Development 128, 2233-2241 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV3351

Pre-pattern in the pronephric kidney field of zebrafish

Fabrizio C. Serluca and Mark C. Fishman*

Cardiovascular Research Center and Developmental Biology Laboratory, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, MA 02129, USA
*Author for correspondence (e-mail: fishman@cvrc.mgh.harvard.edu)

Accepted 26 March 2001

SUMMARY

Vertebrate embryos use a series of transient kidneys to regulate fluid balance, osmolarity and metabolic waste during development. The first kidney to form in the embryo is the pronephros. This kidney is composed of several cell types with very different functions and is organized into discrete segments: glomerulus, tubules and nephric duct. The site of origin of these cells is poorly understood, as are their lineage relationships. We have defined regions of the intermediate mesoderm as candidates for the pronephric field by expression patterns of the Wilms' Tumor suppressor gene (*wt1*), *single-minded 1* (*sim1*) and *pax2.1*. All of these potential kidney markers are expressed in a stripe of intermediate mesoderm, with distinct, overlapping antero-posterior borders. We labeled small groups of cells in this area by laser uncaging of a fluorescent dextran, and then tracked their fates. We found that there was a bounded contiguous region of the intermediate mesoderm that provides pronephric progenitors. As is true for other organ fields, the pronephric field regulates after focal destruction, such that a normal pronephros forms after laser-mediated removal of the *wt1* domain. The progenitors for podocytes, tubular cells and duct are restricted to subdomains within the pronephric field. The most anterior cells in the pronephric field give rise to podocytes. This corresponds to the *wt1*-expressing region. The next more posterior cells contribute to the tubule, and express both *wt1* and *pax2.1*. The most posterior cells contribute to the nephric duct, and these express *pax2.1* and *sim1*, but not *wt1*. Thus, there is a field for the pronephric kidney with classical attributes of defined border, pre-pattern and regulation. The pattern of the fate map reflects particular combinations of transcription factors.

Key words: Pronephros, Kidney field, Glomerulus, Cell fate, Regulative development, Zebrafish

INTRODUCTION

The first kidney to form in embryos of all vertebrate species is the pronephros. It has three components: glomerulus, tubules and nephric duct. This embryonic kidney is replaced later in development by the mesonephros, and in birds and mammals, by a metanephros (Saxén, 1987). The mesonephric and metanephric kidneys arise along the course of the nephric duct and, in fact, the metanephros is generated by mutual inductions between duct and mesenchyme (Saxén, 1987). The pronephric kidney, however, appears to arise de novo from cells in the intermediate mesoderm (IM) (Goodrich, 1930). The origin of the elements of the pronephric kidney have not been defined by lineage analysis. Fashioning of this organ requires the generation and apportionment of cells with at least three distinctive cell fates that represent the three components of the kidney, and their appropriate migration and positioning with respect to each other during morphogenesis.

Organ fields in vertebrate embryos are regions defined classically by explantation and transplantation as regions that contain organ precursors (Huxley and de Beer, 1934). Based on the assumption that tubule formation is indicative of kidney formation, explantation and transplantation in *Ambystoma* points to the intermediate mesoderm as a site of renal origin, at least for the tubule progenitors (Fales, 1935). The positions of molecular markers characteristic of particular cell fates do not necessarily correspond to precursor location defined by lineage analysis (e.g. Serbedzija et al., 1998). Hence, lineage analysis, by marking of cells with dye or virus (Price et al., 1987) is the only definitive way to determine precursor location, migration and cell fate assignment.

There are three modular components to the pronephros. The glomerulus is the site of blood filtration in the kidney. It consists of a capillary tuft in apposition to podocytes of the kidney. Fenestrations in the capillary endothelium and slit diaphragms in the podocytes allow the passage of the filtrate into Bowman's space. The cells of the tubule are the site of selective reabsorption and secretion. The nephric duct carries the modified urine to the exterior (Saxén, 1987).

The pronephros of the zebrafish consists of paired glomeruli at the midline ventral to the dorsal aorta, and a set of pronephric tubules that project laterally from the glomeruli to the nephric ducts that run along the side of the embryo (Drummond et al., 1998). Histologically, the first evidence of the pronephros is a cell aggregate derived from the coelomic epithelium located ventral to the anterior somites (Goodrich, 1930). This aggregate will form the paired pronephric primordia which remain visible as flattened epithelial sacs between the 30 to

36 hours post-fertilization (hpf) stages in the zebrafish (Drummond et al., 1998). Unlike the pronephros of amphibia, the connection to the coelomic cavity in teleosts is transient and the nephrocoel of the primordia is closed to the body cavity (Drummond et al., 1998; Goodrich, 1930). The primordia then fuse at the midline and will form the sole paired nephrons of the pronephros. The glomerular blood supply is derived from the dorsal aorta and filtration begins at the 40 hpf stage (Drummond et al., 1998; Majumdar and Drummond, 1999).

Ablation experiments in *Xenopus* demonstrate that the tubular and duct progenitors in the organ primordia are spatially distinct (Vize et al., 1995). However, explant studies indicate that the kidney cell fate is specified much earlier in the intermediate mesoderm (Brennan et al., 1998; Brennan et al., 1999). The origins of these structures from undifferentiated intermediate mesoderm and the lineage relationship among these cells has not been determined in the normal pronephric kidney. In explanted metanephric mesenchyme, induced to initiate nephron morphogenesis but lacking vessels, retroviral tagging indicates that there is a shared lineage between glomerular and tubular cells (Herzlinger et al., 1992). In vivo labeling of the pronephric duct indicates that in some species the duct grows by accretion of cells from surrounding mesoderm, and in others by caudal migration of a duct rudiment (Cornish and Etkin, 1993; Fox and Hamilton, 1964; Obara-Ishihara et al., 1999; Poole and Steinberg, 1981).

There are suggestions from studies of other organ fields that the distinctive modular elements of an organ may be set aside quite early in development and reflected in spatial domains of gene expression and cell fate in organ fields. Interestingly, single gene defects indicate that structural elements of organs may be selectively removed leaving the rest of the tissue to develop relatively normally (Fishman and Olson, 1997). Hence, both embryological and genetic lines of evidence suggest a relative independence to development of structural modules of organs. For example the *no isthmus* mutation removes the pronephric tubules but does not appear to affect glomerular or duct development (Majumdar et al., 2000). Thus, it is crucial to define where cell fate assignments are made.

We address the origin, patterning and regulatory attributes of the pronephric field in vivo in the zebrafish by use of an uncaging technique we used previously to define the heart field (Serbedzija et al., 1998). We find that there is a bounded region of the intermediate mesoderm that constitutes a pronephric field. Within this region, there is an antero-posterior pattern to the fate map, with pre-glomerular cells most anterior, pre-duct cells most posterior, and pre-tubular cells in between. Interestingly, this fate map corresponds to particular combinations of transcription factor gene expression in the intermediate mesoderm.

MATERIALS AND METHODS

Zebrafish lines

Wild-type zebrafish were maintained and raised as described (Westerfield, 1995). Dechorionated embryos were kept in 30% Danieau's solution and staged according to somite number or hours post-fertilization (hpf) (Kimmel et al., 1995). The wild-type lines were of the TL background obtained from the laboratory of Dr Christianne Nusslein-Volhard in Tübingen, Germany.

Cloning of *wt1* and *sim1*

wt1 was cloned using nested PCR on a 24 hpf embryo lambda-ZAP Express (Stratagene) cDNA library. The first reaction used the vector T3 primer at the 5′ end and WTR1 (GGTTGACCGCACAATGGCAGGAGTC), a reverse primer made against the zinc-finger region (Accession Number X85734). No clear bands were visible by ethidium bromide staining, but Southern blotting using a probe for human *WT1* (a gift from Dr Jerry Pelletier, McGill University) confirmed a 1.1 kb product. That area of the gel was excised and used as the template in the second reaction with a second forward primer made against the pBK-CMV vector and WTR2 (GCCCGGTACTCTCCGCACATCATG) as the reverse primer. A 900 bp product was subcloned in pBluescript (Stratagene) and several clones were sequenced. We noted discrepancies at the 5′ end of the clones and so a primer, WT1F-Eco (CCGAATTCAACGGTAACTGTACTCTTCCAGTG), was made to reamplify the *wt1* clone beginning where the sequences all agreed. The PCR product was cloned into pBluescript, resequenced and used in all in situ hybridizations of this study. *Eco*RI was used to linearize the template and T7 RNA polymerase to transcribe antisense riboprobes.

Zebrafish *single-minded* homologs were cloned using a degenerate nested PCR using adult cDNA as a template. In the first round of PCR, the primers were ATGAARGAGAARTC(A/T/C)AARAAYGCNGCNAA (forward) and TGGAA(A/T/G)GTGTCRCANCCGTG(A/T/G)GCGTGGTGGTA (reverse), the primers for the second PCR were GAGAARGAGAACGCNGARTTCTAYGA (forward) and TCRAT(A/C/G)AGCTCCTGIGGCTCGTANGG (reverse). The resulting product was a 700 bp fragment containing the DNA-binding domain, as well as the PAS domains, this was then used to screen a 24 hpf library at medium stringency (0.2× SSC, 0.1% sodium dodecyl sulphate at room temperature) to obtain full-length clones. *Eco*RI and T7 RNA polymerase were used to linearize and transcribe antisense probes. The sequences for *sim1* and *wt1* were analyzed using the BLAST and CLUSTAL algorithms. The nucleotide sequence and predicted amino acid sequence of the clones have been deposited in GenBank (*sim1*, AY028626; *wt1*, AY028627).

In situ hybridization and histology

Whole-mount in situ hybridization was performed as previously described (Oxtoby and Jowett, 1993) with some minor modifications. The probes used in this study have been previously described: pax2.1 (Krauss et al., 1991), pax8 (Pfeffer et al., 1998) and alpha-tropomyosin (Ohara et al., 1989). For the double label experiments, we adapted an existing protocol from mouse (Cygan et al., 1997). Embryos were hybridized with both digoxigenin- and fluorescein-labeled riboprobes. Anti-DIG AP (1:2000) and BM Purple (Boehringer Mannheim) were used to detect the first probe. The phosphatase was inactivated by incubating in 10 mM EDTA while heating to 65°C for 30 minutes. The fluorescein-labeled probe was detected using anti-fluorescein AP (1:5000) and the INT/BCIP substrate (Boehringer Mannheim). After the color reaction, embryos were equilibrated in 70% glycerol and photographed on a Leica dissecting microscope or a Zeiss Axiophot microscope equipped with DIC optics. For histological analysis, the stained embryos were equilibrated in PBS with 0.1% Tween-20 (PBT), dehydrated in series into ethanol, embedded in JB-4 (Polysciences) and sectioned.

Cell lineage tracing

Cell tracing experiments were performed essentially as described (Serluca and Fishman, 1999) with some modifications. Embryos obtained from natural spawning were dechorionated using Pronase (Sigma) and injected with approximately 1 to 5 pl of 1% DMNB-caged fluorescein dextran (Molecular Probes) in 160 mM KCl at the one- to four-cell stage and raised until early somitogenesis. At the eight-somite stage, embryos were placed on a 2% agarose ramp and positioned to allow a dorsal view of the notochord and developing somites. The lineage tracer was activated using a pulsed nitrogen laser

(LaserScience) tuned to a wavelength of 362 nm mounted on a Zeiss Axioskop microscope equipped with a 40× water-immersion lens (Photonics, Arlington Heights, IL). The activation was followed using a fluorescein filter until patches of the desired size appeared labeled. The embryos were allowed to develop at 28.5°C until the 40-48 hpf stage and then fixed in 4% formaldehyde/PBS. In vivo visualization of the label is complicated at this stage as it lies in deep tissues and is quenched by autofluorescence from the yolk. For visualization in fixed tissue, the embryos were first permeabilized in acetone for 30 minutes at –20°C, then rehydrated in series in phosphate buffered saline+0.1% Tween-20 (PBT). Endogenous peroxidase activity was eliminated by incubating in 0.5% normal sheep serum (NSS) and 0.5% hydrogen peroxide for 30 minutes in PBT. The embryos were blocked for 30 minutes using 10% NSS in PBT and then incubated with a 1:500 dilution of anti-fluorescein-POD (Boehringer Mannheim) in 10% NSS for 2 hours. After several quick washes and one overnight wash in PBT, the antibody was detected using 3,3′ diaminobenzidine (DAB) SigmaFast tablets (Sigma). To facilitate the identification of labeled cells, the embryos were bleached in 3% hydrogen peroxide and 1% potassium hydroxide for 30 minutes at room temperature. Embryos were scored by visual inspection on a dissecting microscope and either photographed or embedded in JB4 plastic for further histological analysis. Serial section were stained with Methelyne Blue-Azure II and coverslipped (Humphrey and Pittman, 1974). In each set of experiments, approximately one third of the embryos were sectioned, in order to confirm the identity of the labeled structure.

Field ablation

For the tissue ablation studies, embryos were mounted and positioned on agarose ramps at the six- to eight-somite stage as described above. The laser was set to 452 nm, and we focused on the intermediate mesoderm cells adjacent to the first and second somite of the left side of the embryo. We used a low pulsing frequency in order to carefully monitor changes in cell shape. The ablated patch contracted, then retained a granular appearance as described (Serbedzija et al., 1998).

RESULTS

wt1 and *sim1* are early markers of zebrafish kidney development

We cloned *wt1* as a potential marker of glomerular podocytes, because it is known to mark these cells in both mice and humans (Armstrong et al., 1993; Pritchard-Jones et al., 1990). *wt1* is a zinc-finger transcription factor involved in the development of multiple kidney forms in many species (Carroll and Vize, 1996; Pelletier et al., 1991; Pritchard-Jones et al., 1990; Sainio et al., 1997). We examined the developmental pattern of *wt1* and compared it with that of *pax2.1*/*no isth*mus (*noi*), a zebrafish ortholog of the *Pax2* gene, known to be expressed in the pronephric kidney, and with *sim1*, which we

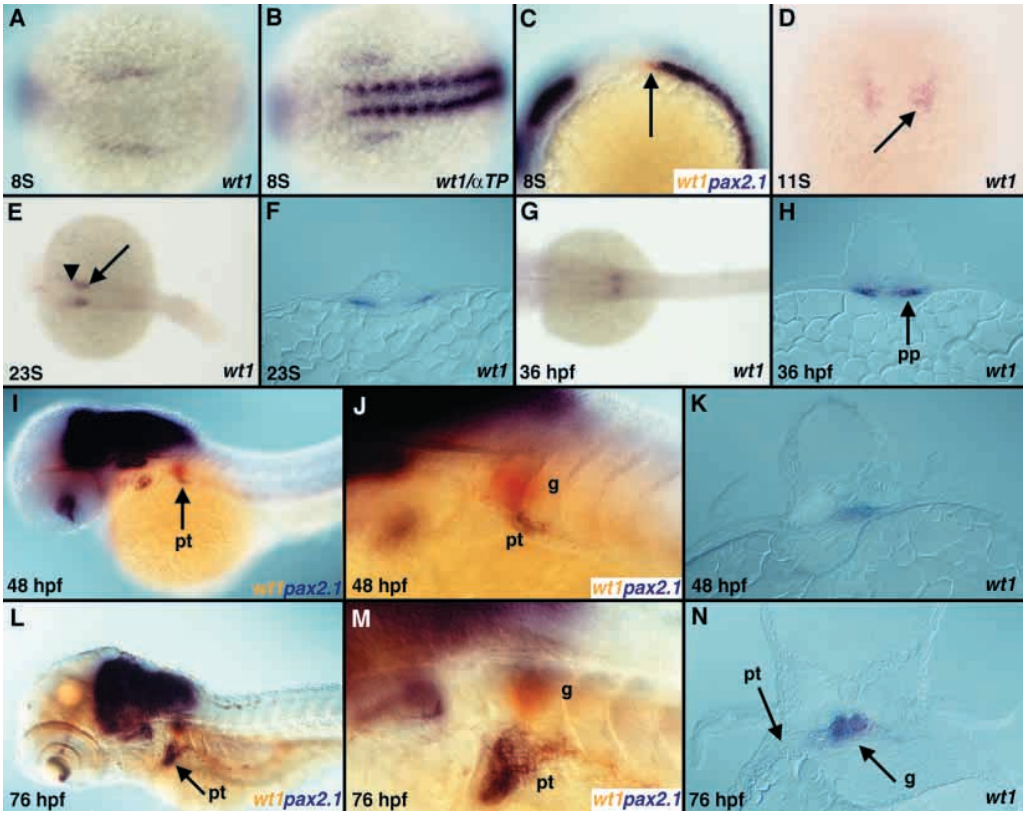

Fig. 1. Expression of *wt1* in the developing kidney. *wt1* first appears as bilateral stripes in the intermediate mesoderm at the two- to three-somite stage. (A) An eight-somite stage embryo showing *wt1* expression in the anterior intermediate mesoderm. (B) Co-staining for *alpha-tropomyosin* indicates that the anterior border lies at the level of the first somite and extends caudally to the level of the fourth somite. (C) Two-color in situ hybridization with *wt1* (orange) and *pax2.1* (purple) labeling the intermediate mesoderm; the anterior-most cells express only *wt1* (arrow). Posteriorly, we find a cell population that expresses both *wt1* and *pax2.1*. (D) At the 11-somite stage, *wt1* expression medial to the two original stripes is observed (arrow). (E,F) *wt1* staining in pronephric cell aggregates ventral to the somites at the 23 somite stage in (E, arrow) and shown in cross-section in F. (G,H) Expression of *wt1* is observed in the paired pronephric primordia at the 36 hpf stage (G), shown in cross-section in H. (I) *wt1* (orange) localizes to the glomerulus while *pax2.1* (purple) marks the pronephric tubule and anterior duct at the 48 hpf stage, a higher magnification of the same photograph is shown in J. (K) Cross-sections of a 48 hpf embryo with midline glomerular *wt1* staining ventral to the dorsal aorta. (L) Co-staining with *wt1* (orange) and *pax2.1* (purple) at 76 hpf reveals that while *wt1* still localizes to the glomerulus, *pax2.1* expression is only observed in the tubules, a higher magnification of the same photograph is shown in M. (N) *wt1*-stained 76 hpf embryo shown in cross-section, note the absence of *wt1* transcripts in the pronephric tubule and the restriction to the glomerulus. Anterior is towards the left in all panels except D, where it is at the top of the panel. (A,B,D,E,G) Dorsal views; (C) Lateral view; (I,J,L,M) Dorsolateral views. g, glomerulus; pp, pronephric primordia; pt, pronephric tubule. Arrowhead in E indicates where the wt1 stripes begin to fade.

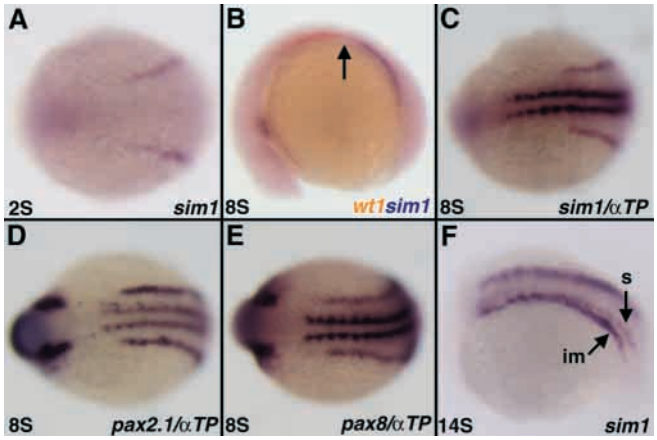

Fig. 2. Nephric expression of *sim1*. (A) Dorsal view showing *sim1* expression in the intermediate mesoderm. This is first evident at the two-somite stage. (B) Double in situ hybridization using *sim1* and *wt1* probes indicates that the posterior limit of *wt1* expression coincides with the anterior limit of *sim1* expression (arrow). (C) The anterior limit of *sim1* expression lies at the level of somite four or five. Similar labeling experiments with *pax2.1* (D) and *pax8* (E) reveal that their anterior limit lies at the level of somite two. (F) *sim1* continues to be expressed in the intermediate mesoderm and the ventrolateral portion of the somite throughout the segmentation period. (A,C,D,E) Dorsal views; (B) Lateral view; (F) Dorsolateral view. Anterior is towards the left in all panels. im, intermediate mesoderm; s, somite.

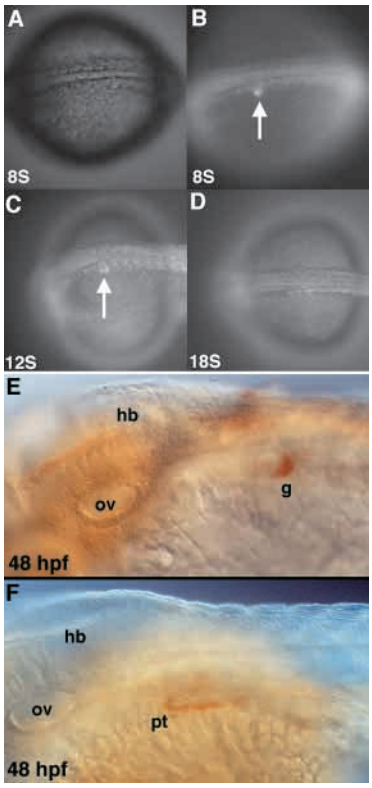

Fig. 3. Origin of glomerular and tubular precursors. (A-D) Time lapse analysis of a single embryo labeled at the eight-somite stage at the level of the second somite. (A) A phase image; (B) the corresponding fluorescence image – arrows indicate the labeled patch. (C) Fluorescence image indicating that cells have started to move in by the 12-somite stage. However, by the 18-somite stage, the cells have become difficult to visualize using fluorescence microscopy (D). In order to detect these cells in older embryos, we have used an anti-fluorescein antibody coupled to a peroxidase and stained with DAB. (E) Whole-mount DAB-stained 48 hpf embryo showing that cells labeled at the level of the first somite give rise to the glomerular segment of the pronephros. (F) Cells labeled at the level of the third somite can give rise to the pronephric tubule. Anterior is towards the left in all panels. hb, hindbrain; g, glomerulus; pt, pronephric tubule; ov, otic vesicle.

cloned because it is a marker of the developing duct in other species (Brand et al., 1996; Fan et al., 1996; Krauss et al., 1991; Pfeffer et al., 1998; Pourquie et al., 1996). *sim1* is a zebrafish homologue of the *Drosophila single-minded* gene and encodes a basic helix-loop-helix transcription factor containing a PAS (*period*, *ARNT*, *single-minded*) motif (Crews and Fan, 1999).

wt1 expression first appears at the two- to three-somite stage as bilateral stripes in the intermediate mesoderm, extending from the first to fourth somite, as shown in Fig. 1A,B. We have used the somitic pattern of *alpha-tropomyosin* as an axial reference (Fig. 1B). Double staining with *wt1* and *pax2.1* indicates that the anterior limit of *wt1* expression extends further than that of *pax2.1* (Fig. 1C).

At the 11 somite stage, *wt1* expression additionally appears in cells medial to the original stripes (Fig. 1D). It is in this cell population, ventral to the somites (arrow in Fig. 1E,F) that *wt1* expression persists as, by midsomitogenesis, the *wt1*-expressing lateral stripes begin to fade (arrowhead in Fig. 1E). Their location suggests that this aggregate (Fig. 1F) may include those cells that will give rise to the paired pronephric primordia and which later express *wt1* (Figs 1G,H), as shown previously (Drummond et al., 1998). Between 36 and 48 hpf, the pattern of *wt1* expression is refined to include only the more medial cells until, from 40 hpf onwards, *wt1* expression becomes limited to the cells of the glomerulus, presumably the podocytes (Fig. 1I-K). This expression is consistent with that observed during mouse and human kidney development, where *WT1* is ultimately restricted to the podocytes (Armstrong et al., 1993; Pritchard-Jones et al., 1990). At the 48 hpf stage, *pax2.1* labels the tubules and the anterior portion of the duct, but not the glomerulus (Fig. 1I,J). By 72 hpf, expression of *pax2.1* recedes from the duct, and becomes confined to the pronephric tubule, while *wt1* still marks the glomerulus (Fig. 1L-N).

sim1 is first expressed at the two somite stage (Fig. 2A). Double labeling experiments using *wt1* and *sim1* probes indicate that the posterior end of *wt1* expression coincides with the rostral end of *sim1* expression (Fig. 2B). Co-staining *alpha-tropomyosin*, indicates that *pax2.1* expression extends to the second somite, while *sim1* expression extends to somite four or five (Figs 2C,D). We have also looked at the expression of a second paired-box gene expressed in the kidney, *pax8*. The anterior border of *pax8* expression is identical to that of *pax2.1* (Fig. 2E). Both *pax2.1* and *pax8* expression in the kidney begins prior to somitogenesis and thus precedes the onset of both *wt1* and *sim1* expression (Krauss et al., 1991; Pfeffer et al., 1998). The intermediate mesoderm continues to express *sim1* throughout the segmentation period. At this stage it is expressed also in the lateral portion of the somite (Fig. 2F). *sim1* expression is absent from the pronephros after 30 hpf.

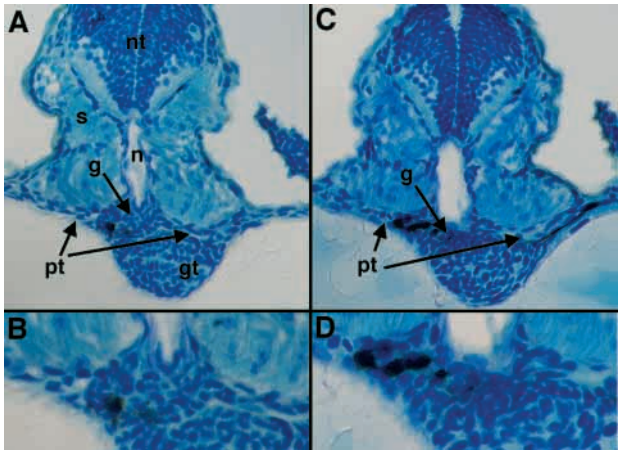

Fig. 4. Histological analysis of labeled embryos. All panels are cross-sections of DAB-stained embryos. (A,B) The intermediate mesoderm lateral to the first somite contains progenitors of the glomerulus (A), shown in higher magnification in (B). (C,D) Labeled cells lateral to the second somite can give rise to tubular cells (C), shown in higher magnification in (D). g, glomerulus; gt, gut; n, notochord; nt, neural tube; pt, pronephric tubule; s, somite.

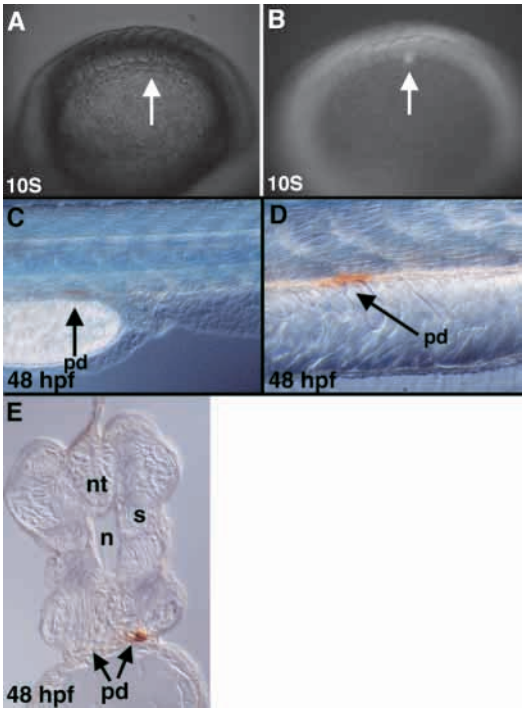

Fig. 5. The nephric duct forms by in situ differentiation of intermediate mesoderm cells. (A) Phase image of an 11-somite stage embryos, the arrow indicates the intermediate mesoderm adjacent to the somites. (B) Fluorescence image of the same embryo in A after the labeling of a patch of cells at the level of somite 9. Embryos at the 48 hpf stage where cells were labeled in the undifferentiated IM (C) or the growing duct (D) at the 10-somite stage, give rise to the nephric duct. Shown here are two typical examples of labeled duct segments, note that the label is continuous, and is the width is never greater than the width of one somite. (E) Cross-section of an embryo with a labeled patch in the duct. Anterior is towards the left in A-D. n, notochord; nt, neural tube; pd, pronephric duct; s, somite.

We were curious as to whether this region of presumptive renal markers corresponds to the site of origin of pronephric precursors, and whether the anterior-posterior patterning reflected elements of a cell fate map. In other words, we were interested in knowing if this region constituted a pronephric field.

Origin of pronephric precursors in the intermediate mesoderm

We used laser activation of a caged lineage tracer to follow the fate of cells and to construct a fate map for the zebrafish embryonic kidney. We injected caged fluorescein in early cleavage stage embryos, which then develop until early (8-10S) somitogenesis. Using a pulsed laser, we then uncaged the fluorescein-dextran in a patch of three to five cells. We found that, as anticipated by the expression studies, cells destined to become the pronephros lay in the intermediate mesoderm adjacent to the somites. We found no cells outside the field that contribute to the pronephros. Lateral to the pronephric area we found that mesodermal cells were fated to become blood, while more anterior cells adopt mesenchymal fates in the pharyngial arches (S. J. Childs and F. C. S. and M. C. F., unpublished).

Patches of cells were activated lateral to the somite at the eight- to ten-somite stage (Fig. 3A,B). At the 12-somite stage, a portion of the labeled patch is found to lie below the somite, indicating a medial movement of the cells (Fig. 3C). However, by the 18 somite stage, the cells are difficult to detect by fluorescence (Fig. 3D) as they probably lie below the somitic mesoderm and above the developing gut. In order to visualize the labeled cells in these later stages, we used an antibody directed against fluorescein. The timing of the early cell movement correlated well with the medial appearance of *wt1*-expressing cells.

Patterning of glomerular, tubule and duct precursors

Using smaller regions of activation within the area we are defined as the pronephric field, we discovered that cell fate is predicted by axial position. These data are summarized in Table 1. Intermediate mesoderm adjacent to the first somite contains cells contributing exclusively to the glomerular segment (Figs 3E, 4A,B). At the level of somite two, we found both tubular progenitor cells (Fig. 4C,D) and glomerular progenitors. In some embryos, we observed label in both the glomerulus and tubules. We cannot determine whether these two fates derive from individual progenitors because we cannot be completely confident that we label only single cells. Cells adjacent to somite three contributed to the pronephric tubules (Fig. 3F) and, to a lesser extent, the anterior nephric duct. Caudally, we found only duct progenitors. Intermediate mesoderm at the level of the first three somites also contains progenitors for fin and gut mesenchyme. The rare labeling of periderm directly along the tract of the laser seems clearly to result from inexact focusing of the laser beam.

Development of the nephric duct

The mechanism of nephric duct elongation in other species involves a combination of directed cell migration and in situ generation of the duct from intermediate mesoderm cells (Cornish and Etkin, 1993; Poole and Steinberg, 1981). We examined this process in the zebrafish embryo by labeling

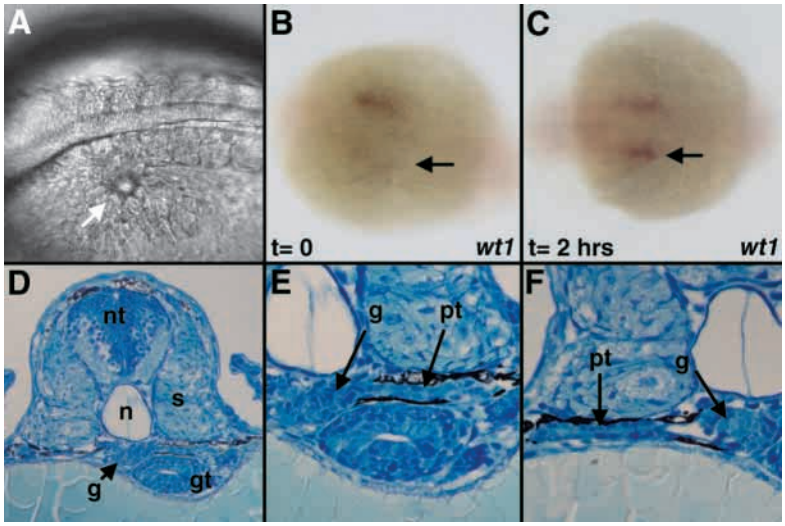

Fig. 6. Regulative development of the zebrafish pronephros. Glomerular and tubular precursors in the intermediate mesoderm were ablated (arrow in A). Embryos were fixed immediately after unilateral (left side) ablation and processed for in situ hybridization with a probe for *wt1* (B). All of the *wt1* stripe is missing from the operated side (arrow). After 2 hours of recovery time, *wt1* expression returns to normal (arrow in C). (D) Histology of a 48 hpf embryo that had glomerular progenitor cells unilaterally ablated at the 9-somite stage. A normal midline glomerulus and tubules are clearly visible. (E) A higher magnification of the section in (D). (F) Unoperated control side of the same embryo in (D,E). Anterior is towards the left in A-C; E-G are cross-sections. g, glomerulus; gt, gut; n, notochord; nt, neural tube; pt, pronephric tubule; s, somite.

intermediate mesoderm cells at somite five (S5), to see if they later migrated to form part of the more caudal duct. In separate experiments, we labeled more posterior undifferentiated mesodermal cells, at the level of the somite ten (S10), in order to assess the role of in situ differentiation (Fig. 5A,B). In some experiments, we labeled adjacent myocytes as a positional reference.

In both types of experiments, either labeling duct or posterior undifferentiated mesoderm, the labeled patch gave rise to a discrete continuous segment of the duct, typically less than one somite width in length. The labeled cells remained contiguous, without appearance of gaps (Fig. 5C,D) and the labeled cells in the duct remained in the same position as the site of IM labeling. Histological analysis of the embryos confirmed the identity of the labeled structure as nephric duct (Fig. 5E). Thus, in the zebrafish embryo the pronephic duct appears to elongate entirely by the recruitment of a subset of intermediate mesoderm cells and their in situ epithelialization without involvement of cell migration from the duct.

Regulation in the kidney field

Embryonic fields have the capacity to regulate (Huxley and de Beer, 1934). That is, local injury or destruction of cells within the field is compensated and there is restoration of the field and eventual formation of a normal organ. To investigate the regulatory capacity of the zebrafish kidney field, we performed unilateral tissue ablations between the eight- and ten-somite stages. We focused on cells within the intermediate mesoderm that form the *wt1*-expressing region. We confirmed the extent of the injury to both glomerular and tubular precursors by in situ hybridization with the *wt1* probe (Fig. 6B). Following the ablation procedure, the patch first contracted and became granular in appearance (Fig. 6A). Two hours following the operation, the ablated side has recovered its normal *wt1* expression (Fig. 6C). We allowed the embryos to develop until the 48 hpf stage. The surviving embryos (28/31) were not edematous and did not exhibit any of the abnormal morphology associated with kidney defects (Brand et al., 1996; Drummond et al., 1998). Histological analysis of the post-ablated embryos revealed the presence of a complete pronephric kidney, including the glomerulus and tubules (Fig. 6D-F), indicating that this pronephric progenitor region of the intermediate mesoderm has regulatory ability characteristic of other embryonic fields.

Table 1. Cell lineage tracing along the AP axis of the intermediate mesoderm

Site of activation	Segment identity					
	G	PT	ND	G and PT	PT and ND	*n*
S1	66	0	0			80
S2	37	43	0	6		131
S3	0	38	7		5	51
S5	0	0	23			23
S10	0	0	18			18

G, glomerulus; PT, pronephric tubule; ND, nephric duct.

Embryos were labeled along the AP axis of the intermediate mesoderm at the eight- to ten-somite stage and allowed to develop until 48 hpf. The lineage tracer was visualised by immunological labeling, and kidney segments were identified by inspection of whole-mount stained embryos or by histology.

DISCUSSION

By use of in vivo lineage markers, we define here the mesodermal site of origin and fate of cells that fashion the pronephros of the zebrafish. These cells constitute a classical organ field: a region of progenitors with internal pattern and regulative ability (Huxley and de Beer, 1934). The pronephric field is in the intermediate mesoderm. Axial position within the field predicts ultimate pronephric fate. Pre-glomerular cells, pre-tubular and pre-nephric duct cells occupy progressively more posterior domains within the field. There is little evidence for intermixing between the populations. This fate map corresponds to compartments of gene expression, such that pre-glomerular cells are *wt1+/pax2.1–/sim1–*; pre-tubular cells are *wt1+/pax2.1+/sim1–*; and pre-nephric duct cells are *wt1–/pax2.1+/sim1+*, indicating that molecular distinctions between the subpopulations are in place prior to nephrogenesis.

Lineage and fate map of the pronephros

The origin of the pronephros has not been previously defined by lineage analysis. Fales demonstrated that explants from the anterior intermediate mesoderm of *Ambystoma* can give rise to

tubules when grafted to an ectopic location, but this corresponds to only one component of the pronephros (Fales, 1935). In particular we were interested in the origin of the glomerulus. There was reason to believe that it might arise from a location distinctive from tubules, despite the proximity and association in the final organ. In evolutionary terms, the glomerular podocytes appear to be derived from coelomic epithelium, a specialization that arose presumably so adjacent vessels could excrete ultrafiltrate into the coelom (Smith, 1943). This direct coelomic excretion is, in fact, the case in lower chordates such as the lamprey (de Beer, 1928). It is also the situation for the pronephros of certain amphibia in which tubules are separated from a glomerulus (or glomus) by the coelom (Vize et al., 1995).

This work indicates that there is a contiguity of pre-podocyte, pre-tubular and pre-nephric duct cells in the IM, but that they are spatially separated. Ablation experiments have shown that the organ primordia is subdivided into discrete domains giving rise to tubules and duct (Vize et al., 1995). However, there are no prior data for direct comparison, because analysis of lineage in the kidney has been largely confined to the duct segment, most particularly the mechanism by which it elongates (Cornish and Etkin, 1993; Obara-Ishihara et al., 1999; Poole and Steinberg, 1981). Indeed observations here on the pronephric field may not be directly comparable with the development of mesonephric or metanephric kidneys, because in species where such kidneys occur they form later in development and by processes of mesenchymal induction from a pre-existing duct. It has been noted, using explants of metanephric mesenchyme organ cultures labeled by retrovirus, that a single cell can contribute to both the glomerulus and tubule if marked prior to the induction event (Herzlinger et al., 1992). In the zebrafish we did observe a few cases (6/131) with mixed fates from one labeling, but because we label three to five cells at a time we suspect that cells are destined for one fate and the rare instances of multiple fates are due to labeling at the borders of these domains.

Thus, it appears that pronephric cell fates are assigned in the intermediate mesoderm prior to or during early somitogenesis. This is similar to the situation for the heart, where progenitors for atrium or ventricle are spatially segregated while still in the lateral plate mesoderm heart field (Fishman and Chien, 1997) and for the limb where proximal-distal identities are specified within the limb field (Murray, 1926).

Regulative ability has been described for the limb, ear and cardiac fields (Copenhaver, 1926; Harrison, 1918; Serbedzija et al., 1998; Tokura, 1925) and for migratory neural crest (Raible and Eisen, 1996). We did not determine whether the embryo can replace an entire pronephric field, because embryos died immediately after ablation of very large areas of the zebrafish intermediate mesoderm. We believe this to be due to the inadvertent ablation of the yolk cell membrane. Ablations of smaller areas, however, were well tolerated. We focused our attention upon the anterior-most portion of the field: those cells that express *wt1* and that normally give rise to the glomerular and tubular segments. The destruction of these cells did not prevent the formation of a glomerular structure, as confirmed by histological criteria.

Duct elongation of the zebrafish pronephros occurs by recruitment of cells from the intermediate mesoderm. This process appears to have a species-dependent mechanism. In axolotl, the duct elongates due to migration of cells from the pronephric duct rudiment (Poole and Steinberg, 1981). In *Xenopus*, the elongation involves both cell migration and a contribution from IM cells located posteriorly, cells that are recruited into the elongating duct structure (Cornish and Etkin, 1993; Fox and Hamilton, 1964).

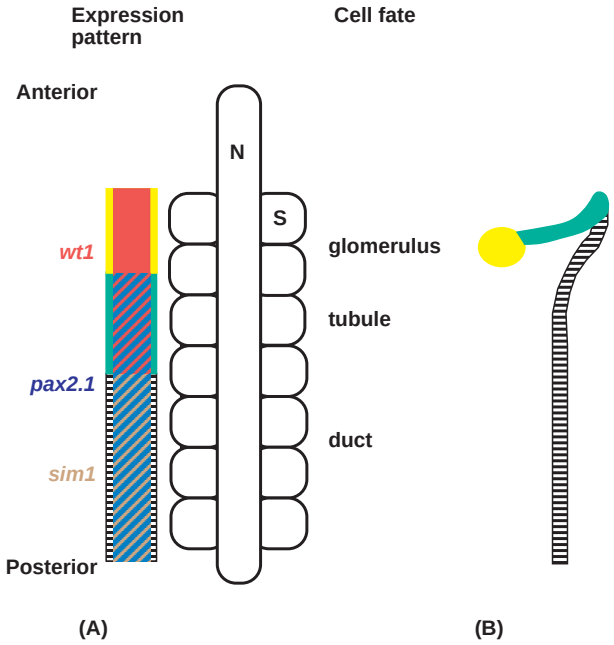

Fig. 7. Pre-patterning in the intermediate mesoderm directs pronephric kidney cell fate. (A) Expression patterns of *wt1* (orange), *pax2.1* (blue) and *sim1* (brown) at the eight- to ten-somite stage. The notochord (N) and somites (S) are shown as axial references. Glomerular progenitors, bordered by yellow lines, lie in the anterior-most nephrogenic mesoderm and express *wt1*; tubule progenitors, bordered by green lines, are found caudally in a zone that expresses both *wt1* and *pax2.1*; and duct cells, horizontal lines, develop from cells that express *pax2.1* and *sim1*. (B) Diagram of the mature pronephric kidney (unilateral) indicating the final position of the segment. The midline glomerulus shown in yellow, the tubule drawn in green and the nephric duct diagrammed using horizontal lines. Anterior is at the top of the figure.

Compartmental gene expression

The clustering of cell fates into spatially distinct zones correspond to different patterns of expression of three transcription factors: *wt1*, *pax2.1* and *sim1* (Fig. 7).

In mice, *Wt1* is expressed in the intermediate mesoderm that will form the mesonephros and in the uninduced mesenchyme which gives rise to the metanephros (Armstrong et al., 1993). Targeted inactivation of *Wt1* leads to an absence of the metanephric kidney and gonads as well as a defect in the caudal mesonephric segments (Kreidberg et al., 1993; Sainio et al., 1997). *Pax2* in the mouse is found in the nephric duct and later in induced mesenchyme. Mouse embryos homozygous for a null allele manifest a failure of normal duct growth and epithelialization of the mesenchyme (Torres et al., 1995). Overexpression of *Pax2* causes an abnormal and dysfunctional renal epithelium, similar to that observed in congenital nephrotic syndrome (Dressler et al., 1993). Thus, *Pax2* in the mouse appears to be involved in the generation and

maintenance of the kidney tubular epithelium. *sim1* is expressed in the developing duct of the mouse and chick. Inactivation of the *sim1* locus does not lead to any overt kidney defects (Michaud et al., 1998). However this analysis may be complicated by the presence of a closely related homolog, *sim2*, in that same tissue (Fan et al., 1996). The roles of these genes in the pronephric kidney are not known.

In zebrafish, *wt1* begins as a broad stripe in intermediate mesoderm, from which a cluster appears to break off and migrate medially. The position of this cluster corresponds roughly to that of glomerular progenitors. After formation of the glomerulus, *wt1* expression in the kidney is limited to the podocytes. Whereas it appears that expression of *wt1* alone is indicative of the glomerular fate, co-expression of *pax2.1* with *wt1* seems to preclude the glomerular fate, and rather is associated with the tubular cell fate. Naturally, we examined the effect of interfering with *wt1* function by morpholino antisense oligonucleotides, but found no effect (F. C. S. and M. C. F., unpublished). There is genetic evidence to indicate a direct role for the *pax2.1* gene in control of a cell fate switch. *no isthmus* zebrafish embryos have a mutation in the *pax2.1* gene and lack pronephric tubules. Tissue found in its place has characteristics of podocytes, including expression of vascular endothelial growth factor (VEGF) (Majumdar et al., 2000). Although *pax2.1* also is expressed in the duct-forming cell, it is not required for duct formation as *noi* embryos do form a nephric duct.

The signals that partition the IM into different zones remain to be determined. The fact that there appears to be an antero-posterior organization suggests that axial information might be used. There is precedent for linkage between axial information and organ field patterning. The anterior notochord regulates the posterior extent of the heart field in zebrafish (Goldstein and Fishman, 1998) and Hox gene expression is reflected in the pattern of the limb field in the chick (Tabin, 1995).

We are greatly indebted to Sarah Childs for assistance, discussions and encouragement especially in the early stages of the project and for comments on the manuscript. We also thank John Mably and Iain Drummond for insightful discussions and criticisms. The pax2.1 and pax8 probes were provided by Iain Drummond. Peter Moffett and Jerry Pelletier kindly provided a mouse sim2 probe prior to publication. This work was supported in part by NIH grants RO1HL49579, RO1DK55383 and RO1HL63206 to M. C. F. This work was carried out in partial fulfillment of the requirements for the PhD degree (F. C. S.).

REFERENCES

Armstrong, J. F., Pritchard-Jones, K., Bickmore, W. A., Hastie, N. D. and Bard, J. B. (1993). The expression of the Wilms' tumor 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.

Brennan, H. C., Nijjar, S. and Jones, E. A. (1998). The specification of the pronephric tubules and duct in Xenopus laevis. *Mech Dev* **75**, 127-137.

Brennan, H. C., Nijjar, S. and Jones, E. A. (1999). The specification and growth factor inducibility of the pronephric glomus in Xenopus laevis. *Development* **126**, 5847-5856.

Carroll, T. J. and Vize, P. D. (1996). Wilms' tumor suppressor gene is involved in the development of disparate kidney forms: evidence from expression in the Xenopus pronephros. *Dev. Dyn.* **206**, 131-138.

Copenhaver, W. M. (1926). Experiments on the development of the heart of *Amblystoma punctatum*. *J. Exp. Zool.* **43**, 321-371.

Cornish, J. A. and Etkin, L. D. (1993). The formation of the pronephric duct in Xenopus involves recruitment of posterior cells by migrating pronephric duct cells. *Dev. Biol.* **159**, 338-345.

Crews, S. T. and Fan, C. M. (1999). Remembrance of things PAS: regulation of development by bHLH-PAS proteins. *Curr. Opin. Genet. Dev.* **9**, 580-587.

Cygan, J. A., Johnson, R. L. and McMahon, A. P. (1997). Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants. *Development* **124**, 5021-5032.

de Beer, G. R. (1928). *Vertebrate Zoology*. London: Sidwick & Jackson.

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.

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.

Fales, D. (1935). Experiments on the development of the pronephros of *Amblystoma punctatum*. *J. Exp. Zool.* **72**, 147-173.

Fan, C. M., Kuwana, E., Bulfone, A., Fletcher, C. F., Copeland, N. G., Jenkins, N. A., Crews, S., Martinez, S., Puelles, L., Rubenstein, J. L. et al. (1996). Expression patterns of two murine homologs of Drosophila single-minded suggest possible roles in embryonic patterning and in the pathogenesis of Down syndrome. *Mol. Cell. Neurosci.* **7**, 1-16.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2117.

Fishman, M. C. and Olson, E. N. (1997). Parsing the heart: genetic modules for organ assembly. *Cell* **91**, 153-156.

Fox, H. and Hamilton, L. (1964). Origin of the pronephric duct in Xenopus laevis. *Arch. Biol.* **75**, 245-251.

Goldstein, A. M. and Fishman, M. C. (1998). Notochord regulates cardiac lineage in zebrafish embryos. *Dev. Biol.* **201**, 247-252.

Goodrich, E. S. (1930). *Studies on the Structure and Development of Vertebrates*. London: Macmillan.

Harrison, R. G. (1918). Experiments on the development of the fore limb of amblystoma, a self-differentiating equipotential system. *J. Exp. Zool.* **25**, 413-461.

Herzlinger, D., Koseki, C., Mikawa, T. and al-Awqati, Q. (1992). Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development* **114**, 565-572.

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.

Huxley, J. S. and de Beer, G. R. (1934). The mosaic style of differentiation. In *The Elements of Experimental Embryology* (ed. J. Barcroft and J. T. Saunders), pp. 195-270. London: Cambridge University Press.

Kimmel, C., Ballard, W., Kimmel, S., Ullmann, B. and Schilling, T. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

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.

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.

Majumdar, A., Lun, K., Brand, M. and Drummond, I. A. (2000). Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. *Development* **127**, 2089-2098.

Michaud, J. L., Rosenquist, T., May, N. R. and Fan, C. M. (1998). Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. *Genes Dev.* **12**, 3264-3275.

Murray, P. D. F. (1926). An experimental study of the development of the limbs of the chick. *Proc. Linn. Soc. New South Wales* **51**, 187-263.

Obara-Ishihara, T., Kuhlman, J., Niswander, L. and Herzlinger, D. (1999). The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development* **126**, 1103-1108.

Ohara, O., Dorit, R. and Gilbert, W. (1989). One-sided polymerase chain reaction: the amplification of cDNA. *Proc. Natl. Acad. Sci. USA* **86**, 5673-5677.

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.

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

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.

Poole, T. and Steinberg, M. (1981). Amphibian pronephric duct morphogenesis: segregation, cell rearrangement and directed migration of the *Ambystoma* duct rudiment. *J. Embryol. Exp. Morphol.* **63**, 1-16.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.

Price, J., Turner, D. and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* **84**, 156-160.

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

Raible, D. W. and Eisen, J. S. (1996). Regulative interactions in zebrafish neural crest. *Development* **122**, 501-507.

Sainio, K., Hellstedt, P., Kreidberg, J. A., Saxen, L. and Sariola, H. (1997). Differential regulation of two sets of mesonephric tubules by WT-1. *Development* **124**, 1293-1299.

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

Serbedzija, G. N., Chen, J.-N. and Fishman, M. C. (1998). Regulation in the heart field of zebrafish. *Development* **125**, 1095-1101.

Serluca, F. C. and Fishman, M. C. (1999). Cell lineage tracing in heart development. *Methods Cell Biol.* **59**, 359-365.

Smith, H. W. (1943). *Lectures on the Kidney*. Baltimore: Waverly Press.

Tabin, C. (1995). The initiation of the limb bud: growth factors, Hox genes, and retinoids. *Cell* **80**, 671-674.

Tokura, R. (1925). Entwicklungsmechanische untersuchungen über das hörbläschen und das akustische, sowie faciale ganglion bei den anuren. *Folia Anat. Japon* **3**, 173-208.

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

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

Westerfield, M. (1995). *The Zebrafish Book. A guide for the Laboratory use of Zebrafish* (Brachydanio rerio). Eugene, OR: University of Oregon Press.