Developmental Biology 385 (2014) 122–135

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Parallel waves of inductive signaling and mesenchyme maturation regulate differentiation of the chick mesonephros

Sharon Soueid-Baumgarten, Ronit Yelin, Etty K. Davila, Thomas M. Schultheiss *

Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel

ARTICLE INFO

Article history:
Received 29 April 2013
Received in revised form
19 September 2013
Accepted 20 September 2013
Available online 30 September 2013

Keywords:
Kidney
Mesonephros
Chick embryo
Wnt signaling
Induction

ABSTRACT

The mesonephros is a linear kidney that, in chicken embryos, stretches between the axial levels of the 15th to the 30th somites. Mesonephros differentiation proceeds from anterior to posterior and is dependent on signals from the nephric duct, which migrates from anterior to posterior through the mesonephric region. If migration of the nephric duct is blocked, markers of tubule differentiation, including Lhx1 and Wnt4, are not activated posterior to the blockade. However, activation and maintenance of the early mesonephric mesenchyme markers Osr1, Eya1 and Pax2 proceeds normally in an anterior-to-posterior wave, indicating that these genes are not dependent on inductive signals from the duct. The expression of Lhx1 and Wnt4 can be rescued in duct-blocked embryos by supplying a source of canonical Wnt signaling, although epithelial structures are not obtained, suggesting that the duct may express other tubule-inducing signals in addition to Wnts. In the absence of the nephric duct, anterior mesonephric mesenchyme adjacent to somites exhibits greater competence to initiate tubular differentiation in response to Wnt signaling than more posterior mesonephric mesenchyme adjacent to unsegmented paraxial mesoderm. It is proposed that mesonephric tubule differentiation is regulated by two independent parallel waves, one of inductive signaling from the nephric duct and the other of competence of the mesonephric mesenchyme to undergo tubular differentiation, both of which travel from anterior to posterior in parallel with the formation of new somites.

Introduction

Vertebrate kidney tissue is derived from the intermediate mesoderm (IM), a region of mesoderm located lateral to the somites (James and Schultheiss, 2003; Saxen, 1987). Amniote vertebrates form three types of kidney tissue during embryonic development: the pronephros, the mesonephros, and the metanephros. The first to form and most anterior is the pronephros. The pronephros does not form many kidney tubules and is of doubtful functionality in many amniote species, but it is essential because it is the source of the nephric duct, which forms in the pronephric region and subsequently extends into the mesonephric and metanephric regions, where it forms the drainage system for those kidneys.

The nephric duct also plays an essential role in the induction of kidney tubules in the mesonephros and metanephros. The most well-studied model for investigating kidney induction has been the formation of tubules in the mammalian metanephros (Dressler, 2002, 2009; Yu et al., 2004), and indeed the mammalian metanephros is one of the best-established experimental systems for studying the general phenomenon of embryonic induction (Grobstein, 1955; Saxen et al., 1968). If cultured alone, metanephric mesenchyme (MM) will not undergo tubular differentiation and quickly degenerates. However, if cultured together with the ureteric bud (UB), a branch of the nephric duct, the UB and the MM undergo mutual inductive interactions such that the UB undergoes branching to form the collecting system of the kidney and the MM undergoes localized aggregation and mesenchymal to epithelial transition (MET) to form tubules (Cho and Dressler, 2003; Costantini and Kopan, 2010; Dressler, 2006). Eventually the MM-derived tubules and the UB-derived collecting system connect to each other to form a tree-like arrangement of functional nephrons.

Wnt signaling has been found to be necessary and sufficient for the induction of tubules from the MM. Several Wnts, including Wnt9b and Wnt11 are expressed in the nephric duct and the branching UB (Carroll et al., 2005; Qian et al., 2003). Wnt9b knockout mice fail to undergo tubule formation and fail to activate the earliest genes associated with MET, including Wnt4 and Pax8 (Carroll et al., 2005). In addition Wnt4, which is expressed in tubule precursor structures, is required for further nephron development (Stark et al., 1994). Supplying exogenous Wnt signals can rescue tubule formation in isolated MM (Herzlinger et al., 1994; Park et al., 2007).

Despite the significant progress that has been made in understanding kidney induction using the MM–UB induction model, there are some limitations to this experimental system. Perhaps most significantly, it is not possible to identify and isolate the MM

* Corresponding author. Fax: +972 4 8535514.
E-mail address: tschulth@tx.technion.ac.il (T.M. Schultheiss).

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.09.026

before it has begun to interact with the UB (Sainio, 2003). Thus it is not clear whether transient interactions with the UB influence the expression of genes including Pax2, Eya1, Six2, and Osr1, which are activated before the initiation of tubule formation and are essential for subsequent metanephros development (Dressler et al., 1990; James and Schultheiss, 2005; Kalatzis et al., 1998; Ohto et al., 1998; Sajithlal et al., 2005; So and Danielian, 1999; Xu et al., 1999, 2003). Evidence that the nephric duct and the UB may not be required for the expression of at least some early kidney genes comes from Gata3−/− mice, which express Pax2 in the metanephros despite defects in nephric duct migration and UB formation (Grote et al., 2006). In Wnt9b knockout mice, early metanephric markers are expressed (Carroll et al., 2005), but that does not exclude a possible inductive role for other signals originating in the UB.

The mesonephros is formed earlier and more anteriorly than the metanephros and constitutes the main fetal kidney in amniotes and the adult kidney in non-amniote vertebrates. Although less studied than the metanephros, the mesonephros has some experimental advantages for studying kidney induction. In the avian embryo it is possible to completely block interaction of the nephric duct with the mesonephric mesenchyme in vivo. Such classic studies have found that mesonephric tubules do not form in the absence of the nephric duct (Gruenwald, 1937; Waddington, 1938). However the response of the mesonephric mesenchyme to the nephric duct has not been analyzed at the molecular level, and thus it is not clear whether there are molecular aspects of mesonephric induction that are not duct-dependent. In addition, the avian mesonephros is a simple linear organ consisting of several tubules per embryonic segment that differentiates in a regular anterior-to-posterior sequence during development. This feature, together with the fact that the nephric duct migrates through the mesonephric region from anterior to posterior at a regular pace, offers the opportunity to study the relationship between the timing of the nephric duct's arrival at a particular axial level within the mesonephros and the expression of mesonephric genes.

The current study investigates the early events of tubule induction in the chick mesonephros. Using a set of molecular markers, we find that, with one interesting exception, gene expression in the chick mesonephros is very similar to that in the mammalian metanephros, thus making the avian mesonephros a good model for kidney induction in general. By blocking interaction between the nephric duct and the mesonephric mesenchyme in vivo, it was possible to distinguish between a set of genes, including Wnt4 and Lhx1, that requires interaction with the nephric duct, and a group of genes, including Osr1, Eya1, and Pax2, whose expression is not duct-dependent. Both in vivo and in an explant culture system, canonical Wnt signaling could partially rescue duct-dependent differentiation events, validating a role for Wnt signaling in mesonephric differentiation but also suggesting that non-Wnt factors supplied by the duct may be important for normal mesonephros induction. Finally, evidence is presented indicating that, in parallel to the anterior-to-posterior migration of the nephric duct, the mesonephric mesenchyme independently undergoes an anterior-to-posterior acquisition of competence to undergo tubular differentiation. Thus mesonephros differentiation appears to require both inductive signaling from the duct and, independently, a process of maturation within the mesonephric mesenchyme that renders it competent to undergo tubulogenesis.

Materials and methods

Embryo culture

Fertile White Leghorn chick eggs were incubated at 38.5 °C in a humidified incubator and staged according to Hamburger and Hamilton (1951). A Whatman-paper ring was placed on top of the embryos, and embryos were excised together with a portion of the attached vitelline membrane, as previously described (James and Schultheiss, 2003). Embryos designated for whole mount in situ staining were fixed overnight in 4% PFA. Embryos designated for surgical manipulation were placed dorsal side up on an agar–albumin 35 mm culture dish.

Duct barrier – blockage insertion

A thin aluminum foil sheet was placed between two layers of parafilm and small rectangles (about 1.5 mm × 3 mm) were cut using a scalpel knife. A small crosswise cut was made on one side of stage 9–10 embryos (cultured dorsal-side up) at the axial level of about two somites after the most posterior somite using a thin tungsten needle (EMS, 0.005 in. diameter). The barrier, isolated from the parafilm slices, was located close to the cut and gently pushed to its proper location using a thicker tungsten needle (EMS, 0.01 in. diameter). Embryos were cultured in a 38.5 °C humidified incubator.

DiI injection

DiI was diluted in 0.3 M sucrose to a final concentration of 0.1 μg/μl and was injected into embryos using a glass capillary (Drummond, 1.0 mm outer diameter, with a 20 μm diameter tip). The needle was attached to a Sutter manual microinjector through a flexible capillary containing mineral oil (Sigma) as a mediator. DiI was injected three to four times into the region of the nephric duct primordium (adjacent to somites 8–10).

Cell pellet preparation and injection

Rat fibroblast cells transfected with Wnt1 (Herzlinger et al., 1994) or empty vector were cultured in DMEM containing 4.5 g/ml glucose, 10% FBS, 2 mM Glutamine, 50 μg/ml PenStrep and 400 μg/ml G418 (Geneticin) in a humidified incubator at 37 °C, 5% CO_2. Prior to pellet preparation, the cells were stained for 15 min at 37 °C with 1 μg/ml DiI in PBS; washed twice with PBS; trypsinized and re-suspended in growing medium. Aliquots of 600 cells per 20 μl medium were transferred into small PCR tubes and incubated for 24 h at 37 °C. The stained cells were centrifuged for 10 min at full speed in a tabletop mini-centrifuge and incubated for an additional 24 h. For pellet injections, a small crosswise cut was made on one side of the embryos at the desired location using a thin tungsten needle. The cell pellet suspended in growing medium was located close to the cut utilizing a mouth-pipette and gently pushed to its proper location using a thicker tungsten needle. Embryos were cultured in a 38.5 °C humidified incubator.

Bio injection

Bio (6-bromoindirubin-3′-oxime, Calbiochem) was diluted in DMSO to a final concentration of 2 mM and injected into duct-blocked embryos posterior to the barrier using a pulled glass capillary (20 μm tip diameter) via a Picospritzer III pressure injector (General Valve Corp.) at settings pressure = 10 psi, duration = 20 ms. Bio was injected at several locations along the embryo starting posterior to the blockage until the caudal end of the embryo. DMSO was injected as a control. 1 μg/ml DiI was added to both Bio and DMSO for visualization of the injected substance.

Explant culture

Isolated embryos at the stage of interest were pinned to a silicone dish dorsal side up and tissues were dissected with a micro-scalpel (Micro Feather). Removed explants were transferred with a P20 pipette to a 10 cm culture dish cover containing 20 μl medium drops and maintained on ice. A Millicell filter (Millipore) was gently laid on growth medium: DMEM 4.5 g/ml glucose; 10% FBS; 2% chick extract; 2 mM Glutamine, 50 μg/ml PenStrep. Alternatively, for low-serum conditions, the FCS and chick extract percentage was reduced to 1% FBS and 0.2% chick extract. The explants were transferred to the filter and incubated at 37 °C for 48 h. In some cultures Bio (50 μM) or IWR-1-endo (50 μM) was added to growth medium.

In situ hybridization

Whole-mount in situ hybridization was performed as described (Schultheiss et al., 1995), using probes to chick Eya1 (MRC Gene Service Clone ChEST843e6, Genbank BU315153), Lhx1 (Tsuchida et al., 1994), Nkx-2.5 (Schultheiss et al., 1995), Osr1 (James and Schultheiss, 2005), Pax2 (Burrill et al., 1997), cRet (Schuchardt et al., 1995), Sim1 (Obara-Ishihara et al., 1999), Six2 (MRC Gene Service Clone ChEST70o11, Genbank BU131501), Wnt4 (MRC Gene Service Clone ChEST843e6, Genbank BU315153), Wnt9b (this paper), and WT1 (Kent et al., 1995). For Wnt9b, an 850 bp fragment was amplified from day 6 chick mesonephros cDNA using the primers 5′-CGTGAAGCAGTGTGCCTTGC-3′ and 5′-TTCTTCCTGCATGCACTGTT-3′ and cloned into pGEMMT-Easy (Promega). Following development, 20 μm cryostat sections were cut on gelatin-embedded embryos and examined and photographed on a Zeiss Axioimager M1 microscope with DIC optics and a Qimaging ExiBlue digital camera with RGB filter.

Analysis of apoptosis

Duct migration was blocked by insertion of foil barriers on one side of Stage 9 embryos as described above. Embryos were cultured for 48 h (until approximately Stage 17) and then fixed, processed and sectioned as described (James and Schultheiss, 2005). Slides were washed in PBS for 15 min at 37 °C and stained with primary antibodies to activated Caspase3 (CM1, Promega, 1:100) and Lhx1 (4F2, DSHB, 1:10) followed by the secondary antibodies Cy3-conjugated affinity purified Donkey-anti-rabbit Fab and Dylight488 affinity purified Donkey-anti-mouse Fab (Jackson Immunoresearch). Slides were mounted and coverslipped with fluorescent mounting medium (Dako) and photographed using a Zeiss Axioimager microscope and Qimaging ExiBlue monochrome camera. Imaris imaging software (Bitplane) was used to trace the mesonephric mesenchyme in photographed sections, which was defined as a triangular region bounded by the nephric duct laterally, the aorta medially, and the line between the dorsal-most edges of the nephric duct and aorta dorsally. Within the defined region, the spot detection module was used to count nuclei on the Dapi channel and apoptotic cells on the activated Caspase3 channel. Statistical analysis was performed with the Intercooled Stata package of statistical software (Stata Corporation), using the two-sample test of proportions.

Results

Characterization of normal gene expression patterns during mesonephros formation

The chick mesonephros develops in the intermediate mesoderm (IM) at the axial level of approximately somites 15–30, with the bulk of the organ located at axial levels 20–30 (Hamilton, 1952). The mesonephros, like the metanephros, is comprised of cells with different origins. In particular, the tubular component of the mesonephros derives from cells that differentiate in place from the intermediate mesoderm of the axial level of somites 15–30. In contrast, the drainage component of the mesonephros is derived from the nephric duct, which forms from intermediate mesoderm adjacent to somites 8–10, and subsequently extends posteriorly into the mesonephric region (Attia et al., 2012; Obara-Ishihara et al., 1999; Schultheiss et al., 2003). In order to obtain a molecular picture of chick mesonephros differentiation, in situ hybridization was performed for a set of genes known to be important for kidney formation in other vertebrates.

Markers of the undifferentiated IM

In the mouse metanephros, Osr1, Eya1, Pax2, and Six2 are all expressed in undifferentiated metanephric mesenchyme, with Osr1, Eya1, and Six2 being down-regulated as nephron formation is initiated (Dressler et al., 1990; James and Schultheiss, 2005; Kalatzis et al., 1998; Ohto et al., 1998; Sajithlal et al., 2005; So and Danielian, 1999; Xu et al., 1999, 2003).

In the chick mesonephros, Osr1 is the earliest of these genes to be expressed. Osr1 expression initiates at HH stage 5 (mid-gastrula), lateral to Henson's node (James et al., 2006). In stage 11 embryos (13 somites), Osr1 is expressed throughout the intermediate mesoderm and medial part of the lateral plate, from the axial level of the first somite until the primitive streak (Fig. 1A). Unlike other genes examined in this study (see below) Osr1 is not activated in an anterior-to-posterior wave that parallels the appearance of new somites, but extends throughout the length of the IM already from Stage 9. As kidney differentiation proceeds, Osr1 expression is down-regulated anteriorly, while it remains expressed at high levels in more posterior, undifferentiated regions of the IM (James et al., in press) (Fig. 1B). On sections taken from two different axial levels of the same embryo, it can be seen that Osr1 is expressed in undifferentiated IM (Fig. 1C). When tubule formation is initiated, Osr1 is down-regulated in the regions adjacent to the nephric duct where tubule formation has initiated, but is maintained in the neighboring non-differentiated IM (Fig. 1D).

Eya1 (Kalatzis et al., 1998; Sajithlal et al., 2005; Xu et al., 1999) is expressed in the intermediate mesoderm and in the dermamyotome of the somite (Fig. 1E–H). It is activated in an anterior-to-posterior sequence, with the posterior-most border of expression typically located approximately 4 somite-lengths posterior to the last somite (Fig. 1E). Similar to Osr1, Eya1 is expressed in undifferentiated IM and is down-regulated upon the initiation of tubule differentiation (Fig. 1F–H).

Transcriptional activation of the transcription factor Pax2 (Dressler et al., 1990) occurs in two stages. Initially, Pax2 is expressed weakly throughout the IM from the axial level of the sixth somite until the posterior end of the embryo (Fig. 1I and J caret). Subsequently, Pax2 is up-regulated in an anterior-to-posterior sequence, with the posterior-most border of strong expression located at an axial level approximately 2–3 somite-lengths posterior to the most newly-formed somite (Fig. 1I and J arrow). Unlike Osr1 and Eya1, Pax2 is expressed in both the nephric duct and the nephrogenic mesenchyme (Fig. 1K). The anterior-to-posterior up-regulation of Pax2 seen in whole mount situ hybridization is partially due to posterior extension of the nephric duct (the posterior limit of which is located approximately 2–3 somite-length posterior to the most posterior somite), but also due to up-regulation of Pax2 expression in the mesonephric IM itself (Fig. 1K). Also, unlike Osr1 and Eya1, Pax2 expression is maintained upon differentiation and can be detected in the

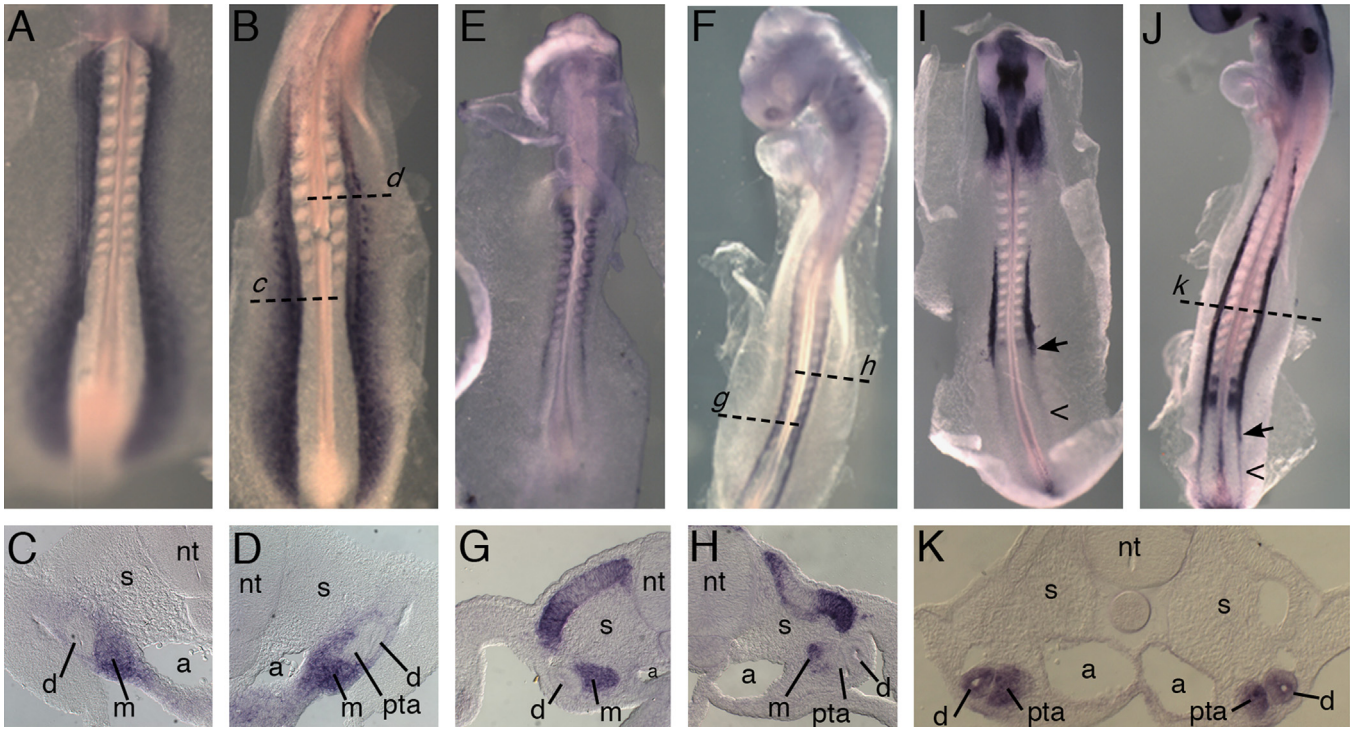

Fig. 1. Gene expression during chick mesonephros formation: early genes. In situ hybridization for Osr1 (A–D), Eya1 (E–H), and Pax2 (I–K) in Stage 11 (A, E, and I) and Stage 15 (B–D, F–H, J–K) chick embryos. C, D, G, H, and K are sections taken at the approximate levels indicated by the dotted lines in B, F, and J. All three genes are expressed in the undifferentiated mesonephric mesenchyme (m) adjacent to the nephric duct (d) (C, G, and K), while Pax2 is also expressed in the nephric duct (K). As differentiation initiates in the mesonephric mesenchyme, Osr1 (D) and Eya1 (H) are down-regulated in forming pretubular aggregates, while Pax2 expression (K) is maintained. In I and J, note that strong Pax2 staining (arrow) extends posteriorly until somewhat posterior to the last formed somite, while weaker staining extends posterior to that point (caret). a, Aorta; d, nephric duct; m, mesonephric mesenchyme; pta, pretubular aggregate; nt, neural tube; s, somite.

nephric duct and developing tubules of the mesonephros (Fig. 1K). Pax2 is also expressed transiently in a cyclical pattern in two stripes in the most anterior part of the paraxial mesoderm, a pattern that is associated with the formation of new somites (Fig.1I and J) (Suetsugu et al., 2002).

The expression of Osr1, Eya1, and Pax2 in undifferentiated chick mesonephric IM and the down-regulation of Osr1 and Eya1 upon the initiation of nephron formation, are similar to the dynamics of the expression of these genes in the mouse metanephros (Dressler et al., 1990; James et al., in press; Kalatzis et al., 1998; So and Danielian, 1999; Xu et al., 1999). Interestingly Six2, which is expressed in the undifferentiated mouse MM and plays an important role in regulating nephron differentiation in the metanephros (Self et al., 2006), was found to be expressed at very low levels and without a distinct expression pattern in the chick mesonephros (Supplementary Fig. S1A).

Markers of the differentiating IM

In the mammalian metanephros, Wnt4 is one of the earliest markers of the initiation of tubule differentiation in response to ureteric bud induction (Carroll et al., 2005; Park et al., 2007; Stark et al., 1994). In the chick mesonephros, Wnt4 expression is first detected at Stage 15–16, appearing as a thin line in the intermediate mesoderm, extending in an anterior-to-posterior sequence from the axial level of somite 15 until 3 to 4 somites anterior to the most newly formed somite (Fig. 2B). On sections, it can be seen that in the mesonephros Wnt4 is expressed in the condensing mesenchyme (Fig. 2C), as in the mammalian metanephros. Strong Wnt4 expression is also seen in the neural tube (Fig. 2C).

The transcription factor Lhx1 (Lim1) is expressed specifically in differentiating epithelial components of the mouse kidney (Shawlot and Behringer, 1995; Tsang et al., 2000). In the chick, Lhx1 expression is seen in the nephric duct as it differentiates from the IM (James and Schultheiss, 2003), and it is activated in the mesonephros as mesenchymal cells begin to differentiate into nephrons (Fig. 2D–F). Lhx1 expression is initiated in tubule precursors slightly after the initiation of Wnt4 expression.

The nephric duct

During the initial stages of mesonephros formation, cRet is a specific marker of the nephric duct (Attia et al., 2012; Pachnis et al., 1993). As seen in Fig. 2G–I, the nephric duct migrates in parallel with the formation of new somites, such that the posterior-most tip of the duct is typically located approximately 3 somite-lengths posterior to the most newly-formed somite.

Based on these descriptive studies, a temporal and spatial portrait was constructed of gene expression during mesonephros formation (Fig. 2J). Because mesonephros differentiation proceeds from anterior to posterior, a snapshot of gene expression at any particular developmental stage gives a picture of the temporal order of mesonephros gene activation, with the expression domains of early-activated genes extending more posteriorly than those of later-activated genes. Osr1 and Pax2 (the early, weak component of Pax2 expression) are expressed throughout the IM. In parallel with the arrival of the nephric duct in the mesonephric region, Eya1 and Pax2 are activated, with Eya1 detectable a few hours before the arrival of the nephric duct, and Pax2 upregulated at approximately the same time as the duct arrives at a particular region of the mesonephros. Subsequently, Wnt4 and then Lhx1 are activated as tubule formation is initiated. This descriptive model is important for designing and interpreting experiments aimed at investigating the mechanisms of mesonephros gene regulation, as described below.

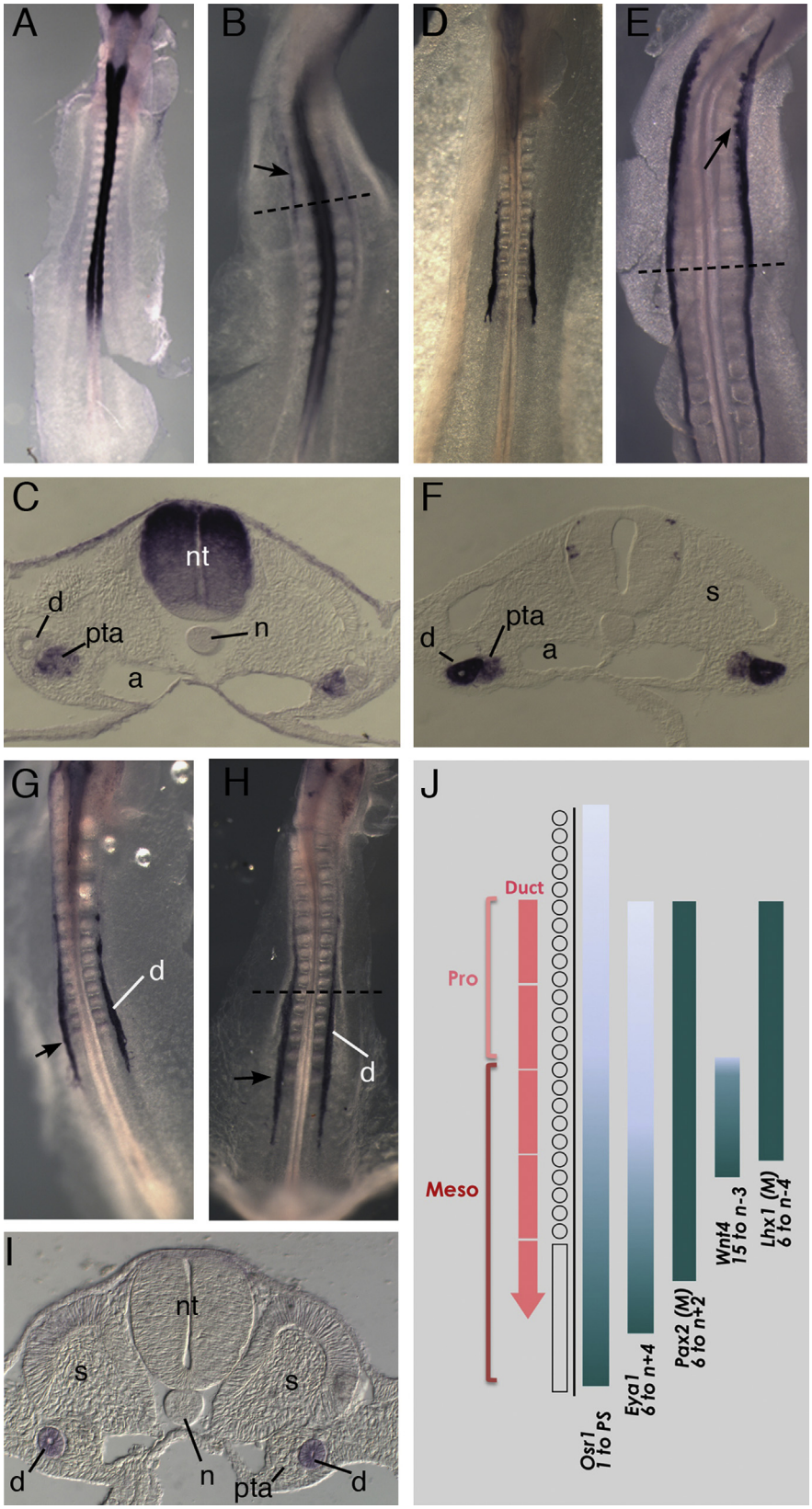

Fig. 2. Gene expression during chick mesonephros formation: markers of differentiation, nephric duct, and summary. (A–F) In situ hybridization for Wnt4 (A–C), and Lhx1 (D–F) in Stage 12 (A and D) and Stage 15 (B, C, E, and F) chick embryos. C and F are sections taken at the approximate levels indicated by the dotted lines in B and E, respectively. Wnt4 expression is absent in the mesonephric region at Stage 12 (A) and at Stage 15 is found in condensing pretubular aggregates (B arrow, C). Lhx1 is expressed in the nephric duct (d in D–F) and is activated in the mesenchyme in condensing pretubular aggregates (E arrow, F). (G–I) In situ hybridization for cRet at Stages 11 (G and I) and 13 (H). cRet is expressed specifically in the nephric duct. Arrows in G,H indicate the most posterior (most recently-formed) somite. The posterior end of the nephric duct is located approximately 3 somite length posterior to the most recently-formed somite in both stages. Dashed line in H indicates approximate axial level of section in I. (J) Summary of expression patterns of mesonephric markers. See text for details. a, Aorta; d, nephric duct; n, notochord; nt, neural tube; pta, pretubular aggregate; s, somite.

Duct-dependent and duct-independent genes during mesonephros differentiation

The gene expression studies of Figs. 1 and 2 found that activation of Eya1, Wnt4 and Lhx1, and upregulation of Pax2, all occur in parallel to the migration of the nephric duct through the mesonephric region. In order to determine whether the nephric duct is required for these events, as well as for the maintenance of Osr1 expression, microsurgical manipulation was conducted to block nephric duct extension into the mesonephric area. Classical studies have found that the chick mesonephros does not develop in the absence of the nephric duct (Gruenwald, 1937; Waddington, 1938). However, those studies reported only on the development of morphologically recognizable nephrons, while expression of mesonephric genes was not examined.

A foil barrier was inserted into Stage 9–10 (7–10 somites) chick embryos in the intermediate mesoderm at the axial level of the future 12th somite (Fig. 3A). Since the nephric duct primordia forms at the axial level of somites 8–10 and begins extending posteriorly at stage 10–11 (Attia et al., 2012), the barrier should block extension of the duct into the region of the mesonephros (which forms at the axial level of somites 15–30) (Fig. 3B). The barrier was inserted into one side of the embryo, with the opposite side serving as a control. In initial experiments, the fluorescent dye DiI was injected into the nephric duct primordia adjacent to somite 10. As can be seen in Fig. 3C, nephric duct migration was blocked by the barrier insertion, a fact that was confirmed by expression of the duct marker Sim1 (Fig. 3D and E).

Osr1 (Fig. 4A and B), Eya1 (Fig. 4C and D), and Pax2 (Fig. 4E and F) expression in the mesonephros were not affected by the duct blockage (Osr1 $N=14$, Eya1 $N=10$, Pax2 $N=7$ embryos). The pattern and timing of gene expression were similar between the control and operated sides. It should be noted that Pax2 is normally expressed in both the duct and mesenchyme. As a result of duct blocking, there is a reduction in Pax2 expression on the blocked side, but this is due to absence of the nephric duct, while mesenchymal expression of Pax2 is maintained. In addition, both the weak posterior and the stronger anterior Pax2 expression patterns were maintained (Fig. 4E), indicating that the upregulation of Pax2 in the mesonephric mesenchyme that occurs approximately two somite-lengths posterior to the most newly-formed somite (at approximately the same location as the posterior tip of the migrating nephric duct) does not in fact depend on signals from the duct. It can also be noted that both mesonephric and somite expression of Eya1 is maintained in duct-blocked embryos (Fig. 4C and D), implying that blockage of the nephric duct does not interfere with somite formation.

In contrast to the duct-independence of Osr1, Eya1, and Pax2, activation of Wnt4 and Lhx1 expression was inhibited in the absence of the nephric duct (Wnt4 $N=8$, Lhx1 $N=12$ embryos). Wnt4 expression was not observed posterior to the barriers (Fig. 4G and H). Since Wnt4 is the earliest known marker of mesonephric tubule differentiation (Carroll et al., 2005; Stark et al., 1994), this indicates that tubule differentiation is not initiated in the absence of the duct. Similarly, Lhx1 expression was absent below the barrier in most duct-blocked embryos (Fig. 4I and J). In some embryos, a few small dots of Lhx1 expression were seen posterior to the barriers (Supplementary Fig. S2), but even in these cases the overall level of Lhx1 expression was greatly reduced compared to the control side. Since Lhx1 is expressed in the duct as well as the tubules, and since Wnt4 expression was not seen posterior to the blockade, these dots of Lhx1 are most likely the progeny of a few duct cells that escaped blockage by the barrier.

Rates of apoptosis in the IM in the absence of the nephric duct

Mouse metanephric mesenchyme cultures undergo apoptosis in the absence of the UB or alternative source of inductive signals

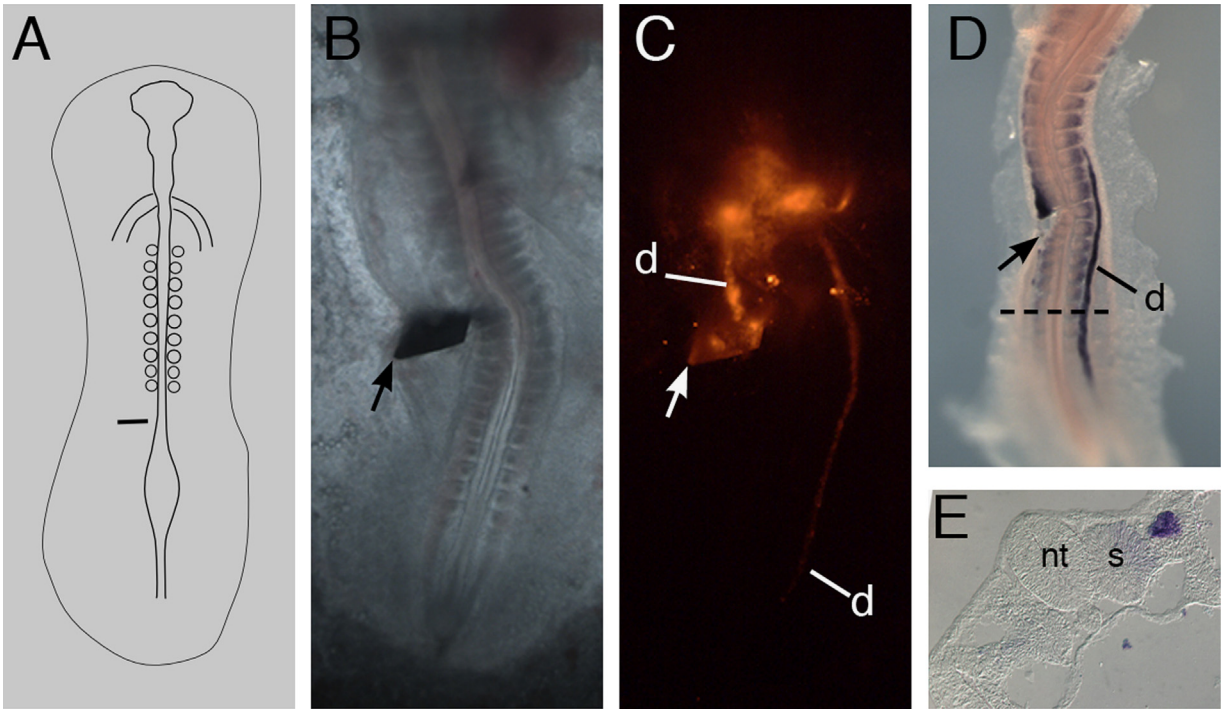

Fig. 3. Blocking of nephric duct migration. (A) Scheme of procedure. A foil barrier was placed posterior to the duct rudiment before the initiation of duct migration. After 48 h, the embryo developed normally (B). (C) DiI was injected into the duct rudiments on both sides prior to insertion of the barrier. After 48 h, the duct on the control (right) side extended normally, whereas the duct on the experimental side (left) was blocked by the barrier. (D and E) Expression of Sim1 in duct-blocked embryo. Sim1 is expressed in the duct and in the lateral border of the somites. On the side of the barrier (left), migration of the duct was blocked, but expression of Sim1 in the lateral somite remained normal. Arrow in B–D indicates position of the barrier. Dotted line in D indicates approximate plane of section in E. d, Nephric duct; nt, neural tube; s, somite.

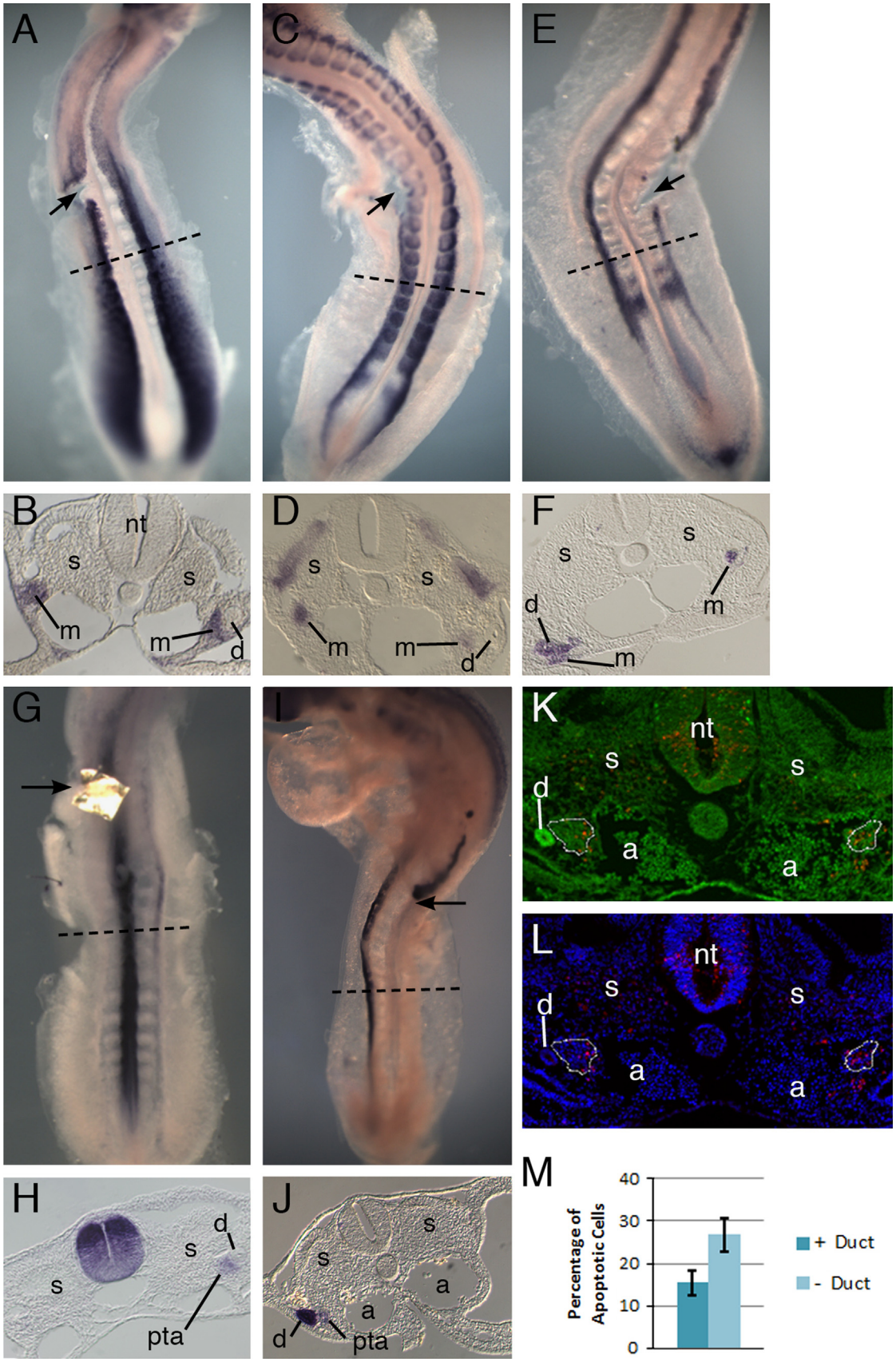

Fig. 4. (A–J) Effect of duct blocking on mesonephric gene expression. Duct extension was blocked on one side of the embryo as depicted in Fig. 3. After 48 h, embryos were analyzed by in situ hybridization for expression of Osr1 (A and B), Eya1 (C and D), Pax2 (E and F), Wnt4 (G and H) or Lhx1 (I and J). Arrows indicate location of barriers. Dotted lines in A, C, E, G, and I indicate approximate levels of sections in B, D, F, H, and J, respectively. Osr1, Eya1, and Pax2 are still activated in the nephrogenic mesenchyme in the absence of the nephric duct, whereas Wnt4 and Lhx1 expression requires the presence of the nephric duct. Note that in Pax2-stained embryos, on the control side expression is found in the duct as well as in the nephrogenic mesenchyme (E and F left side), while on the experimental side expression is seen only in the mesenchyme (E and F right side). (K–M) Effect of duct blocking on apoptosis in the mesonephric mesenchyme. 48 h after barrier insertion, embryos were analyzed by immunofluorescence for activated caspase3 to mark apoptotic cells (K and L, red), for Lhx1 to mark the nephric duct (K, green) and for Dapi to mark nuclei (L, blue). Statistical analysis of the effect of duct blockage on the percentage of activated caspase3-expressing cells in the mesonephric mesenchyme. The white outline in K and L shows the analyzed area in a representative sample. a, Aorta; d, nephric duct; m, mesonephric mesenchyme; nt, neural tube; pta, pretubular aggregate; s, somite.

(Grobstein, 1955; Herzlinger et al., 1994), and the metanephric mesenchyme undergoes apoptosis in several mouse mutants in which the ureteric bud fails to invade the metanephric mesenchyme (Xu et al., 1999, 2003; Sajithlal et al., 2005). Therefore it was of interest to determine whether mesonephric mesenchyme undergoes apoptosis in the absence of the nephric duct. A total of 462 cells on the blocked side of embryos and 618 cells on control sides of embryos were counted on 12 sections taken from 5 separate embryos. As shown in Fig. 4K–M, a relatively high rate of apoptotic cells (as marked by the presence of activated Caspase 3) was observed in the mesonephric region both in the presence and the absence of the nephric duct, likely due to artifacts of embryo culture, but the rate of apoptosis on the blocked side was higher (27% vs. 15%, $p < 0.0001$). It should be recalled that the expression of Osr1, Eya1, and Pax2 in the mesonephric mesenchyme was essentially normal in the absence of the nephric duct (Fig. 4A–F). Thus mesonephric precursor cells do not appear to be lost selectively in the absence of the duct. Rather, the increase of apoptosis on the blocked side is most likely connected to the differentiation of the mesonephros, a process that is duct-dependent (Fig. 4G–J).

Canonical Wnt signaling partially rescues mesonephros formation in duct-blocked embryos

Canonical Wnt signaling can rescue tubule formation in cultures of isolated mammalian metanephric mesenchyme (Carroll et al., 2005; Herzlinger et al., 1994; Park et al., 2007), and Wnt9b expression in the nephric duct and ureteric bud is required for initiation of tubule formation in the mouse mesonephros and metanephros (Carroll et al., 2005). However, in the case of metanephric cultures, the metanephric mesenchyme was potentially exposed to ureteric bud signals prior to its isolation; and in the Wnt9b knockout, the metanephric mesenchyme was potentially exposed to other, non-Wnt9b signals from the ureteric bud. We wished to determine whether Wnt signaling could rescue tubule initiation under conditions in which the mesenchyme had never been exposed to duct signals. The chick nephric duct, like the mouse duct, expresses Wnt9b in the duct in the mesonephric region (Supplementary Fig. S1B and C).

Chick nephric duct migration was blocked by a foil barrier and the following day a pellet of cells expressing Wnt1 was inserted into the embryos posterior to the duct blockage (Fig. 5A; Wnt1, like Wnt9b, is thought to signal through the canonical Wnt pathway (Giles et al., 2003; Karner et al., 2011)). After another 24 h of development, embryos were analyzed for expression of the differentiation markers Lhx1 and Wnt4. Only embryos in which duct elongation was completely blocked were analyzed further (evidence for a complete block was taken as a break in the Lhx1 staining between the barrier and any Lhx1 expression posterior to the barrier, and/or by absence of the duct on section). Embryos receiving control cells did not exhibit activation of Lhx1 or Wnt4 expression (0/20, 0%; Fig. 5B). In embryos receiving pellets of Wnt1 cells, expression of Lhx1 was detected posterior to the barrier in 10 out of 13 cases (77%; $p < 0.0001$) and Wnt4 was detected in 3 of 5 cases (60%; $p < 0.01$) (Fig. 5C–K). In embryos with Wnt cell implants, Lhx1 and Wnt4 on the operated side were typically expressed in a short line (Fig. 5C and F). These lines were significantly longer than the dots of Lhx1 that were sometimes

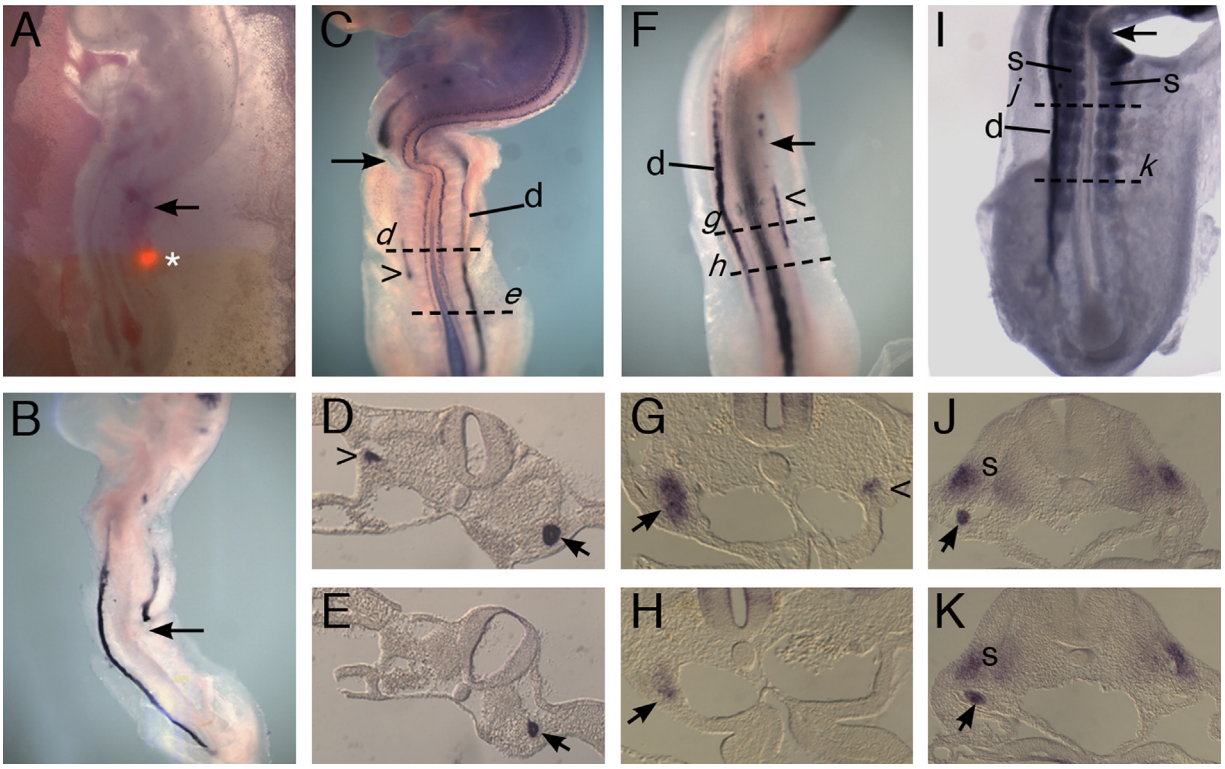

Fig. 5. Rescue of nephrogenesis by Wnt signaling in duct-blocked embryos. Duct extension was blocked as in Fig. 3 (arrows in A–C, F, and I indicate position of barriers) and the following morning rat fibroblasts expressing Wnt1 (A and C–K) or control cells (B) were inserted posterior to the blockade. Asterisk in A shows position of implanted cells. Following an additional 24 h of incubation, embryos were analyzed by in situ hybridization for Lhx1 (B–E), Wnt4 (F–H) or Sim1 (I–K). Embryos with control rat fibroblast inserts did not exhibit Lhx1 staining posterior to the barrier (B). Insertion of Wnt-expressing cells resulted in partial rescue of Lhx1 and Wnt4 expression (C–H; carets indicate rescued expression on duct-blocked sides). Dotted lines in C, F and I indicate approximate planes of section in D and E, G and H, and J and K, respectively. Lhx1-expressing cells on the rescued side were typically not found in an epithelial morphology (caret in D), as compared to the Lhx1-expressing nephric duct on the control side (arrow in D). Insertion of Wnt1-expressing cells did not rescue the duct marker Sim1 (arrow in J and K shows duct expression of Sim1 on the control side; note that expression of Sim1 in the somite was not affected by the duct blockage). d, Nephric duct; s, somite.

seen in duct-blocked embryos (Supplementary Fig. S2). Induced Lhx1 and Wnt4 were seen only within the intermediate mesoderm region, and not in the somitic or lateral plate regions, indicating that the competence to express Lhx1 and Wnt4 in response to Wnt signaling was restricted. Sections of rescued embryos stained for Lhx1 expression revealed that the induced Lhx1 staining was typically found in a cylindrical structure in the dorsal part of the IM (Fig. 5D), in the location where the nephric duct would be expected to be found. However, unlike the nephric duct on the control side of the same embryo, these Lhx1-expressing structures, did not appear to be epithelialized or contain a lumen, nor did Wnt1 cells induce expression of the duct marker Sim1 (Fig. 5I–K). Thus, while canonical Wnt signaling can induce initiation of mesonephros differentiation, the rescued tissue is not identical to normal nephric duct or tubule.

Differential competence of IM to respond to inductive signals

In complementary experiments to those of Fig. 5, rescue of duct-blocked embryos was performed by injection of the GSK-3 inhibitor and canonical Wnt pathway activator Bio (6-bromoindirubin-3′-oxime) (Meijer et al., 2003; Sato et al., 2004). Interestingly, although Bio was injected all along the anterior–posterior axis, from shortly behind the barrier to the primitive streak region, rescue of Lhx1 and Wnt4 was only observed in the anterior-most region of the mesonephric region (Fig. 6; 10/22 embryos (45%) exhibited induced Lhx1 or Wnt4 in the anterior mesonephric region, 0/22 (0%) embryos exhibited induction in the posterior mesonephric region). This suggested that mesonephric mesenchyme at different positions along the A–P axis may differ in its competence to respond to Wnt inducing signals.

In order to further investigate this issue, an explant culture system was developed. Regions of approximately 3-somites in length (approximately 450 μm) including the intermediate mesoderm as well as some adjacent lateral somite and medial lateral plate were manually cut from Stage 15 to 16 embryos at three axial levels: the most caudal somites ("som"), the anterior presomitic mesoderm ("apsm"), and the posterior presomitic mesoderm ("ppsm") (Fig. 7A). Neural tube and notochord were not included in the explants because Wnt4 is expressed strongly in the neural tube (Fig. 2C). Explants were grown on filters at the air-medium interface for 48 h in the presence or absence of Bio, and analyzed for gene expression by in situ hybridization. Staining for Lhx1 allowed for the detection of both the nephric duct and incipient tubules, while staining for Wnt4 allowed detection of differentiating tubules more specifically. In preliminary control experiments, Bio was found to block the expression of the gene Nkx2.5 in explants of precardiac mesoderm (data not shown), a known canonical Wnt-dependent effect (Marvin et al., 2001; Tzahor and Lassar, 2001). Som level cultures expressed high levels of Lhx1 and Wnt4 in the presence or absence of Bio (Fig. 7B, E, H, and K), which was not surprising because som level explants contained a segment of the nephric duct. When Bio was added to cultures of apsm cultures at the same doses shown to be effective in inhibiting cardiac gene expression, a modest increase in Lhx1 and Wnt4 expression was observed (Fig. 7C, F, I, and L), although significantly lower than the levels seen in control som level explants (Fig. 7E and K). Ppsm cultures exhibited an even more limited response to Bio than apsm cultures (Fig. 7D, G, J, and M).

The results of Fig. 7 indicate that activation of a canonical Wnt signaling pathway is not sufficient to strongly induce mesonephric differentiation from psm-level explants. This result could be attributable to the lack of a non-Wnt duct signal that was absent in psm-level cultures (which contained only the posterior tip of the duct in the case of apsm cultures or no duct at all in the case of ppsm cultures), or to a lack of competence to respond to Wnt signaling in psm-level explants. In order to gain further insight into this issue, cultures were prepared from embryos which lacked a nephric duct owing to the placement of a barrier to block duct migration. Cultures were grown in reduced serum medium in order to minimize the concentration of potential inducers in the culture medium. As seen in Fig. 8A–D, in the absence of the nephric duct, control apsm-level cultures did not express Lhx1 (Fig. 8D), while control som-level cultures were variable, with some not expressing Lhx1, and others expressing Lhx1 at low levels (Fig. 8B). Addition of Bio to duct-blocked explants resulted in activation of Lhx1 in both som and apsm cultures, with higher levels of Lhx1 expression obtained in the som-level cultures (Fig. 8A and C). In both the som and apsm-level Bio-induced

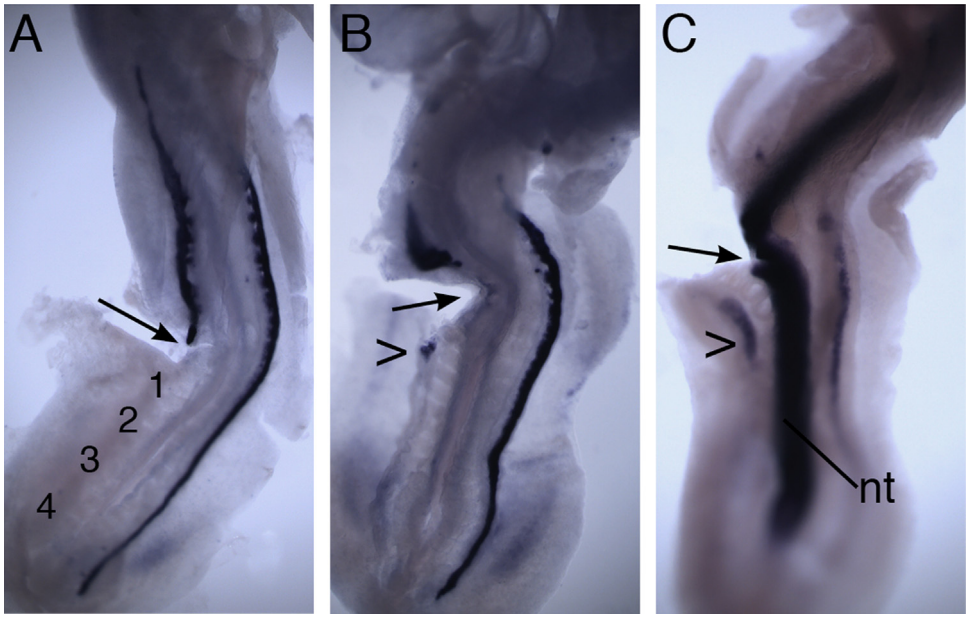

Fig. 6. Rescue of tubule markers in duct blocked embryos by injection of Bio. Migration of the nephric duct was blocked on one side of the embryo (A–C, arrows) and Bio (B and C) or DMSO (A) was injected posterior to the barrier at 4–5 places along the anterior–posterior axis adjacent to the somites (as indicated by numbers 1–4 in A). After 24 h, embryos were analyzed for expression of Lhx1 (A and B) or Wnt4 (C). Rescue was observed only in the anterior injected regions (carets in B and C). Note that the neural tube also expresses Wnt4. nt, Neural tube.

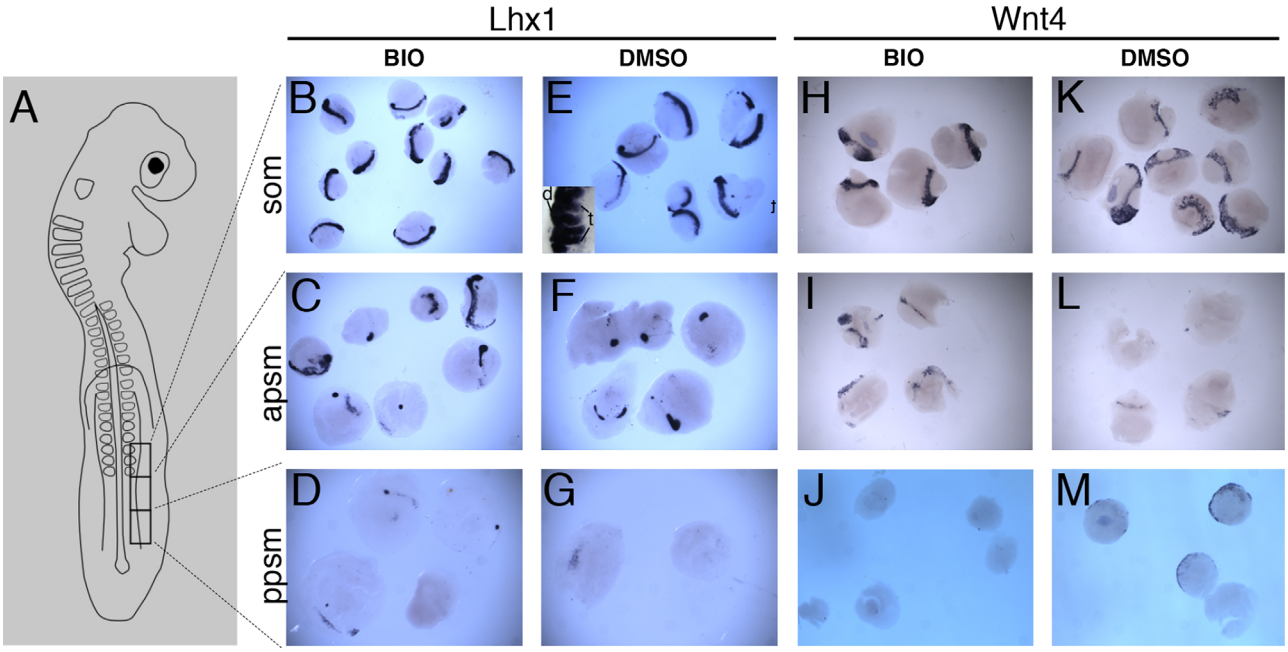

Fig. 7. Response of mesonephric mesenchyme to Wnt signaling in explant culture. Regions from the axial level of the posterior-most somites (som: B, E, H, and K), anterior presomitic mesoderm (apsm: C, F, I, and L), and posterior presomitic mesoderm (ppsm: D, G, J, and M) were dissected from Stage 15–16 embryos as indicated in A, cultured in the presence of Bio (B–D, H–J) or DMSO (E–G, K–M), and analyzed for expression of Lhx1 (B–G) or Wnt4 (H–M) by in situ hybridization. Ppsm cultures did not respond to addition of Bio, apsm cultures exhibited a moderate induction of Lhx1 and Wnt4, and som cultures expressed both markers strongly in control as well as Bio-treated cultures. Inset in E is higher power view of one som level control explant showing tubule formation. d, Nephric duct; t, tubules.

cultures, Lhx1 expression was typically diffuse and did not exhibit the compact tubular morphology typically seen in som-level cultures containing a nephric duct (compare Fig. 8A and C with Fig. 7E and K). Thus, both in vivo (Fig. 6) and in culture (Figs. 7 and 8), the ability to activate mesonephric genes in response to Wnt signaling is greater in more anterior regions of the mesonephric mesenchyme.

In order to characterize the requirement for Wnt signaling during initiation of kidney differentiation, Stage 15–16 som-level explant cultures were treated with the canonical Wnt pathway inhibitor IWR-1-endo (som-level explants were used because control cultures of more posterior psm regions did not generate high levels of tubule markers, as seen in Fig. 7F and L). At the start of the culture period, the intermediate mesoderm of the explants had not yet initiated expression of Wnt4 or Lhx1. In preliminary control experiments to test the efficacy of the reagent, IWR-1-endo at 20–50 μM was found to activate expression of the cardiac marker Nkx-2.5 in posterior embryonic lateral mesoderm (data not shown), a known effect of Wnt inhibition (Marvin et al., 2001). Fig. 8E and F shows that treatment with IWR did not inhibit expression of Wnt4, suggesting that Wnt signaling is not required to obtain initial differentiation of som-level mesonephric mesenchyme. As discussed below, one possible interpretation of this result is that the mesonephric mesenchyme receives sufficient Wnt signaling at psm axial levels, when the nephric duct first comes into contact with the mesonephric mesenchyme, and that Wnt inhibition at later stages can no longer prevent the initiation of tubulogenesis.

Discussion

Duct-independent and duct-dependent aspects of mesonephros induction

The genes Pax2, Eya1, Wnt4, and Lhx1 are all activated in the mesonephric IM in parallel with the passage of the nephric duct through the region. This raised the question of the role of the nephric duct in the activation of these genes. The current study found that expression of Wnt4 and Lhx1, which are associated with the initiation of tubule formation, is dependent on the nephric duct, while expression of Eya1 and Pax2, which are expressed in the undifferentiated mesenchyme, is duct-independent. Maintenance of Osr1, which is expressed earlier than the other genes and more generally in the IM and the lateral plate, is also not dependent on the presence of the nephric duct. Consistent with these findings, mouse Gata3 −/− embryos, which exhibit defects in nephric duct migration such that the duct often does not reach the mesonephric or metanephric regions, still activate Pax2 in the mesonephric and metanephric IM (Grote et al., 2006). Past studies have identified several factors that regulate initial IM formation, including intermediate levels of BMP (James and Schultheiss, 2005), Nodal/Vg1 (Fleming et al., 2013), and Retinoic Acid (Asashima et al., 2000; Preger-Ben Noon et al., 2009). These factors, and possibly others, are likely to act during this first phase of kidney induction to activate Osr1, Eya1, and Pax2.

It is noteworthy that, despite the fact that Eya1 and Pax2 are activated along the A–P axis in parallel with the migration of the nephric duct, this activation was not dependent on the presence of the duct. Thus, Pax2 and Eya1 are activated at the same rate along the A–P axis regardless of whether or not the duct is present (Fig. 4). Although the factors that regulate this A–P aspect of mesonephros gene activation are not yet known, it may be significant that the activation of these genes is temporally linked to the formation of new somites. The posterior limit of Eya1 is found approximately 4 somite-lengths posterior to the most posterior somite, and the posterior border of high levels of Pax2 is located 2 somite-lengths posterior to the last somite (Fig. 2J). Consistent with this possibility, previous studies have found a role for somites or psm in the activation of IM genes (Mauch et al., 2000; Seufert et al., 1999). Alternatively, the IM and the psm/somites may be responding independently to signals that regulate A–P differentiation. Studies have identified Retinoic Acid and FGF signaling as playing roles in the timing of somite formation along

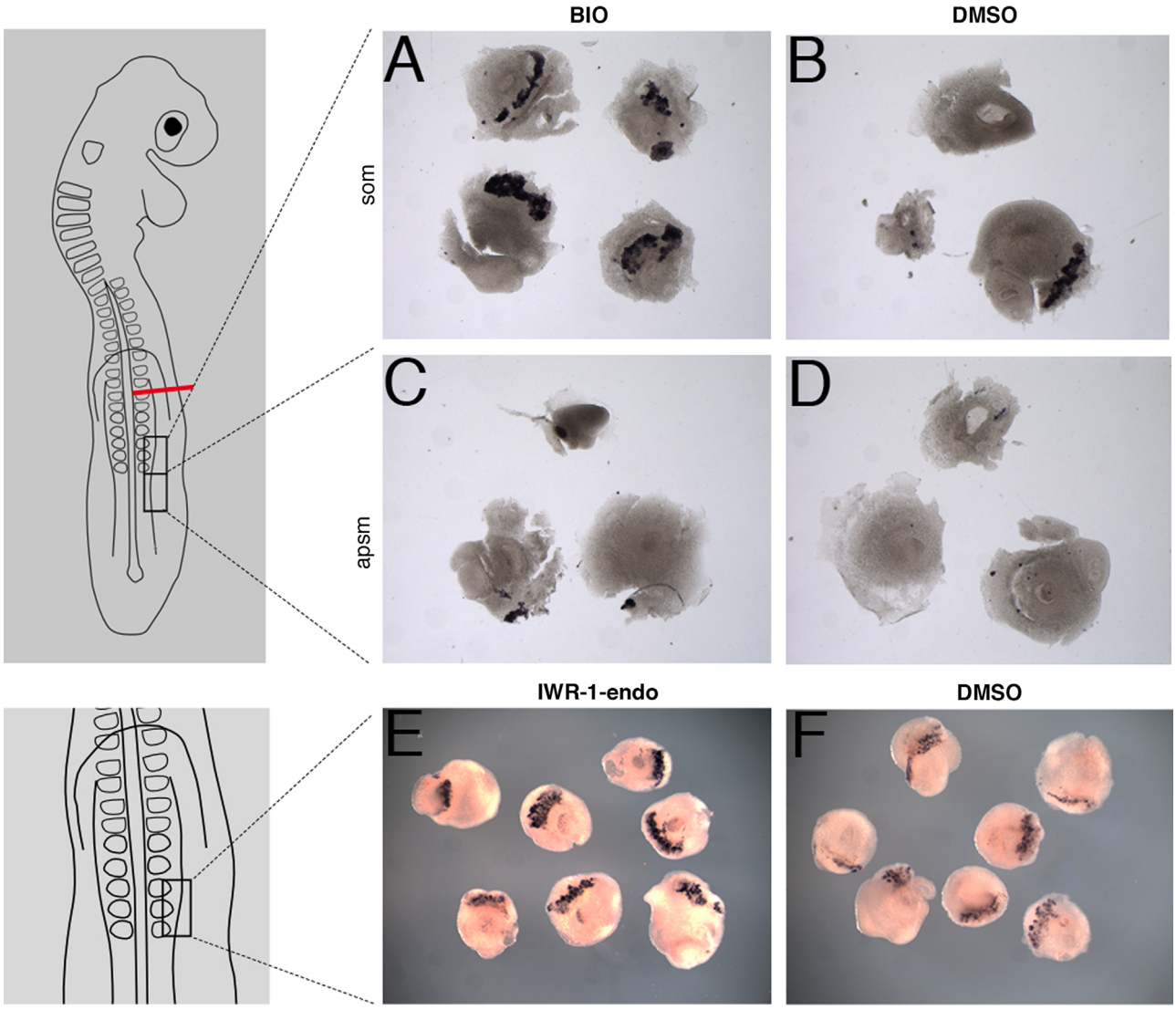

Fig. 8. (A–D) Response of cultured mesonephric mesenchyme to Wnt signaling in the absence of the nephric duct. Nephric duct migration was blocked as in Fig. 3, and at Stage 15–16 explants were taken from somite (A and B) or apsm-level (C and D) levels and treated with Bio (A and C) or DMSO carrier (B and D). After culture for 48 h, cultures were analyzed by in situ hybridization for expression of Lhx1. Addition of Bio resulted in increase of Lhx1 expression in som-level as well as apsm-level cultures, with som-level cultures responding more strongly (A and C). (E and F) Som-level mesonephric mesoderm does not require the continued activity of Wnt signaling. Som-level explants including the nephric duct were cultured with (E) or without (F) the Wnt inhibitor IWR-1-endo (50 μM). Expression of the tubule marker Wnt4 was equal under both conditions.

the A–P axis (Dubrulle et al., 2001; Dubrulle and Pourquie, 2004; Vermot and Pourquie, 2005), and it will be interesting to determine if these molecules influence early IM gene expression either directly or through their influence on somite formation.

The linear nature of the mesonephros allows some estimation of the duration of exposure to the nephric duct that is necessary to initiate activation of mesonephric tubule formation. The first marker of tubule differentiation, Wnt4, is detectable approximately 3–4 somites anterior to the border between the somites and the psm (Fig. 2), while the posterior tip of the duct is located approximately 3 somite-lengths posterior to the somite–psm border (Fig. 2G and H). Since somites form at a rate of approximately one somite per 90 min (Hamilton, 1952), this indicates that Wnt4 is first detectable in a given region 9–10.5 h after the posterior tip of the duct has passed through. This figure is a maximum for the time of exposure to duct signals that is necessary to initiate tubule formation. Since Wnt4 is likely detectable by in situ hybridization only some time after induction has already started, the actual inductive events are likely to take place fewer than 9 h from the first exposure to duct signals (and perhaps even earlier – see below). In mouse metanephric cultures, it has been estimated that induction requires exposure to the ureteric bud of at least 10 h, with full induction requiring 30 h of exposure (Ekblom et al., 1981; Saxen and Lehtonen, 1978).

Wnt signaling and the induction of mesonephros differentiation by the nephric duct

Studies in the mouse have indicated that Wnt signaling is an important component of the tubule-inducing properties of the nephric duct. A source of canonical Wnt signaling is sufficient to replace the ureteric bud in the induction of tubules from cultured metanephric mesenchyme rudiments (Herzlinger et al., 1994), and Wnt9b, which is expressed in the nephric duct, is required for formation of both mesonephric and metanephric tubules (Carroll et al., 2005). In agreement with these studies, the current work found that Wnt9b is expressed in the chick nephric duct (Supplementary Fig. S1), and that a source of canonical Wnt signaling can rescue expression of the early mesonephric tubulogenesis markers Wnt4 and Lhx1 in embryos lacking a nephric duct

(Figs. 5, 6 and 8). However, unlike in mouse metanephric cultures, the rescue was not complete, in that robust tubulogenesis did not occur in Wnt-rescued embryos or mesonephric cultures. One difference between the mouse and chick experimental systems is that in the mouse system, the metanephric mesenchyme has been in contact with the ureteric bud prior to its isolation, while in the chick mesonephros system used in the current experiments the mesonephric mesenchyme has never been exposed to the duct. Thus it is possible that in the mouse system the mesenchyme has already received a second duct-derived signal prior to its being placed in culture. Since BMP signaling is known to be required for the proper maturation of the nephric duct (Obara-Ishihara et al., 1999), we considered the possibility that duct blockage may have interfered with normal BMP expression, which could then have interfered with the inductive properties of the duct. However, BMP4 was expressed normally in duct blocked embryos (Supplementary Fig. S3). Another possible explanations for differences between the chick and mouse systems is that in the chick system, there is a time interval between when duct migration is blocked and when Wnt-expressing cells are implanted or when the mesonephric mesenchyme is harvested and placed in culture. During this time, changes in the mesonephric mesenchyme may occur which affect its ability to undergo tubulogenesis.

The current studies found that in the chick, inhibition of Wnt signaling in som-level mesonephric mesenchyme did not prevent initiation of mesonephric tubule gene expression (Fig. 8E and F). One possibility for the discrepancy is that in chick, unlike in mouse, Wnt signaling may not be required for mesonephric tubule induction. This possibility seems unlikely, given the similarities between many other aspects of chick and mouse kidney induction documented in this report, including the ability of Wnt signaling to initiate expression of tubule genes in the absence of the nephric duct (Fig. 8; (Herzlinger et al., 1994)). A more likely possibility is that the som-level mesonephric mesoderm tested in the current study has already received sufficient Wnt signaling such that Wnt inhibition can no longer prevent subsequent activation of tubule genes. The nephric duct normally begins to interact with the mesonephric mesenchyme at the psm axial level. By the time the wave of somite formation has progressed so that the mesonephric mesenchyme is adjacent to somites, the mesonephric mesenchyme has already been in contact with the Wnt-expressing nephric duct for approximately 5 h. This may be sufficient exposure to Wnt signal to permit future mesonephros differentiation. Note that this hypothesis could not be tested directly in the current experimental system, since psm-level control explants did not activate Lhx1 or Wnt4 robustly in culture (Fig. 7F and L) and thus it would be difficult to detect a reduction upon treatment with Wnt inhibitors. Future studies in which Wnt signaling is broadly inhibited in vivo may be able to shed further light on the requirement for Wnt signaling during these earlier phases of mesonephros induction.

Competence of the mesonephric mesenchyme to express markers of differentiation

The current study revealed that psm-level and som-level mesonephric mesenchyme differ in their competence to initiate tubule expression (Figs. 7 and 8). This difference in competence was not established by signals from the nephric duct, since differences in the differentiation competence between psm- and som-level mesonephric mesenchyme were found in embryos in which the duct had been blocked (Fig. 8). Rather, it appears to be due to a maturation of the mesonephric mesenchyme between psm and som compartments. The molecular basis of this maturation of the mesonephric mesenchyme between psm and som axial levels is not currently known, but it does not appear to consist of acquisition of a competence to respond to Wnt signaling since, as discussed above, Wnt signaling likely acts already on psm-level mesenchyme. It is possible that other inductive signals act on som-level mesenchyme. Consistent with this idea, we observed that explant cultures from duct-blocked embryos exhibited much more robust activation of tubule genes when grown in high-serum than in low-serum medium, potentially indicating the existence of other secreted factors that affect the activation of tubule genes. Mesonephric mesenchyme transcription factors including Eya1 and Pax2 could play a role in acquisition of competence to undergo

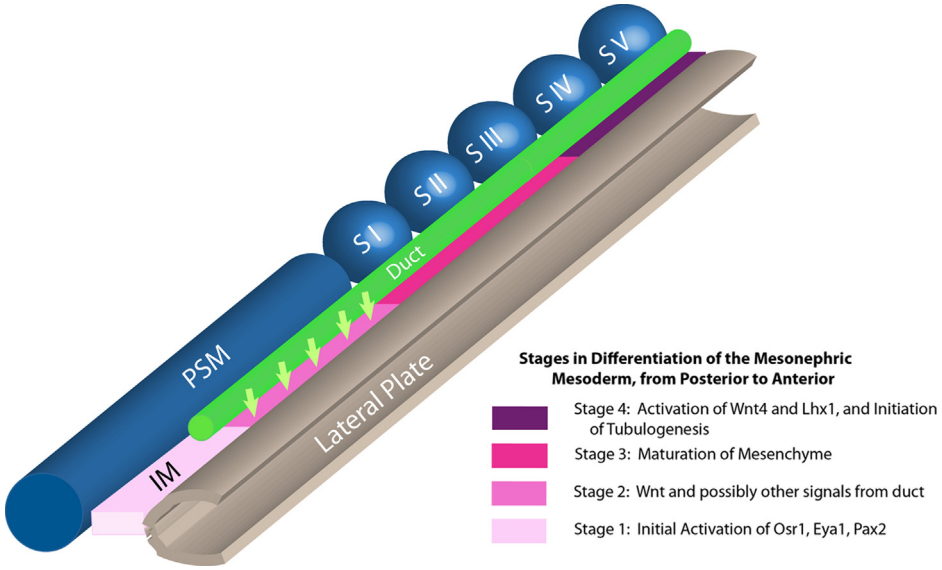

Fig. 9. Model of the stages of induction of mesonephric tubules. In the diagram, anterior is at the top right, and posterior is at the bottom left. The nephric duct (green) extends from anterior to posterior, and the paraxial mesoderm (blue) undergoes segmentation, with new anterior-most psm converting into somites. The intermediate mesoderm is in shades of pink/red/purple, with the least differentiated regions of the mesonephric IM located posteriorly. Four stages in the maturation of the IM can be identified: (1) activation of Osr1, Pax2, and Sim1 in the IM adjacent to the posterior psm; (2) arrival of the nephric duct to the anterior psm region and transmission of Wnt and possibly other duct-derived signals; (3) maturation of the mesonephric mesenchyme, which occurs at approximately the axial level where the psm is transitioning to somite; (4) as a result of the activities of Stages 1–3, tubular genes including Wnt4 and Lhx1 are activated beginning at approximately the axial level of the 4th-to-last somite. IM, intermediate mesoderm; psm, presomitic mesoderm; s, somite.

differentiation. However, other factors are likely to be involved, since the apsm and the som regions of the intermediate mesoderm express Eya1 and Pax2 at similar levels yet differ in their competence to differentiate. An important future area of investigation will be to understand the molecular basis for the competence of the mesonephric mesenchyme to differentiate, and how it is linked to the activation of Osr1, Eya1, Pax2, and other early mesonephric genes.

A working model for induction of mesonephric tubules

Fig. 9 presents a model of the induction of mesonephric tubules in the chick embryo, based on the current study. Because the mesonephros is a linear organ that differentiates from anterior to posterior, the stages in its differentiation can be laid out on the anterior–posterior axis, with the earliest stages located most posteriorly. In the model, at least four stages in the induction of mesonephric tubules can be recognized: (1) activation of a set of early IM genes, including Osr1, Eya1, and Pax2, whose expression is not dependent on the nephric duct; (2) the duct makes contact with the mesonephric mesenchyme at the psm axial level and supplies Wnt and possibly other signals; (3) the mesonephric mesenchyme undergoes maturation at the transition between the psm and the som axial levels. This maturation is independent of the presence of the duct. Continued expression of the genes of Step 1 may contribute to IM maturation; (4) as a result of Steps 1–3, tubulogenesis (as represented by activation of Wnt4 and tubular Lhx1 expression) is initiated at the axial level of approximately the 4th to last somite. Future studies will aim to better understand the molecular regulation of these transitions, studies which should have relevance not only for the formation of the avian mesonephros but also potentially for the mammalian metanephros.

Acknowledgments

The authors would like to thank Inbar Reshef for technical assistance, and members of the Schultheiss lab for discussions. We also thank F. Costantini, M. Goulding, T. Jessell, and V. van Heynigen for probes. This work was funded by Grants to T.M.S. from the Israel Science Foundation (Grants 1328/08 and 1573/12), the March of Dimes, and the Rappaport Family Foundation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.09.026.

References

Asashima, M., Ariizumi, T., Malacinski, G.M., 2000. In vitro control of organogenesis and body patterning by activin during early amphibian development. Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology 126, 169–178.
Attia, L., Yelin, R., Schultheiss, T.M., 2012. Analysis of nephric duct specification in the avian embryo. Development 139, 4143–4151.
Burrill, J.D., Moran, L., Goulding, M.D., Saueressig, H., 1997. PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. Development 124, 4493–4503.
Carroll, T.J., Park, J.S., Hayashi, S., Majumdar, A., McMahon, A.P., 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Developmental Cell 9, 283–292.
Cho, E.A., Dressler, G.R., 2003. Formation and development of nephrons. In: Vize, P., Woolf, A.S., Bard, J.B.L. (Eds.), The Kidney: From Normal Development to Congenital Disease. Academic Press, San Diego, pp. 195–210.
Costantini, F., Kopan, R., 2010. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. Developmental Cell 18, 698–712.
Dressler, G., 2002. Tubulogenesis in the developing mammalian kidney. Trends in Cell Biology 12, 390–395.
Dressler, G.R., 2006. The cellular basis of kidney development. Annual Review of Cell and Developmental Biology 22, 509–529.
Dressler, G.R., 2009. Advances in early kidney specification, development and patterning. Development 136, 3863–3874.
Dressler, G.R., Deutsch, U., Chowdhury, K., Nornes, H.O., Gruss, P., 1990. Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. Development 109, 787–795.
Dubrulle, J., McGrew, M.J., Pourquie, O., 2001. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell 106, 219–232.
Dubrulle, J., Pourquie, O., 2004. fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. Nature 427, 419–422.
Ekblom, P., Miettinen, A., Virtanen, I., Wahlstrom, T., Dawnay, A., Saxen, L., 1981. In vitro segregation of the metanephric nephron. Developmental Biology 84, 88–95.
Fleming, B.M., Yelin, R., James, R.G., Schultheiss, T.M., 2013. A role for Vg1/Nodal signaling in specification of the intermediate mesoderm. Development 140, 1819–1829.
Giles, R.H., van Es, J.H., Clevers, H., 2003. Caught up in a Wnt storm: Wnt signaling in cancer. Biochimica et Biophysica Acta 1653, 1–24.
Grobstein, C., 1955. Inductive interactions in the devlopment of the mouse metanephros. Journal of Experimental Zoology 130, 319–340.
Grote, D., Souabni, A., Busslinger, M., Bouchard, M., 2006. Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. Development 133, 53–61.
Gruenwald, P., 1937. Zur entwicklungsmechanick der Urogenital-systems beim Huhn. Wilhem Roux Archiv für Entwicklungsmechanik der Organismen 136, 786–813.
Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. Journal of Morphology 88, 49–92.
Hamilton, H.L., 1952. Lillie's Development of the Chick. Henry Holt, New York.
Herzlinger, D., Qiao, J., Cohen, D., Ramakrishna, N., Brown, A.M., 1994. Induction of kidney epithelial morphogenesis by cells expressing Wnt-1. Developmental Biology 166, 815–818.
James, R.G., Kamei, C.N., Wang, Q., Jiang, R., Schultheiss, T.M., 2006. Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. Development 133, 2995–3004.
James, R.G., Schultheiss, T.M., 2003. Patterning of the avian intermediate mesoderm by lateral plate and axial tissues. Developmental Biology 253, 109–124.
James, R.G., Schultheiss, T.M., 2005. Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. Developmental Biology 288, 113–125.
Kalatzis, V., Sahly, I., El-Amraoui, A., Petit, C., 1998. Eya1 expression in the developing ear and kidney: towards the understanding of the pathogenesis of Branchio-Oto-Renal (BOR) syndrome. Developmental Dynamics 213, 486–499.
Karner, C.M., Das, A., Ma, Z., Self, M., Chen, C., Lum, L., Oliver, G., Carroll, T.J., 2011. Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development. Development 138, 1247–1257.
Kent, J., Coriat, A.M., Sharpe, P.T., Hastie, N.D., van Heyningen, V., 1995. The evolution of WT1 sequence and expression pattern in the vertebrates. Oncogene 11, 1781–1792.
Marvin, M.J., Di Rocco, G., Gardiner, A., Bush, S.M., Lassar, A.B., 2001. Inhibition of Wnt activity induces heart formation from posterior mesoderm. Genes and Development 15, 316–327.
Mauch, T.J., Yang, G., Wright, M., Smith, D., Schoenwolf, G.C., 2000. Signals from trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm. Developmental Biology 220, 62–75.
Meijer, L., Skaltsounis, A.L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X.P., Vonica, C.A., Brivanlou, A., Dajani, R., Crovace, C., Tarricone, C., Musacchio, A., Roe, S.M., Pearl, L., Greengard, P., 2003. GSK-3-selective inhibitors derived from Tyrian purple indirubins. Chemistry and Biology 10, 1255–1266.
Obara-Ishihara, T., Kuhlman, J., Niswander, L., Herzlinger, D., 1999. The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. Development 126, 1103–1108.
Ohto, H., Takizawa, T., Saito, T., Kobayashi, M., Ikeda, K., Kawakami, K., 1998. Tissue and developmental distribution of Six family gene products. International Journal of Developmental Biology 42, 141–148.
Pachnis, V., Mankoo, B., Costantini, F., 1993. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119, 1005–1017.
Park, J.S., Valerius, M.T., McMahon, A.P., 2007. Wnt/beta-catenin signaling regulates nephron induction during mouse kidney development. Development 134, 2533–2539.
Preger-Ben Noon, E., Barak, H., Guttmann-Raviv, N., Reshef, R., 2009. Interplay between activin and Hox genes determines the formation of the kidney morphogenetic field. Development 136, 1995–2004.
Qian, J., Jiang, Z., Li, M., Heaphy, P., Liu, Y.H., Shackleford, G.M., 2003. Mouse Wnt9b transforming activity, tissue-specific expression, and evolution. Genomics 81, 34–46.
Sainio, K., 2003. Experimental methods for studying urogenital development. In: Vize, P., Woolf, A.S., Bard, J.B.L. (Eds.), The Kidney: From Normal Development to Congenital Disease. Academic Press, San Diego, pp. 327–342.

Sajithlal, G., Zou, D., Silvius, D., Xu, P.X., 2005. Eya1 acts as a critical regulator for specifying the metanephric mesenchyme. Developmental Biology 284, 323–336.
Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., Brivanlou, A.H., 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature Medicine 10, 55–63.
Saxen, L., 1987. Organogenesis of the Kidney. Cambridge University Press, London.
Saxen, L., Koskimies, O., Lahti, A., Miettinen, H., Rapola, J., Wartiovaara, J., 1968. Differentiation of kidney mesenchyme in an experimental model system. Advances in Morphogenesis 7, 251–293.
Saxen, L., Lehtonen, E., 1978. Transfilter induction of kidney tubules as a function of the extent and duration of intercellular contacts. Journal of Embryology and Experimental Morphology 47, 97–109.
Schuchardt, A., Srinivas, S., Pachnis, V., Costantini, F., 1995. Isolation and characterization of a chicken homolog of the c-ret proto-oncogene. Oncogene 10, 641–649.
Schultheiss, T.M., James, R.G., Listopadova, A., Herzlinger, D., 2003. Formation of the nephric duct. In: Vize, P., Woolf, A.S., Bard, J.B.L. (Eds.), The Kidney. Academic Press, Amsterdam.
Schultheiss, T.M., Xydas, S., Lassar, A.B., 1995. Induction of avian cardiac myogenesis by anterior endoderm. Development 121, 4203–4214.
Self, M., Lagutin, O.V., Bowling, B., Hendrix, J., Cai, Y., Dressler, G.R., Oliver, G., 2006. Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. EMBO Journal 25, 5214–5228.
Seufert, D.W., Brennan, H.C., DeGuire, J., Jones, E.A., Vize, P.D., 1999. Developmental basis of pronephric defects in Xenopus body plan phenotypes. Developmental Biology 215, 233–242.
Shawlot, W., Behringer, R.R., 1995. Requirement for Lim1 in head-organizer function. Nature 374, 425–430.
So, P.L., Danielian, P.S., 1999. Cloning and expression analysis of a mouse gene related to Drosophila odd-skipped. Mechanisms of Development 84, 157–160.
Stark, K., Vainio, S., Vassileva, G., McMahon, A.P., 1994. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372, 679–683.
Suetsugu, R., Sato, Y., Takahashi, Y., 2002. Pax 2 expression in mesodermal segmentation and its relationship with EphA4 and Lunatic-fringe during chicken somitogenesis. Gene Expression Patterns 2, 157–161.
Tsang, T.E., Shawlot, W., Kinder, S.J., Kobayashi, A., Kwan, K.M., Schughart, K., Kania, A., Jessell, T.M., Behringer, R.R., Tam, P.P., 2000. Lim1 activity is required for intermediate mesoderm differentiation in the mouse embryo. Developmental Biology 223, 77–90.
Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., Pfaff, S. L., 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 79, 957–970.
Tzahor, E., Lassar, A.B., 2001. Wnt signals from the neural tube block ectopic cardiogenesis. Genes and Development 15, 255–260.
Vermot, J., Pourquie, O., 2005. Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. Nature 435, 215–220.
Waddington, C.H., 1938. The morphogenetic function of a vestigal organ in the chick. Journal of Experimental Biology 15, 271–377.
Xu, P.X., Adams, J., Peters, H., Brown, M.C., Heaney, S., Maas, R., 1999. Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. Nature Genetics 23, 113–117.
Xu, P.X., Zheng, W., Huang, L., Maire, P., Laclef, C., Silvius, D., 2003. Six1 is required for the early organogenesis of mammalian kidney. Development 130, 3085–3094.
Yu, J., McMahon, A.P., Valerius, M.T., 2004. Recent genetic studies of mouse kidney development. Current Opinion in Genetics and Development 14, 550–557.