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Defects of urogenital development in mice lacking *Emx2*

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SUMMARY

The homeobox gene *Emx2* is a mouse homologue of a *Drosophila* head gap gene *empty spiracles* (*ems*) and is essential for the development of dorsal telencephalon (Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S. (1997) *Development* 124, 101-111). At the same time, *Emx2* is expressed in the epithelial components of the developing urogenital system and, in *Emx2* mutant mice, the kidneys, ureters, gonads and genital tracts were completely missing. *Pax-2* and c-*ret* expressions in the Wolffian duct and *WT-1* and *GDNF* expressions in the metanephric blastema were initially normal in the mutant. The ureteric bud grew and invaded the metanephric mesenchyme where *Pax-2* expression was normally induced. Subsequently, however, *Pax-2*, c-*ret* and *Lim1* expressions in the ureteric bud and *GDNF* expression in the mesenchyme were greatly reduced. *Wnt-4* expression was never found in the mesenchyme. The tip of the ureteric bud never dilated and branching of the bud did not occur. Neither pretubular cell aggregates nor epithelialization were found in the mesenchyme. Instead the ureteric bud soon degenerated and apoptotic figures were prominent in mesenchymal cells. In explant culture, the mutant ureteric bud did not induce the epithelial transformation of the wild-type mesenchyme, and branching of the mutant ureteric bud was not induced by wild-type mesenchyme. In contrast, defects were not apparent in the mutant mesenchyme by co-culture with wild-type ureteric bud or spinal cord. These results suggest that, in metanephrogenesis, *Emx2* is essential for the ureteric bud functions subsequent to *Pax-2* induction in the metanephric mesenchyme. Degeneration of the Wolffian duct and mesonephric tubules was also abnormally accelerated without the formation of the Müllerian duct.

Key words: *Emx2*, homeobox, mutant mouse, ureteric bud, metanephrogenesis, kidney development, gonadal development

INTRODUCTION

The kidney has been widely exploited as a model system for the study of tissue interactions regulating organogenesis. Its development in mammals proceeds in three major stages: pronephros, mesonephros and metanephros. The nephric duct (Wolffian duct) develops in craniocaudal succession from the intermediate mesoderm and acts upon surrounding mesenchyme as an 'inducer' of epithelial transformation to nephric tubules (Saxén, 1987). The pronephric tubules, mesonephric tubules and the anterior portion of the Wolffian duct eventually degenerate, and it is the metanephros that becomes the permanent kidney. In metanephrogenesis, the metanephric blastema induces the sprouting of the ureteric bud from the caudal region of the Wolffian duct and the growth of this bud into the mesenchyme. The bud invades the mesenchyme and then induces epithelialization of the mesenchyme and its differentiation into the nephron. In turn this metanephric mesenchyme induces the branching of the ureteric bud. Thus, the permanent kidney develops by the reciprocal induction of the mesenchyme upon the epithelium and the epithelium upon the mesenchyme. Both are organized into functional units called uriniferous tubules, which are composed of duct-derived collecting system and mesenchyme-derived nephron (Grobstein, 1953, 1955; Saxén, 1987).

Information has accumulated especially with mutant mice about molecular events underlying the mutual interactions between the ureteric bud and mesenchyme (Bard et al., 1994; Sariola, 1996; Davis and Bard, 1996; Rothenpieler, 1996; Ekblom, 1996; Schofield and Boulter, 1996). In the null mutants of Pax-2, a transcriptional regulator of the paired-box family, the Wolffian duct develops only partially and metanephric development does not take place (Torres et al., 1995). The metanephrogenic mesenchyme deficient in Wilms tumor gene encoding a transcriptional factor with zinc finger motif, WT-1, lacks the ability to induce the formation of the ureteric bud (Kreidberg et al., 1993). The null mutants of c-Ret, a receptor tyrosine kinase, and of GDNF, a member of the transforming growth factor (TGF)-β superfamily, also fail to form ureteric bud (Schuchardt et. al., 1994, 1996; Moore et al., 1996; Sánchez et al., 1996; Pichel et al., 1996). In *Danforth's short tail* mutants, the ureteric bud is initiated, but does not enter the metanephrogenic mesenchyme (Gluecksohn-Schoenheimer, 1943; Mesrobian and Sulik, 1992). Mice lacking Wnt-

4, a secreted glycoprotein, do not form nephron (Stark et al., 1994). The disruption of *BF-2* encoding a Winged Helix transcription factor reduces the rate of differentiation of the condensed mesenchyme into tubular epithelia as well as the rate of growth and branching of the ureter and collecting system (Hatini et al., 1996). Bmp-7 is another member of the TGF-β superfamily of secreted growth factors and, in its mutants, early metanephrogenesis is unaffected, but the mesenchyme cannot continue to proliferate, differentiate and survive (Dudley et al., 1995; Luo et al., 1995).

The homeobox genes *Emx2* and *Emx1* are mouse homologues of a *Drosophila* head gap gene *ems* (Simeone et al., 1992a,b). Homozygous *Emx2* mutant mice display defects in development of the dorsal telencephalon that develops from the *Emx2*-positive and *Emx1*-negative domain in the pallio-choroidial boundary (Yoshida et al., 1997). *Emx2* mutant mice die due to failure of urogenital system development and we have focused our present study on this kidney defect.

MATERIALS AND METHODS

The generation of *Emx2* mutant mice

Emx2 mutant mice were previously generated (Yoshida et al., 1997) and the genotype of each mouse or embryo was routinely determined on tail or yolk sac extracts by PCR (polymerase chain reaction) with the described primers (Yoshida et al., 1997). The mice were housed in an environmentally controlled room of the Animal Facility in Kumamoto University School of Medicine under the guidelines for recombinant and animal experiments of the School.

Embryo sampling

Fetus by crosses among heterozygous mice were collected in PBS and fixed in Bouin's fixative for histological analysis or in 4% paraformaldehyde (PFA) solution for in situ hybridization analysis and germ cell analysis. For the histological and in situ hybridization analyses, embryos were embedded in paraffin wax (Paraplast) and sectioned at 8 and 10 μm thickness, respectively. Sections were stained with Haematoxylin-Eosin. In the analysis of germ cells, the fixed embryos were soaked in 30% sucrose in phosphate-buffered saline (PBS), embedded in OCT compound (TISSUE-TEK, USA) and frozen. Frozen samples were sectioned at 10 μm thickness with a microtome cryostat.

Sex determination of embryos

Sexual genotypes of mutant embryos were determined by PCR analysis with primers specific for the Y chromosome-encoded *Sry* gene as described (Hogan et al., 1994). PCR primers used were 5′-GAGAGCATGGAGGGCCAT-3′ and 5′-CCACTCCTCTGTGACACT-3′ to amplify a 200 base pair (bp) fragment. A fragment from the *Zfy* gene, which is present in both male and female genomes, was amplified as a control; PCR primers were 5′-GACTAGACATGTCTTAACATCTGTCC-3′ and 5′-CCTATTGCATGGACTGCAGCTTATG-3′ to detect a 120 bp fragment.

In situ hybridization

In situ hybridization analysis was performed using digoxigenin-labeled antisense riboprobe as described (Wilkinson, 1993). The probes used were as described for *Emx2* (Yoshida et al., 1997), *WT-1* (Kudoh et al., 1995), *Pax-2* (Dressler et al., 1990), *Lim1* (Barnes et al., 1994) and *Wnt-4* (Stark et al., 1994). pKSmcRetXR is a plasmid for c-*ret* riboprobe synthesis and was kindly provided by Dr M. Yanagisawa. *GDNF* cDNA was isolated by PCR from a cDNA pool prepared with 9.5 dpc (days post coitus) ICR mouse embryos and subcloned into pBluescript SK. Sequence data of *GDNF* was obtained from GenBank (Accession Number; U37549).

Analysis of germ cells

Endogenous alkaline phosphatase activity of primordial germ cells (PGCs) was determined using the nitroblue tetrazolium reaction with frozen sections of 11.5 dpc embryos as described (Harlow and Lane, 1988).

Kidney explant cultures

Cultures were set up essentially as described by Saxén and Lehtonen (1987). The isolated metanephros was soaked in PBS containing 1 mM ethylendiamine tetraacetic acid (EDTA, pH 8.0) for 15 minutes, and the ureteric bud and metanephric mesenchyme were mechanically separated with a sharpened tungsten needle and co-cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum.

RESULTS

Homozygous *Emx2* mutants lack urogenital system

The *Emx2* mutants were previously generated (Yoshida et al., 1997). In brief, the gene was disrupted in front of its homeodomain by inserting neomycine phosphotransferase gene. The homozygous mutants obtained by intercrosses of heterozygotes died soon after birth. PCR analysis with primers specific for the Y chromosome-encoded *Sry* gene proved that both male and female mutants exhibited the same phenotype. They lacked kidneys, ureters, gonads and genital tracts completely; vas deferens, epididymis, ductulus efferentes and seminal vescicles were lost in male mutants, and oviducts, uterus and the upper part of the vagina in female (Fig. 1). Adrenal glands and bladder developed normally. No defects were found in the heterozygous urogenital system.

Urogenital development in *Emx2* mutant mice

The development of metanephros was grossly normal till 11.5 dpc in the mutants. The metanephric blastema was apparently normal at 10.5 dpc (Fig. 2A,B), and the invasion of the ureteric bud to the metanephric blastema was found at 11.5 dpc mutants (Fig. 2C,D). However, the dilation of the tip of the ureteric bud

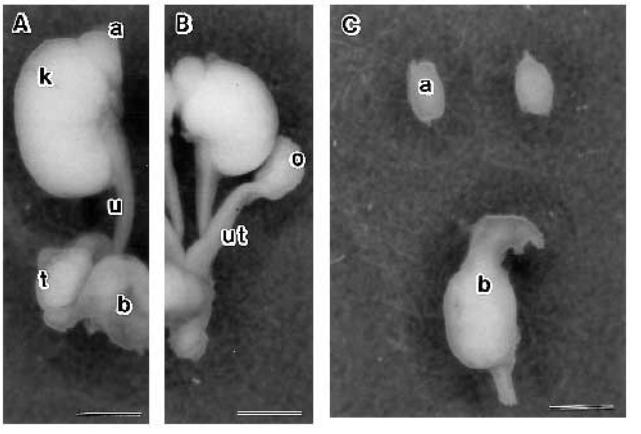


Fig. 1. Urogenital defects in homozygous *Emx2* mutants. 16.5 dpc normal male (A), female (B) and homozygous mutant (C) embryos. Mutants lack kidneys, ureters, gonads and genital tracts completely, while the adrenal glands and bladder develop normally. The mutant phenotype is the same in both sexes. Heterozygous *Emx2* mutants displayed no defects in the urogenital system. Abbreviations: a, adrenal gland; b, bladder; k, kidney; o, ovary; t, testis; u, ureter; ut, uterus. Bars: 1 mm.

was never found (Fig. 2D). In 12.5 and 13.5 dpc wild-type embryos, the invading ureteric bud branches several times, the metanephric mesenchyme cells are induced to transform into epithelial cells around each bud and the nephron is actively formed (Fig. 2E,G). Neither the branching of the ureteric bud nor the epithelial transformation of mesenchyme was observed in the mutant metanephros (Fig. 2F,H); pretubular cell aggregates, comma- or S-shaped bodies, were never observed in the mutant blastema. Instead, the invading ureteric bud had begun to degenerate at 12.5 dpc (Fig. 2F) and had disappeared in the metanephric blastema by 13.0 dpc (Fig. 2H). The size of the mutant blastema did not increase at 12.5 dpc (Fig. 2F), and a number of apoptotic cells with dark nuclear fragments were found at 13.0 dpc (Fig. 2H,I).

In mesonephros, the Wolffian duct developed normally along the urogenital ridge in 10.5 dpc homozygous mutant embryos (Fig. 3B). Mesonephric tubules adjacent to the Wolffian duct were also apparently normal at 10.5 dpc. The 11.5 dpc mutant mesonephros was grossly normal (Fig. 3D), but degeneration of the Wolffian duct was found in several sites (Fig. 3E). Degeneration of the duct never occurs at this stage in the wild-type embryos (Fig. 3C). In wild-type 11.5 dpc embryos, the thickening of the epithelium on the coelomic surface of the urogenital ridge marks the first stage of the gonadal development (Fig. 3C). Thickening of the coelomic epithelium was not prominent in the 11.5 dpc mutants (Fig. 3D,E); gonadal cells were sparse and the coelomic surface was rough. At 13.0 dpc, the invaginations of the mesonephros and

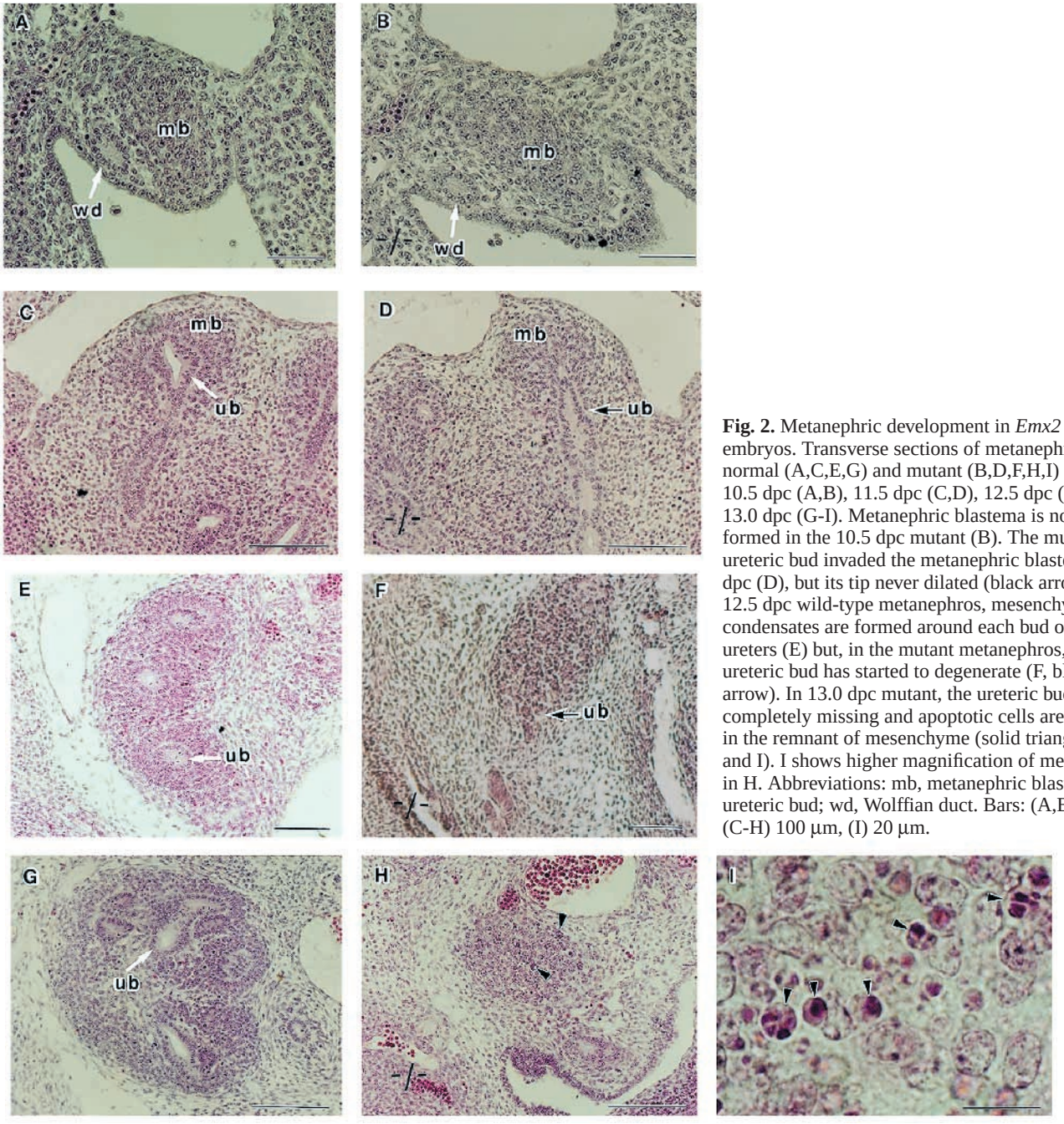


Fig. 2. Metanephric development in *Emx2* mutant embryos. Transverse sections of metanephros in normal (A,C,E,G) and mutant (B,D,F,H,I) embryos at 10.5 dpc (A,B), 11.5 dpc (C,D), 12.5 dpc (E,F) and 13.0 dpc (G-I). Metanephric blastema is normally formed in the 10.5 dpc mutant (B). The mutant ureteric bud invaded the metanephric blastema at 11.5 dpc (D), but its tip never dilated (black arrow). In the 12.5 dpc wild-type metanephros, mesenchymal condensates are formed around each bud of branched ureters (E) but, in the mutant metanephros, the ureteric bud has started to degenerate (F, black arrow). In 13.0 dpc mutant, the ureteric bud is completely missing and apoptotic cells are prominent in the remnant of mesenchyme (solid triangles in H and I). I shows higher magnification of metanephros in H. Abbreviations: mb, metanephric blastema; ub, ureteric bud; wd, Wolffian duct. Bars: (A,B) 50 μm, (C-H) 100 μm, (I) 20 μm.

gonad into the coelom are prominent in the wild-type embryos (Fig. 3F), but these invaginations were very poor in the mutants (Fig. 3G). The PGCs migrate into the gonad from the endodermal region of the yolk sac at 11.5 dpc. Alkaline phosphatase staining showed that the migration of the PGCs to the genital ridge normally occurred in the mutants (Fig. 3H,I). In both male and female, the Müllerian duct normally develops in parallel with the Wolffian duct around 13.0 dpc (Fig. 3F), from which the oviducts, uterus and upper part of the vagina are formed in female. Müllerian duct was never formed in the mutants (Fig. 3G).

Emx2 expression during normal nephrogenesis

To assess the site affected by the *Emx2* mutation, the *Emx2* expression was examined in the developing kidney. At 8.5 dpc, this expression was detected in the nephrogenic cord (Fig. 4A) and, at 9.5 dpc, it was observed in the Wolffian duct (Fig. 4B). In the developing mesonephros of wild-type mouse embryos at 10.5 and 11.5 dpc, *Emx2* transcripts were found in the Wolffian duct, mesonephric tubules and coelomic epithelium (Fig. 4C,D). The expression was not detectable in the mesenchyme cells of the mesonephros. In metanephros at 11.5 dpc, the invading ureteric bud hybridized with the *Emx2* probe, whereas

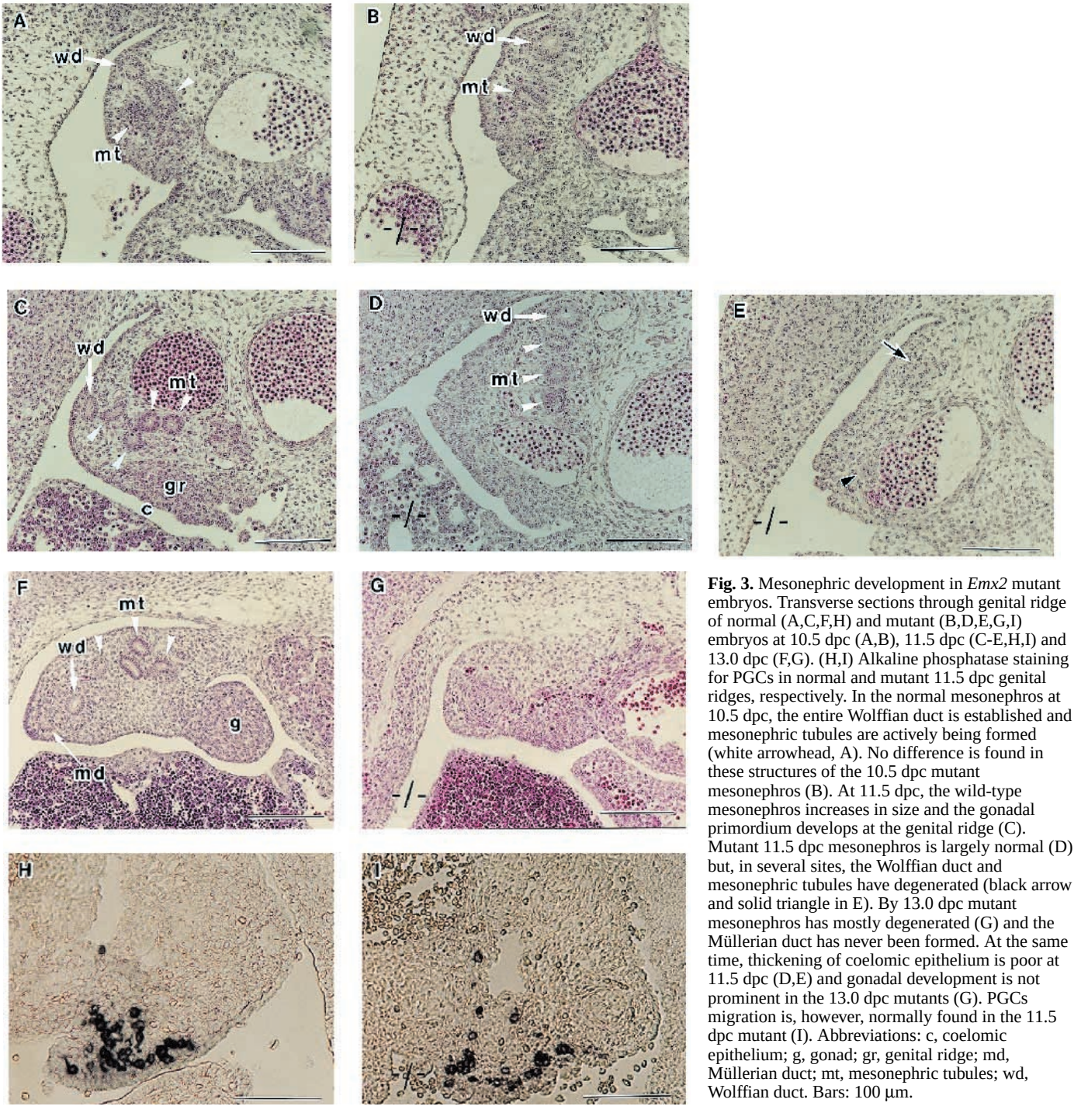


Fig. 3. Mesonephric development in *Emx2* mutant embryos. Transverse sections through genital ridge of normal (A,C,F,H) and mutant (B,D,E,G,I) embryos at 10.5 dpc (A,B), 11.5 dpc (C-E,H,I) and 13.0 dpc (F,G). (H,I) Alkaline phosphatase staining for PGCs in normal and mutant 11.5 dpc genital ridges, respectively. In the normal mesonephros at 10.5 dpc, the entire Wolffian duct is established and mesonephric tubules are actively being formed (white arrowhead, A). No difference is found in these structures of the 10.5 dpc mutant mesonephros (B). At 11.5 dpc, the wild-type mesonephros increases in size and the gonadal primordium develops at the genital ridge (C). Mutant 11.5 dpc mesonephros is largely normal (D) but, in several sites, the Wolffian duct and mesonephric tubules have degenerated (black arrow and solid triangle in E). By 13.0 dpc mutant mesonephros has mostly degenerated (G) and the Müllerian duct has never been formed. At the same time, thickening of coelomic epithelium is poor at 11.5 dpc (D,E) and gonadal development is not prominent in the 13.0 dpc mutants (G). PGCs migration is, however, normally found in the 11.5 dpc mutant (I). Abbreviations: c, coelomic epithelium; g, gonad; gr, genital ridge; md, Müllerian duct; mt, mesonephric tubules; wd, Wolffian duct. Bars: 100 μm.

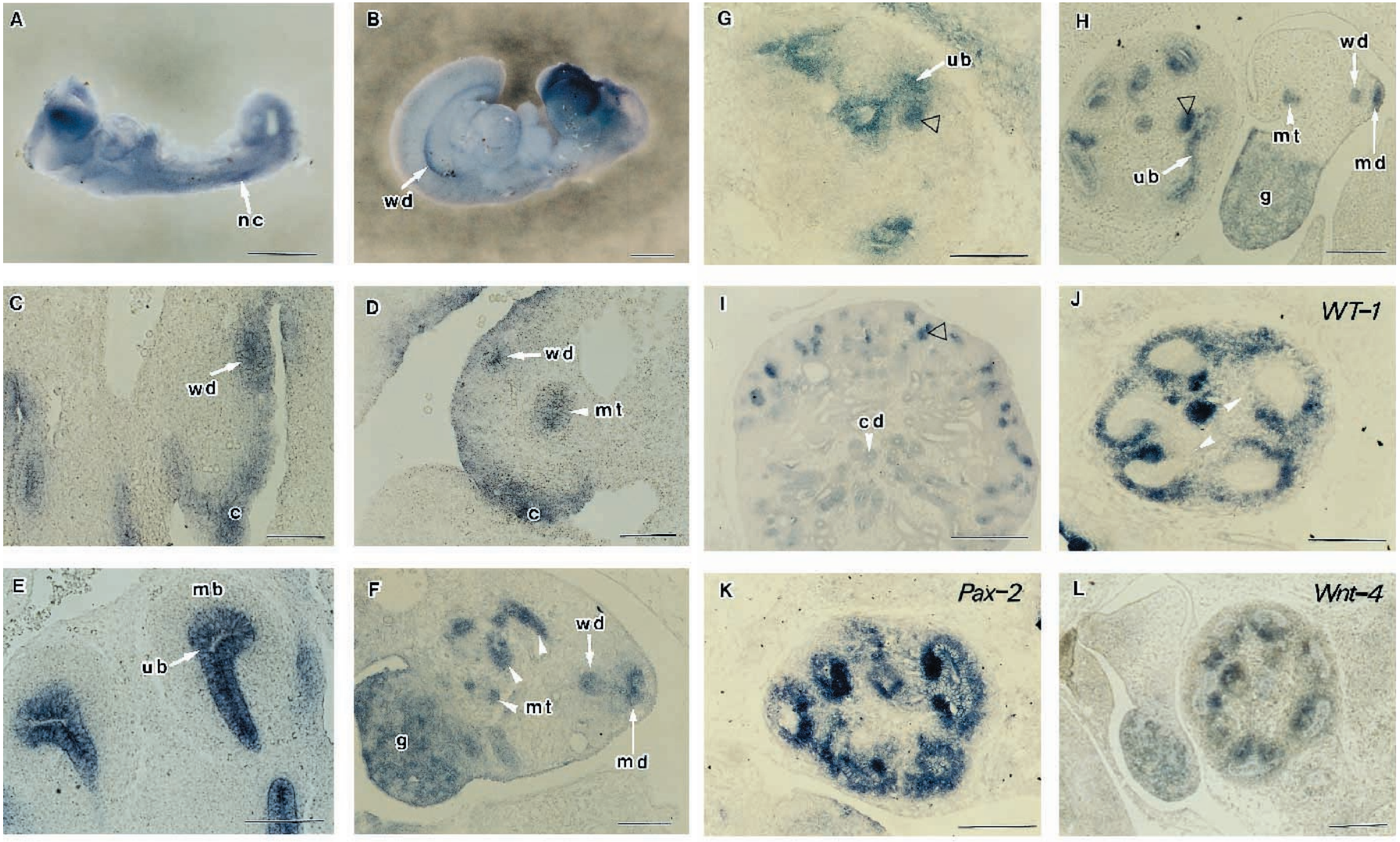


Fig. 4. *Emx2* expression during normal urogenital development. Lateral views of whole-mount 8.5 dpc (A) and 9.5 dpc (B) embryos. Staining is detected in the presumptive nephrogenic cord (white arrow in A) and in the Wolffian duct (white arrow in B). Transverse sections in 10.5 dpc (C), 11.5 dpc (D,E), 12.5 dpc (F,G), 13.5 dpc (H) and 16.5 dpc (I) wild-type mesonephros (C,D,F,H) and metanephros (E,G-I). In the mesonephros, *Emx2* expression at 10.5 and 11.5 dpc is found in Wolffian duct, mesonephric tubules and coelomic epithelium, while mesenchyme cells do not show *Emx2* expression (C,D). Later, *Emx2* expression is also observed in gonad and newly formed Müllerian duct (F,H). In the metanephros, *Emx2* is expressed in the ureteric bud, which has invaded the blastema at 11.5 dpc (E), whereas the surrounding mesenchyme, including pretubular cell aggregates, do not express *Emx2*. Later, *Emx2* expression is found in the branched ureter and mesenchyme cells, which are undergoing epithelialization (G,H; open triangles). At 16.5 dpc, *Emx2* expression becomes restricted to the cortical zone of the kidney where new nephric tubules are being formed (open triangle in I). In the collecting ducts, the *Emx2* expression has diminished (white arrowhead). (J-L) *WT-1*, *Pax-2* and *Wnt-4* expression, respectively, in the 13.5 dpc metanephros. *WT-1* is expressed in the condensed mesenchyme (Armstrong et al., 1992), but not in the *Wnt-4*-positive epithelializing mesenchyme (white arrowheads in J). *Pax-2* expression is found not only in condensed mesenchyme, but also in epithelializing mesenchyme and branched ureters (K; Dressler et al., 1990). *Wnt-4* is expressed in pretubular cell aggregates, the comma- and S-shaped bodies (L; Stark et al., 1994). The *Emx2* expression in the mesenchyme is more restricted than those in the cells at a later stage of epithelialization, possibly in the S-shaped body (H). Abbreviations: c, coelomic epithelium; cd, collecting duct; g, gonad; mb, metanephric blastema; md, Müllerian duct; mt, mesonephric tubules; nc, presumptive nephrogenic cord; ub, ureteric bud; wd, Wolffian duct. Bars: (A,B) 400 μm, (C,D,F) 50 μm, (E,G,H,J-L) 100 μm, (I) 200 μm.

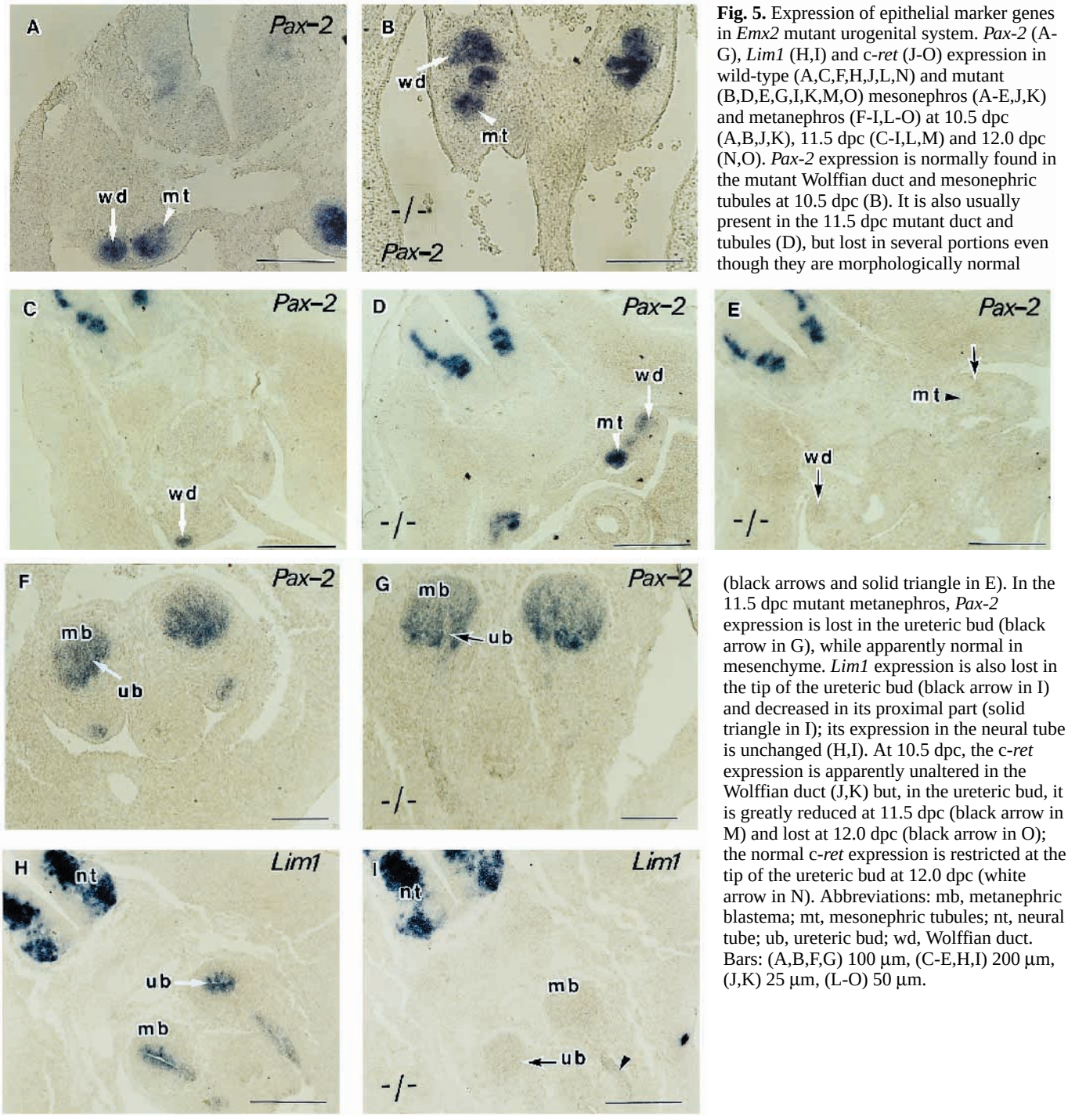
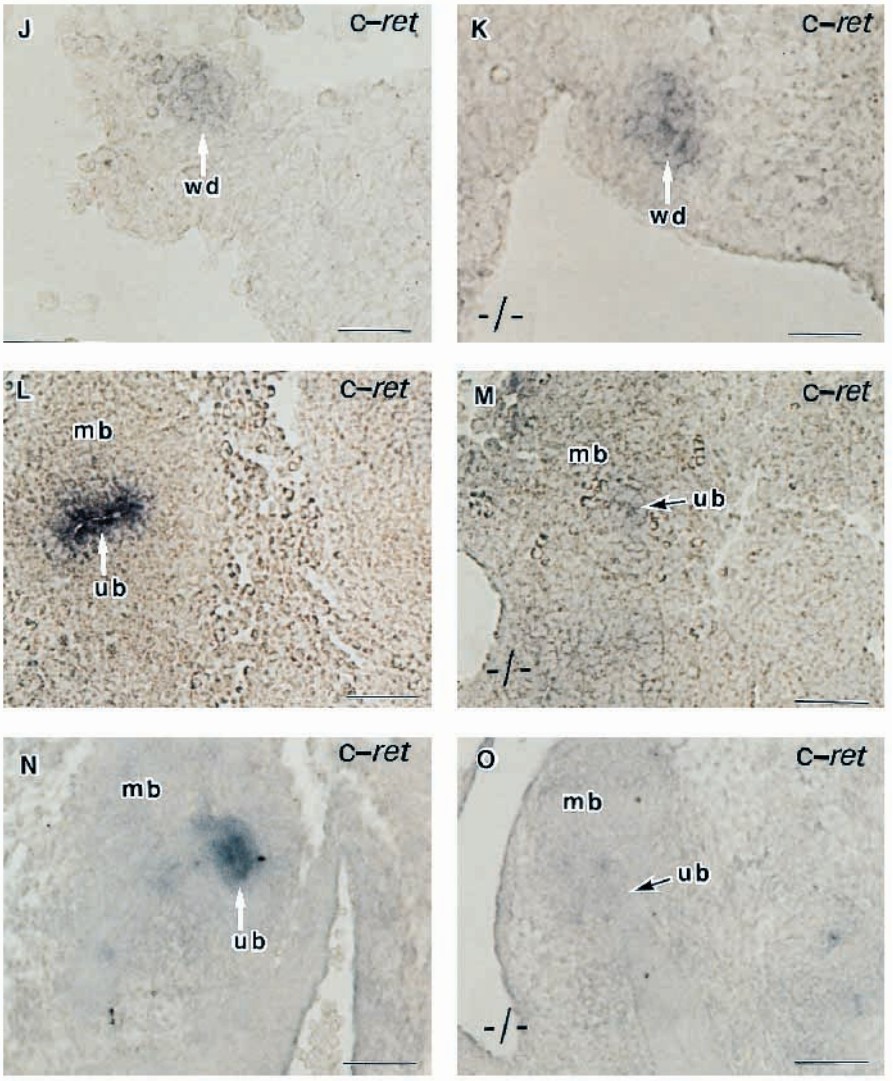


Fig. 5. Expression of epithelial marker genes in *Emx2* mutant urogenital system. *Pax-2* (A-G), *Lim1* (H,I) and c-*ret* (J-O) expression in wild-type (A,C,F,H,J,L,N) and mutant (B,D,E,G,I,K,M,O) mesonephros (A-E,J,K) and metanephros (F-I,L-O) at 10.5 dpc (A,B,J,K), 11.5 dpc (C-I,L,M) and 12.0 dpc (N,O). *Pax-2* expression is normally found in the mutant Wolffian duct and mesonephric tubules at 10.5 dpc (B). It is also usually present in the 11.5 dpc mutant duct and tubules (D), but lost in several portions even though they are morphologically normal (black arrows and solid triangle in E). In the 11.5 dpc mutant metanephros, *Pax-2* expression is lost in the ureteric bud (black arrow in G), while apparently normal in mesenchyme. *Lim1* expression is also lost in the tip of the ureteric bud (black arrow in I) and decreased in its proximal part (solid triangle in I); its expression in the neural tube is unchanged (H,I). At 10.5 dpc, the c-*ret* expression is apparently unaltered in the Wolffian duct (J,K) but, in the ureteric bud, it is greatly reduced at 11.5 dpc (black arrow in M) and lost at 12.0 dpc (black arrow in O); the normal c-*ret* expression is restricted at the tip of the ureteric bud at 12.0 dpc (white arrow in N). Abbreviations: mb, metanephric blastema; mt, mesonephric tubules; nt, neural tube; ub, ureteric bud; wd, Wolffian duct. Bars: (A,B,F,G) 100 μm, (C-E,H,I) 200 μm, (J,K) 25 μm, (L-O) 50 μm.

the surrounding mesenchyme including pretubular cell aggregates did not express *Emx2* at all (Fig. 4E). At 12.5 dpc, *Emx2* expression was also detected in the newly formed Müllerian duct (Fig. 4F). The expression was continuous in 13.5 dpc Wolffian duct, mesonephric tubules, the Müllerian duct and gonad (Fig. 4H). In the 12.5 and 13.5 dpc metanephros, *Emx2* was expressed not only in the branched ureter, but also in an unique population of metanephric mesenchyme (Fig. 4G,H). Later, at 16.5 dpc, the *Emx2* expression became confined to the cortical zone of the kidney, where new nephric tubules are being formed (Fig. 4I). At this stage, the expression in the collecting ducts of the medial zone was decreased.

Of note is that, in the process of epithelialization of the metanephric mesenchyme, *Emx2* expression in the mesenchyme occurs at a later stage of epithelialization. *WT-1* and *GDNF* are expressed in the metanephric blastema in advance of the invasion of ureteric bud (Fig. 6C; Armstrong et al., 1992), and their expressions at 11.5 dpc are found througout the metanephric mesenchyme with the invading ureteric bud (Fig. 6A,E; Armstrong et al., 1992; Sánchez et al., 1996). At subse-



quent stages, their expressions are found in the condensed mesenchyme surrounding the bud of each ureteric branch (Fig. 4J; Pelletier et al., 1991; Armstrong et al., 1992; Moore et al., 1996; Sánchez et al., 1996; Hellmich et al., 1996). *Pax-2* expression is first observed in the metanephric mesenchyme when the ureteric bud invades (Fig. 5F; Dressler et al., 1990). Thereafter, it is continuously expressed in the condensed and epithelializing mesenchyme as well as ureter (Fig. 4K; Dressler et al., 1990; Dressler and Douglass, 1992). *Wnt-4* expression is restricted to pretubular cell aggregates on both sides of the 11.5 dpc invading ureteric bud (Fig. 6G; Stark et al., 1994). At 12.0 and 13.5 dpc, it was found in an increasing number of pretubular cell aggregates, comma- and S-shaped bodies (Figs 4L, 6I; Stark et al., 1994). In contrast, *Emx2* was not expressed in pretubular cell aggregates at 11.5 dpc (Fig. 4E), and was expressed at 12.5 and 13.5 dpc in more limited populations of mesenchyme that would be in a later stage of epithelialization (Fig. 4G,H).

Expressions of epithelial and mesenchymal marker genes

As the next step in determining the defect caused by the *Emx2* mutation, the expressions of several molecular marker genes during nephrogenesis were examined. *Pax-2* is normally expressed in the Wolffian duct and mesonephric tubules (Fig. 5A,C; Dressler et al., 1990). Its expression at 10.5 dpc was apparently normal in the mutant mesonephros (Fig. 5B). At 11.5 dpc, however, the *Pax-2* expression was lost in several portions even where the Wolffian duct and mesonephric tubules were morphologically normal (Fig. 5E); its expression in the neural tube was unaffected (Fig. 5D,E). In metanephros, *Pax-2* is expressed not only in the ureteric bud but also in the mesenchyme (Fig. 5F; Dressler et al., 1990). In the *Emx2* mutants, *Pax-2* expression in the mesenchyme was normally found, but that in the ureteric bud was greatly reduced; it was faint at the tip of the bud and lost in the proximal part (Fig. 5G). *Lim1* transcripts are also present in the wild-type ureteric bud (Fig. 5H; Fujii et al., 1994). Its expression was completely lost in the tip of the mutant ureteic bud and was weak in the proximal part of the bud (Fig. 5I). The c-*ret* is another epithelial marker (Fig. 5J,L,N; Pachinis et al., 1993; Tsuzuki et al., 1995). The c-*ret* expression in the mutant Wolffian duct was apparently normal at 10.5 dpc (Fig. 5K), but its expression in the 11.5 dpc mutant ureteric bud was greatly diminished (Fig. 5M). No c-*ret* expression was found in the tip of the 12.0 dpc mutant bud that had begun to degenerate (Fig. 5O). The *WT-1* expression in the metanephric mesenchyme was normally found (Fig. 6A,B). The *GDNF* expression in the 10.5 dpc metanephric blastema before the invasion of the ureteric bud was normal (Fig. 6C,D), but the 11.5 dpc expression around the invading ureteric bud was greatly reduced in the mutant (Fig. 6E,F). The *Wnt-4* expression in pretubular cell aggregates was never found in the mutant metanephric mesenchyme (Fig. 6H,J).

Epithelial transformation of *Emx2* mutant metanephric blastema by wild-type ureter in culture

To determine whether it is the ureteric bud or the mesenchyme that is lesioned by the *Emx2* mutation, ureteric bud and metanephric blastema were cultured in vitro (Fig. 7A,B; Grobstein, 1955). Neither epithelialization of the mesenchyme nor branching of the ureteric bud occurred in co-culture of the 11.5 dpc mutant ureteric bud and mesenchyme (Fig. 7C,D). In addition, when the mutant ureteric bud was co-cultured with the wild-type metanephric blastema, neither the epithelial transformation of the wild-type mesenchyme nor the branching of the mutant bud was observed (Fig. 7E,F). In contrast, when the isolated wild-type ureteric bud was co-cultured with the mutant metanephric blastema, epithelial transformation occurred in the mutant mesenchyme and the branching was

induced in the wild-type ureteric bud (Fig. 7G,H), though the number of branchings was somewhat less than that in co-culture of wild-type counterparts (Fig. 7A,B). Spinal cord is also known to stimulate epithelial transformation of the metanephric blastema (Fig. 7I; Grobstein, 1955). Indeed, the isolated mutant metanephric mesenchyme was successfully epithelialized by the wild-type dorsal spinal cord (Fig. 7J).

DISCUSSION

In metanephrogenesis, the ureteric bud sprouts from the caudal region of the Wolffian duct and invades the metanephric mesenchyme, possibly guided by a chemotactic signal from the mesenchyme (Kreidberg et al., 1993; Moore et al., 1996; Sánchez et al., 1996; Pichel et al., 1996; Schuchardt et al., 1996). The invading ureteric bud activates the mesenchyme to transform into the epithelia (Grobstein, 1955). The tip of the bud dilates and, at both sides of this dilated tip, the bud induces the mesenchymal cells to form cell aggregates. These pretubular cell aggregates elongate into a ‘comma’ shape and then form a characteristic S-shaped body. The body fuses with the ureter yielding the nephron. At the same time, the growth and dichotomous branching of the ureteric bud is supported by the nephrogenic mesenchyme to give rise to the collecting duct (Saxén, 1987). The present analysis strongly suggests that the defect in metanephrogenesis by the *Emx2* mutation resides in the ureteric bud, and the *Emx2* function in the epithelium is crucial in the reciprocal induction with the mesenchyme for nephrogenesis.

The *WT-1* is expressed in the metanephric blastema (Fig. 6A; Armstrong et al., 1992) and is believed to be involved in outgrowth of

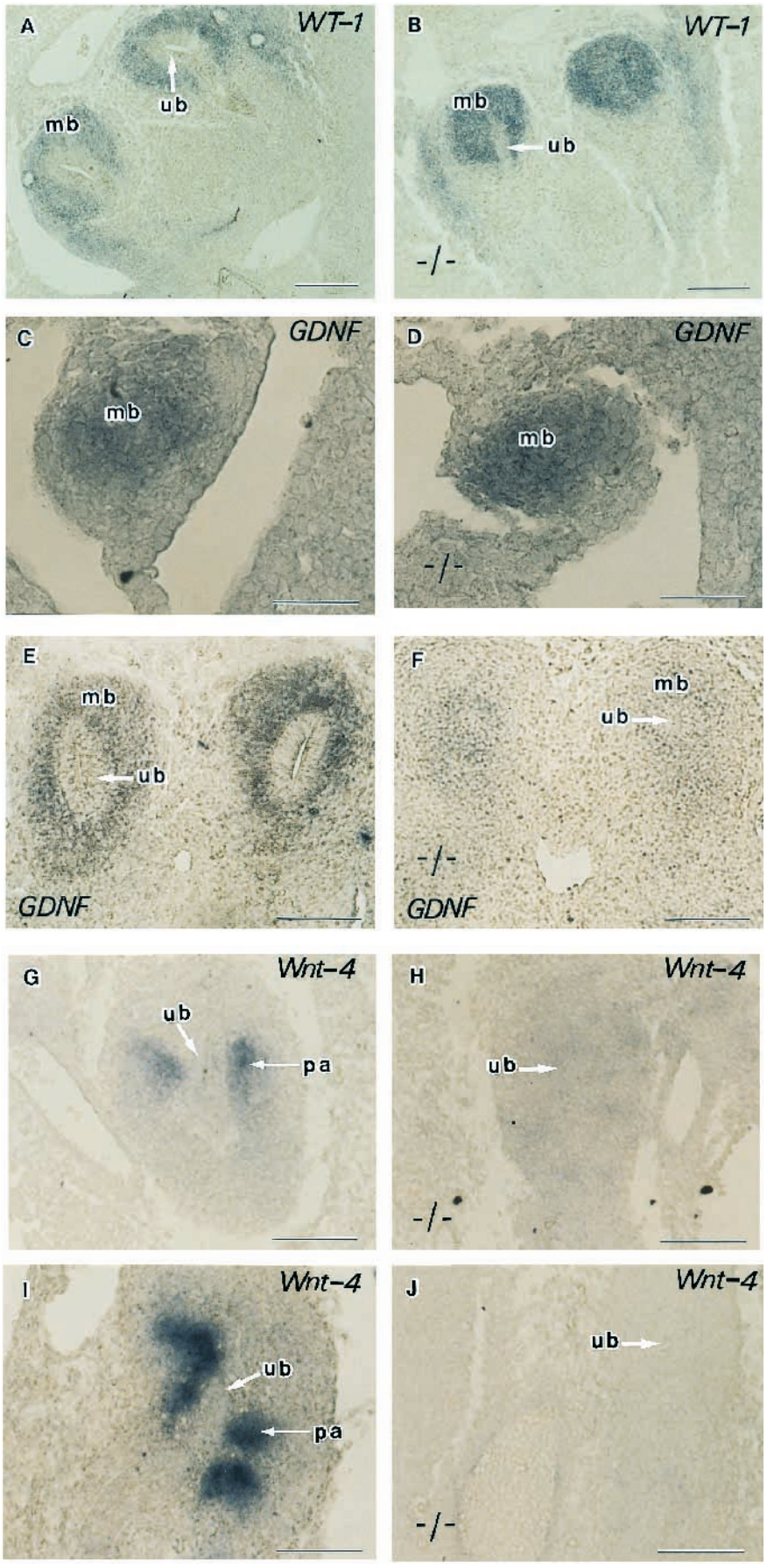


Fig. 6. Expressions of mesenchymal marker genes in *Emx2* mutant urogenital system. *WT-1* (A,B), *GDNF* (C-F) and *Wnt-4* (G-J) expressions in wild-type (A,C,E,G,I) and mutant (B,D,F,H,J) metanephros at 10.5 dpc (C,D), 11.5 dpc (A,B,E-H) and 12.0 dpc (I,J). The *WT-1* expression is unaffected (A,B). *GDNF* expression is also normal in the 10.5 dpc mutant mesenchyme (D), but is greatly reduced in the 11.5 dpc mutant metanephros (F). *Wnt-4* expression is normally found in pretubular cell aggregates along the invaded ureteric bud at 11.5 dpc (G) and in an increasing number of the aggregates, comma- and S-shaped bodies, at 12.0 dpc (I). Its expression is not found in the mutant mesenchyme (H,J). Abbreviations: mb, metanephric blastema; pa, pretubular cell aggregates. ub, ureteric bud. Bars: (A,B) 100 μm, (C-J) 50 μm.

the ureteric bud (Kreidberg et al., 1993). Recently, *GDNF*-deficiency has also been reported to fail to induce the ureteric bud (Moore et al., 1996; Sánchez et al., 1996, Pichel et al., 1996); *GDNF* is expressed in the mesenchyme of metanephros (Fig. 6C, E; Moore et al., 1996; Sánchez et al., 1996; Hellmich et al., 1996). c-Ret activity is stimulated by GDNF (Durbec et al., 1996; Jing et al., 1996; Trupp et al., 1996; Treanor et al., 1996). The receptor type tyrosine kinase is initially expressed in the Wolffian duct and, in the developing metanephros, its expression is gradually restricted to the tip of the ureteric bud (Fig. 5N; Pachinis et al., 1993; Tsuzuki et al., 1995). c-*ret* knockout mice fail to form the ureteric bud (Schuchardt et al., 1994). Thus the *WT-1*, *GDNF* and c-*ret* mutant phenotypes are closely related to each other; it is most plausible that the GDNF secretion from the mesenchyme is regulated by WT-1 and its signaling in the ureteric bud epithelium is mediated by c-Ret. In *Emx2* mutants, the *WT-1* expression was unaffected. The *GDNF* expression appeared also normal in the metanephric blastema before the invasion of ureteric bud (Fig. 6D). The c-*ret* was also expressed normally in the Wolffian duct before the budding of the bud (Fig. 5K). In all the *Emx2* mutants, the ureteric bud was induced and invaded the metanephrogenic blastema. Thus, defects are not present in the inductive capability of the mesenchyme or in the reactivity of the epithelium for the initial growth of ureteric bud.

Pax-2 is one of the earliest responding genes in the

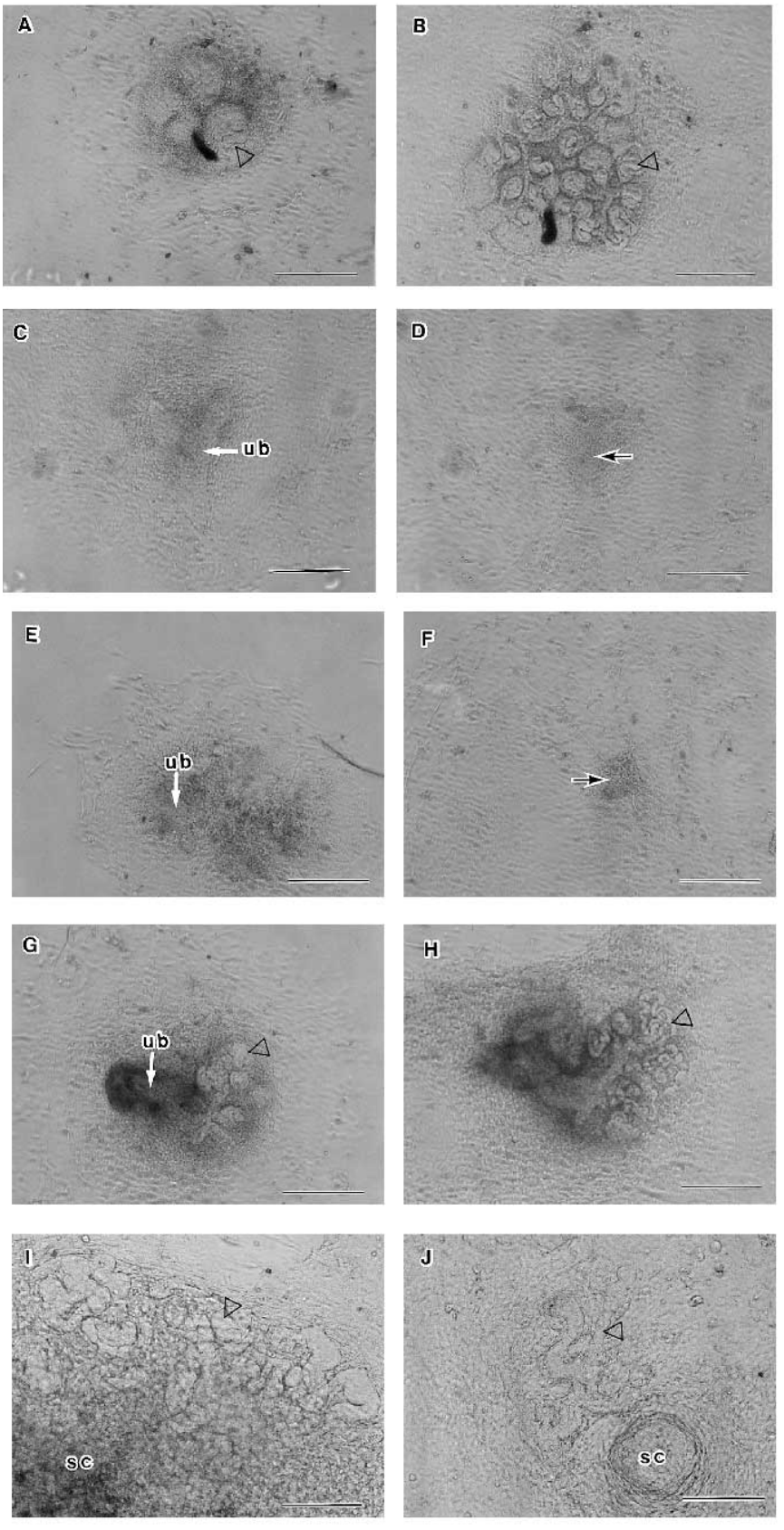


Fig. 7. Developmental potential of the mutant ureteric bud and mesenchyme in culture. 11.5 dpc wild-type and mutant metanephros were separated into ureteric bud and mesenchyme and cultivated in different combinations for 2 days (A,C,E,G) or 4 days (B,D,F,H). (A,B) Wild-type bud is co-cultured with wild-type mesenchyme. In six out of nine experiments, they underwent nephrogenesis. (C,D) Mutant bud is co-cultured with mutant mesenchyme; as in the in vivo observation, neither branching of the bud nor epithelialization of the mesenchyme occurred in any of four experiments. (E,F) Mutant bud is co-cultured with wild-type mesenchyme and, again, neither branching of the bud nor epithelialization of the mesenchyme occurred in any of the five experiments. (G,H) Wild-type bud is co-cultured with mutant mesenchyme. Epithelialization of the mesenchyme and branching of the bud occurred in seven and four cases out of ten experiments, respectively, suggesting that the mutant mesenchyme is normal. (I) Wild-type mesenchyme is co-cultured with dorsal spinal cord for 8 days; epithelialization of mesenchyme was induced in seven out of ten experiments (Grobstein, 1955). (J) Mutant mesenchyme is co-cultured with wild-type dorsal spinal cord. The epithelialization was induced in two out of three experiments, confirming that the mutant mesenchyme is normal. Black arrows indicate the ureteric bud remnants, and open triangles the epithelialized mesenchyme. Abbreviations: sc, spinal cord; ub, ureteric bud. Bars: (A,B,E-H) 500 μm, (C,D) 200 μm, (I,J) 100 μm.

metanephric mesenchyme upon induction by the ureteric bud (Fig. 5F; Dressler et al., 1990) and it is continuously expressed throughout the process of epithelialization. The antisense oligonucleotides to *Pax-2* inhibits the condensation of mesenchyme cells in culture (Rothenpieler and Dressler, 1993). The *Emx2* mutant bud could induce *Pax-2* expression in the mesenchyme. In contrast, *Wnt-4* expression that normally occurs in pretubular cell aggregates was never found in the *Emx2* mutant metanephros. None of the mutants examined showed any histological evidence of mesenchymal aggregation, nor did we ever observe the comma-shaped or S-shaped body. Normal *Emx2* expression is also found in mesenchyme cells, although here it occurs at a later stage of epithelialization, possibly in the S-shaped body, and the *Emx2* mutant mesenchyme had deteriorated much earlier than this stage. Thus, the *Emx2* mutant ureteric bud has a signal to induce *Pax-2* expression in the mesenchyme, but lacks a signal to induce further epithelialization there.

The *Wnt-4* mutant metanephros fails to form pretubular cell aggregates but undergoes considerable branching of the ureter (Stark et al., 1994), whereas the branching was never found in the *Emx2* mutant metanephros. GDNF/c-Ret signaling is thought essential not only in the initial growth of the ureteric bud but also in branching of the bud (Schuchardt et al., 1996). In *Emx2* mutant, *GDNF* and c-*ret* expressions were reduced in the mesenchyme and ureteric bud, respectively, when the bud invaded the blastema. A signal from the bud invading the metanephric blastema may be necessary to maintain *GDNF* expression in the blastema; this signal may be lost in the *Emx2* mutant ureteric bud. At the same time, *Emx2* may act to retain c-*ret* expression in the invading bud.

It is known that, in the absence of a signal(s) from the ureteric bud, metanephric mesenchyme cells not only fail to differentiate but they undergo apoptosis (Koseki et al., 1992; Kreidberg et al., 1993; Coles et al., 1993; Herzlinger, 1995; Camp and Martin, 1996). In the *WT-1* and c-*ret* mutants, all of which fail to form ureteric bud, the metanephric mesenchyme undergoes prominent apoptosis (Kreidberg et al., 1993; Schuchardt et al., 1996). In contrast, the apoptoic figures are not prominent in *Wnt-4* mutants that fail to epithelialize mesenchyme but have normal ureteric epithelium (Stark et al., 1994). These findings implicate a survival factor(s) from the ureteric bud that inhibits apoptosis of the mesenchyme (Camp and Martin, 1996). In *Emx-2* mutants, the ureteric bud invaded the mesenchyme, but apoptotic figures were prominent. The *Emx2* mutant ureteric bud may also be unable to generate the survival factor.

The results of co-culture experiments are also consistent with the deterioration of the *Emx2* mutant ureteric bud, its mesenchyme being normal. The *Emx2* mutant ureteric bud did not induce epithelialization of normal mesenchyme, whereas epithelialization of the mutant mesenchyme was induced by the isolated wild-type ureteric bud and dorsal spinal cord. Branching of the wild-type ureteric bud was also induced by the mutant mesenchyme, while it was not induced in the mutant ureteric bud by wild-type mesenchyme. In the wild-type ureteric bud growing into the mesenchyme not only c-*ret* but also *Pax-2* and *Lim1* are expressed. In *Emx2* mutants, the expressions of these genes in the invading ureteric bud were greatly reduced or completely lost. The ureteric bud was lost in the *Emx2* mutant embryos soon after its invasion into the metanephric blastema. The most conspicuous morphological feature was the non-dilatation of the tip of the invading bud, which is an early branching feature and may be no different from subsequent branchings, regulated by GDNF/c-Ret signaling. Alternatively, it might require a specific process in which *Emx2* has a crucial function so that the gene is essential for all the subsequent steps regulating the epithelial functions of expressions of c-*Ret*, *Pax-2* and *Lim1* as well as signalings to the mesenchyme.

The *Emx2* defects were apparent at 11.5 dpc not only in metanephros but also in mesonephros; no defects were detected at 10.5 dpc either morphologically or molecularly. Degeneration of the Wolffian duct and mesonephric tubules was found in several sites at 11.5 dpc. Studies of *Pax-2* expression have shown that the degeneration progressed more widely in the epithelia that appeared morphologically normal. In contrast to *Pax-2*, *Emx2* (cf. Fig. 4I) as well as *Lim1* (Fujii et al., 1994) are expressed only in earlier phases of the nephrogenic epithelium, suggesting that they are not essential in the epithelium once established. However, in *WT-1*, c-*ret* and *GDNF* mutants that cannot form ureteric bud, Wolffian duct degeneration is not accelerated (Kreidberg et al., 1993; Schuchardt et al., 1994; Moore et al., 1996; Sánchez et al., 1996; Pichel et al., 1996), suggesting that the loss of the ureteric bud in the *Emx2* mutant itself is not the cause of the mesonephric degeneration. It is possible that *Emx2* acts to sustain the overall epithelium in the earlier stages, not specifically the ureteric bud. Then the question would be raised as to why the deterioration happened at the stage when the ureteric bud invades the metanephric blastema.

In several kidney mutants, the homozygous phenotype exhibits considerable variability and the defects were also found by haplo-insufficiency. Most *GDNF* homozygous mutants do not develop ureteric bud as noted above, but some do, while some heterozygotes do not (Moore et al., 1996; Sánchez et al., 1996; Pichel et al., 1996). Only one third of the c-*ret* newborn mutants display a complete absence of ureter and kidney bilaterally, and one-tenth have kidney rudiments bilaterally (Schuchardt et al., 1994, 1996). Several *Wnt-4*-deficient metanephroi develop limited small cell aggregates (Stark et al., 1994). The variability in phenotype may implicate the presence of closely related molecules with functions that overlap. In contrast, the *Emx2* mutant phenotype displayed little variability. The *Emx1*, a cognate of *Emx2*, is also expressed in the kidney (Briata et al., 1996; our unpublished data). No defects were found in the kidney development by the *Emx1* mutation that we previously generated, however (unpublished data).

Relatively little is known about the genetic basis of gonadal development at early stages when sexual differentiation by SRY has not yet occurred. *WT-1* and an orphan nuclear receptor *SF-1/Ftz-F1* are the only two genes known to be involved in this process (Kreidberg et al., 1993; Luo et al., 1994; Jiménez et al. 1996). The defect in gonadal development by *WT-1* deficiency is apparent at 11.5 dpc as a markedly reduced thickening of the epithelium (Kreidberg et al., 1993). *Ftz-F1*-deficient phenotype is characterized by apoptosis in both the epithelium and mesenchyme of the gonadal primordium at 12.0 dpc when sexual differentiation normally becomes manifest. *Emx2* transcripts are observed in the developing gonad. The thickening of the coelomic epithelium was poor at 11.5 dpc,

although PGCs migrated from the endodermal region of the allantois to the genital ridge, and subsequent gonadal development was impaired in the *Emx2* mutant embryos. The interaction between the mesonephros and gonad has to be kept in mind. Mesonephric mesenchyme cells contribute to the gonadal development (Buehr et al., 1993), and the mesenchyme was poor in *Emx2* mutants. The *Emx2* may function in proliferation, differentiation and/or survival of gonadal cells, subjects for future studies.

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