Pax-2 controls multiple steps of urogenital development

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SUMMARY

Urogenital system development in mammals requires the coordinated differentiation of two distinct tissues, the ductal epithelium and the nephrogenic mesenchyme, both derived from the intermediate mesoderm of the early embryo. The former give rise to the genital tracts, ureters and kidney collecting duct system, whereas mesenchymal components undergo epithelial transformation to form nephrons in both the mesonephric (embryonic) and metanephric (definitive) kidney. Pax-2 is a transcriptional regulator of the paired-box family and is widely expressed during the development of both ductal and mesenchymal components of the urogenital system. We report here that Pax-2 homozygous mutant newborn mice lack kidneys, ureters and genital tracts. We attribute these defects to dysgenesis of both ductal and mesenchymal components of the developing urogenital system. The Wolffian and Müllerian ducts, precursors of male and female genital tracts, respectively, develop only partially and degenerate during embryogenesis. The ureters, inducers of the metanephros are absent and therefore kidney development does not take place. Mesenchyme of the nephrogenic cord fails to undergo epithelial transformation and is not able to form tubules in the mesonephros. In addition, we show that the expression of specific markers for each of these components is de-regulated in Pax-2 mutants. These data show that Pax-2 is required for multiple steps during the differentiation of intermediate mesoderm. In addition, Pax-2 mouse mutants provide an animal model for human hereditary kidney diseases.

Key words: Pax-2, urogenital development, gene targeting, mouse

INTRODUCTION

Pax genes encode a family of transcriptional regulators specifically expressed during the development of a wide range of structures and organs (Gruss and Walther, 1992). As indicated by both human and mouse mutations, at least five Pax genes have critical morphogenetic functions during the development of complex tissues (Chalepakis et al., 1992; Strachan and Read, 1994). These functions include the development of paraxial mesoderm derivatives (Pax-1) (Balling et al., 1988), neural crest cells and pre-myoblast migration (Pax-3) (Epstein et al., 1991; Bober et al., 1994), B-cell and midbrain development (Pax-5) (Urbanek et al., 1994), and eye and nose development (Pax-6) (Hill et al., 1991; Hadler et al., 1995). A remarkable characteristic is the semidominant character of loss-of-function mutations for three of the members of the family, Pax-1, Pax-3 and Pax-6. This feature suggests that the dosage of Pax protein is essential to ensure wild-type function.

The urogenital system is derived predominantly from the intermediate mesoderm of the early embryo. This mesoderm lies between paraxial and lateral mesoderms and undergoes extensive epithelial transformation to generate the ducts and tubules that compose urogenital tracts and kidneys (see Saxén, 1987, for review). The first event in the differentiation of intermediate mesoderm is the formation of the Wolffian duct, which progresses rostrocaudally from the cervical level towards the cloaca. The duct runs parallel to a tract of intermediate mesoderm, the nephrogenic cord. Subsequently, three embryonic kidneys, pronephros, mesonephros and metanephros, develop in spatial and temporal sequence. First and most anterior, the pronephros forms by an epithelial transformation of the nephrogenic cord mesenchyme and results in a linear array of tubules emptying into the Wolffian duct. While the pronephros seems to represent a true excretory organ in fish and amphibians, it remains a rudimentary and transitory structure in the mouse. The mesonephros appears after and just posterior to pronephros and develops in a very similar manner. However, mesonephric tubules may be functional during the embryonic life of mammals. Metanephric development starts when the ureter buds out from the posterior Wolffian duct and contacts the metanephric mesenchyme in the caudal part of the nephrogenic cord. Signals from the ureter induce metanephric mesenchyme to condense and proliferate at the ureter tips and reciprocal signals from the mesenchyme induce the ureter to grow and branch, thus forming the kidney collecting system (Grobstein, 1953, 1955). The induced mesenchyme at the tips of the branches undergoes transformation to generate glomerular, proximal tubular and distal tubular epithelium.

Mutations in genes expressed during urogenital development disrupt different aspects of metanephros development.
The transcription factor WT-1 is expressed in uninduced mesenchyme and is required for the induction response (Kreidberg et al., 1993). The tyrosine kinase receptor c-ret (Schuchardt et al., 1994) is essential for ureter growth and branching. The secreted factor Wnt-4 is required for metanephric tubule formation (Stark et al., 1994). However, besides a reduction in the number of mesonephric tubules reported in WT-1 mutants, other epithelial derivatives of intermediate mesoderm develop normally in these mutants.

In the male, mesonephric tubules and the Wolffian duct lose their excretory function and are transformed into the genital tract. Genital tracts originate in females from the Müllerian ducts, which are also derived from the intermediate mesoderm and develop parallel to the Wolffian duct much later in development. Soon after ducts appear, sexual dimorphism is evident and the Wolffian duct and mesonephros degenerate in the female while the Müllerian duct degenerates in the male (Jost and Magre, 1984).

Pax-2 is expressed in both ductal and mesenchymal components deriving from the intermediate mesoderm during the development of pronephros, mesonephros and metanephros (Dressler et al., 1990). In vitro experiments have suggested a role for Pax-2 in the conversion of mesenchyme to epithelium during metanephros development (Rothenpieler and Dressler, 1993). Recently, kidney hypoplasia has been reported associated with heterozygosity for a human PAX-2 point mutation (Sanyanusin et al., 1995) and a mouse chromosome 19 deletion (Krd) spanning about 400 genes, which includes the Pax-2 gene (Keller et al., 1994). These data suggest a role for Pax-2 in kidney organogenesis and adds Pax-2 to the class of haploinsufficient Pax genes.

We report here the generation and analysis of a mouse Pax-2 null mutation (Pax-2<sup>-/-</sup>). Our results indicate that Pax-2 is essential for multiple steps during intermediate mesoderm development including the differentiation of both ductal and mesenchymal components of mesonephros and metanephros and their derivatives. All mutations reported to date affecting urogenital development exclusively disrupt metanephros development with only one of the two components involved, ureter or mesenchyme. These data show that Pax-2 is a primary regulator of urogenital development. We suggest that Pax-2 may control a regulatory hierarchy of genes involved in the differentiation of intermediate mesoderm.

MATERIALS AND METHODS

Targeting Pax-2 locus

A positive-negative selection construct was designed. R1 ES cells were electroporated and selected as described (Wurst and Joyner, 1993) 234 independent resistant clones were screened by genomic southern blot using probe ‘a’ (Fig. 1) and BamHI digestion. One clone (no. 132) gave the expected recombinant band. Clone 132 was used to generate chimeras by morula aggregation (Nagy and Rossant, 1993) and germline transmission of the Pax-2 mutation. F<sub>1</sub> and F<sub>2</sub> generations were genotyped by genomic southern blot using either probe ‘a’ and BamHI digestion or probe ‘b’ and HindIII digestion.

Embryo dissections

Fetuses were collected in PBS and yolk sacs or tails were removed for DNA analysis by Southern blot. Urogenital systems were dissected out and cleaned from main blood vessels.

Histology

E12.5 embryos were dissected out in PBS and embryos were fixed in

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**Fig. 1.** Generation of Pax-2 mutant mice. (A) The targeting construct and expected recombination event with sizes of restriction enzyme digests characteristic for wild-type and mutant alleles. Homologous recombination would remove about 4 kb between the NotI site in exon one, 20 nucleotides before the ATG, and the PvuII site inside the paired box-encoding region in exon two. B, BamHI; N, NotI; H, HindIII; P, PvuII; Bg, BglII; WT, wild type; Mut, mutant. (B) Comparative Southern Blot analysis shows the wild-type bands in the parental ES cell line and both wild-type and expected mutant bands in clone 132. (C) Results obtained from an F2 litter genotyped with probe ‘b’ and HindIII digestion. Mendelian segregation of the wild-type and mutant allele is observed at birth.
Fig. 2. Analysis of the urogenital system defects in Pax-2 mutant mice at E17.5. (A-D) Whole mounts of dissected complete urogenital systems at E17.5 in male (A,C) and female (B,D) homozygous and heterozygous mutants and wild-type fetus. Note the reduced size of kidneys in heterozygous female shown in B. (E,F) Detail of the distal region of male urogenital tracts. (G,H) Detail of gonads and proximal part of genital tracts in female and male, mutant and wild-type fetus. Note the absence of seminal vesicles and vas deferens in F and the normal appearance of gonads in both sexes (G,H). Note that endoderm-derived components: bladder, urethra and prostatic glands (not shown) are unaffected. Mutant gonads are surrounded by a remnant of the urogenital ridge (*) which is blunt at both ends. ad, adrenal glands; ur, ureter; u, urethra; b, bladder; t, testis; e, epididymis; ut, uterus; o, ovary; od, oviduct; mt, mesonephric tubules developing into ductuli efferentes; v, vas deferens; sv, seminal vesicle.
4% paraformaldehyde (PFA) and, after dehydration, embedded in paraffin and sectioned at 8 to 10 μm. Sections were stained with haematoxylin and Eosin. E16.5 embryos were treated similarly except they were fixed in Bouin’s fixative and stained by Mallory’s tetra-chromic procedure.

**Antibody staining**

E13.5 embryos were fixed overnight in PFA, immersed in sucrose 30% for 24 hours, embedded in tissue tek and cryostat sectioned at 10 μm. Sections were incubated with a Pax-2 polyclonal antibody as described (Dressler and Douglas, 1992) using a Cy3-conjugated secondary antibody.

Whole-mount in situ hybridizations were performed as described (Wilkinson, 1992) using probes previously described for Pax-2 (Dressler et al., 1990) and Six-2 (Oliver et al., 1995), and a 700 bp EcoRI fragment from the pmcret10 plasmid of c-ret (Pachnis et al., 1993). After in situ hybridization embryos were embedded in gelatin and vibratome sectioned at 30 μm.

**RESULTS**

**Pax-2 mutants lack kidneys, ureters and genital tracts**

In order to study Pax-2 function, we have generated a null allele by homologous recombination in embryonic stem (ES) cells (Fig. 1). Pax-2 heterozygous (Pax-2+/−) and homozygous (Pax-2−/−) mutant animals are born at the expected Mendelian proportions. Homozygous mutant animals invariably lack both ureters and kidneys while heterozygous mutants frequently show a reduction in kidney size, which ranges from 1/10 of the normal size to wild-type size (Fig. 2). In addition, homozygous mutants of both sexes completely lack the entire genital tracts; females lack oviducts, uterus and vagina and males lack ductuli efferentes, epididymis, vas deferens and seminal vesicles. Gonads appear, in mutants from both sexes, surrounded by a blunt-ended remnant of the genital ridge. Kidney defects seem to be the only trait affected in the urogenital system of heterozygous mutants, genital tracts appear normal in these embryos. All the structures missing in the mutants are intermediate mesoderm-derived. In contrast, the endoderm-derived structures: urethra, bladder and prostatic glands appear normal.

**Development of genital tracts in Pax-2 mutants**

In males, the epididymis, vas deferens and seminal vesicles are derived from the Wolffian duct and in females, the oviducts, uteri and upper part of the vagina are derived from the Müllerian duct. To study the primary cause of the defects observed, we
Fig. 5. Histological analysis of wolffian and Müllerian ducts development at E17.0. Cross sections of female wild-type (A), female mutant (B), male wild-type (C) and male mutant embryos (D). Note in B the degeneration of the Wolffian (*) and Müllerian (arrow) ducts in females and the normal appearance of the ovary. (D) Wolffian duct (*) presents a similarly degenerated aspect in males. E to H show details of the Wolffian ducts in wild-type and mutant females (E,F, respectively) and wild-type and mutant males (G,H, respectively). Degenerated ducts are found inside the remnant of the urogenital ridge shown in Fig. 2. wd, Wolffian duct; md, Müllerian duct; k, kidney; vd, vas deferens.
followed the development of these ducts using histological analysis. In addition, we used the \textit{c-ret} gene as a marker, a tyrosine kinase receptor expressed in the Wolffian duct and ureters (Pachnis et al., 1993). At 9.5 days of embryonic development (E9.5), when most rostral parts of Wolffian duct are being generated, there are no differences between mutant and wild-type embryos in morphology and expression of \textit{c-ret} (Fig. 3). However, while the Wolffian duct has reached the cloaca and strongly expresses \textit{c-ret} at E10.5 in \textit{Pax-2}^{+/+} embryos, in \textit{Pax-2}^{-/-} littermates the Wolffian ducts do not reach the cloaca and have lost \textit{c-ret} expression (Fig. 3). A similar situation is observed in E11.5 embryos where the Wolffian ducts do not extend beyond somite 25 in the mutants (Fig. 3).

In E12.5 embryos, the parts of the Wolffian duct initially formed are degenerating in discontinuous spherical epithelial bodies (Fig. 4). Müllerian ducts develop in parallel to Wolffian ducts around E13.0. At E13.5 Müllerian ducts are found in wild-type embryos along the entire length of the urogenital...
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ridge parallel to the Wolffian ducts and reaching the cloaca. At the same stage, Müllerian ducts are only present at the upper most levels of the genital ridge in Pax-2−/− embryos (data not shown). By E16.5 the part of the Müllerian duct initially formed has degenerated and the spherical bodies remaining from the degeneration of the Wolffian duct have lost the epithelial morphology in both sexes (Fig. 5). The degenerated remains from both ducts are contained within the remnant of the urogenital ridges flanking the gonads (Fig. 5). Sections at a lower level at this stage show the absence of uterine horns and vagina in mutant females (Fig. 6).

We have analysed the expression of Pax-2 protein in the developing urogenital ridge at E13.5 when both Wolffian and Müllerian ducts are present. At this stage, Pax-2 is expressed in both developing Wolffian and Müllerian ducts, suggesting an autonomous function of Pax-2 in the development of both ducts (Fig. 7).

Development of mesonephros in Pax-2 mutant embryos

The mesonephros is a transient embryonic kidney consisting of a linear array of mesonephric tubules connecting to the Wolffian duct (Saxén, 1987). Formation of the mesonephric tubules requires epithelial transformation of the nephrogenic cord mesenchyme flanking the Wolffian duct and is concomitant with Pax-2 expression (Dressler et al., 1990 and Fig. 8). Pax-2−/− embryos do not develop mesonephric tubules (Fig. 4), therefore, it is likely that Pax-2 is needed for the epithelial transition in the nephrogenic cord.

We have analyzed tubule formation using the homeobox gene Six-2 as a marker (Oliver et al., 1995). While initially weakly expressed in the nephrogenic cord, Six-2 is up-regulated precluding tubule formation at about E10 (Fig. 8). In contrast, Pax-2−/− embryos keep a low expression level of Six-2 at E10, not showing the up-regulation typical of this stage. Mesonephric tubules regress in the female but persist in the male where they form the ductuli efferentes, also missing in the mutants (Fig. 2).

Metanephros development in Pax-2 mutants

Development of the definitive kidney starts at about E11 when the ureter buds out from the posterior part of the Wolffian duct and contacts the metanephric mesenchyme, a morphologically distinct group of posterior nephrogenic cord cells (Grobstein, 1953, 1955). Ultimately, the mesenchyme will undergo epithelial transformation to generate the definitive nephrons (Eckblom et al., 1981). Pax-2 is expressed in both the ureter and induced mesenchyme (Dressler et al., 1990). In Pax-2−/− embryos, metanephric development does not take place since ureter buds are absent (Fig. 9), and the ureter marker c-ret is not expressed (Fig. 10). However, there is a morphologically distinct metanephric mesenchyme (Fig. 9). Paralleling mesonephric development, Six-2 is expressed weakly in uninduced mesenchyme and strongly up-regulated after invasion of the mesenchyme by the ureter bud at E11.5. In contrast, mutant embryos still express Six-2 at similar levels to the uninduced mesenchyme (Fig. 10).

The function of Pax-2 in metanephric development appears

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Fig. 9. Metanephric development in Pax-2 mutants. (A,B) Cross sections at a metanephric level of E12.5 wild-type and mutant embryos, respectively. wd, Wolffian duct; ur, ureter; mm, mesonephric mesenchyme. Arrowhead marks the place of the missing Wolffian duct and asterisk marks metanephric mesenchymes in the mutants. (C,D) Kidney cross sections at the level where the ureter stems from the calyx of, respectively, wild-type and heterozygous E17.0 embryos. c, calix; ur, ureter.

Fig. 10. Expression analysis of Six-2 and c-ret during metanephros development in Pax-2 mutant embryos. Vibratome cross sections at the metanephric level of E11.5 embryos hybridized with c-ret (A,B) or Six-2 probes (C,D). (A,C) Wild-type embryos; (B,D) mutant embryos. ur, ureter; w, Wolffian duct at the level where the ureter buds out.
so crucial that even one wild-type copy of the gene is not enough to provide normal kidney development. Pax-2<sup>−/−</sup> animals frequently develop hypoplastic kidneys mainly due to reduced calyces and upper part of the ureters, suggesting defects in ureter branching and/or cell proliferation, but also to a thinner cortical region where a reduced number of developing nephrons is found, suggesting a decreased rate of epithelial transformation and/or cell proliferation (Fig. 9).

**DISCUSSION**

**Pax-2 and the development of epithelial components of the intermediate mesoderm**

The defects found in urogenital system development in Pax-2 mutant mice point to a critical function during intermediate mesoderm differentiation. This mesoderm undergoes a series of sequential inductions that transform it into different epithelial components of the kidney and the urogenital tracts. The mesenchymal cells of the intermediate mesoderm are unique in this respect, as most other epithelial tissues are formed by branching morphogenesis of pre-existing epithelium. Pax-2 is expressed during formation and differentiation of all epithelial structures derived from intermediate mesoderm, consistent with the developmental defects in Pax-2 mutants. In Pax-2 mutants, we observe defects not only in the formation of the Wolffian duct and ureter but also in tubule formation during mesonephric development. The Wolffian duct appears normal at E9-10 but fails to extend caudally towards the cloaca. Rather, by E11, the duct begins to degenerate and appears as discontinuous epithelium with enlarged lumens. Although duct formation appears normal in the region of the mesonephros, Pax-2 mutant mice fail to form mesonephric tubules. The mutant Wolffian duct may not be competent to induce tubule formation despite its normal morphology. However, it is likely that the Pax-2 mutant mesonephric mesenchyme is unable to undergo epithelial transformation, as mesonephric tubule formation can occur independent of the Wolffian duct (Etheridge, 1968; Croisille et al., 1976). Therefore, the defects in mesonephric tubules observed in Pax-2<sup>−/−</sup> embryos most probably result from an autonomous function of Pax-2 in tubule formation. Likewise, the defects observed in the Wolffian duct formation and posterior degeneration are most likely due to an autonomous function of Pax-2 in the duct cells. However, we cannot exclude the possibility that the defects in the Wolffian duct may result at least partially from an impaired interaction with the mutant mesenchyme.

Because the ureter fails to form, it is not possible to study tubule formation during metanephric development in Pax-2 mutant embryos. However, in vitro experiments using Pax-2 antisense oligonucleotides (Rothenpieler and Dressler, 1993) have suggested that Pax-2 is also necessary for epithelial transformation in metanephric tubule formation. In our studies and those by Keller et al. (1994), heterozygous mice carrying a normal Pax-2 allele show reduced kidney size and an attenuated nephrogenic zone. Thus, a Pax-2 function during proliferation and epithelial differentiation of the metanephric mesenchyme is clearly indicated.

The function of Pax-2 is thus essential for the development of all epithelial components derived from the intermediate mesoderm, irrespective of their time of appearance or mechanism of induction. Supporting this view, the development of the Müllerian duct is also impaired. The Müllerian duct and the Wolffian duct both originate from the intermediate mesoderm; however, each duct consists of unique cell types since they react in opposite ways to sex hormones (Jost and Magre, 1984). Epithelial components are not the only derivatives from the intermediate mesoderm. Mesenchymal cells from the mesonephros migrate during gonadal development and populate testis where they contribute to interstitial cell populations (Buehr et al., 1993). Quite remarkably, in Pax-2 mutants, testis appear completely normal in structure and cell type composition (data not shown), supporting the view that Pax-2 function is specifically needed for development of the epithelial components of the intermediate mesoderm.

Among the genes required for urogenital development are c-ret, WT-1 and Wnt-4. WT-1 is expressed in the uninduced metanephric mesenchyme and enables these cells to respond to induction by the ureteric bud. In the absence of WT-1 function in the mesenchyme, there is no ureteric bud outgrowth from the adjacent Wolffian duct. Thus, the outgrowth of the duct may depend on positional cues originating from the metanephric mesenchyme. These cues may be received by the receptor-type kinase c-ret, which is expressed in the Wolffian duct and subsequently in the tips of the growing ureter bud. Finally, in the absence of the secreted factor Wnt-4, tubule formation does not take place in metanephros, despite normal ureter growth and branching (Stark et al., 1994).

Because Pax-2 is a transcription factor, it may be required to regulate the expression of genes essential for the differentiation of the epithelial components of the urogenital system. We have observed alterations in the expression pattern of c-ret and Six-2 during the development of the affected structures. However, it is likely that these alterations are the result of impaired development of the structures implicated, rather than implying direct control by Pax-2 of the expression of these genes. In support of this view, expression of the c-ret gene was normal in the Wolffian duct and altered only after the duct started degeneration.

**Pax-2 and kidney disease**

There is suggestive evidence that links human kidney diseases with altered Pax-2 expression. During normal kidney development, Pax-2 is repressed after tubule formation (Dressler et al., 1990). Overexpression of Pax-2 is incompatible with normal kidney development in transgenic mice and results in kidney abnormalities similar to human nephrotic syndromes (Dressler et al., 1993). Persistent Pax-2 expression is found in Wilm’s tumor (Dressler and Douglas, 1992; Eccles et al., 1992) and in adult renal carcinoma where it is required for continued proliferation (Gnarra and Dressler, 1995). Partial loss of function by reducing the Pax-2 gene dosage through mutation of one of the alleles, either in humans or mice, results in hypoplastic kidneys (Keller et al., 1995; Sanyanusin et al., 1995; this report). Appropriate timing and dosage of Pax-2 expression is therefore essential to accomplish proper development of the kidney. There are at least four haplo-insufficient Pax genes, Pax-1, Pax-2, Pax-3 and Pax-6. Single gene haplo-insufficiency is rather rare; for the majority of genes one copy is enough to ensure wild-type function. The unusual high incidence of haplo-insufficient phenotypes among Pax genes suggests that, despite the heterogeneity in their developmental functions, an underlying common molecular mechanism for
Pax-2 protein function may exist that depends quantitatively on the level of protein present in the cell. Pax-2 is also expressed during central nervous system (CNS) development in eye, ear and midbrain (Nornes et al., 1990; Püschel et al., 1993). We have observed defects in the development of these three structures in Pax-2 mutant mice (unpublished data). Eye defects have also been reported in Krd mice and human Pax-2 mutants. Therefore, Pax-2 mutant mice provide an excellent animal model for the study of both CNS and urogenital system human hereditary diseases.

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