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

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Accepted 29 February; published on WWW 18 April 2000

#### 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*<sup>-</sup> 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

#### 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

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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 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

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 commaand 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  $pax^2$  gene, the observed absence of mesonephric tubule differentiation indicates a role for  $pax^2$ 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  $pax^2$  during later stages of nephrogenesis. The function of  $pax^2$  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 Sshaped 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 wtl 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 lossof-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  $noi^{ib21}$  allele behaves as a hypomorphic mutation in *pax2.1* and is associated with deletion of amino acids 263-303 (Lun and Brand, 1998). The  $noi^{ib21}$  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  $\mu$ m 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  $\mu$ m, 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,  $\alpha$ 1-subunit Na<sup>+</sup>/K<sup>+</sup> 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 ( $1 \times$  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  $\alpha$ -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*<sup>tu29a</sup> (*noi*<sup>-</sup>; null allele) and *noi*<sup>tb21</sup>, 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

#### no isthmus and pax2.1 in zebrafish tubulogenesis 2091

 $noi^{tb21}$  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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 subunit which can be visualized with the monoclonal antibody  $\alpha$ -6F (Fig. 2B). In 3 dpf *noi*<sup>tb21</sup> 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<sup>+</sup>/K<sup>+</sup> 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 wildtype 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) wildtype and (G-I) noi<sup>-</sup> 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  $noi^{u29}$  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*<sup>tb21</sup>, and (D) *noi*<sup>-/tb21</sup>embryos with mAb 3G8 (A,C), and α-6F mAb (B,D), which recognizes Na<sup>+</sup>/K<sup>+</sup> 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*<sup>tb21</sup> (C). α-6F marks the pronephric duct in wild type (arrows in B) and anterior duct expression is absent in *noi*<sup>-/tb21</sup>(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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 subunit is another marker of nephron patterning and is expressed in the pronephric duct. At 25 hpf, the Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 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 12somite 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.



Correlating spatially with an expansion of wt1 expression, we observed a loss of Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 subunit expression in the anterior duct cells, demonstrating that duct epithelial cell differentiation is altered in *noi*<sup>-</sup> 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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 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<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ 1 subunit expression

#### no isthmus and pax2.1 in zebrafish tubulogenesis 2093

Fig. 4. noi affects patterning of the nephric primordia. (A,C,E,G) Wildtype and (B,D,F,H) noi<sup>tb21</sup> embryos in situ hybridized with pax2.1 (A,B,G,H), wtl (C,D) and  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup> 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<sup>tb21</sup> mutants, even though CNS expression still remains. (C) Whole-mount in situ hybridization with  $wt\hat{l}$  marks the nephric primordia in wild type. wtl expression is caudally expanded in *noi*<sup>tb21</sup> (arrows in D). Sagittal section through a *noi*<sup>tb21</sup> pronephros stained with *wt1* antisense probe (inset in D) reveals ectopic wtl expression in lateral cells of the nephron primordia (arrows). Anterior duct expression of α-subunit of Na<sup>+</sup>/K<sup>+</sup> 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<sup>tb21</sup>.

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<sup>-/tb21</sup>*, 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<sup>+</sup>/K<sup>+</sup> ATPase protein is localized to the basolateral surface of wild-type tubule and duct epithelial cells (Fig. 6A). In *noi*<sup>tb21</sup> mutants, Na<sup>+</sup>/K<sup>+</sup> 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<sup>+</sup>/K<sup>+</sup> ATPase expression is detectable adjacent to cells that express Na<sup>+</sup>/K<sup>+</sup> ATPase in a delocalized manner. In contrast to the pronephric ducts, Na<sup>+</sup>/K<sup>+</sup> 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

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<sup>tb21</sup> (C,D,G,H) and  $noi^{-/tb21}$ (K,L) embryos in situ hybridized with wtl (A, C),  $\alpha$ -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  $\alpha$ -subunit (E) but not wt1 (A). Anterior duct cells are outlined with WGA in the corresponding FITC images (arrows in F,B). In noi<sup>tb21</sup> mutants, anterior duct cells lose Na<sup>+</sup>/K<sup>+</sup> ATPase αsubunit 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<sup>-</sup> 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<sup>-</sup> 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<sup>+</sup>/K<sup>+</sup> ATPase α-subunit. (B) In noi mutants, mAb  $\alpha$ -6F staining is diffuse throughout the duct cells with mAb  $\alpha$ -6F positive cells sitting adjacent to duct cells showing no mAb  $\alpha$ -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). Ductspecific expression of ret1 is absent in noi- even though CNS expression remains unaffected (F).

cells never express pax2.1 and podocyte-specific marker expression is expanded, suggesting that presumptive tubule cells follow an alternative developmental pathway in pax2.1mutants (Fig. 7). The result suggests that pax2.1 expression serves as an instructive signal for lateral cells to assume a



**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.

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 podocytespecific 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<sup>+</sup>/K<sup>+</sup> ATPase, reflecting a defect in terminal differentiation. This phenotype is not limited to the region affected by ectopic wtl 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 wtl expression. Delocalized Na<sup>+</sup>/K<sup>+</sup> 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 wtl 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 wtl 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 wtl podocyte nuclear localization and display an upregulation of *pax2* in podocytes, suggesting that in normal tissue, wtl 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, wtl 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 wtl reporter plasmid used their experiments may not contain the complete set of cis-regulatory elements controlling wtl 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 wtl expression is independent of pax2.1 at this stage (Toyama and Dawid, 1997).

It is also possible that  $pax^2$  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  $pax^{2.1}$ splice forms are expressed in the pronephros, our results demonstrate that inclusion of exon 7 is essential for  $pax^{2.1}$ function in the pronephros given that the  $noi^{ib^{21}}$  allele is essentially an exon 7 deletion (Lun and Brand, 1998).  $noi^{ib^{21}}$ 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*<sup>tu29a</sup> 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<sup>+</sup>/K<sup>+</sup> ATPase is specifically missing in the presumptive tubules and anterior pronephric ducts where wtl is ectopically expressed. From this observation, we would argue that in the absence of a functional pax2.1 gene, ectopic expression of wtl (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, pax2.1mRNA is detectable in the anterior duct cells, which ectopically express wtl, it would appear that wtl 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 midbrainhindbrain 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.

We are grateful to Dr Mark Fishman and Colleen Boggs for use of the MGH CVRC fish facility. We also thank members of the Fishman

#### no isthmus and pax2.1 in zebrafish tubulogenesis 2097

laboratory for helpful discussions. Special thanks go to Mary McKee for technical help with electron microscopy. This work was supported by NRSA fellowship DK09763 to A. M. and NIH R01 DK53093 and PO1 DK 54711 to I. A. D. Support by the DFG Schwerpunktprogramm 'Evolutionentwicklungsbiologischer Prozesse', the Förderprogramm Neurobiologie Baden-Württemberg and the Max Planck Society is acknowledged by M. B. and K. L.

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