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Hoxd11 specifies a program of metanephric kidney development within the intermediate mesoderm of the mouse embryo

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ABSTRACT

The mammalian kidney consists of an array of tubules connected to a ductal system that collectively function to control water/salt balance and to remove waste from the organisms' circulatory system. During mammalian embryogenesis, three kidney structures form within the intermediate mesoderm. The two most anterior structures, the pronephros and the mesonephros, are transitory and largely non-functional, while the most posterior, the metanephros, persists as the adult kidney. We have explored the mechanisms underlying regional specific differentiation of the kidney forming mesoderm. Previous studies have shown a requirement for *Hox11* paralogs (*Hoxa11, Hoxc11* and *Hoxd11*) in metanephric development. Mice lacking all *Hox11* activity fail to form metanephric kidney structures. We demonstrate that the *Hox11* paralog expression is restricted in the intermediate mesoderm to the posterior, metanephric level. When Hoxd11 is ectopically activated in the anterior mesonephros, we observe a partial transformation to a metanephric program of development. Anterior Hoxd11$^+$ cells activate *Six2*, a transcription factor required for the maintenance of metanephric tubule progenitors. Additionally, Hoxd11$^+$ mesonephric tubules exhibit an altered morphology and activate several metanephric specific markers normally confined to distal portions of the functional nephron. Collectively, our data support a model where *Hox11* paralogs specify a metanephric developmental program in responsive intermediate mesoderm. This program maintains tubule forming progenitors and instructs a metanephric specific pattern of nephron differentiation.

Introduction

The vertebrate kidney functions to maintain the organism's homeostasis by controlling water/salt balance, eliminating nitrogenous waste and regulating blood pressure and blood composition. The kidney serves as a well-established model system to study multiple aspects of organogenesis such as reciprocal interactions between mesenchymal and epithelial populations (Grobstein, 1953; Vega et al., 1996), mesenchymal to epithelial transitions (MET) (Barasch et al., 1999; Carroll et al., 2005) and branching morphogenesis (Basson et al., 2006; Majumdar et al., 2003).

Around embryonic day 8.0 (E8.0), the murine intermediate mesoderm (IM) gives rise to two lateral paired ducts, the nephric ducts (ND), that extend caudally along the anterior–posterior (A–P) axis of the embryo (Grote et al., 2006; Pedersen et al., 2005). Three different kidney structures, the pronephros, the mesonephros and the metanephros, form in association with, and are dependent on, the ductal epithelium (Saxén, 1987). The pronephros forms around E8.5 in the most anterior portion of the IM, but quickly degenerates (Bouchard et al., 2002; Saxén, 1987). The mesonephros arises just caudal to the pronephros beginning around E9.5 and consists of arrays of cranial and caudal tubules that lie adjacent to the ND (Sainio et al., 1997; Sainio and Raatikainen-Ahokas, 1999; Saxén, 1987). Cranial tubules form connections to the ND, and in the mouse, caudal tubules remain disconnected. Sainio et al. (1997) have suggested that cranial tubules are derived from outgrowths of the mesonephric ND whereas caudal tubules form due to a MET within the adjacent IM-derived mesonephric mesenchyme. However, direct evidence of a ND contribution to cranial tubules has not been demonstrated. The mesonephric kidney of the mouse is not thought to play a role in embryonic physiology. The eventual fate of the mesonephros depends on the sex of the animal. Cranial tubules are remodeled to form epididymal ducts around E12.5 in the male mouse embryo. In contrast, the female mesonephric duct and mesonephric tubules undergo apoptosis (Sainio and Raatikainen-Ahokas, 1999; Saxén, 1987; Tilmann and Capel, 2002; Vize et al., 2002).

The metanephric kidney, the functional kidney of the fetus and permanent kidney in amniotes, develops from the posterior IM at the level of the hindlimb around E10.5 of mouse development. Several lines of evidence support a model whereby *Gdnf* expression is restricted to the metanephric mesenchyme (Grieshammer et al., 2004;

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Kume et al., 2000; Sanchez et al., 1996). GDNF then induces the metanephric (posterior) portion of the ND to invade (Basson et al., 2005; Michos et al., 2007; Pepicelli et al., 1997; Vega et al., 1996) and thereafter, branch repetitively within the mesenchyme, forming the network of the collecting duct system (Basson et al., 2006; Majumdar et al., 2003). An inductive interaction at the tips of the branching ureteric epithelium induces a sub-population of adjacent metanephric mesenchyme capping each tip to undergo a MET establishing the renal vesicle, the epithelial precursor of the main body of the nephron (Barasch et al., 1999; Carroll et al., 2005; Grieshammer et al., 2005; Kobayashi et al., 2005b; Park et al., 2007; Stark et al., 1994). Importantly, maintenance of a nephron progenitor population requires the activity of a transcriptional regulator, *Six2* (Self et al., 2006). Upon MET, a portion of the cap mesenchyme downregulates *Six2* (Self et al., 2006), while the remainder maintains *Six2* expression, ensuring that each future ureteric tip will be associated with a tubule precursor population.

The ND is critical for the induction of both mesonephric and metanephric tubules (Carroll et al., 2005; Grobstein, 1953; Gruenwald, 1952; Saxén, 1987). Recent studies indicate that ND-derived *Wnt9b* is a key factor required for induction of both tubule types (Carroll et al., 2005). *Wnt9b* acts via the canonical Wnt signaling pathway (Park et al., 2007) to induce a common, tubule promoting developmental program involving *Wnt4* (Stark et al., 1994; Vainio et al., 1999) and *FGF8* (Crossley et al., 1996; Perantoni et al., 2005). While both the mesonephric and metanephric kidneys share a common regulatory pathway of tubulogenesis, the nephrons that form are distinct in organization, scale and differentiation as mesonephric tubules lack both a juxtaglomerular apparatus and loop-of-Henle (Vize et al., 2002). What determines the differences in response to a common inductive input is unclear.

One model supposes that the IM mesenchyme is differentially patterned along its A–P axis and that this pre-pattern instructs either a mesonephric or metanephric response to inductive signals. Amongst candidate pattern regulators, *Hox* genes are of particular interest given that *Hox* gene function has been widely demonstrated to regulate differential patterning along the body axis during metazoan development (Deschamps and van Nes, 2005; Krumlauf, 1994; Wellik, 2007). Evidence in favor of a potential role for a *Hox* mediated pre-patterning of the IM mesenchyme comes from analysis of *Hox11* paralog mutants (Patterson et al., 2001; Wellik et al., 2002). When activity of all three *Hox11* paralogs (*Hoxa11*, *Hoxc11* and *Hoxd11*) is removed in the mouse, there is a complete failure of both *Gdnf* and *Six2* expression in the position where metanephric mesenchyme normally forms, resulting in renal agenesis (Wellik et al., 2002). In contrast, mesonephric development is largely unaffected. Though mutations in *Pax2* (Bouchard et al., 2002; Grote et al., 2006; Narlis et al., 2007; Torres et al., 1995), *Osr1* (James et al., 2006; Wang et al., 2005), *Sall1* (Nishinakamura et al., 2001; Ott et al., 2001), and *Six1* (Kobayashi et al., 2007; Xu et al., 2003) result in similar metanephric phenotypes, mesonephric development is also perturbed. Sajithlal et al. (2005) hypothesize that *Eya1* specifies the metanephros, however they also demonstrate *Eya1* expression in mesonephric mesenchyme, suggesting a more general role for *Eya1* in IM development. Thus, *Hox11* paralogs are strong candidates for the specific regulation of a metanephric response in the IM.

We have investigated the differential specification of mesonephric and metanephric kidneys. We demonstrate that mesonephric tubules are formed by MET of the mesonephric mesenchyme and that these tubules do not express markers of distal metanephric segments. Unlike other transcription factors required for metanephric development, *Hox11* paralogs are not expressed in the mesonephros, only in the metanephros. We demonstrate that ectopic Hoxd11 activity in the mesonephric mesenchyme is sufficient to activate cell-autonomous ectopic expression of *Six2*. Furthermore, Hoxd11^{+} mesonephric tubules adopt a morphology and genetic profile reminiscent of metanephric tubules. Taken together, our data support a model where the posterior metanephric mesenchyme is specified as such by *Hox11* paralogs. These paralogs simultaneously activate distinct molecular programs, the first being required for the maintenance of the cap mesenchyme while the second instructs metanephric-specific tubule development in response to inductive signals.

Materials and methods

Ontolology

All urogenital ontological terms are used as defined in Little et al. (2007).

Animals and genotyping

Animal care and research protocols were performed in accordance with Harvard University's institutional guidelines, following approval by Harvard University's institutional committee on animal use. For staging of embryos, the morning of vaginal plug was designated as embryonic day 0.5 (E0.5). Swiss Webster mice (Taconic) were used for all wild type whole mount *in situ* hybridization. *Tg*(*Hoxb7-cre*)*12Amc* (Yu et al., 2002), *Tg*(*Rarb-cre*)*1Bhr* (Kobayashi et al., 2005a), *Gt*(*ROSA*)*26*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001) and *Gt*(*ROSA*)*26*^{tm1Sor} (Soriano, 1999) (Jackson Laboratories) were genotyped as previously described. The *Osr1*$^{eGFPCreERt2/+}$ line was generated by knocking an *eGFP-CreERt2* construct into the endogenous *Osr1* locus and will be described in more detail in a future publication. *Osr1*$^{eGFPCreERt2/+}$ mice and embryos were genotyped with the primers Osr1ER3′GenoFw: ACCCGTGATATTGCTGAAGAGCTTG and Osr1ER3′GenoRv: TGAAGAGCGCTGAAACCATAC. The eGFPCreERt2 protein driven by the *Osr1* promoter was activated by intraperitoneal injection of Tamoxifen (Sigma) dissolved in corn oil (Sigma) into dams at a dose of 3 mg Tamoxifen per 40 g mouse body weight at day E7.75.

The *R26Hoxd11* line was created by inserting a cassette containing mouse *Hoxd11*::IRES2::*nuclear LacZ* into the NheI and XhoI sites of pBigT (Srinivas et al., 2001), releasing the construct with AscI and PacI and subcloning it into pRosa26PAS (Mao et al., 2005) cut with AscI and PacI. The construct was linearized with SwaI and electroporated into YFP 3.1 embryonic stem cells (Mao et al., 2005). Two neomycin resistant colonies demonstrated ubiquitous YFP expression and no β-gal activity. Nuclear β-gal activity was detected in these two clones only upon addition of 4OH-Tamoxifen to the ES cell media. The clones were expanded and confirmed on the 5′ end by PCR using the primers Rosa26-5armFlanking: CCTAAAGAAGAGGCTGTGCTTTGG and Rosa26-SA: CATCAAGGAAACCCTGGACTACTG. The presence of the targeted Hoxd11 transcript was also detected only with addition of 4OH-Tamoxifen with the following primers: cHD11Fw: AAAAGCGCTGTCCCTACACCAAGTAC and cHD11Rv: TCAACAGACCTTGCATTCCTTTGGC. One clone was injected into host (C57BL/6J) blastocysts by the Genome Manipulation Facility, MCB, Harvard University. The line was maintained on a C57BL/6J background (Jackson Laboratories). Mice and embryos were genotyped by PCR with the cHD11Fw and cHD11Rv primers.

Generation of a Hoxd11 polyclonal antibody

Rabbits were immunized with a KHL-conjugated peptide PEGAADKGDPKPG corresponding to amino acids 202–214 of mouse Hoxd11 and anti-serum was affinity purified against immobilized Hoxd11 peptide (Covance Research Products). The purified anti-serum was tested by transiently transfecting (Lipofectamine 2000, Invitrogen) COS7 cells with a mouse *Hoxd11* expression plasmid followed by immunostaining. Immunostaining was also performed on E15.5 metanephric cryosections. Specificity was tested by pre-incubation of purified anti-serum with Hoxd11 peptide followed by immunostaining on E15.5 metanephric cryosections.

Whole mount analysis

For *in situ* hybridization, embryos were collected in PBS, fixed in 4% paraformaldehyde at 4 °C overnight, washed thoroughly in PBS, dehydrated through a methanol series into 100% methanol and stored at −20 °C. Prior to *in situ* hybridization, head and trunk regions above the forelimb were removed from E9.5 or E10.5 embryos and embryos were split in half along the dorsal–ventral axis. E11.5 to E15.5 mesonephros, gonads and metanephros were dissected from the rest of the embryo prior to *in situ* hybridization. Whole mount *in situ* hybridization was performed as previously described (Park et al., 2007). For whole mount X-gal staining, embryos were dissected in PBS and fixed (1% formaldehyde, 0.2% gluteraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40) for 30 min and washed thoroughly in PBS + 0.02% NP-40. Prior to staining, embryos were dissected as described above. Staining was carried out in staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% NaDeoxycholate, 0.02% NP-40, 1 mg/ml X-gal) at 37 °C for 6–8 h. Staining was stopped by thorough washing in PBS followed by fixation in 4% paraformaldehyde + 0.02% gluteraldehyde at 4 °C overnight. Whole mount samples were cleared in 80% glycerol and imaged on a Nikon SMZ1500 Stereoscope with a Nikon DXM1200C camera (Nikon Instruments).

Histological analysis

For immunofluorescence, embryos were dissected in PBS, fixed for 1 h on ice in cold 4% PFA, washed thoroughly in PBS and cryopreserved overnight at 4 °C in 30% sucrose.

Tissue was frozen in OCT (Tissue-Tek) on a dry ice/ethanol bath. 20 µm cryosections were collected on Superfrost coated slides (VWR), dried and used immediately or frozen at −20 °C. Slide mounted sections were incubated in PBS for 5 min, blocked for 30 min in 3%BSA, 1% serum in PBS+0.25% TritonX100 and incubated with anti-Hoxd11 (1:500), anti-Six2 (1:1000, J.W. Mugford and A.P. McMahon, unpublished), anti-Pax2 (1:500, Covance), anti-GFP (1:500, AvesLabs), anti-cytokeratin (1:500, Sigma) or anti-β-galactosidase (1:500, AbCam) in block solution overnight at 4 °C. Sections were washed 3 times in PBS+0.25% TritonX100 (PBTX) and then incubated at room temperature with appropriate Cy2 (1:500, Jackson Immuno), Alexa488, Alexa568 or Alexa647 (1:500, Invitrogen) conjugated secondary antibodies in block solution for 1.5 h. Sections were washed 3 times in PBTX, rinsed once in PBS, stained with 1 µg/ml Hoechst 33342 (Invitrogen) for 5 min and rinsed once in PBS. Sections were coverslipped in Vectashield (Vector Labs) and imaged on a Zeiss LSM 510 META confocal microscope (Zeiss). After the whole mount *in situ* hybridization or X-gal staining procedure, 50 µm vibrotome sections were cut, mounted in 80% glycerol and imaged on a Nikon Eclipse 90i compound microscope with a Nikon DXM1200C camera (Nikon Instruments).

Results

Cranial mesonephric tubules are primarily derived from mesonephric mesenchyme

As a first step towards understanding the differential specification of the IM towards a mesonephric or metanephric tubulogenic program, we first sought to clarify the origin of mesonephric tubules. Sainio et al. (1997) proposed a ND origin for cranial mesonephric tubules, in contrast to more posterior mesonephric tubules that are physically detached from the ND. We addressed the fate of cranial mesonephric tubules using *Tg*(*Hoxb7-cre*)*12Amc* (Yu et al., 2002) and *Tg*(*Rarb-cre*)*1Bhr* (Kobayashi et al., 2005a) Cre driver lines, referred to here as Hoxb7-Cre and Rarb-Cre, respectively. Hoxb7-Cre expresses Cre recombinase throughout the entire ND prior to either mesonephric or metanephric tubule formation whereas Rarb-Cre expresses Cre recombinase in the mesonephric (from E9.5) and also the metanephric (from E10.5) mesenchyme. To fate map the mesonephric tubules, we crossed Hoxb7-Cre and Rarb-Cre to the *Gt*(*ROSA*)*26*^{tm1Sor} (Soriano, 1999) reporter line, referred to here as R26R, and examined the location of X-gal$^+$ cells in the mesonephros of both Hoxb7-Cre;R26R and Rarb-Cre;R26R embryos.

In the E10.5 mesonephros of Hoxb7-Cre;R26R embryos, X-gal$^+$ cells are located mainly in the ND (Fig. 1A black arrowhead). Additionally, X-gal$^+$ ND outgrowths project into the mesonephric mesenchyme (Fig. 1A arrow) toward developing X-gal$^-$ cranial mesonephric tubules (Fig. 1A white arrowheads). Conversely, developing mesonephric tubules of Rarb-Cre;R26R embryos are X-gal$^+$ (Fig. 1B black arrowheads). In addition, a small number of X-gal$^+$ cells are found in or around the ND (Fig. 1B white arrowhead). At E12.5, cranial and caudal mesonephric tubules of Hoxb7-Cre;R26R embryos are largely X-gal$^-$ (Fig. 1C white arrowheads); X-gal$^+$ cells are located in the ND (Fig. 1C black arrowhead). Conversely, X-gal staining in E12.5 Rarb-Cre;R26R embryos is complimentary to that of Hoxb7-Cre;R26R embryos; all mesonephric tubules are X-gal$^+$ (Fig. 1D black arrowheads), while the ND remains X-gal$^-$ (Fig. 1D white arrowhead).

To examine cell fates with single cell resolution, we next crossed mice carrying Hoxb7-Cre and Rarb-Cre alleles to the *Gt*(*ROSA*)*26*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001) reporter line, referred to here as R26YFP, and performed immunofluorescence for Cytokeratin, Pax2 and YFP on transverse sections through cranial mesonephros. At E10.5, the ND and ND outgrowths are both Cytokeratin$^+$ and Pax2$^+$ (Figs. 1I, J, M, N asterisks and arrowheads, respectively), whereas the developing mesonephric tubules produce only Pax2 (Figs. 1I, J, M, N arrows). In the E10.5 mesonephros of Hoxb7-Cre;R26YFP embryos, YFP is detected in Cytokeratin$^+$ and Pax2$^+$ cells (Figs. 1E, M arrowheads) and is rarely observed in cells that only produce Pax2 (Figs. 1E, M arrows). Conversely, YFP is largely restricted in E10.5 Rarb-Cre;R26YFP embryos to cells that are only Pax2$^+$ (Figs. 1F, N arrows), in addition to a few cells of the surrounding mesonephric mesenchyme. Minimal YFP is observed in cells that produce both Cytokeratin and Pax2 (Figs. 1F, N arrowheads).

At E12.5, the ND and all mesonephric tubules are both Cytokeratin$^+$ and Pax2$^+$ (Figs. 1K, L). The ND is located lateral to the mesonephric tubules (Figs. 1G, H, K, L, O, P asterisks). YFP is detected in the connections between anterior cranial tubules and the ND in the E12.5 cranial mesonephros of Hoxb7-Cre;R26YFP embryos (Figs. 1G, O arrowheads), whereas the majority of the remaining mesonephric tubules are YFP$^-$ (Figs. 1G, O arrows). Interestingly, YFP is also detected in the connections between the cranial mesonephric tubules and the ND in the E12.5 mesonephros of Rarb-Cre;R26YFP embryos (Figs. 1H, P arrowheads). Additionally, YFP is present throughout the remainder of both cranial and caudal E12.5 mesonephric tubules of Rarb-Cre;R26R embryos (Figs. 1H, P arrows and data not shown). In summary, these results demonstrate that the majority of cranial mesonephric tubules are derived from the mesonephric mesenchyme. The ND contributes to a connecting segment attaching the ND to a mesonephric tubule, creating a structure that is likely a mosaic of ND and mesonephric mesenchyme-derived cells.

Mesonephric tubules do not express markers of metanephric distal segments

Next, we screened for molecular markers that may potentially distinguish between a mesonephric and metanephric program of renal tubule development. Initially, we examined mesonephric tubules for genes with known segment-specific expression patterns along the proximal–distal (glomerular-collecting duct) axis of the metanephric nephron. Of these, six genes are expressed in both mesonephric and metanephric tubules, forming two classes according to their relative expression levels. Both classes initiate expression in developing mesonephric tubules at E11.5 (Figs. 2A, B, E, F arrowheads). Class one genes are expressed at high levels and include *Slc12a1* (Figs. 2A–D), a marker of the thick ascending loop-of-Henle (Nakai et al., 2003), *Brn1* (data not shown), a marker of the thin ascending loop-of-Henle (Nakai et al., 2003) and *Slc34a1* (data not shown), a marker of proximal convoluted tubules (Murer et al., 2003). Class two genes are expressed at lower levels than class one genes and include *Barttin* (Figs. 2E–H), a maker of the ascending loop-of-Henle (Wolf et al., 2003), *Ihh* (data not shown), a marker of proximal straight tubules (Valentini et al., 1997) and *Gsh1* (data not shown), a marker of early podocytes (McMahon et al., 2006). Neither class one nor class two genes are expressed in the mesonephros after mesonephric tubule remodeling (data not shown).

Three genes were expressed exclusively in metanephric nephrons. This group includes *Calb3* (Figs. 2I–L), a marker of connecting tubules (Chen et al., 2006), *Clck1a* (Figs. 2M–P), a marker of the thin ascending loop-of-Henle (Wolf et al., 2003) and *Slc12a3* (Figs. 2Q–T), a marker of distal convoluted tubules (Nakai et al., 2003). Expression of these genes was never observed in developing mesonephric tubules (Figs. 2I–K, M-O, Q–S arrowheads) but each was expressed with a distinct temporal program in metanephric tubules (Figs. 2L, P, T); *Calb3* expression initiates at E13.5 (Fig. S1A, B arrowhead), and *Slc12a3* (Fig. S1C, D arrowhead) and *Clck1a* (Fig. S1E, F arrowhead) at E14.5. In general, the genetic profile of mesonephric and metanephric tubules are remarkably similar. The principle difference reflects genes associated with distal metanephric distal segments that arise late in metanephric nephron patterning.

Hox11 paralog expression implicates their role in metanephric specification

In order to clearly define *Hox11* paralog expression in conjunction with kidney development, we compared the expression of *Hoxa11*, *Hoxc11* and *Hoxd11* in the IM with that of known regulators of metanephric development such as *Osr1* (James et al., 2006; Wang et al.,

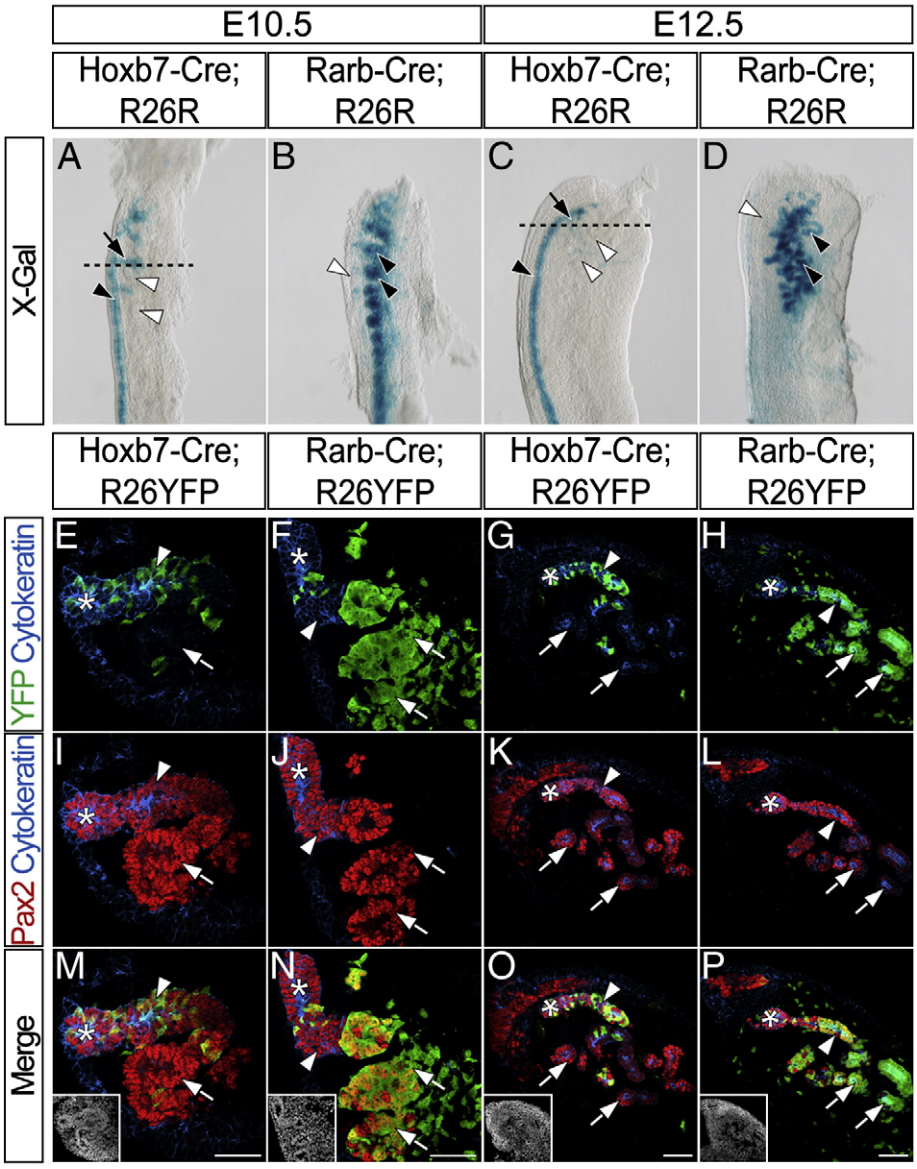

Fig. 1. Cranial mesonephric tubules are primarily derived from anterior intermediate mesoderm mesenchyme. (A–D) X-gal staining in the mesonephros of E10.5 (A) and E12.5 (C) Hoxb7-Cre;R26R or E10.5 (B) and E12.5 (D) Rarb-Cre;R26R embryos. Black arrowheads (A, C) indicate X-gal staining in the ND. Black arrows (A, C) indicate X-gal staining in ND outgrowths. Black arrowheads (B, D) indicate X-gal staining in the mesonephric tubules. White arrowheads (B, D) indicate lack of X-gal staining in the ND. Dashed lines (A, C) indicate approximate planes of section (E–T). (E–T) Immunofluorescent confocal microscopy of transverse sections of anterior mesonephros in E10.5 (E, I, M) and E12.5 (G, K, O) Hoxb7-Cre;R26YFP or E10.5 (F, J, N) and E12.5 (H, L, P) Rarb-Cre;R26YFP embryos stained for YFP, Cytokeratin and Pax2. Insets (M–P) indicate nuclei. Asterisks indicate the ND. Arrowheads indicate ND outgrowths (E, F, I, J, M, N) or mesonephric tubule connecting segments (G, H, K, L, O, P). Arrows (E–P) indicate developing mesonephric tubules. Scale bars in panels M–P=50 μm.

2005), *Pax2* (Narlis et al., 2007; Torres et al., 1995), *Eya1* (Sajithlal et al., 2005; Xu et al., 1999), *Six1* (Kobayashi et al., 2007; Xu et al., 2003), *WT1* (Donovan et al., 1999; Sainio et al., 1997), *Six2* (Self et al., 2006) and *Sall1* (Nishinakamura et al., 2001; Nishinakamura and Osafune, 2006).

Our results demonstrate that the transcription factors examined can be grouped into three classes. The first class includes *Osr1* (Fig. S2A–E), *Pax2* (Fig. S2F–J), *Sall1* and *WT1* (data not shown). As exemplified by *Osr1* and *Pax2*, class one genes are expressed from E9.5 through E11.5 in both the developing mesonephros (Fig. S2A–J, arrowheads) and metanephros (Fig. S2C, H, E, J arrows). Differences exist among the mesonephric expression patterns of the different transcription factors. For instance, *Osr1* is expressed in mesenchymal populations (Fig. S2B, D arrowheads), whereas *Pax2* is expressed in both epithelial and mesenchymal populations (Fig. S1G, H, I arrowheads).

The second class includes *Eya1* (Fig. S2K–O), *Six1* and *Six2* (data not shown). As exemplified by *Eya1*, class two genes are also expressed in both the mesonephros (Fig. S2K, L, N arrowheads) and metanephros (Fig. S2M, O arrows). Unlike class one genes, their expression is down-regulated in the mesonephric mesenchyme at E10.5 (Fig. S2N arrowhead) and absent by E11.5 (Fig. S2O arrowhead). *Eya1* and *Six2* are robustly expressed in the metanephric mesenchyme from E10.5 onwards (Fig. S2O arrow), while *Six1* is completely down-regulated by E11.5 (data not shown).

The third class is restricted to members of the *Hox11* paralog group (Fig. S2P–T and Fig. 3). Within the IM, these three genes are expressed exclusively in the metanephric mesenchyme (arrows in Fig. S2R, T and Figs. 3B–E, G–I, L–O). Interestingly, as the metanephros develops, individual *Hox11* paralog members have slightly differing expression patterns. *Hoxa11* (Figs. 3A–E) and *Hoxd11* (Figs. 3K–O) are consistently expressed at higher levels than *Hoxc11* (Figs. 3F–J). *Hoxc11* is undetectable by E15.5 (Fig. 3J and data not shown). In addition, *Hoxd11* (Fig. 3N arrow and O) is expressed more robustly *Hoxa11* (Fig.

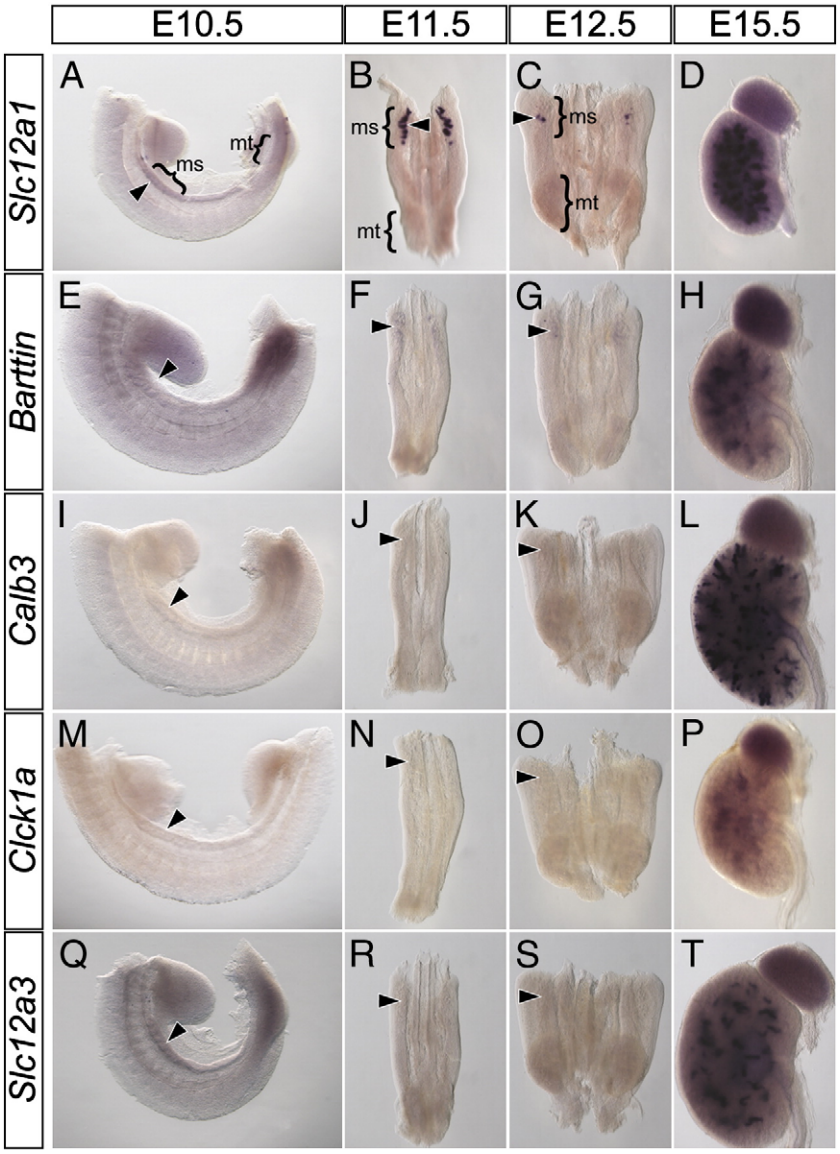

Fig. 2. Mesonephric tubules do not express markers of metanephric distal segments. Whole mount *in situ* hybridization for *Slc12a1* (A–D), *Barttin* (E–H), *Calb3* (I–L), *Clck1a* (M–P) and *Slc12a3* (Q–T) in wild type E10.5 (A, E, I, M, Q) trunks, E11.5 (B, F, J, N, R) and E12.5 (C, G, K, O, S) IM and E15.5 (D, H, L, P, T) metanephros. Arrowheads indicate location of mesonephric tubules. ms – mesonephros, mt – metanephros.

3D arrow and E) in both the cortical stroma and cap mesenchyme. In summary, our results demonstrate that the expression patterns of the *Hox11* paralogs are unique among transcription factors required for metanephric development and suggestive of a class of molecules that may differentially regulate mesonephric and metanephric responses within the IM mesenchyme to inductive signals.

Six2, but not Gdnf, is activated in mesonephric mesenchyme expressing Hoxd11

To directly address the function of *Hox11* genes, we developed two different mouse lines to enable ectopic activation of *Hoxd11* in anterior IM prior to mesonephric tubule induction. *Hoxd11* was selected amongst the three *Hox11* paralogs based on the relative contribution of *Hoxd11* to the metanephric kidney program in compound mutants (Wellik et al., 2002) and the observation that *Hoxd11* shows strong, broad early expression that is maintained throughout subsequent metanephric development (Figs. 3L–O). An *R26Hoxd11* line was generated by introducing a full-length mouse *Hoxd11* cDNA and a *nuclear LacZ* reporter into the *ROSA26* locus in a configuration that enables Cre dependent regulation in any cell type (Fig. S3). A second line, *Osr1*$^{eGFPCreERt2/+}$, referred to here as Osr1-GCE (J.W. Mugford, P. Sipilä, J. McMahon and A.P. McMahon, unpublished), contains an eGFPCreERt2 construct knocked into the *Osr1* locus. When Osr1-GCE;R26R compound heterozygotes were induced with Tamoxifen at E7.75 through intraperitoneal injection of the dam, X-gal^{+} cells contribute to the IM from E8.5, strongly labeling the mesonephros, gonad and metanephros at E12.5 (Fig. S4A and data not shown).

R26Hoxd11 mice were intercrossed with mice carrying Osr1-GCE and Rarb-Cre alleles. In Rarb-Cre;R26Hoxd11 embryos, Hoxd11 is activated in mesonephric tubules and their derivatives beginning at E9.5 (Kobayashi et al., 2005a). In contrast Osr1-GCE;R26Hoxd11 embryos injected with Tamoxifen at E7.75 activate Hoxd11 throughout the IM as early as E8.5. Immunofluorescence using anti-Hoxd11 (Fig. S4C), anti-Cytokeratin and anti-β-gal on sections of E13.5 mesonephros from R26Hoxd11, Rarb-Cre;R26Hoxd11 and Osr1-GCE;HoxD11 embryos demonstrates that both Hoxd11^{+} and β-gal^{+} cells co-localize with Cytokeratin^{+} mesonephric tubules only when Cre alleles are present (Fig. S4B).

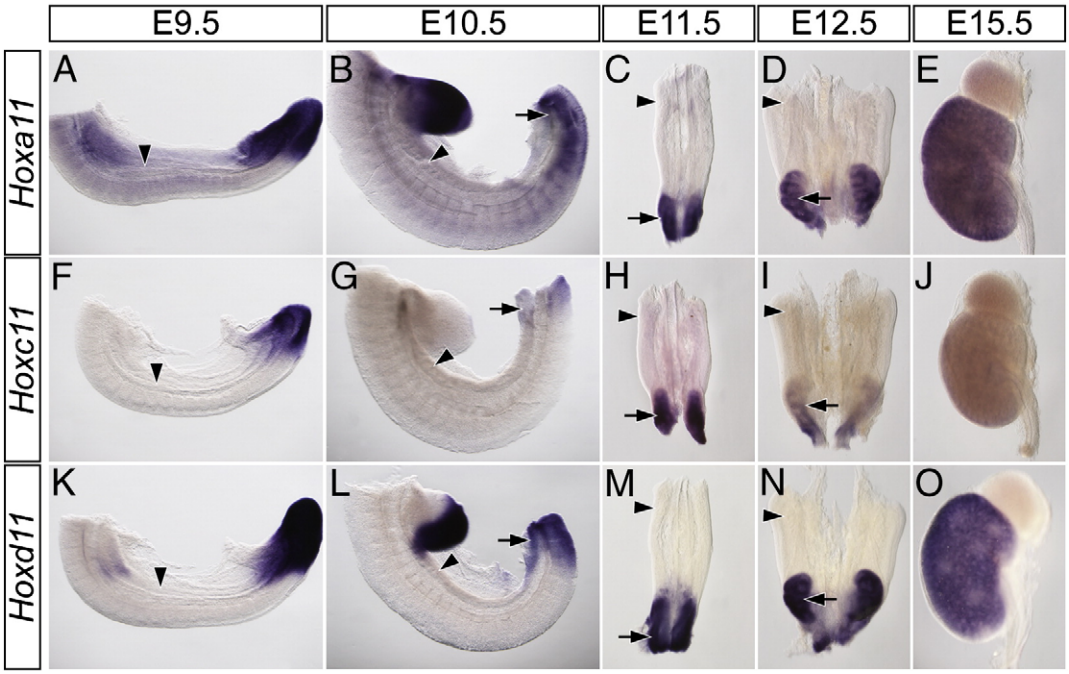

Fig. 3. Hox11 paralogs are exclusively expressed in the metanephros. Whole mount *in situ* hybridization for *Hoxa11* (A–E), *Hoxc11* (F–J) and *Hoxd11* (K–O) in wild type E9.5 (A, F, K) and E10.5 (B, G, L) trunks, E11.5 (C, H, M) and E12.5 (D, I, N) IM and E15.5 (E, J, O) metanephros. Arrowheads indicate location of the mesonephros. Arrows indicate the location of the metanephros.

Both *Six2* and *Gdnf* expression are absent from the posterior IM of *Hox11* mutants (Wellik et al., 2002). We examined their expression in the E10.5 mesonephros of Rarb-Cre;R26Hoxd11 and Osr1-GCE; R26Hoxd11 embryos. Neither was activated in the E10.5 mesonephros of Rarb-Cre;R26Hoxd11 embryos (data not shown). In contrast, *Six2* was cell-autonomously activated in small clusters of cells following Osr1-GCE mediated activation of R26Hoxd11 at E7.75 (Fig. 4D arrows). *Six2* expression is normally absent in the mesonephric region at E10.5 (Figs. 4C, E, G, I) and all Six2$^+$ cells are β-gal$^+$ (Figs. 4F, H, J arrowheads). As expected, β-gal was not detected in the absence of Cre activity (Figs. 4E, G, I). Ectopic Six2 was restricted to mesenchymal mesonephric cells; no Six2 was observed in either the ND or developing mesonephric tubules (Figs. 4F, H, J dashed line and data not shown). These results suggest that the maintenance of *Six2* expression, a distinct feature of the metanephric mesenchyme, is regulated by *Hoxd11* and likely other *Hox11* paralog members (Wellik et al., 2002), consistent with *Hoxd11* regulation of a metanephric mesenchymal program. The differential results between the two IM drivers likely reflect the early activation from the Osr1-GCE driver allele. In contrast to the *Six2* findings, we did not observe activation of *Gdnf* (Figs. 4A, B) using the Osr1-GCE driver allele. In an attempt to increase the levels of Hoxd11, we intercrossed Osr1-GCE;R26Hoxd11 males with R26Hoxd11 homozygous females and examined E10.5 embryos harboring the Osr1-GCE allele and two R26Hoxd11 alleles, but found no evidence of ectopic branching of the ND (data not shown). Thus, *Hoxd11* may not be sufficient for full programming of a metanephric pathway (see Discussion).

Ectopic Hoxd11 activity alters the morphology and differentiation of mesonephric tubules

Hoxd11 expression is normally down-regulated as metanephric tubules develop (Patterson and Potter, 2004). In order to ensure that metanephric tubule development occurs normally in the presence of constitutive *Hoxd11* expression from the *ROSA26* locus, we examined the expression of *Slc12a1*, *Barttin*, *Calb3*, *Clck1a* and *Slc12a3* in the E15.5 metanephros following activation of the R26Hoxd11 allele. All tubule markers were detected (data not shown), thus deregulated *Hoxd11* expression does not alter, in an obvious way, induction or subsequent development of the metanephric tubules.

To examine the affect of ectopic Hoxd11 activity in the mesonephros, we used *Pax2* expression to assess the morphology of E13.5 mesonephric tubules in Osr1-GCE;R26Hoxd11 embryos. While the overall number and spatial arrangement of mesonephric tubules of both males and females is relatively normal, mesonephric tubules of Osr1-GCE;R26Hoxd11 embryos exhibit an altered, more elaborate morphology as compared to control littermates at E13.5 (Figs. 5A, B). Further, *Barttin*, whose expression is absent in control embryos at E13.5, was maintained in transgenic tubules (Figs. 5C, D arrowhead). Additionally, *Calb3* (Figs. 5E,F arrowhead) and *Clck1a* (Figs. 5G, H arrowhead), which are never expressed in mesonephric structures, were activated upon *Hoxd11* expression. No *Slc12a3* expression was observed (Figs. 5I, J arrowhead). *Pax2* expression in the E12.5 mesonephros of Rarb-Cre;R26Hoxd11 and Osr1-GCE;R26Hoxd11 reveals a similarly altered morphology (Figs. 6A–C). Embryos of both genotypes express *Barttin* (Figs. 6D–F arrowheads) and *Calb3* (Figs. 6G–I arrowheads), but not *Clck1a* at this time (Figs. 6J–L arrowheads). Identical results were obtained in the mesonephros of E12.5 embryos harboring the Osr1-GCE allele and two R26Hoxd11 alleles (data not shown). Thus, mesonephric tubules expressing *Hoxd11* activate a transcriptional program reflective of a partial metanephric conversion.

Discussion

Mesonephric and metanephric tubules utilize identical inductive and early developmental programs

The cellular origin of mesonephric and metanephric tubules has been a matter of debate. Sainio et al. (1997) have suggested that cranial mesonephric tubules are derivatives of the ND, whereas the caudal mesonephros forms due to a MET of the mesonephric mesenchyme, implying a distinct, regional specific origin of tubular structures. Our fate mapping analysis solves this issue. The ND only

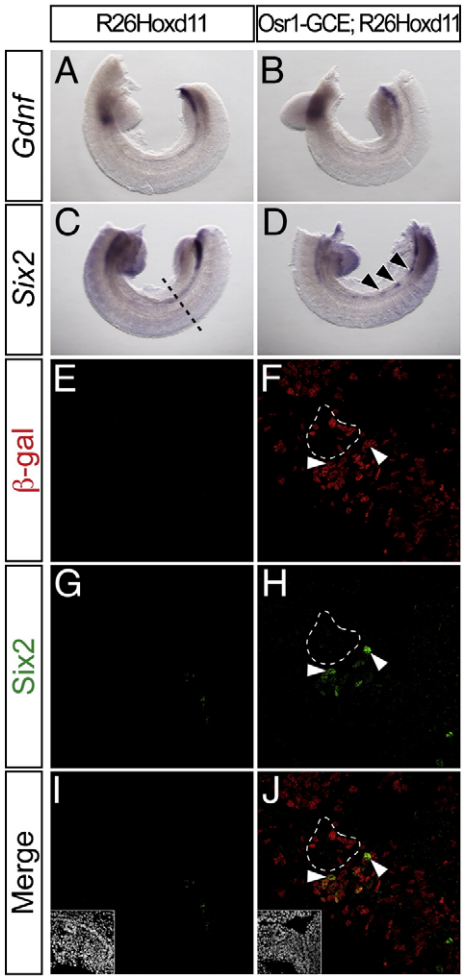

Fig. 4. *Hoxd11* activates cell-autonomous activation of Six2, but not *Gdnf*, in mesonephric mesenchyme. (A–D) Whole mount *in situ* hybridization for *Gdnf* (A, B) and *Six2* (C, D) in E10.5 control (A, C) or Osr1-GCE;R26Hoxd11 (B, D) embryos. Arrowheads (D) indicate sites of ectopic *Six2* expression. Dashed line (C) indicates approximate plane of section (E–L). (E–L) Immunofluorescent confocal microscopy of transverse sections of the mesonephros in E10.5 control (E, G, I) or Osr1-GCE; R26HoxD11 (F, H, J) embryos stained for β-gal and Six2. Arrowheads (F, H, J) indicate examples of β-gal and Six2 co-localization. White dashed line (F, H, J) indicates ND epithelia positive for β-gal, but not Six2. Insets (I, J) indicate nuclei.

contributes to a connecting segment of a cranial mesonephric tubule, whereas the remainder of the tubule is derived from the mesonephric mesenchyme. The connecting segment is a mosaic of ND and mesonephric mesoderm-derived cell types, whereas the remainder of the mesonephric tubule is entirely derived from mesenchymal progenitors. Thus, as in metanephric nephrons, all mesonephric tubules derive from a MET within mesenchyme adjacent to the ND.

Previous studies have demonstrated that *Wnt9b* induces the metanephric mesenchyme to form tubules via the canonical Wnt pathway (Carroll et al., 2005; Park et al., 2007) and mesonephric tubules are absent from *Wnt9b* mutants (Carroll et al., 2005). Thus, it is likely that the mesonephric mesenchyme is also induced to undergo MET by a similar inductive events. That *Wnt4* and *FGF8* are also downstream of *Wnt9b* in both mesonephric and metanephric tubulogenesis lends additional evidence for a common program of tubule formation within distinct regions of the IM (Grieshammer et al., 2005; Perantoni et al., 2005; Stark et al., 1994; Vainio et al., 1999). Further, these findings support the hypothesis that the differences observed between the mesonephros and metanephros are due to a pre-pattern of the IM mesenchyme rather than regionally distinct inductive signals that govern distinct tubule differentiation programs.

Interestingly, mutants in *WT1* (Sainio et al., 1997) and *Osr1* (James et al., 2006) are reported to develop cranial, but not caudal mesonephric tubules. However, these studies fail to distinguish between the connecting segment and the more distal regions of the cranial tubule. In both mutants, the morphology of the cranial

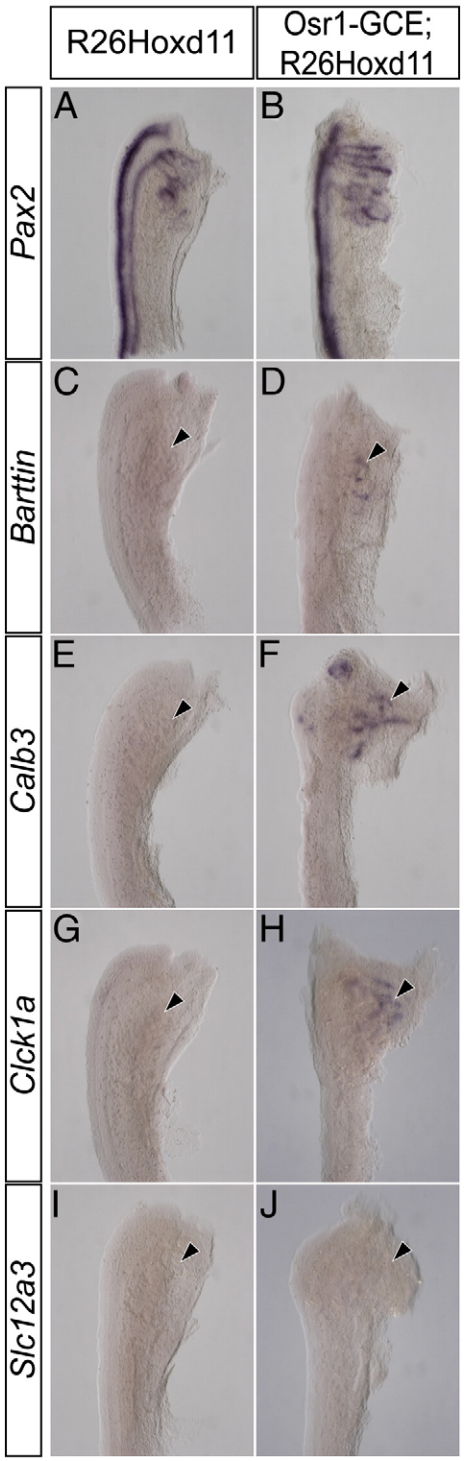

Fig. 5. Mesonephric tubules expressing Hoxd11 have a morphology and gene expression reminiscent of metanephric tubules. Whole mount *in situ* hybridization for *Pax2* (A, B), *Barttin* (C, D), *Calb3* (E, F), *Clck1a* (G, H) and *Slc12a3* (I, J) in the E13.5 mesonephros of control (A, C, E, G, I) or Osr1-GCE;R26HoxD11 (B, D, F, H, J) embryos. Arrowheads indicate location of mesonephric tubules.

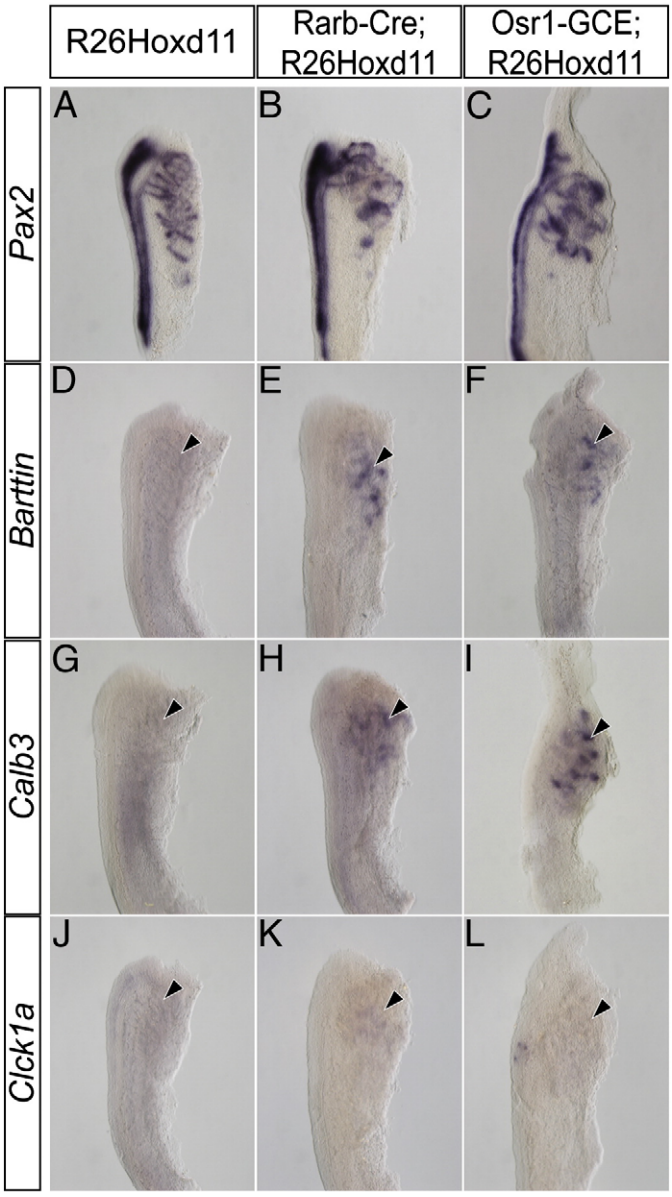

Fig. 6. *Hoxd11* activity in the mesonephros alters the morphology and gene expression of E12.5 mesonephric tubules. Whole mount *in situ* hybridization for *Pax2* (A–C), *Barttin* (D–F), *Calb3* (G–I), and *Clck1a* (J–L) in the E12.5 mesonephros of control (A, D, G, J), Rarb-Cre;R26HoxD11 (B, E, H, K) or Osr1-GCE;R26HoxD11 (C, F, I, L) embryos. Arrowheads indicate location of mesonephric tubules.

tubules is grossly abnormal. Thus, the remaining cranial mesonephric tubules observed in these mutants may actually represent arrested ND outgrowths that lack any mesenchymal contribution. Cell fate analysis in the context of each mutant would shed light on this issue.

Our analysis demonstrates that mesonephric and metanephric tubules express many of the same genes. Interestingly, though mesonephric tubules are reported to lack metanephric structures, notably the loop-of-Henle (Vize et al., 2002), mesonephric tubules do express markers such as *Barttin* and *Brn1*, that are present in the loop-of-Henle (Nakai et al., 2003; Wolf et al., 2003). In contrast, mesonephric tubules do not express *Calb3*, *Clck1a* and *Slc12a3*, markers of connecting tubules, ascending thin loop-of-Henle, and distal convoluted tubules, respectively (Chen et al., 2006; Nakai et al., 2003; Wolf et al., 2003). Thus, mesonephric and metanephric tubules differ largely with respect to distal segment markers.

The Hox11 paralogs specify the metanephric mesenchyme

Remarkably, the *Hox11* paralogs are unique in displaying metanephric mesenchyme specific expression prior to metanephric tubulogenesis. Consistent with roles in the development of both kidney types, *Osr1*, *Pax2*, *WT1*, *Sall1*, *Eya1* and *Six1* are all expressed, at least briefly, in both the mesonephros and later, with the exception of *Six1*, in the metanephric mesenchyme throughout metanephric kidney development. Indeed, mutants in *Osr1*, *Pax2*, *WT1*, *Sall1*, and *Six1* all display mesonephric and metanephric phenotypes (Grote et al., 2006; James et al., 2006; Kobayashi et al., 2007; Narlis et al., 2007; Nishinakamura et al., 2001; Sainio et al., 1997; Torres et al., 1995; Wang et al., 2005; Xu et al., 2003). The role of *Eya1* is less clear. A recent report suggests that *Eya1* specifies the metanephros (Sajithlal et al., 2005) as *Eya1* mutants lack metanephric kidneys (Xu et al., 1999), but mesonephric tubules are present at E10.5. However *Hoxd11* is expressed in *Eya1* mutants (Gong et al., 2007), thus regional specification of the IM is intact in *Eya1* mutants. Interestingly, *Eya1* expression remains in *Hox11* mutants, suggesting that *Eya1* has a more general role in IM nephric development that is at least partially independent of *Hox11* paralog activity.

Consistent with *Hox11* specification of the metanephros, our results demonstrate that ectopic Hoxd11 activity in the mesonephros is sufficient to drive ectopic expression of a key regulator, *Six2*, in mesonephric IM mesenchyme. Several additional lines of evidence support a likely role for *Hox11* regulation of *Six2*. *Six2* expression is absent in *Hox11* mutants (Wellik et al., 2002). Further, a recent report demonstrates that Pax2, Eya1, and Hoxa11 can synergistically activate a reporter under the control of a *Six2* enhancer element in MDCK cells (Gong et al., 2007). Our results demonstrate that Hoxd11 activity in the E10.5 mesonephric mesenchyme activates *Six2* in a cell-autonomous manner. Furthermore, *Six2* expression in the mesonephros was only observed in mesenchymal cells and not in β-gal$^+$ epithelial cells of the ND or in mesonephric tubules. Thus, some other mesenchymally contributed factors must ensure an appropriate cell type specific response in conjunction with Hoxd11.

In the metanephros, Six2 is down-regulated as the cap mesenchyme epithelializes to form nephron precursors, renal vesicles (Self et al., 2006). We have demonstrated that mesonephric tubules are derivatives of the mesonephric mesenchyme. The presence of β-gal$^+$, Six2$^-$ cells within mesonephric tubules indicates that these cells also down-regulate *Six2* expression upon epithelialization. This likely explains why Rarb-Cre;R26Hoxd11 embryos do not activate *Six2* as Rarb-Cre is not active until mesonephric mesenchyme induction is underway. Taken together, these results demonstrate that Hoxd11 activates *Six2*, potentially directly, and that the addition of Hoxd11 activity in cells of the mesonephric mesenchyme causes these cells to behave in a similar manner to metanephric cap mesenchyme maintaining *Six2* activity. *Six2*, in turn, ensures the continued expression of a metanephric nephron progenitor population (Self et al., 2006).

Importantly, the morphology and onset of metanephric marker expression in mesonephric tubules expressing Hoxd11 mimics that of metanephric tubules. During metanephric development, *Calb3* and *Barttin* expression precede that of *Slc12a3* and *Clck1a* by one day. The same timing is observed in developing Hoxd11$^+$ mesonephric nephrons. Though *Slc12a3* was not detected in mesonephric tubules expressing Hoxd11, it is possible that these tubules are still receiving sex-specific molecular cues from the adjacent gonads. Gonad derived signals could compete with the metanephric program activated by Hoxd11 and not allow for the completion of a bona fide metanephric tubule program. Furthermore, the E12.5 mesonephric tubules of Osr1-GCE;R26Hoxd11 and Rarb-Cre;R26Hoxd11 embryos express identical metanephric markers. This suggests that the metanephric tubule program activated by *Hoxd11* is independent of *Six2* function since *Six2* is not ectopically activated in Rarb-Cre;R26Hoxd11 embryos. This indicates that the *Hox11* paralogs simultaneously activate two

separate and independent molecular programs in the metanephric mesenchyme. The activation of Six2 allows for the maintenance of the cap mesenchyme, while an independent program confers a metanephric-specific response downstream of common inductive events.

While ectopic *Hoxd11* expression can result in several features of a metanephric program, we do not observe a whole scale conversion of the mesonephric region into a metanephric kidney. There may be many underlying reasons for this result. For example, the kinetics and mosaicism of *Hoxd11* activation by Osr1-GCE may activate *Hoxd11* too late and in too few cells for a global change. Alternatively, the levels of Hoxd11 may be insufficient; however when Hoxd11 dosage was increased by activating two copies of R26Hoxd11, we did not observe an enhancement of the mesonephric to metanephric, thus suggesting that this explanation is unlikely. Further, the roles of the *Hox11* paralogs may not be simply additive. One critical factor in the failure of a more extensive metanephric conversion is likely to be the failure of *Gdnf* activation in Hoxd11^{+} mesonephric mesenchyme.

Gdnf expression is absent in the metanephric mesenchyme of *Hox11* mutants, suggesting that *Gdnf* is a likely target of *Hox11* action (Wellik et al., 2002). Additionally, Pax2, Eya1 and Hoxa11 can synergistically activate a reporter gene under the control of a *Gdnf* promoter element in MDCK cells (Gong et al., 2007). At the initiation of metanephric development, *Gdnf* expression must be tightly controlled in order to restrict ND invasion to a specific location in the metanephric mesenchyme. *Gdnf* activation in the metanephros requires *Osr1*, *Pax2*/*Pax8*, *Eya1*, *Six1* and *Hox11* paralog function (James et al., 2006; Kobayashi et al., 2007; Narlis et al., 2007; Sajithlal et al., 2005; Wellik et al., 2002; Xu et al., 2003). However, in addition to these activating factors, *FoxC1* activity (Kume et al., 2000) and *Robo2*/*Slit2* signaling (Grieshammer et al., 2004) are also required to restrict *Gdnf* to the metanephric mesenchyme. Hoxd11 activity in mesonephric mesenchyme may be insufficient to override the negative regulation of *FoxC1* or *Robo2/Slit2* signaling *in vivo*, whereas these negative signals may not be present in MDCK cell culture.

It is tempting to speculate that the development of the metanephric kidney requires the convergence of parallel molecular pathways upon the posterior IM. In this model, the early IM is specified by genes such as *Osr1*, *Pax2, WT1* and *Eya1. Hox11* paralog expression in the posterior IM mesenchyme functions with *Pax2* and *Eya1* to activate *Gdnf* and *Six2*, allowing for ND branching and self-renewal of nephron progenitors, respectively. *Gdnf* expression is restricted to a single site by *FoxC1* and *Robo2*/*Slit2* signaling, providing the exact location for ND invasion into the metanephric mesenchyme. Simultaneously, the *Hox11* paralogs regulate the metanephric-specific tubule response of the induced cap mesenchyme, resulting in the formation of an appropriately organized nephron structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.03.044.

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