Zebrafish *no isthmus* reveals a role for *pax2.1* in tubule differentiation and patterning events in the pronephric primordia

Arindam Majumdar[1], Klaus Lun[2], Michael Brand[2] and Iain A. Drummond[1],*

[1]Renal Unit, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129, USA
[2]Department of Neurobiology, University of Heidelberg, and MPI Cell Biology and Genetics, Dresden, Im Neuenheimer Feld 364, D-69120, Heidelberg, Germany
*Author for correspondence (e-mail: idrummon@receptor.mgh.harvard.edu)

SUMMARY

Pax genes are important developmental regulators and function at multiple stages of vertebrate kidney organogenesis. In this report, we have used the zebrafish *pax2.1* mutant *no isthmus* to investigate the role for *pax2.1* in development of the pronephros. We demonstrate a requirement for *pax2.1* in multiple aspects of pronephric development including tubule and duct epithelial differentiation and cloaca morphogenesis. Morphological analysis demonstrates that *noi⁻* larvae specifically lack pronephric tubules while glomerular cell differentiation is unaffected. In addition, *pax2.1* expression in the lateral cells of the pronephric primordium is required to restrict the domains of Wilms' tumor suppressor (*wt1*) and vascular endothelial growth factor (VEGF) gene expression to medial podocyte progenitors. Ectopic podocyte-specific marker expression in pronephric duct cells correlates with loss of expression of the pronephric tubule and duct-specific markers mAb 3G8 and a Na⁺/K⁺ ATPase α1 subunit. The results suggest that the failure in pronephric tubule differentiation in *noi* arises from a patterning defect during differentiation of the pronephric primordium and that mutually inhibitory regulatory interactions play an important role in defining the boundary between glomerular and tubule progenitors in the forming nephron.

Key words: Zebrafish, *no isthmus*, *noi*, Pronephros, *pax2.1*, *wt1*, Tubulogenesis

INTRODUCTION

Kidney organogenesis in vertebrates can be viewed as occurring in four general stages or developmental events. The early patterning of the mesoderm during gastrulation to generate nephrogenic cell populations in the intermediate mesoderm is followed by the formation of a nephric primordium during somitogenesis and the caudal growth of the pronephric or Wolffian duct, which will form the future collecting system of the kidney. Induction of nephron development from nephrogenic mesenchyme and the patterning of nephron primordia into glomerular and tubule domains is closely followed by vascularization of the glomerulus and the onset of blood filtration. Genetic studies have identified a number of regulatory genes that play important roles in nephric development (Lechner and Dressler, 1997; Vize et al., 1997). While a variety of mutants have provided insights into the developmental signals controlling ureteric bud growth and metanephric induction, the molecular mechanisms underlying nephron patterning are still not known.

The *pax2* gene is expressed during multiple stages of vertebrate nephrogenesis and is mutated in human genetic diseases of the genitourinary system (Dressler et al., 1990, 1993; Krauss et al., 1991; Puschel et al., 1992; Sanyanusin et al., 1996; Dressler and Woolf, 1999). In vertebrates, the *pax2*, *pax5* and *pax8* genes have been grouped into a common subfamily based on their sequence similarity and expression pattern. *pax2*/*pax5*/*pax8* members share in common the paired domain, an octapeptide motif, and a partial homeodomain. Mice homozygous for a null mutation in *pax2* display severe defects in genitourinary development (Torres et al., 1995). Specifically, the Wolffian, or pronephric, duct degenerates before it is completely formed, resulting in kidney agenesis thus demonstrating a critical role for this gene in early stages of nephrogenesis.

In addition to its role in pronephric duct growth, *pax2* is likely to function later in kidney development during nephron differentiation. During metanephric development, *pax2* is expressed strongly in the branching ureteric bud and in comma- and S-shaped mesenchymal condensations, suggesting a role for *pax2* during nephron differentiation and morphogenesis (Dressler et al., 1990; Ryan et al., 1995). Deregulated expression of *pax2* in transgenic mice impedes tubular and glomerular differentiation and results in kidney cysts, implying that temporal and spatial control of *pax2* expression is important for proper renal epithelial differentiation (Dressler et al., 1993). In addition, antisense oligonucleotides directed against *pax2* interfere with nephron differentiation in organ culture (Rothenpieler and Dressler, 1993). In mouse embryos

homozygous for a disrupted *pax2* gene, the observed absence of mesonephric tubule differentiation indicates a role for *pax2* in mesenchyme-to-epithelial transitions in forming nephrons (Torres et al., 1995). However, the failure of the pronephric duct to grow to the level of the metanephric mesenchyme removes the normal inductive signal for nephron development (the ureteric bud) and precludes a complete analysis of the role of *pax2* during later stages of nephrogenesis. The function of *pax2* in nephron differentiation and patterning is therefore not known.

The Wilms' Tumor Suppressor gene (*wt1*), like *pax2*, is a key regulator of kidney development and knock out of the *wt1* locus in mouse results in degeneration of the metanephric mesenchyme and failed kidney organogenesis (Pelletier et al., 1991; Armstrong et al., 1993; Kreidberg et al., 1993). The *wt1* gene may also function later during nephrogenesis during nephron patterning events. Following metanephric induction, *pax2* and *wt1* are expressed in spatially complementary patterns in S-shaped bodies during nephron patterning (Ryan et al., 1995). *pax2* is transiently expressed in regions of the S-shaped body adjacent to the branched ureteric bud while high levels of *wt1* are observed in a crescent region of the S-shaped body where podocytes will differentiate. Potential molecular interactions between *pax2* and *wt1* have been explored in cell culture transfection assays where *wt1* was shown to act as a repressor of *pax2* expression (Ryan et al., 1995). These results suggest that interactions between *pax2* and *wt1* are likely to play an important role in regulating the differentiation of cell types within the nephron.

Pronephric development in zebrafish and *Xenopus* has been recently reviewed (Vize et al., 1997; Brandli, 1999; Drummond, 2000). Compared with the metanephric kidney, the zebrafish larval kidney, the pronephros, is a simple structure composed of just two nephrons joined at the embryo midline where they receive vascular input through capillaries from the overlying dorsal aorta. Each nephron is composed of glomerular and pronephric tubule segments and contains the typical cell types found in nephrons from higher vertebrates (Drummond et al., 1998). Mutagenesis screens have produced a large collection of mutants affecting various organ systems, including the pronephros (Brand et al., 1996; Driever et al., 1996; Haffter et al., 1996; Drummond et al., 1998). The zebrafish *no isthmus* (*noi*) mutant has been isolated as a loss-of-function allelic series in *pax2.1* based on a failure to properly form a midbrain-hindbrain boundary (MHB) (Brand et al., 1996; Lun and Brand, 1998). *noi* embryos have also been shown to exhibit a defect in optic tract formation (Macdonald et al., 1995; Brand et al., 1996; Lun and Brand, 1998). *pax2.1* functions, together with *wnt1* and *fgf8* signals, in the formation of the MHB organizer (Lun and Brand, 1998; Reifers et al., 1998). Zebrafish have two *pax2* genes, *pax2.1* and *pax2.2*, with *pax2.1* more closely resembling the mammalian *pax2* based on it expression pattern (Pfeffer et al., 1998). *pax2.1* and *pax2.2* are both expressed in the MHB, neural tube, ears and optic tracts, but only *pax2.1* is expressed in the pronephros suggesting an additional role for this transcription factor in pronephric development.

In this report, we have used the zebrafish *pax2.1* mutant *no isthmus (noi)* to investigate the role for *pax2* in pronephric differentiation. The initial analysis of *noi* suggested a defect in pronephric duct epithelial cell differentiation; however, the full extent of *pax2.1* function in the pronephros has not been described (Brand et al., 1996). We find that *pax2.1* expression in the lateral cells of the pronephric nephron primordia is required for pronephric tubule development and for restricting the domain of the Wilms' tumor suppressor gene expression to medial cells. *pax2.1* is required for tubule cell fate and, in its absence, presumptive tubule and duct cells express podocyte-specific markers. In addition, *pax2.1* is required in the pronephric ducts for complete epithelial differentiation and cloaca morphogenesis.

MATERIALS AND METHODS

Zebrafish stocks

Zebrafish were grown and mated at 28.5°C (Westerfield, 1993). The *noi*[tb21] allele behaves as a hypomorphic mutation in *pax2.1* and is associated with deletion of amino acids 263-303 (Lun and Brand, 1998). The *noi*[tb21] allele gives rise to a truncated protein detectable on western blots (data not shown). The *noi*[tu29a] allele is an RNA and protein null allele of *pax2.1* resulting from a stop codon mutation at amino acid 139 (Brand et al., 1996; Lun and Brand, 1998). In this work, the null allele *noi*[tu29a] homozygotes are referred to as *noi*⁻. *noi* mutant embryos were phenotypically identified under the dissecting microscope based on the absence of a midbrain-hindbrain boundary and reduced or absent tectum.

Histological sections

Zebrafish embryos were fixed in BT fix (Westerfield, 1993), dehydrated and embedded in JB-4 (Polysciences) for plastic sectioning. Sections of 4 μm thickness were cut and stained using methylene blue/azure II (Humphrey and Pittman, 1974; Drummond et al., 1998) and Permount (SIGMA) mounted for light microscopy. Photographs were taken on Kodak Ektachrome 160T film on a Zeiss Axioplan microscope.

In situ hybridization and immunocytochemistry

Whole-mount in situ hybridization was performed as described using digoxigenin-labeled antisense RNA probes and anti-digoxigenin alkaline phosphatase-conjugated antibodies (Boehringer Mannheim (Oxtoby and Jowett, 1993). Hybridized embryos were cleared in benzyl benzoate/benzyl alcohol 2:1 and photographed on Kodak 160 Tungsten film. Tissue sections, made by embedding embryos in JB-4 plastic and sectioning at 4 μm, were photographed using DIC optics. The following templates were linearized and transcribed with RNAP (Boehringer Mannheim) to make antisense probes: *pax2.1 Bam*HI/T7, Wilms' Tumor Suppressor gene (*wt1*) *Not*I/T7, vascular endothelial growth factor (VEGF) *Eco*RI/T3, α1-subunit Na⁺/K⁺ ATPase *Bam*HI/T7, and *ret1 Eco*RI/T7.

Wheat Germ Agglutinin (WGA, Molecular Probes) was used at 1:10 dilution to stain in situ hybridized embryos in PBDT (1× PBS, 1% BSA, 1% DMSO, 1% dry milk, 0.1% Tween-20) and all washes were done in PBDT. Embryos were processed for sectioning as described above.

Whole-mount antibody staining was performed following the BT fixation (Westerfield, 1993) method or the Dent's fixation method (Dent et al., 1989) depending upon the sensitivity of the 1° antibodies. Embryos were Dent's fixed and stained with α-6F antibody as described in Drummond et al. (1989). Alternatively, embryos were BT fixed and stained with a 1:2 dilution of mAb 3G8 (Vize et al., 1995) and staining was visualized with goat anti-mouse Cy3 conjugated 2° antibody (Jackson Labs).

Electron microscopy

Zebrafish embryos were processed for electron microscopic analysis as described (Majumdar and Drummond, 1999).

RESULTS

noi mutants show defects in multiple aspects of pronephric development.

pax2.1 is sequentially expressed during different stages of pronephric development and it expression patterns are summarized in Fig. 1 (Krauss et al., 1991; Puschel et al., 1992). During somitogenesis stages, *pax2.1* is initially expressed in the intermediate mesoderm which includes the nephrogenic cells (Fig. 1A). Afterwards, at the pharyngula stage (24 hours postfertilization (hpf)), strongest *pax2.1* expression is seen in the anterior and posterior pronephric duct cells, with lower levels of expression in between (Fig. 1B). At 30 hpf, *pax2.1* is transiently expressed in the nephron primordia while expression remains strong in anterior duct cells (Fig. 1C).

During normal zebrafish development, glomerular filtration of blood begins between 40 and 48 hpf (Drummond et al., 1998; Majumdar and Drummond, 1999). *no isthmus* (*noi*) homozygotes display severe edema by 48 hpf indicating a failure in osmoregulation and a possible defect in pronephric function (Brand et al., 1996). To investigate the role of *pax2.1* in zebrafish pronephric development, we examined the pronephros histologically in two alleles of *no isthmus* representing the extremes of allele strength; *noi*[tu29a] (*noi*⁻; null allele) and *noi*[tb21], a hypomorphic exon 7 deletion allele showing reduced expression of *pax2.1* mRNA and protein (A. M., unpublished observations; Lun and Brand, 1998).

In wild-type pronephroi, tubules extend laterally off of the medial glomerulus and connect with the caudally extending ducts (Fig. 1D). Importantly, *noi* mutants lack differentiated pronephric tubules while still retaining a midline glomerulus (Fig. 1G). The tubule-loss phenotype is equally severe in the

noi[tb21] and null *noi*⁻ alleles. The *noi* glomerulus is complete with podocytes and surrounding parietal epithelium (Bowman's capsule), suggesting glomerular differentiation and morphogenesis does not require *pax2.1*. Unlike the mouse or human *pax2* mutations, which behave haploinsufficiently, *noi* heterozygotes do not display edema or show a histological pronephric phenotype, suggesting that one copy of the *pax2.1* gene is sufficient for normal development.

In order to determine whether the pronephric phenotype in *noi* mutants resulted from a developmental defect, we followed the differentiation of *noi* pronephroi with tubule and duct molecular markers. mAb 3G8 has been described as a marker for visualizing zebrafish and *Xenopus* pronephric development where it labels pronephric tubules and ducts (Vize et al., 1995; Drummond et al., 1998). In zebrafish, mAb 3G8 stains tubules and ducts at 3 days post fertilization (dpf) and is restricted to the tubules by 3.5 dpf (Fig. 2A and data not shown). The pronephric tubules and ducts also express the Na⁺/K⁺ ATPase α1 subunit which can be visualized with the monoclonal antibody α-6F (Fig. 2B). In 3 dpf *noi*[tb21] mutants, tubule and anterior duct mAb 3G8 expression is not seen, demonstrating a defect in the differentiation of these pronephric structures (Fig. 2C). Furthermore, in *noi*⁻/[tb21] Na⁺/K⁺ ATPase expression can not be detected in tubule or anterior duct, again pointing to a failure in pronephric tubule and duct epithelial differentiation (Fig. 2D).

noi mutants show defects in nephric patterning

We sought to identify the earliest stage when the pronephric defects in *noi* embryos were detectable. During wild-type development, the glomerular and tubular pronephric components arise from a common nephron primordium

Fig. 1. *noi* affects multiple aspects of pronephric development. (A-C) Pattern of *pax2.1* expression in the pronephros during wild-type development. Whole-mount in situ hybridization using the *pax2.1* antisense probe on (A) 12 somite, (B) 24 hpf and (C) 30 hpf wild-type embryos. *pax2.1* is first expressed in intermediate mesoderm (A), and then *pax2.1* expression is maintained in anterior duct (black arrows in B) and forming cloaca (arrowhead in B). Later, *pax2.1* is transiently expressed in forming pronephric tubules (arrows in C). Transverse histological sections of (D-F) wild-type and (G-I) *noi*⁻ mutant embryos at (E,H) 36 hpf and (D,F,G,I) 3 dpf. In wild type, tubules extend laterally from a medial glomerulus and pronephric duct (D). In *noi*⁻ mutants, pronephric tubules are missing even though a midline glomerulus is seen (G). Pronephric duct is not seen at this level of section. Wild-type pronephric ducts (arrows in E) have well-formed epithelial cells and a small central lumen. In *noi*⁻, the pronephric ducts (arrows) are distended and epithelia appear flattened (H). Sagittal histological section of wild-type pronephric cloaca shows cuboidal epithelial cells and a central lumen (F). Cloaca morphogenesis in *noi*[tu29] is affected (I). Epithelial cells are flattened and the lumen is greatly distended. Note the lumen does open to the outside.
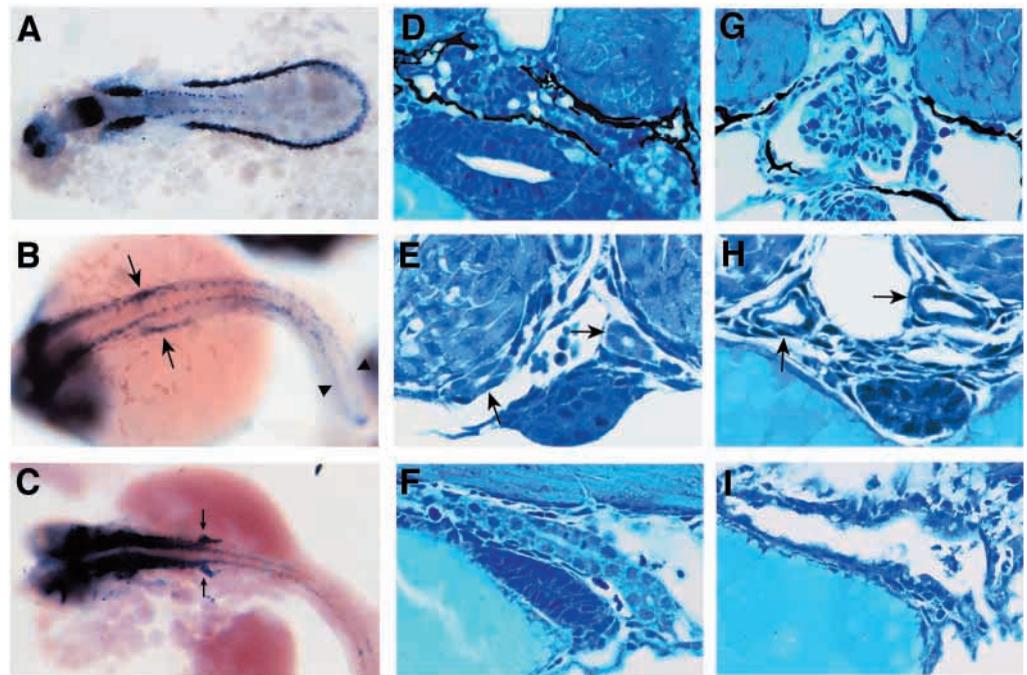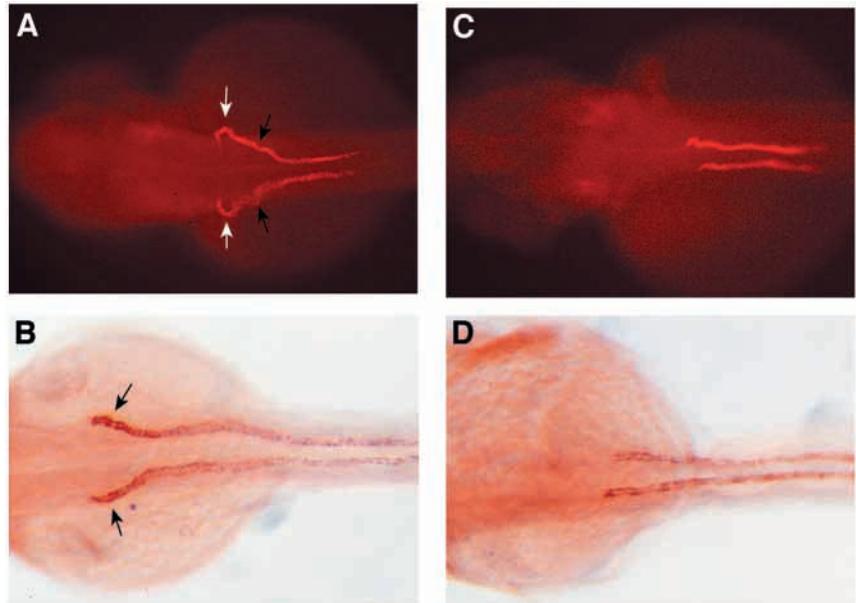
Fig. 2. Tubule and anterior duct differentiation is affected in *noi*. Whole-mount antibody staining of (A,B) wild-type, (C) *noi*[tb21], and (D) *noi*[−/tb21] embryos with mAb 3G8 (A,C), and α-6F mAb (B,D), which recognizes Na+/K+ ATPase α1-subunit. At this time, mAb 3G8 marks the anterior duct (black arrows in A) and tubules (white arrows in A). Anterior duct is visualized as two laterally extending wings of mAb 3G8 reactivity. Tubule and anterior duct staining is gone in *noi*[tb21] (C). α-6F marks the pronephric duct in wild type (arrows in B) and anterior duct expression is absent in *noi*[−/tb21] (D). 3 dpf embryos (A,C) and 2 dpf embryos (B,D) are shown.

(Drummond et al., 1998). Prior to overt morphological differentiation, *pax2.1* is transiently expressed in the lateral cells of the nephron primordium between 30 and 32 hpf and is the earliest known marker of tubule differentiation and nephron patterning (Fig. 4A; refer to Fig. 3 for details on plane of section). In the hypomorphic *noi*[tb21] allele, *pax2.1* mRNA expression can be detected in several tissues during pharyngula stages where *pax2.1* is normally expressed including the optic tracts, spinal cord and anterior pronephric duct cells (Fig. 4H). However, *pax2.1* mRNA expression is specifically lost in the lateral cells of the nephron primordium at 30 hpf where expression would normally occur (Fig. 4B). The loss of *pax2.1* expression in the primordium indicates that the absence of pronephric tubules at later stages is due to a defect at the earliest stages of tubule differentiation. Furthermore, the result indicates that *pax2.1* positively regulates its own expression. In *noi* homozygotes, pronephric primordia cells can be seen histologically (Fig. 4B, D) and by pronephric molecular marker expression (see below and Fig. 5) demonstrating that the lack of *pax2.1* expression is due to the absence of a functional *pax2.1* gene rather than a loss of lateral cells altogether. In addition, expression of earlier markers of pronephric development like *lim-1* and *ret1* were unaffected in *noi* mutants at the 12-somite stage (data not shown). We were not able to detect any cell death in the pronephros using acridine orange while apoptosis in the MHB was easily detected suggesting that cell death was not the basis for the loss of pronephric tubule differentiation (data not shown; Brand et al., 1996).

We next asked whether loss of tubule differentiation might affect the development of glomerular progenitor cells in *noi*. The Wilms' tumor suppressor gene (*wt1*) is expressed in the nephron primordia reflecting the morphological events associated with patterning the pronephric nephron (Drummond et al., 1998). *wt1* is initially expressed in the intermediate mesoderm and then becomes restricted to the nephron primordia cells which lie adjacent to the anterior tips of the pronephric ducts (Fig. 4C; (Drummond et al., 1998).

Coincident with tubular differentiation, *wt1* expression is downregulated in lateral primordium cells. *wt1* expression is later maintained only in differentiated glomerular podocytes. The Na+/K+ ATPase α1 subunit is another marker of nephron patterning and is expressed in the pronephric duct. At 25 hpf, the Na+/K+ ATPase α1 shows a complementary pattern of expression compared to *wt1*, being expressed in the pronephric duct but not in the pronephric primordia (Fig. 4E).

In both *noi*[−] and *noi*[tb21] mutants, the *wt1* expression domain is expanded caudally at 25 hpf implying that *pax2.1* restricts the spatial domain of *wt1* expression (Fig. 4D). No rostral expansion was detected. In situ hybridization during the 12-somite stage on embryos from *noi* crosses did not reveal any differences in *wt1* expression demonstrating that the posterior restriction occurs after this time (data not shown). Expanded *wt1* expression was also observed at later stages (36 hpf) with the onset of podocyte differentiation (data not shown).
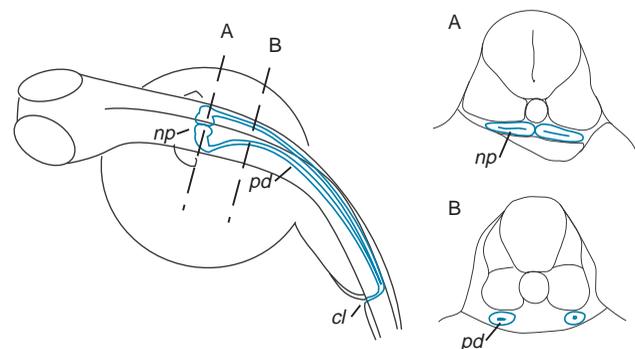

Fig. 3. Diagram of the zebrafish pronephros. The nephric primordia (np), pronephric duct (pd) and cloaca (cl) are shown for a 25-32 hpf embryo. The dashed lines A and B mark the anteroposterior level of histological sections made and correspond to the schematized transverse cross sections to the right. (A) Sections are made at the level of the nephric primordia. (B) Sections are made through the anterior pronephric duct, immediately behind the nephric primordia.
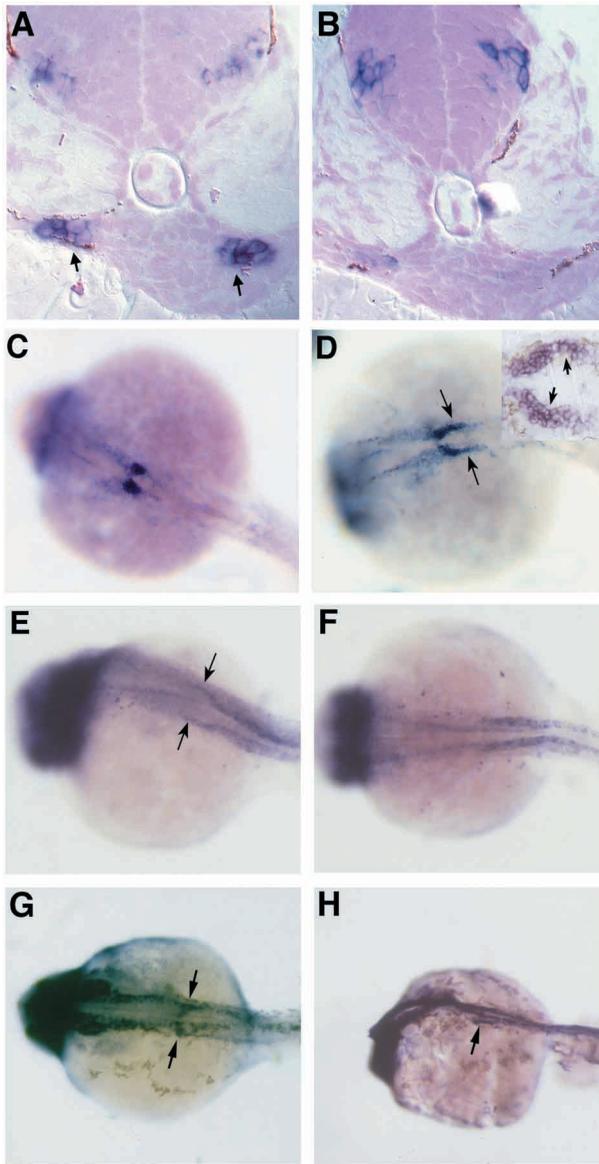
Fig. 4. *noi* affects patterning of the nephric primordia. (A,C,E,G) Wild-type and (B,D,F,H) *noi^tb21* embryos in situ hybridized with *pax2.1* (A,B,G,H), *wt1* (C,D) and α-subunit of Na$^+$/K$^+$ ATPase antisense probes (E,F) at 30 hpf (A,B,G,H) or 25 hpf (C-F). (A) Transverse histological sections (schematized by A in Fig. 3) of in situ hybridized 30 hpf embryos shows expression of *pax2.1* in lateral, but not medial, cells of the pronephric primordia in wild type. (B) *pax2.1* expression in nephric primordia is greatly reduced in *noi^tb21* mutants, even though CNS expression still remains. (C) Whole-mount in situ hybridization with *wt1* marks the nephric primordia in wild type. *wt1* expression is caudally expanded in *noi^tb21* (arrows in D). Sagittal section through a *noi^tb21* pronephros stained with *wt1* antisense probe (inset in D) reveals ectopic *wt1* expression in lateral cells of the nephron primordia (arrows). Anterior duct expression of α-subunit of Na$^+$/K$^+$ ATPase (arrows in E) is lost in *noi^tb21* embryos while more posterior duct expression remains (F). Whole-mount in situ hybridization with *pax2.1* reveals tubule and duct expression in wild type (arrows in G). (H) Tubule expression is gone, but anterior duct *pax2.1* expression remains in *noi^tb21*.

and instead ectopically express *wt1* confirming that anterior duct cell differentiation is affected in *noi* (Fig. 5C,G).

We next asked whether the expression of other podocyte cell fate markers is also expanded in *noi*. The vascular endothelial growth factor (VEGF) gene is expressed in differentiating podocytes but not in tubules or duct cells (Fig. 5I,J; (Liang et al., 1998). In *noi^-/tb21*, VEGF is ectopically expressed in cells in the position of the anterior ducts (Fig. 5K). These results demonstrate that *pax2.1* restricts the domain of *wt1* and VEGF expression to the future glomerulus and that in the absence of *pax2.1* function, presumptive tubule cells and anterior duct cells ectopically express podocyte-specific markers.

Pronephric duct differentiation is abnormal in *noi* mutants

Epithelial cells of the pronephric tubules and ducts form a polarized epithelium with well-defined apical and basal surfaces and a central lumen. The ducts can be visualized histologically in sections (Fig. 1E). In *noi^-* 36 hpf mutants, the ducts appear dilated and the epithelia have a flattened appearance suggesting a defect in duct epithelial morphogenesis (Fig. 1H). These defects are observed prior to vascularization of the glomerulus and are therefore not due to abnormally high filtration pressure.

The Na$^+$/K$^+$ ATPase protein is localized to the basolateral surface of wild-type tubule and duct epithelial cells (Fig. 6A). In *noi^tb21* mutants, Na$^+$/K$^+$ ATPase is not localized to the basolateral surface of duct cells, instead appearing diffusely throughout the cytoplasm (Fig. 6B). In addition, transverse sections reveal duct cells where no Na$^+$/K$^+$ ATPase expression is detectable adjacent to cells that express Na$^+$/K$^+$ ATPase in a delocalized manner. In contrast to the pronephric ducts, Na$^+$/K$^+$ ATPase basolateral subcellular localization in the gut is unaffected in *noi,* demonstrating the organ-specific nature of the defect (data not shown).

In order to assess the extent of epithelial differentiation in *noi* pronephroi, we examined *noi* pronephric duct epithelium by electron microscopy. In wild-type embryos at 2.5 dpf, pronephric duct columnar epithelial cells extend apical microvilli and cilia into the central lumen to form a dense brush border while basally, a basement membrane is visible (Fig. 6C). Duct epithelial cells display well-formed apical cell-cell

Correlating spatially with an expansion of *wt1* expression, we observed a loss of Na$^+$/K$^+$ ATPase α1 subunit expression in the anterior duct cells, demonstrating that duct epithelial cell differentiation is altered in *noi^-* mutants (Fig. 4F). *wt1* has been shown to repress *pax2* transcription in vitro (Ryan et al., 1995). Ectopic *wt1* expression in *noi* could result in repression of *pax2.1* in the anterior pronephric duct. *pax2.1* expression persists in *noi* anterior ducts indicating the ectopic *wt1* expression is not repressing *pax2.1* transcription (Fig. 4H).

In order to determine whether the duct cells that lost Na$^+$/K$^+$ ATPase α1 subunit expression were the same cells ectopically expressing *wt1*, we carried out a double labeling experiment with wheat germ agglutinin (WGA), a lectin that stains basement membrane and allows the morphological identification of the pronephric tubules and ducts in cross section. In wild type, anterior duct cells express the Na$^+$/K$^+$ ATPase α1 subunit but not *wt1* (Fig. 5A,E; refer to Fig. 3 for details on plane of section). In *noi* mutants, cells in the position of the anterior duct lose Na$^+$/K$^+$ ATPase α1 subunit expression

Fig. 5. Anterior pronephric duct cells lose duct marker expression and misexpress the podocyte-specific markers *wt1* and VEGF. Transverse histological sections of (A,B,E,F,I,J) wild-type, *noi*[tb21] (C,D,G,H) and *noi*[−/tb21](K,L) embryos in situ hybridized with *wt1* (A, C), α-subunit Na⁺/K⁺ ATPase (E,G), or VEGF (I,K) antisense probes and double stained with WGA at 28 hpf (A-H) or 48 hpf (I-L). WGA-stained sections (B,D,F,H,J,L) correspond exactly with the DIC images (A,C,E,G,I,K) on their left. Therefore, A and B are pictures of the same section with A taken with DIC optics and B taken on the FITC channel. In wild type, anterior duct cells express Na⁺/K⁺ ATPase α-subunit (E) but not *wt1* (A). Anterior duct cells are outlined with WGA in the corresponding FITC images (arrows in F,B). In *noi*[tb21] mutants, anterior duct cells lose Na⁺/K⁺ ATPase α-subunit expression (G) but misexpress *wt1* (C). (D,H) WGA reactive cells in the position of the anterior duct. (I) Wild-type podocytes but not pronephric tubule or duct cells express VEGF. In *noi*[−/tb21], VEGF expression is laterally expanded (K) into cells that would normally form tubule. All sections were made at the same anteroposterior level, schematized by B in Fig. 3, immediately behind the pronephric glomerulus.
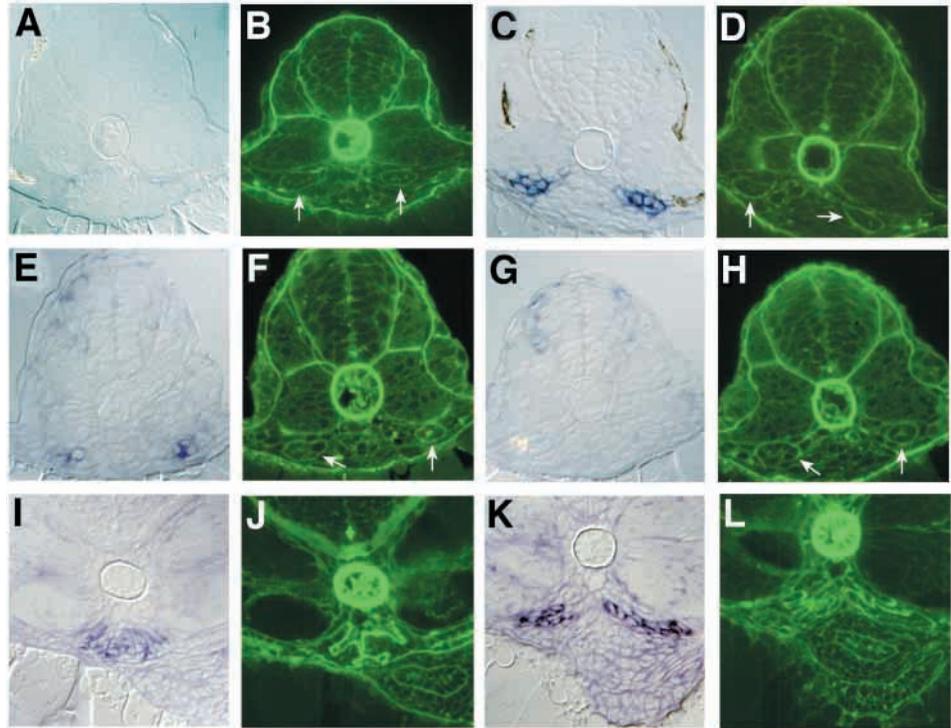
junctions. Mitochondria are found basally and laterally, but are excluded from the terminal web region beneath the microvilli. Ciliated and non-ciliated cells are found throughout the anterior posterior length of the duct. In *noi* mutants, an apical brush border is absent and, unlike wild-type cells, mitochondria can be found at the apical poles of the cells (Fig. 6D). Duct epithelia are not columnar and appear flattened while the duct lumen is greatly distended along its entire length. However, apical cell-cell junctions and basement membranes are visible suggesting that these epithelia do retain some apicobasal polarity. No evidence of nuclear fragmentation or cell death was observed.

Posterior pronephric duct development

The pronephros opens to the environment at the cloaca where the posterior pronephric ducts have fused to form a single tubule. In wild-type 3 dpf embryos, histological sagittal sections at the cloaca reveal the organization of the pronephric duct with cuboidal epithelial cells surrounding a central lumen (Fig. 1F). In *noi*⁻ mutants, the posterior pronephric duct at the cloaca appears cystic with a significantly enlarged lumen and severely flattened epithelial cells (Fig. 1I). The *ret1* tyrosine kinase receptor is known to play an important role in pronephric duct growth and ureteric bud branching during mouse kidney development (Pachnis et al., 1993; Schuchardt et al., 1994). In zebrafish, *ret1* is first detected at the caudal end of the growing pronephric duct and, later, *ret1* is expressed in a discrete group of cells at the caudal end of the pronephric duct (Marcos-Gutierrez et al., 1997); Fig. 6E). This later phase of *ret1* expression is completely missing in *noi* embryos, demonstrating a correlation between the cloaca defect and

missing *ret1* expression (Fig. 6F). The result also suggests that *pax2.1* may positively regulate *ret1* during cloaca formation.

DISCUSSION

pax2.1 functions in nephron patterning

We demonstrate here a requirement for the transcription factor *pax2.1* in multiple aspects of pronephric development including tubule and duct epithelial differentiation and cloaca morphogenesis. Zebrafish have two *pax2* genes, *pax2.1* and *pax2.2*, but only *pax2.1* is expressed in the pronephros. The phenotypic analysis of *noi* mutants demonstrates that defects in tubule, duct and cloaca development correlate with the expression of *pax2.1* in these parts of the pronephros. A related gene, *pax8*, is expressed at the 1-somite stage in the intermediate mesoderm and its expression is later maintained by *pax2.1* (Pfeffer et al., 1998). However, *pax8* expression has not been observed during nephron patterning events making *pax2.1* the only *pax* member thus far to be expressed during tubule differentiation (A. M. unpublished observations). The observation of a fully penetrant pronephric tubule phenotype in *noi* may therefore mean that *pax2.1* is the only member of the *pax2/5/8* genes active in tubule development with no redundant function to compensate for loss of *pax2.1*.

During zebrafish pronephric development, nephron primordia are patterned to give rise to the glomerular and tubular epithelial components. Medial primordia cells express the *wt1* gene while lateral cells express *pax2.1*. In the *noi* mutant, the pronephric primordia are present, but subsequent nephron patterning events are abnormal. Presumptive tubule
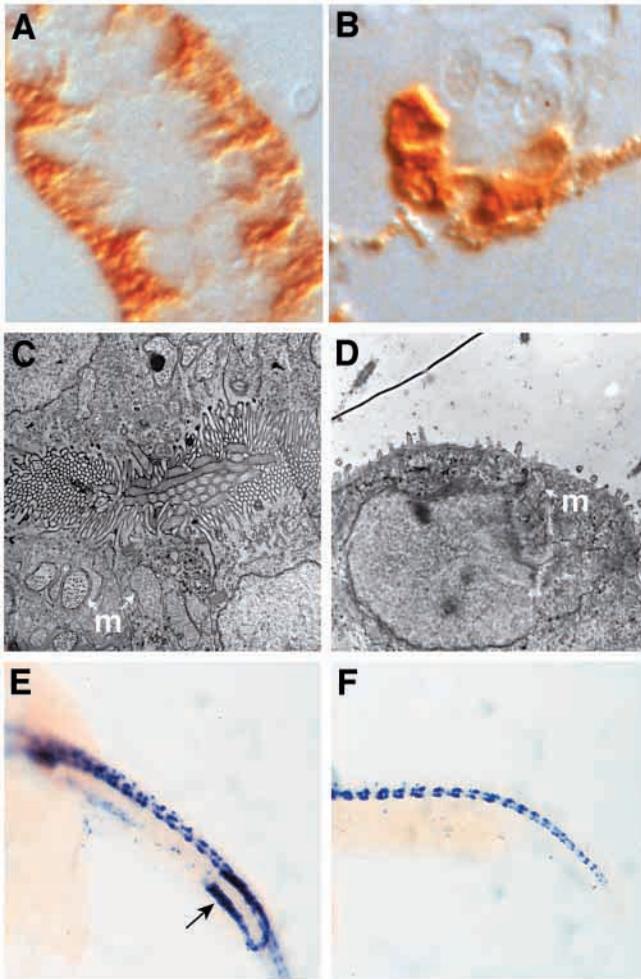
Fig. 6. *noi* mutants show defects in pronephric duct epithelial differentiation and cloaca development. (A,C,E) Wild-type and (B,D,F) *noi*⁻ embryos stained with α-6F mAb at 3 dpf (A,B) and electron micrographs of pronephric duct epithelia from 2 dpf embryos (C,D). (A) In wild-type pronephric tubule and duct, mAb α-6F staining is basolateral reflecting the basolateral subcellular localization of Na⁺/K⁺ ATPase α-subunit. (B) In *noi* mutants, mAb α-6F staining is diffuse throughout the duct cells with mAb α-6F positive cells sitting adjacent to duct cells showing no mAb α-6F reactivity. Some endogenous pigmentation is seen in B and is not HRP reaction product. Electron micrograph of wild-type pronephric duct epithelia shows an abundance of microvilli and cilia packed densely in the lumen. Mitochondria (m) are excluded from the apical terminal web. In *noi*⁻, the pronephric duct lumen is greatly distended and epithelia lack a apical brush border seen in D as a huge reduction in apical microvilli. Some ciliated cells are still present. Apically localized mitochrondria (m) are seen. Cloaca development is abnormal in *noi* mutants. (E,F) In situ hybridization with *ret1* antisense probe at 22 hpf. *ret1* is expressed in a posterior domain of the pronephric duct during cloaca development (arrow in E). Duct-specific expression of *ret1* is absent in *noi*⁻ even though CNS expression remains unaffected (F).

Fig. 7. *pax 2.1/wt1* regulatory interactions are important during nephron patterning. During wild-type pronephric development, *pax2.1* and *wt1* are expressed in mutually exclusive domains. Strongest *pax2.1* expression (violet) is found in the forming pronephric tubule (pt), the anterior pronephric duct and the cloaca (cl). Lower levels of *pax2.1* expression (pink) are observed throughout the pronephric duct epithelium (pd). In contrast, *wt1* (purple) is strongly expressed in the glomerulus (gl) and is excluded from other regions of the pronephric epithelium. *pax2.1* is required to restrict the domain of *wt1* expression. In the absence of *pax2.1* function, *wt1* gene expression is derepressed in presumptive tubule and anterior duct cells resulting in failed tubulogenesis and incomplete differentiation of the renal epithelium.

cells never express *pax2.1* and podocyte-specific marker expression is expanded, suggesting that presumptive tubule cells follow an alternative developmental pathway in *pax2.1* mutants (Fig. 7). The result suggests that *pax2.1* expression serves as an instructive signal for lateral cells to assume a
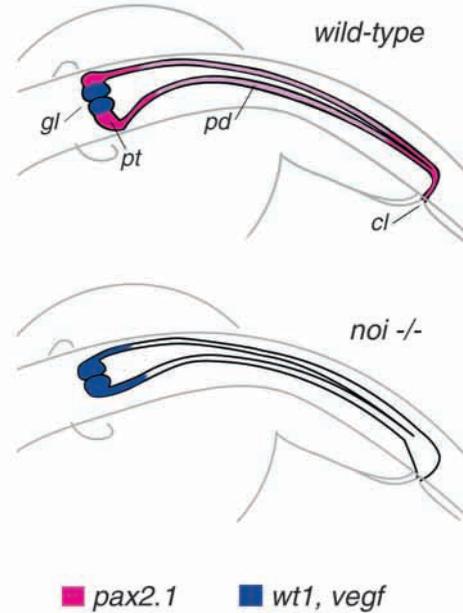
different fate from neighboring cells in the primordium and may function, at least in part, by repressing podocyte-specific gene expression.

Phenotypic analyses of *pax* mutants in *Caenorhabditis elegans*, *Drosophila* and mouse suggest requirements for *pax2* homologs in specific cell fate decisions and patterning events in a variety of developmental contexts. During *C. elegans* vulval development, the *pax2/pax5/pax8* homolog *egl-38* is needed to specify the uterine cell fate and, in its absence, presumptive uterine cells adopt an alternative cell fate resulting in a vulval morphogenesis defect (Chamberlin et al., 1997). In *Drosophila*, the *pax2* homolog, *D-pax2*, similarly functions in multiple cell fate decisions. During ommatidial development, *D-pax2* is required for the differentiation of non-neuronal cone and primary pigment cells (Fu and Noll, 1997). In the pupal notum, *D-pax2* is needed for shaft cell differentiation during bristle sensory organ development (Kavaler et al., 1999). Our analysis of *noi* raises the possibility that *pax2.1* may function in a similar way in nephron patterning by repressing podocyte-specific gene expression in the lateral primordial cells to promote the tubule cell fate.

Alternatively, *pax2.1* could function in lateral pronephric primordium cells as a proliferative signal. In this scenario, a lack of cell divisions in *noi* nephron primordia would underlie the failure to elaborate pronephric tubules. While proliferation may play a role during normal tubule development, several

observations argue that *pax2.1* is not simply regulating cell proliferation. First, *pax2.1* is expressed throughout the pronephric ducts, yet we do not see a decrease in the number of duct cells in comparing *noi* mutant and wild-type embryos (data not shown). In addition, in BrdU-labeling experiments, we observe S-phase cells in pronephric primordia from *noi* mutants (data not shown). Furthermore, no evidence of increased apoptosis was seen in the pronephros of *noi* mutants. Together, these results suggest presumptive tubule and anterior duct cells follow an alternative developmental pathway in the absence of *pax2.1* function.

pax2.1 also functions in renal epithelial differentiation

In addition to a role for *pax2.1* in nephron patterning, the ultrastructural and immunohistochemical studies presented here suggest a more general requirement for *pax2.1* in pronephric terminal epithelial differentiation, consistent with previous morphological analyses (Brand et al., 1996). While *noi* mutant duct cells do have morphologically distinct apical and basal cell surfaces and cellular junctions, they lack apical microvilli and mis-localize the Na$^+$/K$^+$ ATPase, reflecting a defect in terminal differentiation. This phenotype is not limited to the region affected by ectopic *wt1* expression and occurs along the entire length of the duct, demonstrating that the abnormal membrane protein targeting results from a loss of *pax2.1* function and not from ectopic *wt1* expression. Delocalized Na$^+$/K$^+$ ATPase has been observed in other zebrafish pronephric mutants where it correlates with the presence of pronephric cysts and dilated duct lumens (Drummond et al., 1998). We suggest that the epithelial defects in *noi* may reflect the persistence of an immature developmental state. Consistent with this interpretation, previous studies on explanted mouse kidneys have demonstrated that antisense oligonucleotides directed against *pax2* can interfere with the conversion of mesenchyme to epithelium in the process of nephron development (Rothenpieler and Dressler, 1993).

pax2.1 is also required for formation of the cloaca. In *Xenopus* pronephric development, the rectal diverticulum, an anterior outgrowth of the proctodeum, establishes a continuity between the duct and the cloaca (Heller and Brandli, 1997; Brandli, 1999). The existence of a rectal diverticulum in zebrafish is not known. Electron microscopic sections of *noi* mutant ducts (Fig. 6D and data not shown) show that the pronephric duct lumen is continuous with the cloaca suggesting that morphogenesis of the intermediate mesoderm to form a continuous duct tube occurs in *noi*. Furthermore, the early pattern of *c-ret* expression suggests posterior migration of duct cells occurs successfully in *noi*. Together, these data suggest cloacal defects result from a problem in differentiation of the cloacal epithelium rather than a morphogenetic defect.

Regulatory relationships between *pax2* and *wt1* genes

Our analysis suggests *pax2.1/wt1* regulatory interactions are important for proper nephric development. Similar patterns of *pax2* and *wt1* gene expression are observed during *Xenopus* pronephric development with *pax2* transcripts found in more lateral and *wt1* transcripts found in more medial pronephric analage cells (Carroll et al., 1999). During mouse metanephric

development, tubular and podocyte epithelia arise from a common renal vesicle where *pax2* and *wt1* are expressed in mutually exclusive domains of the forming nephrons. *wt1* is expressed in visceral glomerular epithelium while *pax2* is expressed throughout the mesenchymal condensates and comma shaped body but is dramatically downregulated in regions of *wt1* expression (Ryan et al., 1995). The relative expression patterns of *pax2.1* and *wt1* during nephron development are evolutionarily conserved between zebrafish nephron primordia and nephron formation in other vertebrates implying a common mechanism underlying nephron patterning.

The expression analysis in mouse, together with in vitro binding studies demonstrating that *wt1* protein can bind to the *pax2* upstream regulatory region to repress *pax2* transcription, have led to the idea that *wt1* is a direct negative regulator of *pax2* expression (Ryan et al., 1995). This idea has received further support from analysis of *pax2* and *wt1* expression in kidney tissue from Denys-Drash syndrome (DDS) patients (Yang et al., 1999). DDS is a rare human urogenital disorder caused by dominant negative mutations in *wt1* that abolish its ability to bind to its downstream targets (Pelletier et al., 1991; Hastie, 1992). Kidney tissue from Denys-Drash patients show a loss of *wt1* podocyte nuclear localization and display an upregulation of *pax2* in podocytes, suggesting that in normal tissue, *wt1* functions to repress *pax2* expression in podocytes. In the absence of a functional *wt1* gene, the ectopic expression of *pax2* in podocytes may account for some aspects of the glomerulopathy phenotype observed in DDS since glomerular dysgenesis is also observed when *pax2* is constitutively overexpressed in transgenic mice kidneys (Dressler et al., 1993).

Our results suggest a complementary in vivo role for *pax2* in repressing, either directly or indirectly, *wt1* transcription in the pronephric primordium and anterior pronephric ducts during zebrafish development. These results are in contrast to a previous report showing *pax2* transactivation of a *wt1* promoter reporter construct in cell culture transient transfection assays (Dehbi et al., 1996). This apparent discrepancy may reflect differences in experimental systems rather than inherent differences in the function of mouse and zebrafish *pax2* genes. The context in which *pax2* is functioning is different in our experiments compared to the erythroleukemia cell line employed in Dehbi et al. (1996). Also, the target *wt1* reporter plasmid used their experiments may not contain the complete set of *cis*-regulatory elements controlling *wt1* expression. In the case of the zebrafish pronephros, *pax2.1* is not expressed in the forming nephron primordium at 24 hpf when *wt1* is highly expressed, indicating that *wt1* expression is independent of *pax2.1* at this stage (Toyama and Dawid, 1997).

It is also possible that *pax2* function may vary depending on which mRNA splice forms are present in a given tissue. Although at present it is not known which of the twelve *pax2.1* splice forms are expressed in the pronephros, our results demonstrate that inclusion of exon 7 is essential for *pax2.1* function in the pronephros given that the *noi^{tb21}* allele is essentially an exon 7 deletion (Lun and Brand, 1998). *noi^{tb21}* has been characterized as a hypomorphic allele based on the partial development of brain structures (the optic tectum), which are completely missing in embryos homozygous for the

noi^{tu29a} null allele. This characterization of the *noi* allelic series holds true for the pronephros with regard to *pax2.1* transcript expression (A. M., unpublished observations). However, our studies on the pronephros show that the null and exon 7 deletion *noi* alleles result in similar phenotypic outcomes, i.e. a complete absence of pronephric tubules and expansion of podocyte marker gene expression. The exon 7 sequence of *pax2.1* has been shown to encode amino acids adjacent to the C-terminal transactivation domain (Dorfler and Busslinger, 1996; Lechner and Dressler, 1996). While the function of this domain is currently unknown, its absence may result in a reduction in the transactivation potential of *pax2.1*. Whether or not *pax2.1* acts directly or indirectly to inhibit *wt1* expression in the pronephros, our results along with the observations in DDS glomeruli suggest that during nephron differentiation, *pax2* and *wt1* act in a mutually antagonistic fashion to limit their respective expression domains (summarized in Fig. 7). This relationship is likely to be important for proper demarcation of podocyte versus tubule regions of the nephron.

Are the nephron patterning defects in *noi* a result of loss of *pax2.1* function or the ectopic expression of *wt1* in nephron primordia? In *noi* embryos, *pax2.1* function is missing in all cells of the embryo while the expression of the pronephric markers mAb 3G8 and Na$^+$/K$^+$ ATPase is specifically missing in the presumptive tubules and anterior pronephric ducts where *wt1* is ectopically expressed. From this observation, we would argue that in the absence of a functional *pax2.1* gene, ectopic expression of *wt1* (or other podocyte-specific regulatory factors) in the pronephric duct is likely to account for the downregulation of tubule and duct-specific gene expression. This interpretation is consistent with observations by Wallingford et al. (1998) who show that ectopic expression of Xenopus *wt1* is sufficient to inhibit pronephric tubule differentiation. However since, in *noi^{tb21}* embryos, *pax2.1* mRNA is detectable in the anterior duct cells, which ectopically express *wt1*, it would appear that *wt1* is not sufficient by itself to repress pax2.1.

pax2.1 may function in regulating its own expression since only barely detectable levels of *pax2.1* mRNA are found in *noi^{tb21}* lateral primordia cells while expression in other tissues is less affected. A similar requirement for *pax2.1* in maintaining its own expression is observed in the midbrain-hindbrain boundary (Brand et al., 1996; Lun and Brand, 1998). The fact that *pax2.1* mRNA is observed in pronephric duct epithelial cells argues against the idea that *pax2.1^{tb21}* mRNA may be generally unstable in the pronephros. The anterior pronephric duct cells could be the source of a signal that induces *pax2.1* expression in the primordium or alternatively, *pax2.1* protein expressed at a low level earlier during pronephric development may be needed for upregulation of *pax2.1* expression at 30 hpf in the lateral primordium.

In summary, our results suggest that *pax2.1* expression is critical for proper nephron patterning in zebrafish and plays an important role in defining the border between different cell types during pronephric development. Further experiments in higher vertebrates, for example using conditional knockout alleles of *pax2*, may reveal new roles for *pax2* in nephron patterning during metanephric development.

REFERENCES

Armstrong, J. F., Pritchard-Jones, K., Bickmore, W. A., Hastie, N. D. and Bard, J. B. (1993). The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mech. Dev.* **40**, 85-97.

Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Lun, K., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., et al. (1996). Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. *Development* **123**, 179-190.

Brandli, A. W. (1999). Towards a molecular anatomy of the Xenopus pronephric kidney. *Int. J. Dev. Biol.* **43**, 381-395.

Carroll, T. J., Wallingford, J. B. and Vize, P. D. (1999). Dynamic patterns of gene expression in the developing pronephros of Xenopus laevis. *Dev. Genet.* **24**, 199-207.

Chamberlin, H. M., Palmer, R. E., Newman, A. P., Sternberg, P. W., Baillie, D. L. and Thomas, J. H. (1997). The PAX gene egl-38 mediates developmental patterning in Caenorhabditis elegans. *Development* **124**, 3919-3928.

Dehbi, M., Ghahremani, M., Lechner, M., Dressler, G. and Pelletier, J. (1996). The paired-box transcription factor, PAX2, positively modulates expression of the Wilms' tumor suppressor gene (WT1). *Oncogene* **13**, 447-453.

Dent, J. A., Polson, A. G. and Klymkowsky, M. W. (1989). A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. *Development* **105**, 61-74.

Dorfler, P. and Busslinger, M. (1996). C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. *EMBO J.* **15**, 1971-1982.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Dressler, G. R., Wilkinson, J. E., Rothenpieler, U. W., Patterson, L. T., Williams-Simons, L. and Westphal, H. (1993). Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. *Nature* **362**, 65-67.

Dressler, G. R. and Woolf, A. S. (1999). Pax2 in development and renal disease. *Int J Dev. Biol.* **43**, 463-468.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.

Drummond, I. A. (2000). The zebrafish pronephros: a genetic system for studies of kidney development. *Pediatr. Nephrol.* (in press).

Drummond, I. A., Majumdar, A., Hentschel, H., Elger, M., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Stemple, D. L., Zwartkruis, F., Rangini, Z., et al. (1998). Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. *Development* **125**, 4655-4667.

Fu, W. and Noll, M. (1997). The Pax2 homolog sparkling is required for development of cone and pigment cells in the Drosophila eye [see comments]. *Genes Dev.* **11**, 2066-2078.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P., et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1-36.

Hastie, N. D. (1992). Dominant negative mutations in the Wilms tumour (WT1) gene cause Denys- Drash syndrome–proof that a tumour-suppressor gene plays a crucial role in normal genitourinary development. *Hum. Mol. Genet.* **1**, 293-295.

Heller, N. and Brandli, A. W. (1997). Xenopus Pax-2 displays multiple splice forms during embryogenesis and pronephric kidney development. *Mech. Dev.* **69**, 83-104.

Humphrey, C. and Pittman, F. (1974). A simple methylene blue-azure II-

basic fuchsin stain for epoxy-embedded tissue sections. *Stain Technol.* **49**, 9-14.

Kavaler, J., Fu, W., Duan, H., Noll, M. and Posakony, J. W. (1999). An essential role for the Drosophila Pax2 homolog in the differentiation of adult sensory organs. *Development* **126**, 2261-2272.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. *Development* **113**, 1193-1206.

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.

Lechner, M. S. and Dressler, G. R. (1996). Mapping of Pax-2 transcription activation domains. *J. Biol. Chem.* **271**, 21088-21093.

Lechner, M. S. and Dressler, G. R. (1997). The molecular basis of embryonic kidney development. *Mech. Dev.* **62**, 105-120.

Liang, D., Xu, X., Chin, A. J., Balasubramaniyan, N. V., Teo, M. A., Lam, T. J., Weinberg, E. S. and Ge, R. (1998). Cloning and characterization of vascular endothelial growth factor (VEGF) from zebrafish, Danio rerio. *Biochim. Biophys. Acta* **1397**, 14-20.

Lun, K. and Brand, M. (1998). A series of no isthmus (noi) alleles of the zebrafish pax2.1 gene reveals multiple signaling events in development of the midbrain- hindbrain boundary. *Development* **125**, 3049-3062.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Majumdar, A. and Drummond, I. A. (1999). Podocyte differentiation in the absence of endothelial cells as revealed in the zebrafish avascular mutant, cloche. *Dev. Genet.* **24**, 220-229.

Marcos-Gutierrez, C. V., Wilson, S. W., Holder, N. and Pachnis, V. (1997). The zebrafish homologue of the ret receptor and its pattern of expression during embryogenesis. *Oncogene* **14**, 879-889.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

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

Pelletier, J., Bruening, W., Kashtan, C. E., Mauer, S. M., Manivel, J. C., Striegel, J. E., Houghton, D. C., Junien, C., Habib, R., Fouser, L., et al. (1991). Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* **67**, 437-447.

Pfeffer, P. L., Gerster, T., Lun, K., Brand, M. and Busslinger, M. (1998). Characterization of three novel members of the zebrafish Pax2/5/8 family: dependency of Pax5 and Pax8 expression on the Pax2.1 (noi) function. *Development* **125**, 3063-3074.

Puschel, A. W., Westerfield, M. and Dressler, G. R. (1992). Comparative analysis of Pax-2 protein distributions during neurulation in mice and zebrafish. *Mech. Dev.* **38**, 197-208.

Reifers, F., Bohli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. and Brand, M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.

Rothenpieler, U. W. and Dressler, G. R. (1993). Pax-2 is required for mesenchyme-to-epithelium conversion during kidney development. *Development* **119**, 711-720.

Ryan, G., Steele-Perkins, V., Morris, J. F., Rauscher, F. J., 3rd and Dressler, G. R. (1995). Repression of Pax-2 by WT1 during normal kidney development. *Development* **121**, 867-875.

Sanyanusin, P., Schimmenti, L. A., McNoe, T. A., Ward, T. A., Pierpont, M. E., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1996). Mutation of the gene in a family with optic nerve colobomas, renal anomolies and vesicoureteral reflux. *Nat. Genet.* **13**, 129.

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 [see comments]. *Nature* **367**, 380-383.

Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.

Toyama, R. and Dawid, I. B. (1997). lim6, a novel LIM homeobox gene in the zebrafish: comparison of its expression pattern with lim1. *Dev. Dyn.* **209**, 406-417.

Vize, P. D., Jones, E. A. and Pfister, R. (1995). Development of the Xenopus pronephric system. *Dev. Biol.* **171**, 531-540.

Vize, P. D., Seufert, D. W., Carroll, T. J. and Wallingford, J. B. (1997). Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. *Dev. Biol.* **188**, 189-204.

Wallingford, J. B., Carroll, T. J. and Vize, P. D. (1998). Precocious expression of the Wilms' tumor gene xWT1 inhibits embryonic kidney development in Xenopus laevis. *Dev. Biol.* **202**, 103-112.

Westerfield, M. (1993). *The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Brachydanio rerio).* Eugene, OR: M. Westerfield.

Yang, Y., Jeanpierre, C., Dressler, G. R., Lacoste, M., Niaudet, P. and Gubler, M. C. (1999). WT1 and PAX-2 podocyte expression in Denys-Drash syndrome and isolated diffuse mesangial sclerosis. *Am. J. Pathol.* **154**, 181-192.