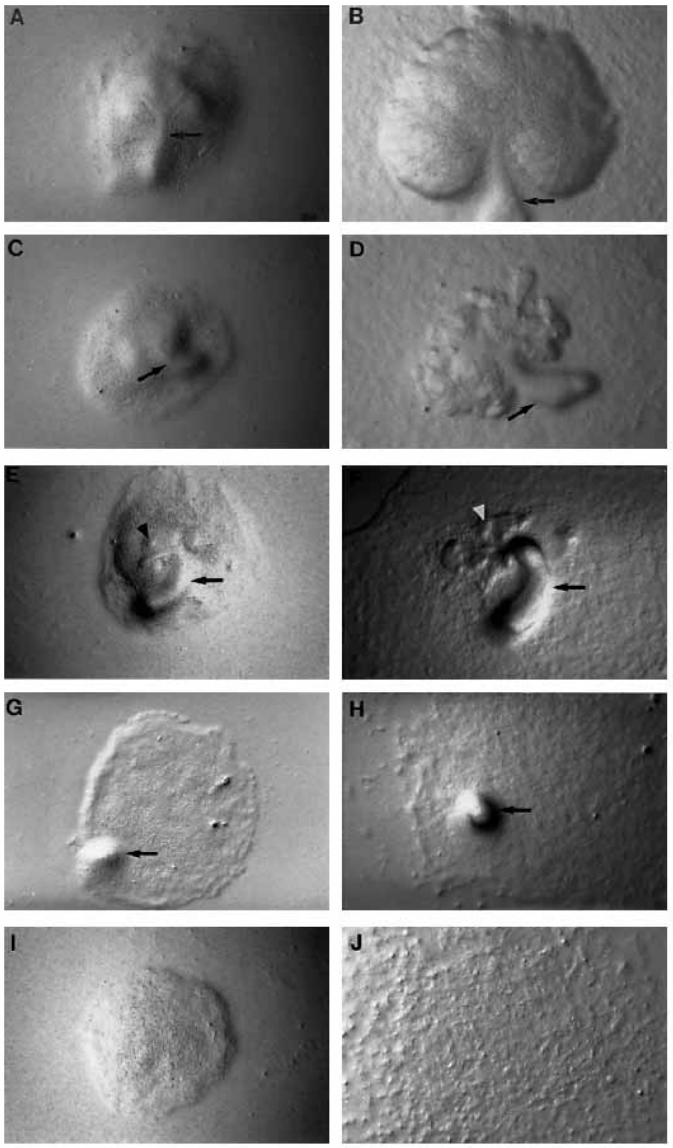


Fig. 4. In vitro organ culture of intact metanephric rudiments isolated from wild-type and mutant embryos. The panels on the left (A,C,E,G,I) display the development after 24 hours in culture, while those on the right (B,D,F,H,J) depict development after 7 days. Arrows point to the ureteric buds. (A,B) Culture of a wild-type metanephric rudiment; (C-J) cultures of four metanephric rudiments isolated from homozygous *ret-k*$^{-}$ embryos, indicating some of the phenotypes observed. The mutant rudiment shown in C and D was the only one to display ureteric bud branching in vitro, but (as was observed in some mutant metanephroi in vivo), the first branch of the ureteric bud was asymmetrical (compare C with A). Although this culture developed farther than any other mutant rudiment, it remained much smaller than the wild-type culture (compare B and D). (E,F) Culture of a mutant rudiment in which the ureteric bud had entered the metanephric mesenchyme prior to isolation. Although the ureteric bud remained unbranched throughout the culture period, mesenchymal differentiation (arrowheads) can be seen at both time points. (G,H) A mutant rudiment in which the ureteric bud had not contacted the metanephric mesenchyme prior to isolation failed to show any mesenchymal differentiation or ureteric bud branching, even after 7 days in culture. (I,J) A mutant metanephros that lacked a ureteric bud displayed no mesenchymal differentiation in vitro. Scale bar in A, 100 μm.

revealed a decrease in the extent of ureteric bud branching and mesenchymal tubulogenesis, although all nephron elements were present (data not shown).

These results indicated that metanephric kidney development in vitro is severely affected by the *ret-k*$^{-}$ mutation, with defects in ureteric bud growth and branching as well as in the extent of mesenchymal tubulogenesis. Therefore, the organ culture system (Erickson, 1968; Grobstein, 1953b, 1955; Saxen, 1970) could be used to evaluate independently the ability of mutant bud and mesenchyme to develop when recombined with wild-type tissues.

Co-culture of mutant and wild-type ureteric bud and metanephric mesenchyme

To evaluate the ability of the mutant bud to grow and branch when induced by wild-type mesenchyme, as well as the ability of mutant mesenchyme to induce development of the wild-type bud, ureteric buds and metanephric mesenchymes from E11.5 embryos were isolated by enzymatic digestion and mechanical manipulation, and co-cultured for 6-7 days. When ureteric bud or mesenchyme was cultured alone, no further development was observed and the cells died (Table 1).

In 30 control co-cultures of wild-type ureteric bud with wild-type mesenchyme, the bud was maintained until the end of the culture period in seven cases, and branched visibly in four of these cases (Table 1; Fig. 5A,D). Mutant metanephric mesenchyme was also capable of inducing the growth and branching of wild-type ureteric bud and, surprisingly, did so at a higher frequency than wild-type mesenchyme: of 19 co-cultures, the bud was maintained in 16 cases and branched in 12 cases (Table 1; Fig. 5B,E). The ureteric bud origin of the branched tubules was confirmed by staining sections with the lectin Dolichos Biflorus, which specifically labels the ureteric bud epithelium (Laitinen et al., 1987) (Fig. 5F). In contrast, in 19 co-cultures of mutant ureteric bud with wild-type mesenchyme, the bud was never seen to branch and always disappeared by the end of the culture period (Table 1; Fig. 5C). Even compared to the low success rate of the wild-type/wild-type co-cultures, the failure of the mutant ureteric buds to persist was significantly different ($P<0.02$, test of the difference between two proportions; Dunn, 1977). Overall, these results strongly suggest that the failure of ureteric bud development in *ret-k*$^{-}$ embryos is due not to a defect in the ability of the mutant metanephric mesenchyme to induce growth and branching, but in the ability of the bud to respond.

In these co-culture experiments, unlike the cultures of intact, wild-type metanephroi, only limited mesenchymal differentiation was observed. Therefore, we used a stronger inducer of tubulogenesis, embryonic spinal cord (Grobstein, 1955; Saxen, 1970), to test the ability of mutant metanephric mesenchyme to differentiate.

Differentiation of mutant metanephric mesenchyme in response to spinal cord

Metanephroi were isolated from E11.5-E12.0 embryos, and the mesenchyme was isolated and cultured adjacent to a fragment of embryonic spinal cord for 6-8 days. Three genotypic combinations were assembled: wild-type mesenchyme with wild-type spinal cord, mutant mesenchyme with wild-type spinal cord and wild-type mesenchyme with mutant spinal cord. As controls, mesenchyme was cultured alone or with embryonic

Table 1. Branching of mutant and wild-type ureteric buds co-cultured with metanephric mesenchyme

Ureteric bud	Metanephric mesenchyme	Number of cultures	Appearance of ureteric bud at end of culture period		
			Absent	Present with no branching	Present with branching
wild type	wild type	30	23	3	4
wild type	mutant	19	3	4	12
mutant	wild type	19	19	–	–
wild type	none	13	13	–	–
none	wild type	14	14	–	–
none	mutant	6	6	–	–

Table 2. Induction of tubulogenesis in cultured mutant and wild-type metanephric mesenchyme

Inducer	Metanephric mesenchyme	Number of cultures	Extent of mesenchymal differentiation		
			Negative	Weak positive	Strong positive
wild-type spinal cord	wild type	16	–	6	10
wild-type spinal cord	mutant	15	–	4	11
mutant spinal cord	wild type	6	–	1	5
none	wild type	6	6	–	–
none	mutant	6	6	–	–
wild-type dorsal root ganglion	wild type	4	4	–	–

Cultures which were classified as 'weak positive' showed less mesenchymal tubulogenesis than those classified as 'strong positive'.

dorsal root ganglia, a tissue that lacks inducing ability (Grobstein, 1955). None of the control cultures showed signs of mesenchymal tubulogenesis (Table 2; Fig. 6D).

In the experimental cultures, although a range of mesenchymal differentiation was observed within each genotypic combination, no differences were seen between genotypic combinations in the extent or timing of mesenchymal differentiation (Table 2; Figs 6, 7). Several cultures containing wild-type or mutant mesenchyme were stained for endogenous β-galactosidase, a marker of mature nephrons (Bard and Ross, 1991), and the number of nephrons detected did not differ significantly between the two genotypic combinations (Fig. 6E,F). Histological analysis revealed that mesenchyme of both genotypes could form nephric tubules and glomeruli (Fig. 7A-C). As a further test of differentiation, cultures containing mutant and wild-type mesenchyme were stained with the lectin Tetragonolobus Lotus, which binds specifically to proximal tubules (Laitinen et al., 1987), and positive-staining tubules were observed in both types of cultures (Fig. 7D,E). These data show that metanephric mesenchyme from homozygous *ret-k⁻* mutant embryos is equivalent to wild-type in its ability to respond to a spinal cord induction and suggest that the reduced number of nephrons formed in vivo is secondary to the defects in ureteric bud development. In these experiments, we also observed that wild-type mesenchyme could be induced by mutant spinal cord (Table 2; Fig. 7C,D), demonstrating that the *ret-k⁻* mutation does not affect the inducing potential of the spinal cord.

DISCUSSION

In these studies, the severe renal abnormalities observed in newborn mice lacking a functional *c-ret* gene have been traced to defects in the formation, growth and branching of the ureteric bud. The ureteric bud is believed to give rise to the collecting system of the metanephric kidney, although recent cell lineage analyses suggest that the bud grows not only by cell proliferation, but also by recruitment of metanephric mesenchymal cells (Qiao et al., 1995). In addition, the ureteric bud induces nephron formation by cells of the metanephric mesenchyme. In the excretory system of homozygous *ret-k⁻* newborns, the most frequent phenotype is renal agenesis, and examination of mutant embryos suggested that this defect results from failure of the ureteric bud to evaginate from the Wolffian duct. The same analyses suggested that blind-ending ureters develop in cases where a ureteric bud forms, but fails to grow into the metanephric mesenchyme, and that hypodysplastic kidney rudiments develop from mutant metanephroi in which the ureteric bud succeeds in reaching the mesenchyme. In the latter cases, not only is the subsequent growth and branching of the bud severely deficient, but so is the formation of nephrons in the mesenchyme. However, organ culture studies demonstrated that the primary defect in *ret-k⁻* kidney development is restricted to the ureteric bud: while the mutant bud failed to respond to induction by wild-type metanephric mesenchyme, mutant metanephric mesenchyme displayed no defects in its capacity for differentiation when co-cultured with embryonic spinal cord, a strong inducer of tubulogenesis, or in its ability to induce the growth and branching of a wild-type ureteric bud. These findings allow us to propose a model of the role of the RET receptor tyrosine kinase during metanephric kidney development (Fig. 8).

c-ret mRNA is expressed along the length of the Wolffian duct during the mesonephric stage and appears at the highest levels at the caudal end of the duct (Pachnis et al., 1993) where the ureteric bud normally forms at E10.5. Because development of the mutant mesonephric kidney is grossly normal and the male reproductive organs are unaffected by the *ret-k⁻* mutation (Schuchardt et al., 1994), *c-ret* does not appear to be important for the growth of the Wolffian duct per se. However, the slight reduction in the number of mesonephric tubules

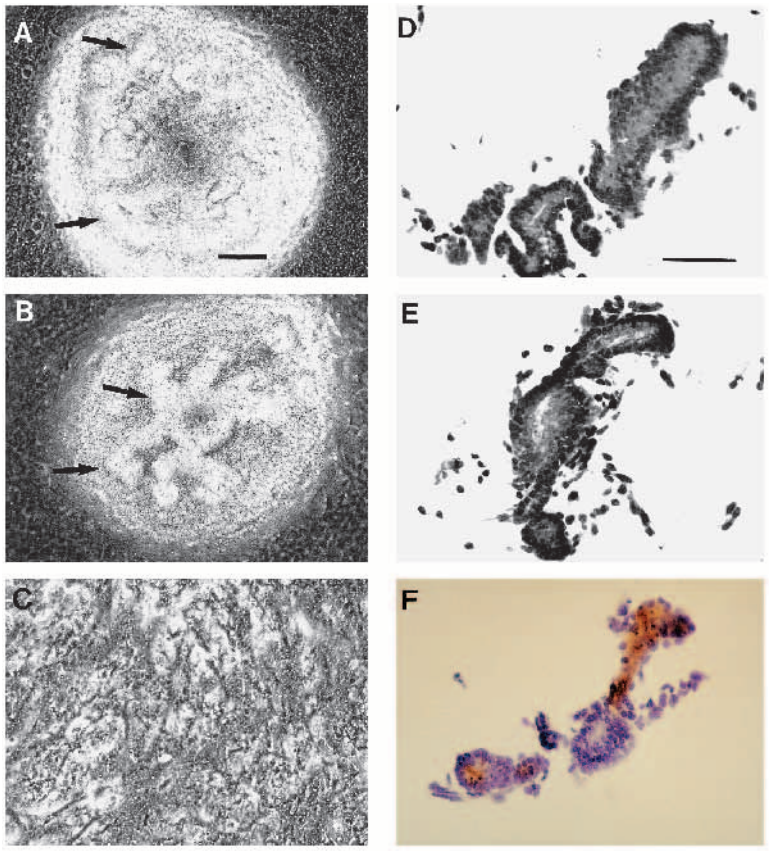


Fig. 5. Ureteric bud branching in vitro in response to wild-type or mutant metanephric mesenchyme. (A-C) Phase-contrast micrographs of ureteric buds, isolated as shown in Fig. 3E or F, following co-culture for 7 days with isolated metanephric mesenchyme. (A) Wild-type ureteric bud co-cultured with wild-type metanephric mesenchyme; (B) wild-type ureteric bud co-cultured with mutant metanephric mesenchyme; (C) mutant ureteric bud co-cultured with wild-type mesenchyme. After 7 days in culture, the mutant ureteric bud is no longer visible (C), but the wild-type ureteric buds have branched (arrows) in response to either wild-type or mutant metanephric mesenchyme (A,B). (D,E) Histological sections through co-cultures of wild-type ureteric bud with wild-type metanephric mesenchyme (D), or wild-type ureteric bud with mutant metanephric mesenchyme (E). The H&E-stained sections reveal epithelial tubules apparently derived from the ureteric bud. (F) Apical tubular staining with the lectin Dolichos Biflorus (brown stain, counter-stained with hematoxylin) confirms the ureteric bud origin of the epithelial tubules in a co-culture of wild-type ureteric bud with wild-type mesenchyme. (A-C) Scale bar, 100 μm; (D-E) scale bar, 50 μm.

suggests that tubule induction by the mesonephric duct is mildly affected.

The defects in development of the metanephros are far more severe: in a large proportion of mutant embryos, one or both ureteric buds fail to evaginate from the Wolffian duct, indicating that *c-ret* plays an important role in this early event in metanephric kidney development. Further insight into the possible role of *c-ret* in this process comes from a mutation in the murine Wilms' Tumor gene, *WT-1*, which also blocks ureteric bud evagination (Kreidberg et al., 1993). Because *WT-1* encodes a nuclear factor expressed in the metanephric mesenchyme but not in the Wolffian duct or the bud (Armstrong et al., 1992; Pelletier et al., 1991; Pritchard-Jones et al., 1990), it was proposed that the *WT-1* mutation interferes with the production of a signal(s) that induces the outgrowth of the bud (Kreidberg et al., 1993). According to such a model, *c-ret* might encode the receptor for this mesenchyme-derived signal (Fig. 8A,B). As the bud still forms in a fraction of *ret-k*$^{-}$ embryos, more than one signaling molecule and receptor appears to be involved in ureteric bud formation.

After the bud has formed, *c-ret* appears to be important for its growth towards the metanephric mesenchyme. Approximately one third of mutant ureteric buds visible by E12.0 have not yet contacted the metanephric mesenchyme, and ureteric buds of this class apparently never grow into the mesenchyme, but develop into blind-ending ureters with no renal tissue. Those mutant ureteric buds that eventually contact the mesenchyme do so at a later stage than wild-type ureteric buds, which could result from a delay in bud formation and/or a reduction in growth rate. It is interesting that, in both of these situations, the mutant ureter continues to grow, since the blind-ending ureters in mutant newborn mice, as well as the ureters associated with dysplastic kidney rudiments, are many times the length of the embryonic ureteric bud (Schuchardt et al., 1994). This suggests that the later stages of ureter elongation, in contrast to collecting duct growth, are controlled by a different, *c-ret*-independent mechanism.

When the ureteric bud has grown into the metanephric mesenchyme, *c-ret* continues to be important for its growth and branching. Growth of the mutant bud was retarded, and branching was greatly reduced or eliminated, both in vivo and in cultured metanephroi. This is consistent with the small size and limited development of the collecting system in the rudimentary kidneys of *ret-k*$^{-}$ newborn mice, and also with the expression of the normal *c-ret* gene at the tips of the branching ureteric bud throughout kidney development (Fig. 8D,E). Interestingly, the symmetry of ureteric bud branching was also dependent on *c-ret*. While in wild-type buds the initial branch is always symmetrical, in many of the mutant ureteric buds that had branched, one branch was shorter than the other or absent. This phenomenon suggests that dichotomous branching is not a unitary event, but that each branch forms independently. Indeed, it has been suggested that the mechanism of branching morphogenesis in the kidney is distinct from that in other organs, such as salivary gland, in which the bifurcating tips of epithelial tubes are cleaved by a 'tourniquet' of extracellular matrix (Nakanishi and Ishii, 1989). In the ureteric bud, in contrast, branches appear to form as bumps evaginating from a smooth tube (Davies et al., 1995). Thus, the arborization of the ureteric bud might occur by the essentially the same

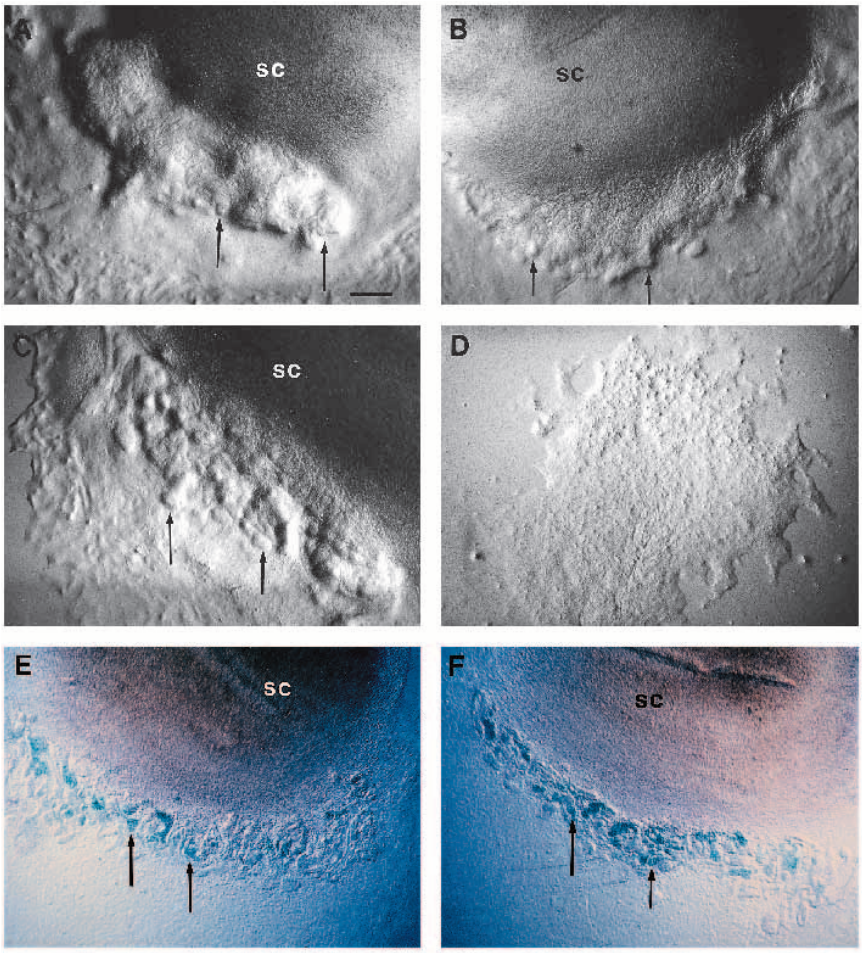
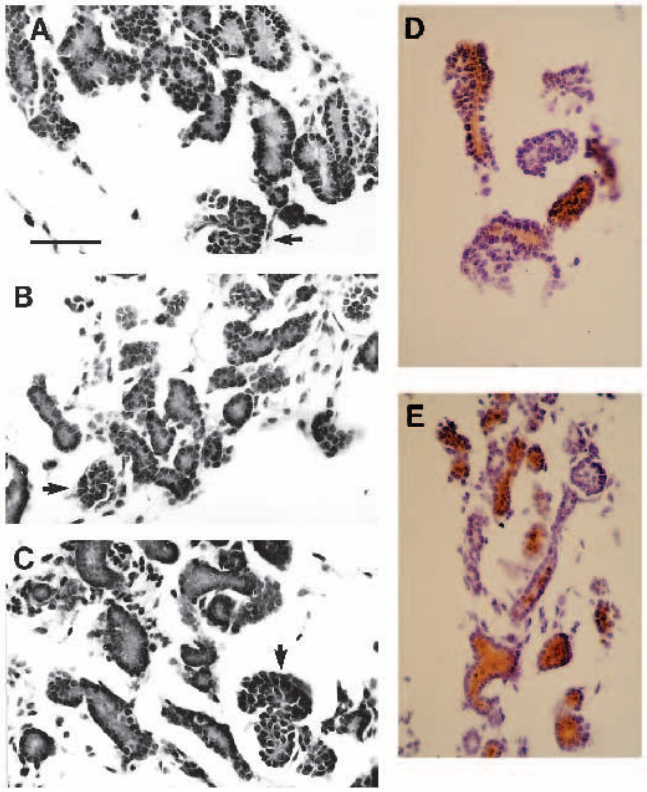


Fig. 6. Differentiation of metanephric mesenchyme in response to induction by spinal cord. Co-culture of (A) wild-type metanephric mesenchyme combined with wild-type spinal cord (sc); (B) mutant mesenchyme with wild-type spinal cord; (C) wild-type mesenchyme with mutant spinal cord, and (D) wild-type mesenchyme with wild-type dorsal root ganglion. Mesenchymal differentiation (i.e., formation of nephric tubules, indicated by arrows) is observed in A-C, but not in D. (E,F) X-gal staining of wild-type (E) and mutant (F) metanephric mesenchyme following induction by spinal cord reveals the presence of mature nephrons (arrows). The average numbers of mature nephrons stained by X-gal was 8 in cultures of wild-type mesenchyme and 10.5 in mutant mesenchyme (P>0.25, students t-test; Dunn, 1977). Original magnification 45× (scale bar, 200 μm).



mechanism as the initial evagination of the bud from the Wolffian duct, and be similarly stimulated by RET and its ligand. In this light, asymmetric branching of the ureteric bud may be viewed as failure of one of the two branches to evaginate, or to elongate normally, and may be closely related to the frequent failure of the mutant ureteric bud to evaginate and grow out from the Wolffian duct.

In addition to the defects in ureteric bud development, the initiation of mesenchymal condensation and tubulogenesis in the metanephroi of *ret-k*⁻ embryos appeared to be delayed. However, mutant metanephric mesenchyme showed no defect in the amount or timing of tubulogenesis when isolated and co-cultured with a strong inducer of tubulogenesis, wild-type embryonic spinal cord. Therefore, the nephrogenic potential of the mesenchyme had not been irreversibly affected at E11.5-E12.0, the time it was isolated. The apparent retardation of

Fig. 7. Histological analysis and Tetragonolobus Lotus lectin staining of induced metanephric mesenchyme. (A-C) Histological sections of wild-type mesenchyme induced by wild-type spinal cord (A) or mutant spinal cord (B), and mutant mesenchyme induced by wild-type spinal cord (C), stained with H&E. Epithelial nephrogenic tubules as well as glomeruli (arrows) are present in all specimens. (D,E) Sections of wild-type mesenchyme induced by mutant spinal cord (D) and mutant mesenchyme induced by wild-type spinal cord (E), stained with the lectin Tetragonolobus Lotus, which specifically labels the apical surface of proximal tubules (brown stain) and counterstained with hematoxylin. Scale bar, 50 μm.

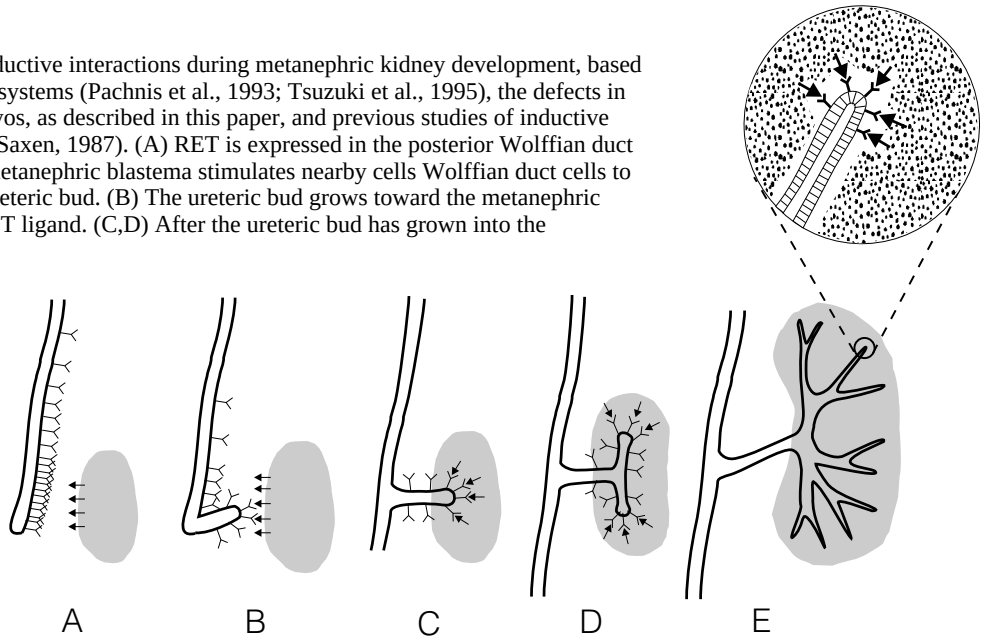


Fig. 8. A model for the role of RET in inductive interactions during metanephric kidney development, based on the normal pattern of *c-ret* expression systems (Pachnis et al., 1993; Tsuzuki et al., 1995), the defects in renal development in *ret-k*$^{-}$ mutant embryos, as described in this paper, and previous studies of inductive interactions during kidney development (Saxen, 1987). (A) RET is expressed in the posterior Wolffian duct and a diffusible ligand produced by the metanephric blastema stimulates nearby cells Wolffian duct cells to proliferate, causing an evagination, the ureteric bud. (B) The ureteric bud grows toward the metanephric blastema, possibly along a gradient of RET ligand. (C,D) After the ureteric bud has grown into the metanephric blastema, RET ligand stimulates the formation and growth of the two symmetrical branches. (E) At later stages, RET expression is restricted to cells at the tips of the ureteric bud, and RET ligand continues to stimulate growth and branching at the bud tips. The identity, as well as the sites of expression, of the RET ligand are unknown.

mesenchymal condensation may have been due in part to the delay in contact with the ureteric bud, or to the reduced number of ureteric bud cells contacting the mesenchyme. However, this difference was apparent even when comparing mutant and wild-type metanephroi in which the ureteric buds had developed to a similar stage, suggesting that the mutant bud may have a reduced capacity to induce mesenchymal condensation. For example, the mutant bud, as a secondary effect of the *ret-k*$^{-}$ mutation, might produce insufficient quantities of a factor involved in mesenchymal induction.

Surprisingly, the mutant metanephric mesenchyme displayed an increased capacity, compared to mesenchyme from wild-type littermates, to support the growth and branching of wild-type ureteric buds in co-culture experiments. It has been previously observed that 'loose' metanephric mesenchyme, but not condensed mesenchyme or nephric tubules, would induce ureteric bud branching (Erickson, 1968). Therefore, our observation might be explained by the decreased mesenchymal condensation in the mutant metanephric rudiments.

The developmental abnormalities in *ret-k*$^{-}$ embryos serve to highlight the importance of timing in the development of the excretory system. Our studies suggest that, if a ureteric bud has not formed by E11.0, it will not form at a later stage, resulting in renal agenesis. Many inductive events display constraints on timing, which can be imposed by limitations in either the responding or the inducing tissue (Gurdon, 1987). According to the model in Fig. 8, temporal restrictions could be caused either by the transient production of a signal by the metanephric mesenchyme to stimulate ureteric bud formation, or by a transient ability of the Wolffian duct to respond to this signal. The failure of some mutant ureteric buds to contact the metanephric mesenchyme suggests the presence of temporal restrictions on this second critical event in metanephric kidney development. The proportion of metanephroi displaying this phenotype remains relatively constant after E12.0. At this time in mouse embryogenesis, the metanephric blastema begins to be displaced anteriorly, and shortly thereafter, mesenchymal apoptosis is apparent in the uninduced mesenchyme (Koseki, 1993; Koseki et al., 1992). Therefore, limitations on the period during which the ureteric bud can enter and interact with the metanephric mesenchyme may be imposed by the anterior displacement of the mesenchyme, combined with mesenchymal apoptosis.

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REFERENCES

Abantaggiato, V., Dathan, N. A., Grieco, M., Fabien, N., Lazzaro, D., Fusco, A., Simeone, A., and Santoro, M. (1994). Developmental expression of the RET protooncogene. *Cell. Growth Diff.* **5**, 305-311.

Armstrong, J. F., Pritchard-Jones, K., Bickmore, W. A., Hastie, N. D., and Bard, J. B. L. (1992). The expression of the Wilms' tumour gene, WT1, in the developing embryo. *Mechan. Devel.* **40,** 85-97.

Bard, J. B. (1992). The development of the mouse kidney--embryogenesis writ small. *Curr. Opin. Genet. Dev.* **2,** 589-595.

Bard, J. B., and Ross, A. S. (1991). LIF, the ES-cell inhibition factor, reversibly blocks nephrogenesis in cultured mouse kidney rudiments. *Development* **113,** 193-198.

Beddington, R. S., Morgernstern, J., Land, H., and Hogan, A. (1989). An in situ transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early organogenesis. *Development* **106,** 37-46.

D'Agati, V., and Trudel, M. (1992). Lectin characterization of cystogenesis in the SBM transgenic model of polycystic kidney disease. *J. Am. Soc. Nephrol.* **3,** 975-983.

Davies, J., Lyon, M., Gallagher, J., and Garrod, D. (1995). Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development. *Development* **121,** 1507-1517.

Dunn, O. J. (1977). *Basic Statistics: A Primer for the Biomedical Sciences.* New York: John Wiley and Sons.

Ekblom, P. (1992). Renal Development. In *The Kidney: Physiology and*

Pathophysiology (ed D. W. Seldin and G. Giebisch), pp. 475-501. New York: Raven Press.

Erickson, R. A. (1968). Inductive interactions in the development of the mouse metanephros. *J. Exp. Zool.* **169,** 33-42.

Gluecksohn-Waelsch, S., and Rota, T. R. (1963). Development in organ culture of kidney rudiments from mutant mouse embryos. *Dev. Biol.* **7,** 432-444.

Grobstein, C. (1953a). Inductive epithelio-mesenchymal interaction in cultured organ rudiments of the mouse. *Science* **118,** 52-55.

Grobstein, C. (1953b). Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. *Nature* **172,** 869-871.

Grobstein, C. (1955). Inductive interaction in the development of the mouse metanephros. *J. Exp. Zool.* **130,** 319-340.

Grobstein, C. (1956). Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* **10,** 424-440.

Gurdon, J. B. (1987). Embryonic induction - molecular prospects. *Development* **99,** 285-306.

Hanks, S. K. (1991). Eukaryotic protein kinases. *Curr. Opin. Struct. Biol.* **1,** 369-383.

Hardman, P., Kolatsi, M., Winyard, P. J., Towers, P. R., and Woolf, A. S. (1994). Branching out with the ureteric bud. *Exp. Nephrol.* **2,** 211-219.

Koseki, C. (1993). Cell death programmed in uninduced metanephric mesenchymal cells. *Pediatric Nephrology* **7,** 609-611.

Koseki, C., Herzlinger, D., and Al-Awqati, Q. (1992). Apoptosis in metanephric development. *J. Cell. Biol.* **119,** 1327-1333.

Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74,** 679-691.

Laitinen, L., Virtanen, I., and Saxen, L. (1987). Changes in the glycosylation pattern during embryonic development of mouse kidney as revealed with lectin conjugates. *J. Histochem. Cytochem.* **35,** 55-65.

Nakanishi, Y., and Ishii, T. (1989). Epithelial shape change in mouse embryonic submandibular gland: modulation by extracellular matrix components. *BioEssays* **11,** 163-167.

Pachnis, V., Mankoo, B. S., and Costantini, F. (1993). Expression of the *c-ret* proto-oncogene during mouse embryogenesis. *Development* **119,** 1005-1017.

Pelletier, J., Schalling, M., Buckler, A. J., Rogers, A., Haber, D. A., and Housman, D. (1991). Expression of the Wilms' tumor gene WT1 in the murine urogenital system. *Genes Dev.* **5,** 1345-1356.

Perry, W. L. I., Vasicek, T. J., Lee, J. J., Rossi, J. M., Zeng, L., Zhang, T., Tilghman, S. M., and Costantini, F. (1995). Phenotypic and molecular analysis of a transgenic insertional allele of the mouse *Fused* locus. *Genetics* **141,** 321-332.

Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., van Heyningen, V., and Hastie, N. (1990). The candidate Wilms' tumour gene is involved in genitourinary development. *Nature* **346,** 194-197.

Qiao, J., Cohen, D., and Herzlinger, D. (1995). The metanephric blastema differentiates into collecting system and nephron epithelia in vitro. *Development* **121,** 3207-3214.

Saxen, L. (1970). Failure to demonstrate tubule induction in a heterologous mesenchyme. *Dev. Biol.* **23,** 511-523.

Saxen, L. (1987). *Organogenesis of the Kidney*. Cambridge: Cambridge University Press.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367,** 380-383.

Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H., and Hiai, H. (1988). Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene* **3,** 571-578.

Takahashi, M., and Cooper, G. M. (1987). ret transforming gene encodes a fusion protein homologous to tyrosine kinases. *Mol. Cell. Biol.* **7,** 1378-1385.

Theiler, K. (1989). *The House Mouse. Atlas of Embryonic Development*. New York: Springer-Verlag.

Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M., and Asai, J. (1995). Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. *Oncogene* **10,** 191-198.

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