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Developmental Biology xx (2006) xxx–xxx
DEVELOPMENTAL BIOLOGY
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FGF is essential for both condensation and mesenchymal–epithelial transition stages of pronephric kidney tubule development

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Received for publication 8 February 2006; revised 10 April 2006; accepted 28 April 2006

Abstract

The pronephros is a transient embryonic kidney that is essential for the survival of aquatic larvae. It is also absolutely critical for adult kidney development, as the pronephric derivative the wolffian duct forms the ductal system of the adult kidney and also triggers the condensation of metanephric mesenchyme into the adult nephrons. While exploring *Xenopus* pronephric patterning, we observed that epidermally delivered hedgehog completely suppresses pronephric kidney tubule development but does not effect development of the pronephric glomus, the equivalent of the mammalian glomerulus or corpuscle. This effect is not mediated by apoptosis. Microarray analysis of microdissected primordia identified FGF8 as one of the potential mediators of hedgehog action. Further investigation demonstrated that SU5402-sensitive FGF signaling plays a critical role in the very earliest stages of pronephric tubule development. Modulation of FGF8 activity using a morpholino has a later effect that blocks condensation of pronephric mesenchyme into the pronephric tubule. Together, these data show that FGF signaling plays a critical role at two stages of embryonic kidney development, one in the condensation of the pronephric primordium from the intermediate mesoderm and a second in the later epithelialization of this mesenchyme into the pronephric nephron. The data also show that in *Xenopus*, development of the glomus/glomerulus can be uncoupled from nephron formation via ectopic hedgehog expression and provides an experimental avenue for investigating glomerulogenesis in the complete absence of tubules.

Keywords: Hedgehog; Induction; Pronephros; Nephrogenesis; Organogenesis; Mesenchymal–epithelial transition; MET

Introduction

Amphibian larvae utilize a simple embryonic kidney called the pronephros up until metamorphosis when it is supplanted by the permanent adult kidney. In essence, each pronephric kidney is a single giant nephron with a large external glomerulus or glomus (Field, 1891; Fox, 1963). In addition to being absolutely essential to the development and function of the adult kidney, the pronephros is also required for survival in amphibian and fish larvae, as bilateral pronephrectomy leads to rapid death due to edema (Howland, 1921). Histologically, the pronephric tubule has many of the same features as a mammalian cortical nephron such as a proximal tubule with a well-developed brush border and a columnar epithelia, followed by a distal tubule with a cuboidal epithelia and a duct with low cylindrical cells (Møbjerg et al., 2000; Vize et al., 2003a). Proximo-distal patterning of the pronephric nephron recapitulates that of the adult kidney nephrons of both amphibians and mammals with a segmented organization and at least six different functional domains specialized in specific ion transport processes (Zhou and Vize, 2004). Similar regulatory genes control development of both pronephroi and complex adult kidneys (Vize et al., 1997; Drummond, 2005) and genetic interactions identified in model systems such as *Xenopus* pronephroi (Carroll and Vize, 1999; McLaughlin et al., 2000) have subsequently been found to hold true in mammalian kidney development (Bouchard et al., 2002; Cheng et al., 2003).

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.04.469

Pronephric development is initiated during gastrulation when inductive interactions between the anterior somites and lateral plate specify the pronephric mesoderm (Seufert et al., 1999; Mauch et al., 2000; James and Schultheiss, 2003). The anterior portion of the pronephros, both tubular and glomerular, is specified in *Xenopus* by Nieuwkoop and Faber (NF) stage 12.5 whereas the more posterior region is specified by stage 14. Once specified, presumptive pronephric tissue can be explanted and grown ex vivo in a basic saline solution and still differentiate into the appropriate kidney structures (Brennan et al., 1998, 1999). At around the time at which specification occurs, Pax8 (XPax-8, Carroll and Vize, 1999) and lim1 (Xlim-1; Taira et al., 1994) gene expression is activated in the presumptive kidney region. A few hours later, WT1 expression initiated in the presumptive pronephric glomus (xWT1, Carroll and Vize, 1996; Drummond et al., 1998; Semba et al., 1996). Some 8–9 h following the activation of early kidney specification genes, pronephric morphogenesis is initiated when the future pronephric nephron mesoderm segregates away from the lateral plate and somites on its borders and begins its posterior migration (Nieuwkoop and Faber, 1994; Vize et al., 2003b). The migrating mesenchymal pronephric duct fuses with the rectal diverticulum, an outgrowth of the cloaca, to complete the tubular portion of the pronephric system. While the duct is still migrating posteriorly, a wave of epithelialization moves along the primordium from anterior to posterior converting the condensate into a hollow tubule (Nieuwkoop and Faber, 1994). Approximately 12 h after the morphogenesis of the tubular portion of the pronephros begins, the external glomerulus of the pronephros, the glomus, pushes inwards into the coelom and is vascularized by the dorsal aorta (Gerth et al., 2005; Nieuwkoop and Faber, 1994). The glomus looks much like an enlarged version of a mammalian glomerulus with a mesangial matrix and well-developed podocytes (Drummond et al., 1998; Kluge and Fischer, 1990). It extends over multiple body segments and filters into the coelom rather than into a Bowman's capsule. From the coelom, the primary filtrate is swept into the proximal portion of the pronephric tubules through thin ciliated funnels known as nephrostomes (Vize et al., 1997, 2003b).

The above-described series of developmental and genetic process is largely recapitulated during mammalian nephron condensation. Rather than forming from the multipotential intermediate mesoderm as pronephroi do, mammalian nephrons condense from the metanephric blastema, a determined but undifferentiated pool of mesenchymal cells awaiting a passive inductive signal from the ureteric bud (Carroll and McMahon, 2003). Despite this potentially important difference, similar genes are associated with, and required for, both processes. As in pronephroi, key players include lim1, notch, Pax2, Pax8, wnt4, and WT1 (Carroll and McMahon, 2003; Jones, 2003).

In this report, the *Xenopus* system was used to explore the role of hedgehog signaling in pronephric patterning. The results indicate that, as previously proposed (Majumdar and Drummond, 1999), hedgehog (hh) itself plays no role in the establishment of the early kidney primordia. However, hh does possess a striking ectopic activity manifested on the developing kidney. When supplied to the pronephros from the overlying epidermis, hh completely suppresses pronephric tubule development while leaving glomeral development unaffected. This hh activity appears to function by ectopic regulation of essential kidney genes and can therefore be used to identify such genes. Microarray analysis of hh transcriptional targets indicates that suppression of FGF8 may contribute to the defects in pronephric primordium development. An essential role for FGF was confirmed through a combination of FGF signaling inhibitor studies and morpholino-mediated inhibition of FGF8 translation. FGF signaling is essential for maintenance of lim1, Pax8, and vHNF1 transcription and condensation of the pronephric primordium. FGF8 also functions during the mesenchymal to epithelial transition that converts the pronephric primordium into the epithelial tubules of the pronephric nephron and the pronephric/wolffian duct. Together these data demonstrate a critical requirement for FGF in pronephrogenesis and show that FGF8-independent glomerular development can occur in the complete absence of pronephric tubules.

Materials and methods

Nomenclature

The standard *Xenopus* nomenclature adopted by the NCBI for frog genes is utilized rather than the original gene names in order to maximize compatibility with data available from other model systems. Where possible *Xenopus* names are the same as the human ortholog.

Embryos, microsurgery, and in situ analysis

Xenopus laevis embryos were generated via standard techniques (Sive et al., 2000). Ectopic hh was supplied to the developing kidney by microinjecting the animal pole of 2-cell stage embryos with 100 pg of banded hedgehog mRNA (Ekker et al., 1995), either in one or both blastomeres.

In situ hybridization protocols for colorimetic staining were derived from Sive et al. (2000). Fluorescent in situs were performed according to Zhou and Vize (2004) and Gerth et al. (2005). Whole-mount TUNEL assays were performed on bleached embryos following the staining protocols of Hensey and Gautier (1998) and Veenstra et al. (1998).

Zebrafish embryos were obtained through timed matings of heterozygous carriers of the smu^{b641} allele of slow muscle omitted/smoothened. This allele changes a glycine to an arginine in the second transmembrane domain of the Smoothened protein (Varga et al., 2001). Embryos were raised at 28°C and staged according to Kimmel et al. (1995) before being fixed overnight at 4°C in 4% paraformaldehyde in PBS. RNA in situ hybridization was performed following Thisse et al. (1993). Digoxigenin or fluorescein-labeled antisense RNA probes were generated for pax2.1 and wt1 (Drummond et al., 1998) and detected using anti-digoxigenin or anti-fluorescein antibodies conjugated to alkaline phosphatase (Roche), followed by incubation with BM purple (Roche) or iodonitrotetrazolium chloride (INT; Sigma) and 5-bromo 4-choro 3-indolyl phosphate (Roche).

Morphant

A morpholino, FGF8-MO, 5′ CCAGGATGGAGGTGATGTAGTTCAT 3′ was generated (GeneTools) targeting both *X. laevis* FGF8A and FGF8B variants, based on a previously published reagent (FGF8-AS) that has been demonstrated to effectively block translation of both these mRNAs (Park et al., 2004). One presumptive pronephric blastomere, V2.2 (Moody, 1987), was injected with 14 ng of morpholino resuspended in 0.1× MR (Sive et al., 2000). FITC-dextran (FLDx; 70 kDa) was coinjected with the morpholino to trace injected blastomeres. When necessary, the tracer was detected using anti-FITC-POD (Roche) and either a red peroxidase substrate (Vector Laboratories) or a

FITC-tyramide (Zhou and Vize, 2004). The control morpholino was an unrelated sequence, GACAAACAACACAGGCACCATA.

Microarray analysis

Pronephric region explants were prepared from stage 24 embryos using a gastromaster (Xenotek) with the tip set to a width of 70 μm. Rectangular explants, approximately 70 μm (dorsal to ventral) × 100 μm (anterior-posterior) and 70 μm (laterally) were cut from the flanks of both sides of embryos and stored in Trizol (Gibco) until sufficient numbers were gathered. Two hundred forty-one explants were removed from control pronephroi and another 241 from the equivalent region in embryos previously bilaterally injected with bhh mRNA as described above. Total RNA was prepared according the manufacturers recommendations. Biotinylated cRNA was prepared using an RNA labeling kit (Ambion). A single round of amplification was performed starting with 1 μg of total RNA according to the manufacturer's instructions. Three replicates each were prepared from the pooled treated and untreated samples to yield six reactions. Fifteen micrograms of each reaction was fragmented using RNA fragmentation reagent (Ambion) and submitted to the University of Florida ICBR Gene Expression Core facility for hybridization and scanning on the Affymetrix GeneChip *X. laevis* genome array according the manufacturers recommendations.

Results

Ectopic expression of banded hedgehog effectively suppresses pronephric nephron development but not glomeral development

Patched-type hedgehog receptors can be activated by multiple hedgehog ligands (Lewis et al., 1999) and are broadly expressed in the early mesoderm (Concordet et al., 1996; Lewis et al., 1999). As the banded hedgehog (bhh) clones available (Ekker et al., 1995) were vastly better at generating mRNA than alternatives, bhh was used in all ectopic hh experiments and is referred to in the remainder of this report simply as "hh". Hh was provided to the developing pronephros by injecting hh mRNA into the animal pole of two- or four-cell stage *Xenopus* embryos. Such injections result in the segregation of the injected molecules into the ectoderm of the embryo, as injected mRNAs do not readily diffuse in the cytoplasm (Kintner, 1988; Vize et al., 1991). Supplying mRNA to the overlying ectoderm is a useful approach with mRNAs encoding secreted factors to avoid gastrulation defects that sometimes occur in marginal zone injected embryos. Such generic defects typically cause kinking of embryos or incomplete closure of the blastopore and make it impossible to establish function in the developing kidney (Vize et al., 1991; Grammer et al., 2000). Embryos injected with 100 pg hh mRNA in the animal pole appear to be normal and display no abnormal external characteristics up until at least stage 40. Embryos injected into only one cell of a two-cell embryo continue to look superficially normal, but embryos injected into both blastomeres begin to become severely edematous at late tadpole stages (Fig. 1). This edema continues to worsen and the tadpoles swell into distended balloon-like shapes and die. In such transparent, late stage embryos, it is clear by visual inspection that there are no pronephric tubules present, thus explaining the edema (Howland, 1921). As tadpoles only require one functional pronephros to control water balance (Howland, 1921), the normal phenotype in unilaterally injected embryos is expected. The penetrance of the phenotype is extremely strong, with approximately 90% of embryos showing complete kidney tubule suppression in otherwise normal tadpoles in > 10 independent experiments. The effect is dose dependent, with lower mRNA amounts generating the loss of kidney phenotype at lower frequencies. Doses as small as 20 pg can still block kidney development in over 85% of injected embryos (Table 1).

To explore what parts of the kidney are absent in response to ectodermal expression of hh, mRNA was injected in the animal pole of one cell of two-cell stage embryos, which were raised to various stages and analyzed by histology, in situ hybridization, and immunohistochemistry (Fig. 1). Unilaterally injected embryos form a completely normal kidney on the uninjected side. On the injected side, however, there are no tubular portions of the kidney present, including proximal and distal tubules or the pronephric duct. In its place, the pronephric mesoderm forms a thin mesothelial-like layer below the skin (Fig. 1). We have not yet identified what this tissue has transfated to, but this is to some extent addressed by the microarray data presented below. The glomus is normal on both sides as assayed by histology (Fig. 1) and by in situ hybridization with crescent (Shibata et al., 2000) and nephrin (Gerth et al., 2005; Fig. 2). In order to determine at what point in pronephrogenesis hh was acting, a series of in situs were performed with different marker genes (Fig. 2). Unilaterally injected embryos show mild phenotypes as early as NF stage 17/18. Exposure to hh at this stage generates a slightly more diffuse staining pattern of lim1 and Pax8 in the intermediate mesoderm. Pronephric expression of all tested primordium markers is completely suppressed by stage 21/22. Interestingly, expression of some genes, for example, Pax8 and wnt4, is not effected in non-kidney tissues on the injected side. This is particularly obvious in regard to Pax8 expression in the otic vesicle, which remains robust at stage 22, despite complete suppression in the pronephros (Fig. 2).

The suppression of pronephric tubule development by hh is not caused through apoptosis of the nephric primordium. This was determined by whole-mount TUNEL staining of embryos that had been unilaterally injected with hh mRNA plus FLDx (Fig. 3). The frequency of TUNEL-positive nuclei was very low in both control and hh-injected embryos and no increase in apoptotic rates in the pronephric region was observed in hh injected embryos examined at a variety of developmental stages from stage 21 (n = 26) through to 30 (n = 12).

Hedgehog is not required for the normal establishment of tubule or glomeral primordia

In order to test whether the tubule suppression phenotype reflected an ectopic hedgehog activity or a role in normal patterning of the pronephros, for instance in promoting glomeral development over tubular development, the importance of hh signaling in zebrafish pronephrogenesis was examined. The role of hh in pronephric patterning has previously been explored to some extent in zebrafish using sonic you (*syu*) sonic hedgehog mutants (Majumdar and

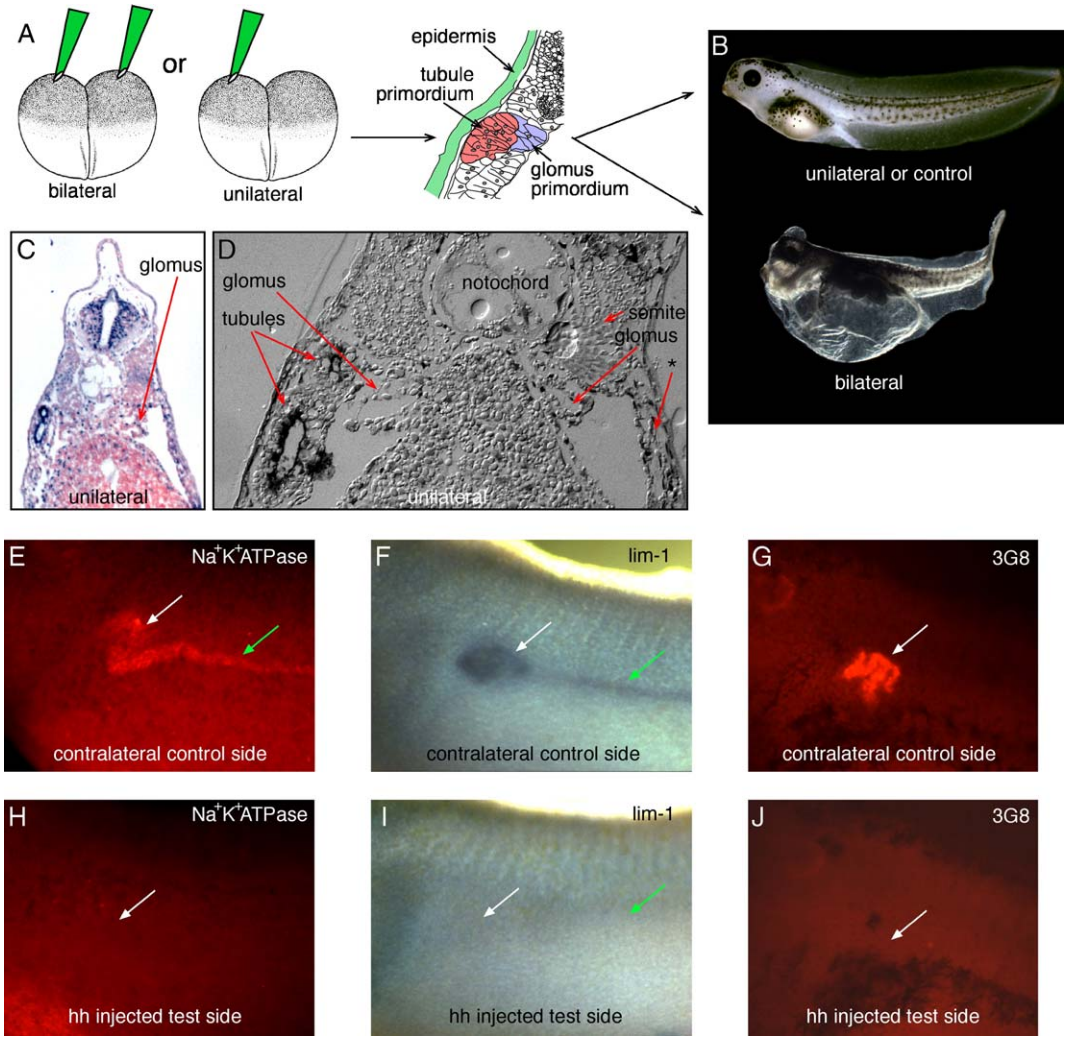

Fig. 1. Ectopic hh suppresses pronephric tubule and duct development but not glomeral development in *Xenopus* embryos. (A) Ectopic hh mRNA was delivered to the animal pole of either one (unilateral) or two (bilateral) cells at the two-cell stage. This results in the mRNA being present in the epidermis overlying the pronephric primordium, not the primordium itself. (B) Embryos injected unilaterally appear superficially normal at stage 42/43. Tadpoles that develop from bilaterally injected embryos lack all pronephric tubules and die from edema. (C, H, and E) Counterstained transverse section through 3G8 stained stage 38/39 embryo. 3G8 stains the apical surface of proximal pronephric tubules on the left. No tubules are present on the hh-injected side (right). A normal glomus is present on both sides of the injected embryo. (D) Transverse section through unilaterally injected embryo. As in C, the uninjected side has both tubules and glomus, but the injected side only has glomeral tissue. In place of tubules, a flat layer of mesoderm is observed (*). (E) Left side (uninjected) of hh unilaterally injected embryo probed with Na^+K^+ ATPase. Proximal tubules (white arrow), distal tubule, and duct (green arrow) are present. (F) As for panel E, except the probe is lim-1. (G) As for panel E, except staining is immunofluorescence with 3G8. (H) The opposite (right) side of the same embryo as shown in panel E. Note the complete absence of all pronephric tubules. (I) The opposite (right) side of the same embryo as shown in panel F. Note the complete absence of all lim-1-positive tissues. (J) The opposite (right) side of the same embryo as shown in panel G. No 3G8-positive tubules are present.

Drummond, 1999). However, as redundant hedgehogs and patched genes exist in zebrafish, and patched receptors can be activated by multiple hh ligands (Lewis et al., 1999), it is possible that not all hh signaling activity is lost in the *syu* mutant. The mutation slow muscle omitted (*smu*) is a loss-of-function allele of smoothened, an essential mediator of all zygotic hedgehog signaling (Barresi et al., 2000; Varga et al., 2001). To determine if hh plays a role in early pronephric development, for instance in determining whether pronephric cells form tubule versus glomus, *smu* mutant embryos were assayed for expression of wt1 and pax2.1, markers of the glomus and tubules, respectively (Drummond et al., 1998). Loss of all zygotic hh signaling in *smu* mutants did not block pronephric patterning and both pronephric glomus and pronephric tubule primordia are readily visible in 26 hpf embryos (Fig. 4). As previously reported for *syu*/sonic hedgehog mutants, in *smu*/smoothened mutants glomeral morphogenesis is abnormal and glomera fail to migrate to the midline and fuse as they do in normal zebrafish pronephric development (Fig. 4). Whereas it is possible that maternal smoothened still provides some signaling in *smu* mutants (Varga et al., 2001), the expression of pax2.1 in the pronephric tubules in both *smu* and *syu* mutants indicates that hh is very likely not required for early specification of the pronephric primordium or its segregation from the intermediate mesoderm (Fig. 4; Majumdar and Drummond, 1999).

Table 1
Ectodermal expression of hh blocks pronephric tubule and duct development

	hh mRNA	No kidney	Total *N*	%
FLDx injected	0	0	24	0
FLDx injected	0	0	15	0
Experiment 1	100 pg	13	13	100
Experiment 2	100 pg	8	14	57
Experiment 3	20 pg	16	18	89
Experiment 4	20 pg	35	40	87
Experiment 5	20 pg	18	20	90
Experiment 6	4 pg	21	30	70
Experiment 7	4 pg	11	47	23

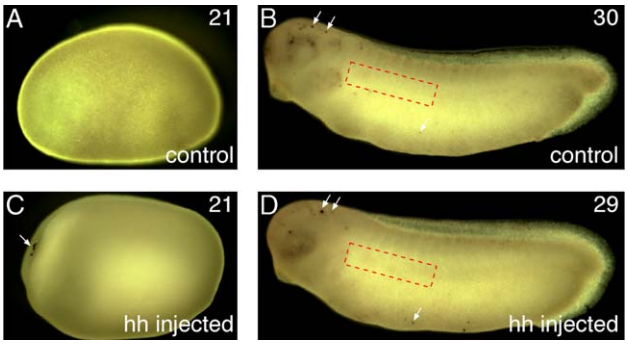

Fig. 3. The hh block to tubule development is not mediated by apoptosis. Embryos unilaterally injected with 100 pg of hh mRNA at the two-cell stage were grown to stage 21/22 or 28–30, fixed and processed for TUNEL staining in whole mount. Random apoptotic nuclei are detected, as expected (for examples, see white arrows). The pronephric region does not show any TUNEL-positive cells (red boxes) at any stage examined. All embryos are oriented with anterior to the left, and dorsal up.

Hedgehog targets

The data presented above indicate that the pronephric hh phenotype (Fig. 1) is most likely an ectopic effect. In order to identify potential hh targets and mediators of normal pronephric patterning and morphogenesis, a microarray approach was used

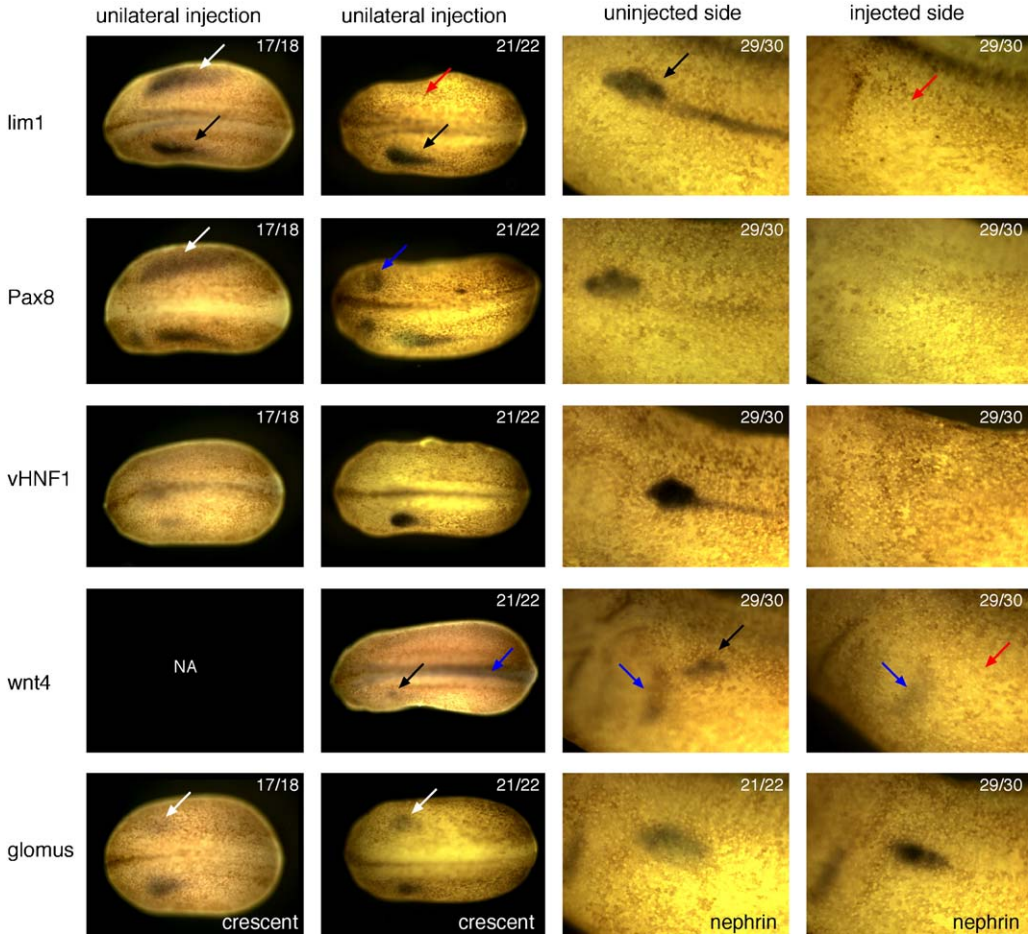

Fig. 2. Hedgehog target genes. Embryonic stage is indicated in the top right of each panel. Unilaterally injected embryos (the injected half of the embryo, identified via fluorescent lineage tracer, is oriented towards the top in all dorsal views) were fixed and processed at stages 17/18 (left), stage 21/22 (center), or stage 29/30 (right). Ectopic ectodermal hh has only a modest effect on the pronephros at stage 17/18, but by stage 21/22 expression of all markers of the pronephric tubule primordium are extinguished. Marker gene expression in other tissues is not affected, despite equivalent exposure to the hh (blue arrows). Black arrows indicate normal pronephric staining, whereas white arrows indicate abnormal and red arrows absent pronephric staining. The white arrow in the glomus at stage 21/22 indicates a more diffuse but still visible crescent staining pattern. wnt4 is only transcribed at extremely low levels in the pronephros at stage 16/17 and so is not shown. All views of stage 17/18 and 21/22 embryos are dorsal with anterior to the left. All stage 29/30 views are lateral, with anterior to the left and dorsal up. The glomus was detected using crescent at stage 17/18 and 21/22, and nephrin at stage 29/30.

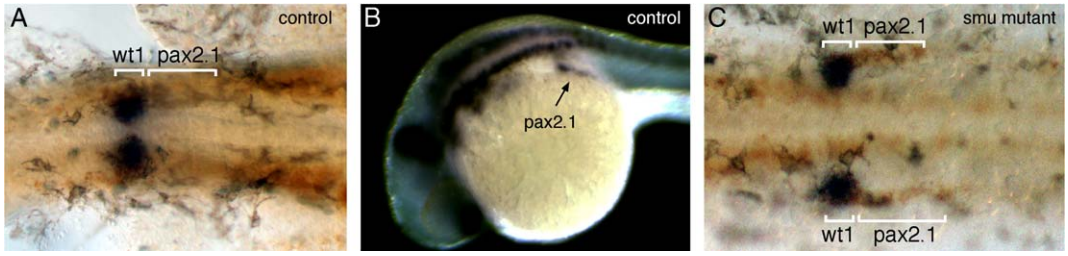

Fig. 4. Hedgehog is not required for establishment of tubule or glomeral primordia in the zebrafish. 26 hpf wild-type (panels A and B) and *smu* mutant (panel C) zebrafish embryos stained with pax2.1 (brown) and wt1 (blue) probes (A and C) or pax2.1 only (panel B). Although glomera fail to migrate to the midline in *smu* mutants, the primordia for both glomus and tubules are clearly present. In panel A pax2.1 expression in the overlying neural tube makes the pronephric expression difficult to visualize from the dorsal view shown in panel A, but it is clearly visible, and of similar size to that in *smu* mutants, in control panel B.

to analyze transcriptional responses to ectopic hh in developing pronephroi.

Either control or bilaterally animal pole hh injected embryos (100 pg/injection) were raised to stage 24 then the presumptive pronephric region excised via microsurgery. This stage corresponds to the point at which the pronephric primordium is just beginning to thicken and separate from the flank of the embryo (Hausen and Riebesell, 1991; Vize et al., 1997) as this seemed an appropriate time to look for changes in gene expression that are causal to the block to kidney development. RNA was prepared from 241 hh treated and 241 control pronephroi. Labeled RNA from each pooled sample (hh-treated pool and control pool) was then used to probe 14,400 transcript Affymetrix *X. laevis* gene array chips in triplicate. Analysis of responses was performed using BRB ArrayTools developed by Richard Simon and Amy Peng (http://www.linus.nci.nih.gov/BRB-ArrayTools.html). To identify differentially regulated genes, we performed significance analysis of microarrays (SAM) (Tusher et al., 2001). The target proportion of false discoveries was set to 0.1 and 100 permutations were performed. Hierarchical clustering was performed using centered correlation and average linkage.

There were 462 genes that were different between samples via SAM (Fig. 5, Table 2, and Supplemental data). SAM performs a false discovery rate-based statistical test to identify genes differentially expressed in a microarray experiment. The greatest difference (79-fold) was, not surprisingly, the injected mRNA itself (banded hedgehog). Of the strongest upregulated genes, hedgehog interacting protein (HIP, 3.5-fold) and Patched-2 (3.4-fold) stand out as known hh feedback regulators (Chuang et al., 2003). A number of transcription factors involved in cell specification are also activated, including NK3 (3.5-fold), FoxF1, -D1, and -D2 (2.1- to 2.4-fold), twist, and GATA5. Upregulated growth factors include wnt8, wnt11, nodal-related BMP1 and BMP2, and the growth factor antagonists WIF-1 and dapper-1. Downregulated growth factors included wnt4, FGF8, and wnt11R and growth factor antagonists, dickkopf-1, follistatin, frzb-1, and gremlin. Notch pathway members Hey1/HRT1 and serrate-1 were also both downregulated. A number of genes associated with mesenchymal to epithelial transitions and epithelial to mesenchymal transitions are also differentially regulated including Cdc42, crumbs, fibronectin, par-6, slug, snail, twist, and ZO2. Full details on all regulated genes are available in the supplemental material and all array data have been submitted to GEO (GSE3712, sample numbers GSM85709 through GSM85714). Regulation of multiple hh targets was confirmed using QPCR on unamplified explant RNA. QPCR-validated upregulated targets include HIP, WIF-1, Nk3, Xwnt-8, and SPR2, and downregulated targets wnt4, POU2, Na^+K^+ ATPase, gremlin, ESR, HESR1, AP-2, FGF8, Dkk1, and serrate (Supplemental data). All genes tested by QPCR (for which the assay worked) confirmed microarray identified regulation without exception.

Validation of the microarray data is also provided by the in situ analysis shown in Fig. 2. All of the genes shown to respond to hh in the in situ analysis were also identified by the microarray. Furthermore, when the array contained multiple spots representing regulated genes, all of the spots showed similar patterns of regulation. Examples include patched-2 (3.4×, 3.19×, 2.78×), HIP (3.45×, 2.0×), BMP2 (1.52×, 1.40×), POU2 (0.15×, 0.35×), and Pax2 (0.60×, 0.62×) (Supplemental data).

Many of the genes regulated by hh may play a role in the observed pronephric tubule phenotype. Some of these genes may, by themselves, play important roles in pronephric development. Downregulation of a growth factor that is essential to pronephric development is one possible mode of action of ectopic hh on tubulogenesis. As the entire pronephric tubular system is lost in response to ectopic hh, and wnt4 is only expressed (Carroll et al., 1999) and required (Saulnier et al., 2002) in the anterior portion of the pronephros, we chose to first examine the role of the microarray downregulated factor FGF8 (0.66× microarray, 0.4× QPCR) as one of the potential mediators of hh action. In *Xenopus*, this gene is expressed transiently in the forming pronephric primordium from stages 20 to 33 (see http://www.xenbase.org/index/kidney/fgf8/fgf8.html), at which point pronephric expression is extinguished (Christen and Slack, 1997; Hayashi et al., 2004). FGF8 is also transcribed in other tissues adjacent to the developing pronephros (Christen and Slack, 1997; Hayashi et al., 2004; Park et al., 2004).

Role of FGFs in pronephric development

The pharmaceutical SU5402 blocks the FGF receptor (FGFR) directly by binding in the ATP pocket (Mohammadi et al., 1997). The importance of FGFs in pronephric development was assayed by testing the effect of SU5402 in various time windows. Pronephric specification occurs around

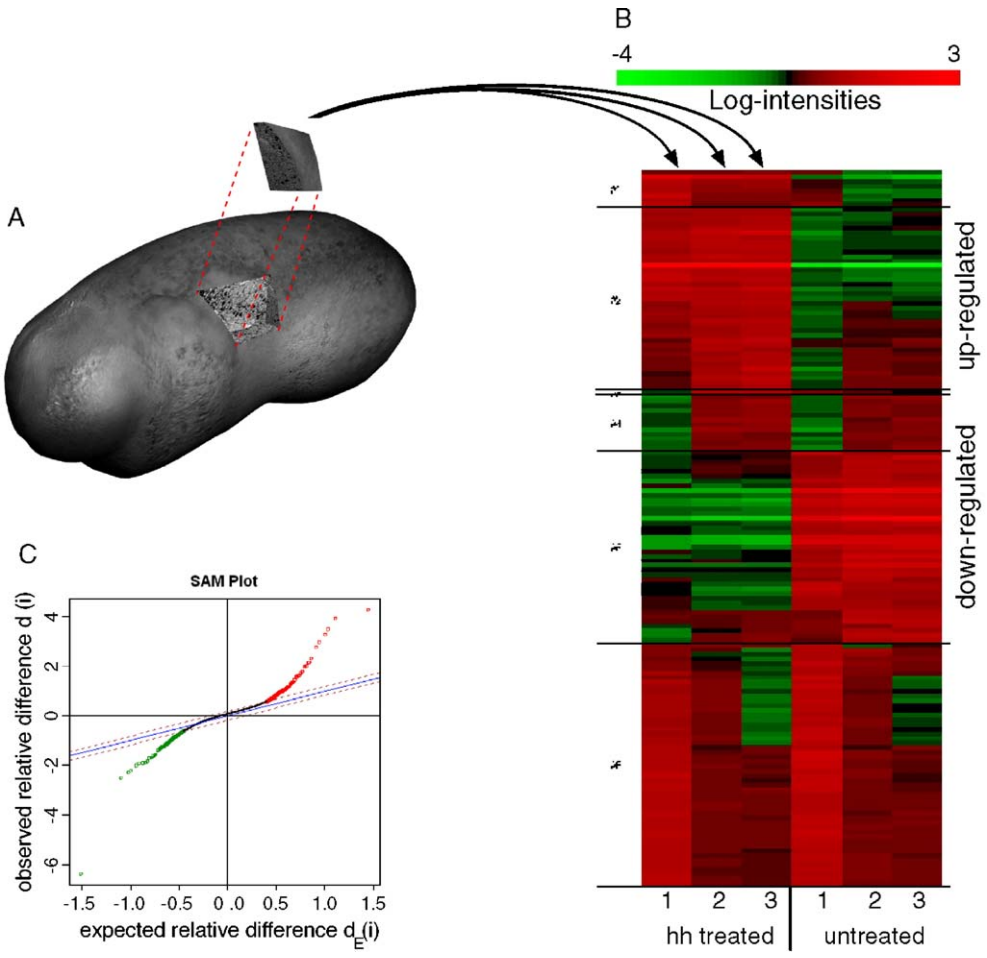

Fig. 5. Microarray analysis of hh response in the pronephric region at stage 24. (A) Schematic of microsurgical removal of the pronephric primordium. (B) RNA prepared from pooled explants was used to probe microarrays. The response of the 75 most highly regulated genes is shown. (C) Scatter plot illustrating the distribution of significantly regulated genes according to a SAM analysis. All data have been submitted to GEO, series GSE3712, sample numbers GSM85709 through GSM85714.

stage 12/13, but pronephric morphogenesis does not begin until stage 21/22. Inhibitor (50 μM) was added to growing embryos and maintained from stages 12.5 to 15 (Table 3). As previously reported, application of SU5402 during gastrulation and axis extension caused defects in both processes and abnormal embryos (Sivak et al., 2005). By adding SU5402 at stage 15, the effects on gastrulation are avoided but tailbud extension remains effected. This time window covers pronephric morphogenesis but not early pronephric specification (Brennan et al., 1998, 1999).

SU5402 treatment blocks all pronephric development when added at either stage 12.5 or stage 15 (Fig. 6, Table 3). In treated stage 36/37 embryos stained with both Na^+K^+ ATPase and NKCC2, there are no pronephric tubules or duct present. As FGF may be a critical target of ectopic hh, and ectopic hh blocks pronephric primordium development by stage 21 without blocking pronephric glomus development (Figs. 1 and 2), we next determined if the same was true for SU5402 treated embryos. As Fig. 7 demonstrates, lim1 expression in the pronephric primordium is effectively blocked by SU5402 treatment from stage 15 to stage 21/22, the same window in which ectopic hh acts. One critical window for FGF function is therefore between stages 15 and 21/22, a period of only 5–6 h in length. SU5402 caused a change in shape in the developing glomus but did not block expression of the podocyte marker, nephrin, in this structure (Fig. 7). The glomus is a flat linear shape in SU5402-treated embryos, as opposed to the rounded structure observed in controls. This may reflect a defect in vascularization. The effects of SU5402 treatment are therefore very similar to ectopic ectodermal hh expression and preferentially impacts the epithelial tubules of the pronephros. As edema in hh-injected embryos is not manifested until much later stages of development (Fig. 1), it is not surprising that SU5402 treated tadpoles are not distended at these stages.

The MEK inhibitor U0126 also inhibits pronephric tubule development when applied at a concentration of 100 μM from stage 14/15 onwards but is more variable than is SU5402. At this concentration, 5/18 stage 34 embryos had no pronephric tubules positive for Na^+K^+ ATPase expression and 8/18 had much fainter than normal pronephric staining (Fig. 8). Similar results were obtained at stage 39/40. Treatment with 150 μM U0126 is lethal, whereas treatment with 50 μM generates the faint Na^+K^+ ATPase expression phenotype (Fig. 8G), but no embryos completely lacking pronephric tubules. The inhibition

Table 2
Representative up- and downregulated genes (also see Supplemental data for complete list) from microarray and QPCR analysis of hh-treated pronephric explants

Gene	Fold change		Gene	Fold change	
	Microarray	QPCR		Microarray	QPCR
α-Tubulin	6.2×		g-Na^+K^+ ATPase	0.06×	0.03×
NK3	3.5×	3.6×	Xlim1/lim1	0.1×	
HIP	3.5×	2.6×	vHNF-1/TCF2	0.13×	
Patched-2	3.4×		POU2	0.15×	0.03×
FoxF1	2.4×		AP-2 beta	0.25×	0.05×
P450 CYP17	2.3×		ARNT2	0.32×	
FoxD2	2.1×		Pax3	0.33×	
FoxD1	2.1×		aldehyde dehydrog. 1	0.36×	
PCD 8	2.1×		Xmyf-5	0.39×	
VITO	2.1×		follistatin	0.43×	
Odd skipped related 2A	2.0×		enolase 3	0.43×	
Twist	2.0×		XlFli	0.45×	
Xwnt-8	2.0×	3.2×	enhancer of split related	0.53×	0.5×
GATA5	1.9×		XDCoH	0.55×	
wnt inhibitor WIF-1	1.8×	1.3×	dickkopf-1	0.56×	0.5×
eHAND1	1.8×		gremlin	0.57×	0.15×
MyoD	1.7×		Xwnt-4/wnt4	0.59×	0.12×
N-myc	1.7×		zona occludens 2	0.60×	
par-6	1.6×		Pax2	0.60×	
Nodal-related 1	1.6×		Egr1	0.60×	
BMP-2	1.5×		HNF4	0.61×	
Snail	1.5×		cold shock protein A	0.62×	
Fibronectin	1.5×		FGF8B	0.66×	0.4×

of MEK signaling by U0126 resulting in a block to kidney development supports a role for the FGFR tyrosine kinase in pronephric morphogenesis.

SU5402 treatment does not identify which FGF is acting on kidney development but does show that FGF signaling is essential for pronephrogenesis. It is also possible that SU5402/U0126 are acting indirectly and the effect on kidney development is downstream of an effect on other tissues, for example somites, which are critical for normal pronephric development (Seufert et al., 1999; Mauch et al., 2000; James and Schultheiss, 2003). However, somites appear to be anatomically normal in treated embryos (Fig. 6F, and data not shown). The SU5402-mediated block to formation of the pronephric mesenchyme is not caused by apoptosis, as whole-mount TUNEL staining of SU5402-treated (stage 15–22, $n = 55$) and control ($n = 57$) embryos did not detect any increase in TUNEL-positive cells in the pronephros (data not shown).

As FGF8 is downregulated in response to ectopic hh (Table 2), and FGF signals are essential for kidney development (Figs. 6–8), we next tested whether FGF8 is a candidate for the key kidney FGF.

FGF8 morphant

A morpholino that can effectively block translation of both FGF8A and FGF8B mRNAs in *X. laevis* has been characterized by Park et al. (2004). This reagent was used to block FGF8 translation in the developing pronephros. Presumptive kidney blastomeres V2.2 or C3 (see http://www.xenbase.org/atlas/xenbasefate.html) were microinjected with the FGF8-MO (14 ng/embryo plus 14 ng FLDx), embryos were raised to different stages, then fixed and processed by in situ hybridization or immunohistochemistry.

Inhibition of FGF8 translation had less severe effects on pronephric tubule development than did treatment with SU5402. This may reflect the action of other critical FGFs in kidney development that are inhibited by SU5402, or may indicate that the morpholino modulates rather than ablates FGF8 production. Also, unlike the situation when embryos are bathed in a solution of SU5402, due to embryo to embryo variations in cell lineage, every microinjected embryo is unique with respect to the region in which FGF8 translation is inhibited. For this reason, only embryos in which the coinjected lineage tracer was distributed within the pronephric region were scored for phenotypic effects and included in the data presented in Table 4 and Fig. 9, which illustrate the morpholino phenotype. The morphants form a normally sized mesenchymal pronephric primordia, but the primordium fails to undergo a normal epithelial transition and to polarize into a pronephric nephron. The transition from a mesenchymal condensate to an epithelialized structure with a consistent 3D form normally begins in the anterior portion of the primordium at around stage 28, a time at which the duct is still a migrating mesenchyme. In controls, three knuckle-like protrusions form on the dorsal aspect of the epithelializing nephron, which will go on to form the dorsal branches that link the nephron to the coelom via the nephrostomes (Fig. 9). However, in FGF8-MO-injected embryos, the pronephric primordium fails to undergo the normal epithelial transition, the associated organ shape change, and the anterior restriction of Pax8 expression. The pronephric mesoderm remains as a mass of Pax8-positive mesenchyme directly ventral to the somites on the injected side of the embryo, whereas the primordium on the control epithelializes (Fig. 9M). The stage 30 FGF-MO injected pronephroi (Figs. 9E and H) continue to look like the uncondensed pronephric mesenchyme of control stage 25 embryos (e.g., compare to Fig. 9A). These effects were noted in three independent experiments scored at stage 30/31 via Pax8 expression, in 3G8-stained samples at stages 33 and 36/37, and Na^+K^+ ATPase expression at stage 36 (Table 4, Fig. 10).

The 3G8 antibody stains only the apical surface of the pronephric proximal tubule lumen and can be used as a stringent marker of epithelialization (Vize et al., 1995). The protein

Table 3
SU5402 blocks pronephric development assayed by Na^+K^+ ATPase (pan-pronephric) and NKCC2 (distal segment specific) double fluorescent in situ hybridization at stage 36/37

No. of kidneys	Pan-pronephric			Distal segment			*n*
	Two	One	None	Two	One	None	
Controls	12	0	0	12	0	0	12
Stage 12.5	0	0	24	0	0	24	24
Stage 15	0	0	45	0	0	45	45

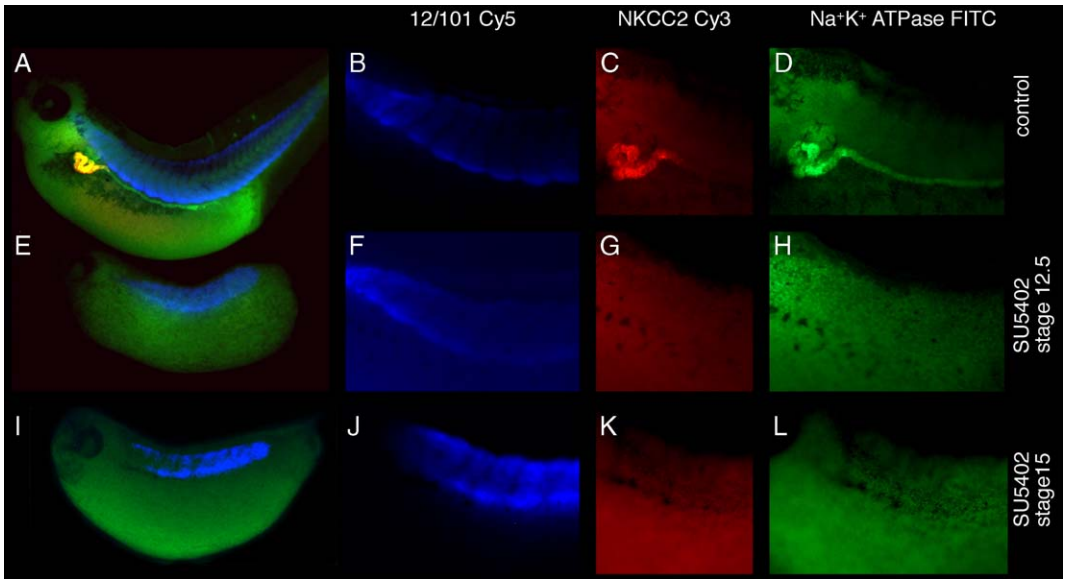

Fig. 6. SU5402 blocks pronephric development. (A) Stage 36 control embryo triple labeled for somites with 12/101 (B), the pronephric distal segment with NKCC2 (C) and pronephric tubules and duct with Na^+K^+ ATPase (D). (E–H) Embryo treated with 50 μM SU5402 from stage 12.5 to stage 36. (I–L) Embryos treated with SU5402 from stage 15 to 36 and processes as described above. No pronephric tubules are present in treated embryos.

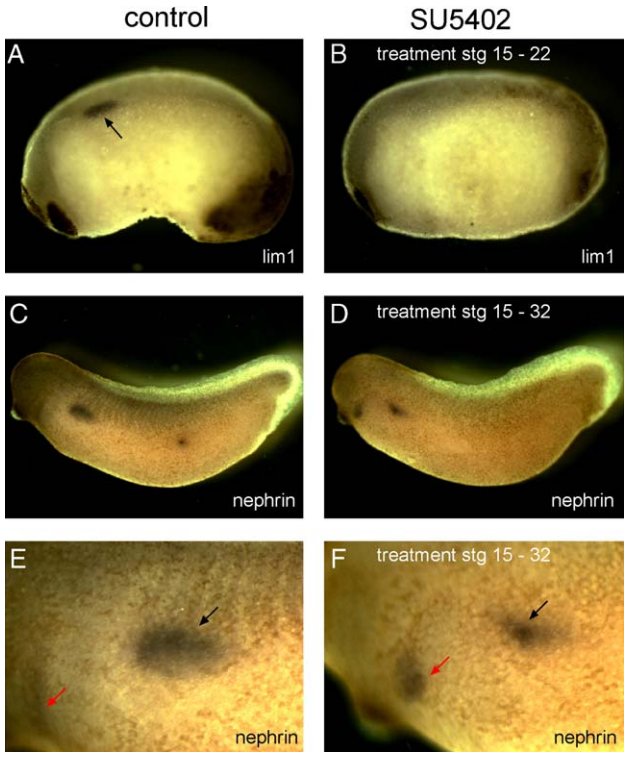

Fig. 7. SU5402 treatment blocks pronephric primordium development by stage 22 but does not block glomeral development. (A, B) Control and SU5402 treated (from stage 15 to 22) embryos stained with lim1. SU5402 treatment has completely blocked lim1 expression in the pronephros. (C–F) Nephrin expression in controls and embryos treated with 50 μM SU5402 from stage 15 to 32. The glomus is abnormally shaped but is still present, as it is in embryos injected with hh. A second, previously unrecorded site of nephrin expression was observed in the branchial arches. This expression is also retained in SU5402-treated embryos but is in an abnormal position indicating an alteration in neural crest migration (red arrows). Panels E and F are enlarged views of the same embryos as shown in panels C and D.

encoded by the alpha subunit of Na^+K^+ ATPase is localized to the basolateral surface of pronephric epithelia and is expressed from stage 31 onwards, as the primordium epithelializes (Zhou and Vize, 2004). As with 3G8 immunoreactivity, Na^+K^+ ATPase expression is not activated in FGF8-MO-injected embryos in the pronephric primodium at any of the stages tested, up until stage 36, and even at this late stage the MO-injected primordia remain Pax8-positive mesenchymal masses (Fig. 10). In control morpholino experiments, abnormal pronephroi were only rarely observed and when defects were present (2/30 embryos) they were very mild (Table 4). mRNA rescue of the morpholino has not been possible due to the multiple developmental effects generated by ectopic FGF8 (e.g., see Hardcastle et al., 2000).

The block to pronephric epithelialization is observed when the morpholino is present in the pronephros but not present in the adjacent somites (Figs. 9F and I). When a portion of the pronephros contained the FGF8-MO and tracer, that portion failed to epithelialize whereas the portion that did not contain the injected morpholino formed a small amount of epithelium (Figs. 9I and K). These data imply that FGF8 acts in a short-range manner, and within the pronephros itself, not from adjacent tissues.

A 3D confocal stack illustrating inhibition of expression of the epithelial polarity marker 3G8 in the kidney by FGF8-MO is depicted in an animation available at http://www.xenbase.org/lab/movies/sample1_37-39.mpg.

Discussion

The adult kidney, or metanephros, grows via a reiterated series of mesenchymal to epithelial transitions as nephrons condense from the metanephric mesenchyme. This process begins when the ureteric bud first contacts the mesenchyme at

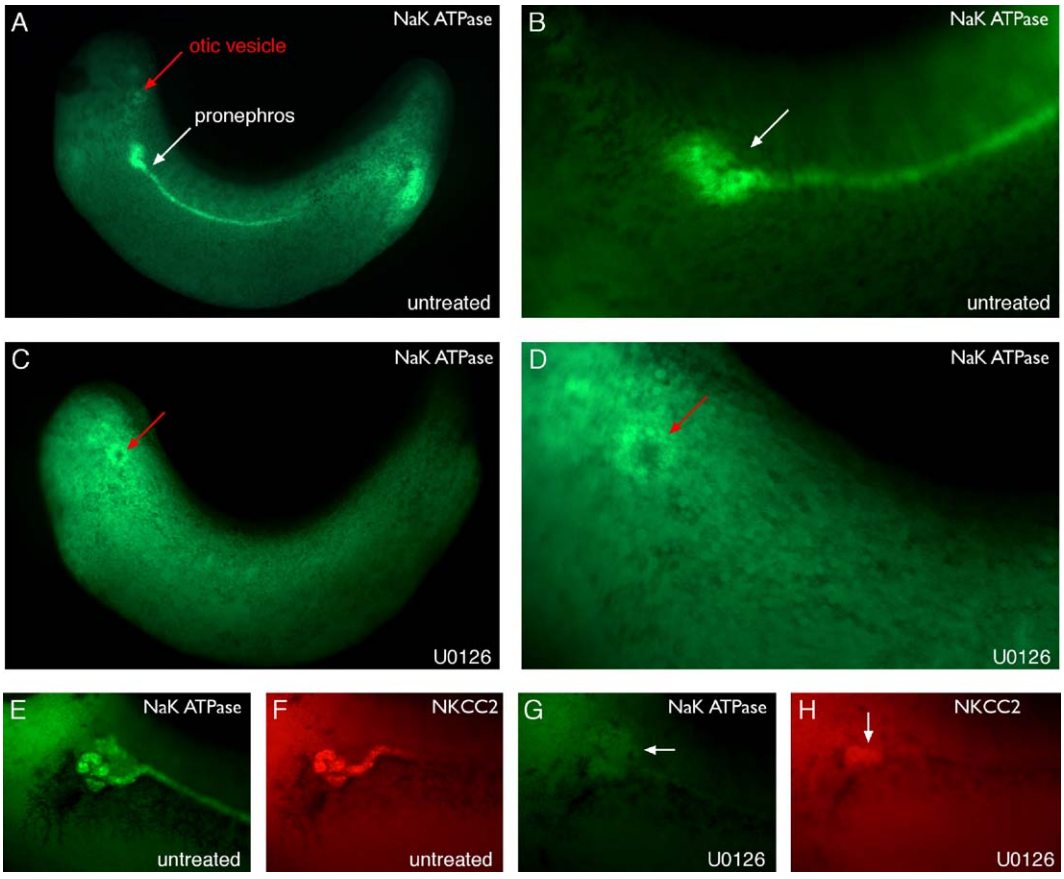

Fig. 8. The MEK inhibitor U0126 blocks pronephric tubule development. (A, B) Untreated control embryos at stage 32/33 stained for Na^+K^+ ATPase. Expression of Na^+K^+ ATPase is much stronger in the pronephros (white arrows) than it is in the otic vesicle (red arrows). (C, D) U0126-treated (from stage 14/15 to stage 32) embryos stained for Na^+K^+ ATPase. Strong expression is retained in the otic vesicle, but no pronephric staining is observed. (E, F) Stage 39 control embryos stained for Na^+K^+ ATPase (E) and NKCC2 (F) expression. (G, H) 50 μM U0126-treated embryos (from stage 12 to stage 39) stained for Na^+K^+ ATPase (G) and NKCC2 (H) expression. Very faint expression of both markers is visible (white arrows). This embryo was fixed, processed, and stained under identical conditions to the embryo shown in panels E and F.

embryonic day 10.5 in the mouse. As the ureter invades the mesenchyme, a reciprocal inductive process occurs whereby the ureter triggers mesenchyme to begin condensing into nephrons whereas the mesenchyme signals the ureter to bifurcate (Carroll and McMahon, 2003). The process works somewhat differently in pronephroi. In this instance, a single giant nephron forms and the pronephric/wolffian duct develops from the same mesenchymal mass as the nephron itself. Once the primordium has segregated from the intermediate mesoderm, the free posterior end of the wolffian duct extends posteriorly towards the cloaca. The anterior portion of the primordium is anchored in place, and the elongation of the mesenchymal mass in this direction results in a ventral buckling. Two distinct phases of polarization then occur within the primordium. The first of these is a transition from a disorganized mass to a radialized intermediate, with the radialization being initiated at NF stage 24. The second phase of

Table 4
FGF8-MO blocks pronephric mesenchymal to epithelial transition

Injected MO	Stage	Assay	No epithelialization		Partial epithelialization		Normal epithelialization		*n*
			n	%	*n*	%	*n*	%	
FGF8-MO	Stage 30	Pax8	24	65	9	24	4	11	37
FGF8-MO	Stage 30	Pax8	7	47	3	20	5	33	15
FGF8-MO	Stage 30	Pax8	7	78	2	22	0	0	9
FGF8-MO	Stage 33	3G8	5	71	2	29	0	0	7
FGF8-MO	Stage 36	3G8	11	31	23	64	2	5	36
FGF8-MO	Stage 36	NaK ATPase	25	80	0	0	6	19	31
Control-MO	Stage 30	Pax8	0	0	1 [a]	11	8	89	9
Control-MO	Stage 33	3G8	0	0	1	5	20	95	21

[a] Very mild phenotype, almost normal.

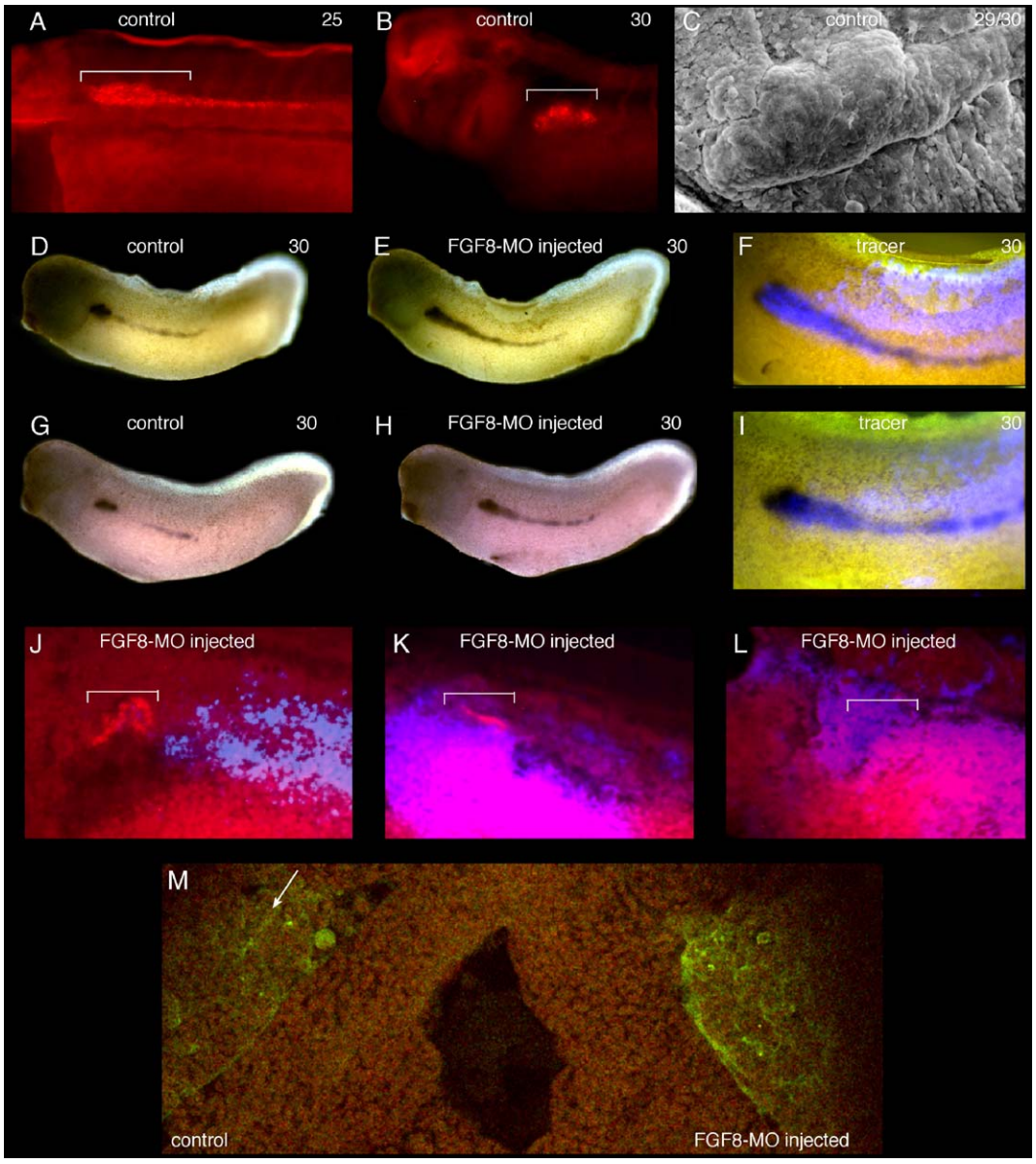

Fig. 9. FGF8-MO blocks the pronephric mesenchymal to epithelial transition. Normal epithelialization and acquisition of form in tailbud embryos visualized via Pax8 fluorescent in situ hybridization (A, B) or SEM (C) are illustrated in the top row. Stage 25 illustrates the mesenchymal stage of pronephric development, and stage 30 illustrates the early epithelialization stage. Test embryos were microinjected with FGF8-MO (14 ng) plus FLDx into blastomere V2.2 then raised to stage 30 or 33, fixed and stained for either Pax8 with a purple substrate (D–I) or 3G8 using a TRITC-coupled secondary antibody (J–L). The pronephric primordium forms (panels E and H) but fails to epithelialize. Note how the MO-injected pronephroi at stage 30 resemble the control pronephroi at the stage 25 mesenchymal period whereas the contralateral control side resembled the epithelialized controls shown in panels B and C. In both MO-injected examples, the lineage tracer (blue) is located in the pronephric mesenchyme (F, I, K, L) and is not present in the overlying somites. In panels H, I, and K, some epithelialization is occurring in the portion of the primordium that lack the tracer and the FGF8-MO. (M) Confocal optical transverse section through FGF8-MO unilateral stage 30/31 embryo stained for Pax8 via FISH. The left control side shows a Pax8-positive pronephros beginning to epithelialize (note forming lumen, white arrow), whereas the right side shows the FGF8-MO containing primordium as a Pax8-positive mesenchymal mass.

polarization begins at stage 28 when the radialized cells near the anterior end of the anlagen complete epithelialization and form a tiny lumen that then extends through the primordium reaching the posterior most end by stage 33/34 (Nieuwkoop and Faber, 1994).

Ectopic hedgehog secreted from the epidermis completely blocks the ability of the underlying intermediate mesoderm to undergo the normal early morphogenesis process required to segregate the pronephric primordium from the intermediate mesoderm. Early pronephric patterning occurs normally, as evidenced by activation of lim1 and Pax8 expression in the intermediate mesoderm in hedgehog expressing neurulae. The phenotype is first observed at NF stage 17/18 when expression of lim1 and Pax8 is more diffuse than in controls. Some 3–4 h prior to the first cellular sign of pronephric mesoderm segregation, the phenotype is fully manifested and expression of pronephric tubule markers lim1, Pax8, and vhnf-1 is extinguished. Following the identification of FGF8 as a

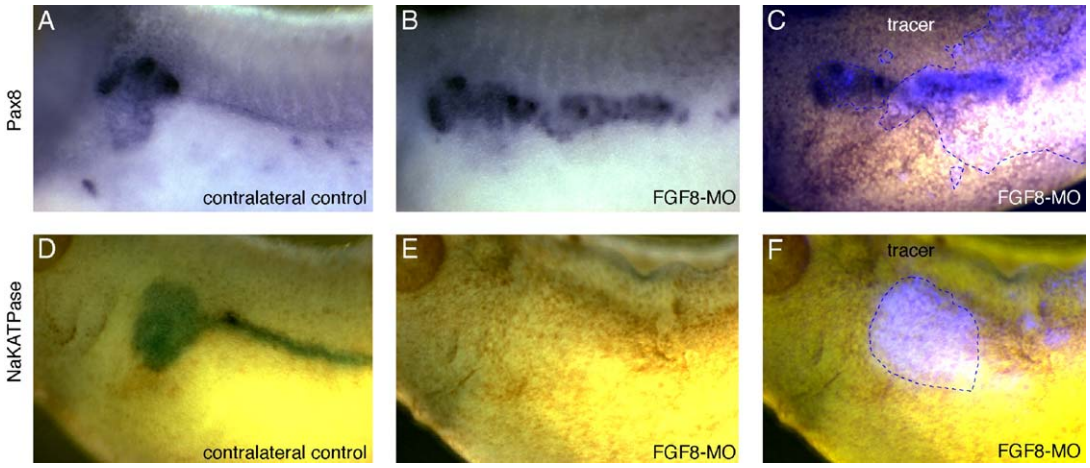

Fig. 10. Inhibition of FGF8 translation leads to retention of the pronephric mesenchymal marker Pax8 but blocks activation of the pronephric epithelial marker Na^+K^+ ATPase. (A–C) Pax8 expression on the contralateral control (A) and FGF8-MO-injected (B, C) sides of an embryo. The morpholino results in pronephric cells retaining Pax8 expression and appearing to be mesenchymal. (D–F) FGF8-MO blocks expression of the epithelial marker Na^+K^+ ATPase on the injected side of the embryo (Table 4).

potential component of the hedgehog phenotype, pharmacological inhibition of FGFR signaling with SU5402 was performed. When applied to embryos at NF stage 12.5 (late gastrula) or 15 (neurula), SU5402 completely blocked the ability to form pronephric tubules but only had a mild effect on glomeral development manifested as a minor change in organ shape. This similarity between the hedgehog and SU5402 phenotypes combined with the downregulation of FGF8 and egr1 in response to ectopic hedgehog suggests that attenuation of the FGF pathway may be a component of the observed suppression of pronephric tubule development. Together with the observation that inhibition of FGF8 translation leads to a block in pronephric epithelialization, these results indicate that FGF signaling plays at least two critical roles in the development of the embryonic kidney—an early role in pronephric mesoderm segregation and a later role in the mesenchymal to epithelial transition.

FGF8-null mouse embryos have severe gastrulation defects and die before kidney development can be analyzed (Sun et al., 1999). Recently, two reports on tissue-specific inactivation of FGF8 in the mouse have circumvented this problem via tissue-specific excision and thereby demonstrated a role for this gene in nephrogenesis (Grieshammer et al., 2005; Perantoni et al., 2005). In one report, a T-cre driver was used to delete FGF8 in essentially all post-gastrulation mesoderm (Perantoni et al., 2005) whereas the other used a Pax3-cre line to generate null mutants of FGF8 and a hypomorphic line to examine partial loss of function (Grieshammer et al., 2005). The murine results are similar to those described here in *Xenopus* using SU5402 and the FGF8-MO. In mice, a complete loss of function results in a very early block to nephron condensation. Condensation begins but does not proceed to the point of epithelialization at which wnt4 or lim1 expression is activated in the mesenchyme (Grieshammer et al., 2005; Perantoni et al., 2005). In *Xenopus*, both the hedgehog-mediated phenotype and early treatment with SU5402 result in a very early block to tubule primordium formation and no pronephric mesenchyme ever segregates from the intermediate mesoderm. Lim1 and wnt4 expression is also blocked in the *Xenopus* phenotypes by stage 21, but earlier expression of lim1 is not effected (Fig. 2). A partial loss of function in the mouse allows condensation to proceed but nephrons are fewer in number, are shorter than normal, and lack a distal segment (Grieshammer et al., 2005). Interestingly, such truncated nephrons still develop glomeruli. In *Xenopus*, the FGF-MO allows pronephric tubule primordia to condense but blocks its epithelialization while not interfering with the establishment of the glomera. In *Xenopus*, the distinction between nephron formation and glomerulogenesis is even more extreme, as normal glomera can form in the complete absence of a nephron in the hh and SU5402 phenotypes. One difference between the mouse mutants and the *Xenopus* data presented here is that in mice FGF8 is acting as a survival factor and inactivation leads to extensive apoptosis (Grieshammer et al., 2005; Perantoni et al., 2005), whereas in *Xenopus* the block to primordium formation or to nephron epithelialization can be effected without activating apoptosis.

The role of other FGFs in kidney development has been extensively studied, largely in the mouse and rat. These experiments have examined the role of FGF proteins and receptors on the survival/condensation of the metanephric mesenchyme and on ureteric bud branching. Two groups of FGFs appear to control different components of metanephric development. In one group, FGFs 1 and 10 promote branching and growth of the ureteric bud (Ohuchi et al., 2000; Qiao et al., 2001) whereas FGFs 2 and 7 may be more important as cell survival and proliferation promoting factors (Perantoni et al., 1995; Barasch et al., 1997; Qiao et al., 2001). These different effects are reflected in different responses in FGF-treated metanephric mesenchyme assayed by microarray (Qiao et al., 2001) but may also overlap to some extent. FGFRs have been studied through targeted mutagenesis and the use of secreted extracellular domains that act as dominant-negative inhibitors by sequestering ligands. These studies have shown that FGFR2 inhibition generates a reduced branching phenotype in the

kidney, an increase in apoptosis, and lower cell survival (e.g., Zhao et al., 2004) whereas FGFR1/FRGR2 double mutants have similar ureteric buds to FGFR2 mutant, but in addition lack all metanephric mesenchyme due to early apoptosis (Poladia et al., 2006). Interestingly, none of the abovementioned FGFs can, on their own, trigger the conversion of the mesenchyme to kidney epithelia, although a combination of FGF2 and pituitary extract can induce such a condensation (Perantoni et al., 1995). Interestingly, the pituitary expresses FGF8 (Norlin et al., 2000). These, and potentially other FGFs, remain candidates for the early FGF signal required in *Xenopus* between stages 15 and 20.

Our results demonstrate that when all signaling through FGFR is blocked, the pronephric primordium fails to segregate from the intermediate mesoderm and when FGF8 levels are reduced using a morpholino the primordium forms but epithelialization stalls. This less penetrant phenotype may reflect the action of alternative FGFs in the earlier condensation stage, or a less effective inhibition by the morpholino than is achieved via SU5402. A number of FGFs were not present on the *Xenopus* microarray used here including fgf3, fgf4, fgf6, fgf9, fgf10 and fgf12. Others, including fgf2, efgf and frl1, were on the array but were not regulated by hh. In mammals, other FGFs have been shown to function in metanephric kidney development, either in branching morphogenesis or proliferation. A large study of this growth factor family and the use of soluble receptor competitors will be required to fully understand the role of this class of molecules in pronephric development, but it is beyond the scope of the present report.

Previous studies (Brennan et al., 1999) have shown that *Xenopus* ectoderm can be induced to form pronephric glomera, but not pronephric tubules, when treated with FGF plus high doses of retinoic acid—the reverse of what one may expect from the data presented here. Our results show that FGFs are required for tubule development at two different stages but are not required for the formation of the glomus. This reversal is very likely due to the very different processes tested in these distinct systems. In animal cap ectoderm experiments, the FGF is acting as a mesoderm inducer. The competence to respond to induction in this manner is lost by stage 10 (Green et al., 1990) some 8–11 h before the windows in which FGFs are shown to function in pronephric development in the present report. As one system is inducing mesoderm with a particular specification that can then be patterned by retinoic acid to form glomera, and the other is testing FGF function during later morphogenesis, differences in the activity of FGFs are not surprising.

This is the first identification of a molecular component of the pronephric mesenchymal to epithelial transition. These data are consistent with those recently reported in the mouse (Grieshammer et al., 2005; Perantoni et al., 2005) but go further in that they show that this requirement can be extended to the development of the wolffian duct and that there are two distinct phases in which FGF signaling is essential. In addition, the microarray analysis performed here provides a rich resource for further exploration of the epithelialization phenotype. A preliminary analysis of regulated genes identify Cdc42, fibronectin, par-6, slug, snail, twist and ZO2 as potential epithelialization participants (Table 2, Supplemental data), but higher level analyses will undoubtedly provide further insights. The pronephros is therefore not only a useful system in which to perform induction assays, our data demonstrate that it is also a powerful system for exploring mesenchymal to epithelial transitions during organogenesis of the kidney and allows for the separation of nephron and glomeral development, something unique to this kidney form. Many of the genes identified by the microarray analysis presented here may be essential for kidney development, in addition to FGF8. Further experiments injecting pools of mRNAs encoding regulated genes may be a powerful new approach to identify the genetic networks regulating the very earliest stages of kidney organogenesis.

Uncited references

Carroll et al., 2005

Acknowledgments

We would like to thank Victor Gerth for graphics support, confocal expertise and for generating the model shown in Fig. 5A, Steve Ekker for *Xenopus* hedgehog clones, Jonathan Slack for frog FGF8, Iain Drummond for zebrafish pax2.1 and wt1, Masanori Taira for crescent, Walter Knochel for XFD clones, Richard Harland for FoxD1 and CS107/108 clones, and Elizabeth Jones for 3G8 and 4A6 antibodies. We would also like to thank the University of Florida ICBR Gene Expression Core facility for microarray data. This work was supported by grants from the Alberta Heritage Foundation for Medical Research (PV) and the Canadian Institutes of Health (MOP-67152, to PV).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.469.

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