odd skipped related1 reveals a novel role for endoderm in regulating kidney versus vascular cell fate

Sudha P. Mudumana¹, Dirk Hentschel², Yan Liu¹, Aleksandr Vasilyev¹ and Iain A. Drummond¹,*

The kidney and vasculature are intimately linked both functionally and during development, when nephric and blood/vascular progenitor cells occupy adjacent bands of mesoderm in zebrafish and frog embryos. Developmental mechanisms that underlie the differentiation of kidney versus blood/vascular lineages remain unknown. The odd skipped related1 (osr1) gene encodes a zinc-finger transcription factor that is expressed in the germ ring mesendoderm and subsequently in the endoderm and intermediate mesoderm, prior to the expression of definitive kidney or blood/vascular markers. Knockdown of osr1 in zebrafish embryos resulted in a complete, segment-specific loss of anterior kidney progenitors and a compensatory increase in the number of angioblast cells in the same trunk region. Histology revealed a subsequent absence of kidney tubules, an enlarged cardinal vein and expansion of the posterior venous plexus. Altered kidney versus vascular development correlated with expanded endoderm development in the same trunk region. The results indicate that osr1 activity is required to limit endoderm differentiation from mesendoderm; in the absence of osr1, excess endoderm alters mesoderm differentiation, shifting the balance from kidney towards vascular development.

KEY WORDS: Odd-skipped related, Endoderm, Pronephros, Vasculature, Glomerulus, Kidney development, Zebrafish

INTRODUCTION

The kidney and vasculature are mesodermal derivatives that originate from adjacent regions of gastrulating fish and frog embryos (Iraha et al., 2002; Kimelman, 2006; Kimmel et al., 1990; Walmsley et al., 2002). Soon after gastrulation is complete, zebrafish kidney progenitors, which are marked by the expression of transcriptional regulators such as pax2a (Krauss et al., 1991; Majumdar et al., 2000) and lim1 (Toyama and Dawid, 1997), and blood/vascular progenitors, which are marked by expression of scl (Gering et al., 1998) and gata1 (Detrich et al., 1995), are found in adjacent stripes of mesoderm lateral to the somites. These tissues, referred to as intermediate mesoderm (IM) in nephric development and lateral plate mesoderm (LPM) in blood vascular development, serve as the source of all pronephric cells, the posterior blood islands, and the main vessels of the trunk, the dorsal aorta and the cardinal vein. Analysis of mesoderm patterning in frog embryos has demonstrated overlapping expression of the vascular marker fli1 and the kidney marker lim1 in intermediate mesoderm (Walmsley et al., 2002), suggesting that, prior to cell differentiation, kidney and blood/vascular cells may share a common progenitor cell population. The close association of kidney and vascular progenitor cells during development is ultimately manifested as a functional relationship in the mature organs, where arterial blood is filtered by the kidney glomerulus and metabolites recovered by kidney tubules are delivered directly back to the venous blood supply. This relationship between kidney and vascular tissues raises the idea that the specification of kidney and vascular progenitor cells in early embryos may be influenced by common developmental regulatory factors.

Both kidney and vascular patterning is strongly influenced by bone morphogenetic proteins (BMPs) during gastrulation (Kimelman, 2006; Kimelman and Griffin, 2000; Pyati et al., 2005; Stickney et al., 2007; Szeto and Kimelman, 2004). Zebrafish mutants defective in BMP signaling, such as swirl/bmp2b (Kishimoto et al., 1997; Nguyen et al., 1998), snailhouse/bmp7 (Dick et al., 2000; Schmid et al., 2000) and somitabun/smad5 (Hild et al., 1999), show a reduced number of both kidney and blood cell progenitors, and an expansion of dorsal somites. However, ventralized/posteriorized mutants that lack BMP inhibitors such as chordino/chordin and the tolloid antagonist ogon/sizzled show an enlargement of kidney and blood precursor cell populations and a loss of anterior somites (Hammerschmidt et al., 1996; Leung et al., 2005; Miller-Bertoglio et al., 1999). Signaling events occurring later in development may also affect kidney versus blood/vascular fates. Post-gastrulation expression of a dominant-negative BMP receptor expands the gata1-positive blood progenitor cell population and reduces the number of pax2a-positive kidney progenitor cells in the ventroposterior mesoderm (Gupta et al., 2006). Mutations in BMP4 affect ventrolateral mesoderm at post-gastrulation stages, favoring blood and kidney development at the expense of vascular development (Stickney et al., 2007). Evidence has also been presented that blood/vascular and kidney fates may be mutually exclusive in the mesoderm. Ectopic overexpression of the blood/vascular transcriptional regulators scl and lmo2 during early development results in expansion of the blood/vascular progenitor cell population at the expense of kidney progenitors, indicating that intermediate mesoderm can be translated to blood/vascular mesoderm (Gering et al., 2003). These findings suggest that the differentiation of the blood/vascular and kidney lineages are linked at multiple stages of development. It is likely that, in addition to BMP signaling, other morphogens and transcriptional circuitry is required to ultimately define lateral mesoderm cell lineages.

The zinc-finger transcription factor odd-skipped related 1 (osr1) is initially expressed in the mesendoderm in gastrulating zebrafish embryos and, later, in a broad domain of lateral plate/intermediate mesoderm. The osr1 gene is initially expressed in the mesendoderm in gastrulating zebrafish embryos and, later, in a broad domain of lateral plate/intermediate mesoderm. The results indicate that osr1 activity is required to limit endoderm differentiation from mesendoderm; in the absence of osr1, excess endoderm alters mesoderm differentiation, shifting the balance from kidney towards vascular development.
mesoderm that encompasses both kidney and vascular mesoderm in chick, mouse and zebrafish embryos (James et al., 2006; Tena et al., 2007; Wang et al., 2005). Mouse embryos lacking a functional Osr1 gene show cardiac defects and kidney agenesis (James et al., 2006; Wang et al., 2005). Knockdown experiments in zebrafish have also revealed a role for osr1 in pronephric development (Tena et al., 2007). We present here evidence that osr1 is not only required for zebrafish kidney development but that it also controls the commitment of mesoderm to the angioblast cell fate. Surprisingly, we find that the function of osr1 in post-gastrulation mesoderm differentiation is linked to an early role in regulating mesoderm versus endoderm differentiation during gastrulation. Our findings reveal a novel role for endoderm in determining the balance of kidney versus angioblast cell differentiation during somitogenesis.

### MATERIALS AND METHODS

#### Plasmid constructs

The zebrafish osr1 gene was identified by tblastn search of zebrafish genomic DNA sequence (Sanger Center zebrafish genome project; http://www.sanger.ac.uk/Projects/D_rerio/) using mouse Osr1 protein sequence as query. Reverse blastx using zebrafish osr1 coding sequence as query against GenBank confirmed the zebrafish gene as the closest osr1 ortholog. Full-length osr1 was amplified by RT-PCR from RNA obtained from 24 hpf wild-type Tü/AB embryos and cloned into pCR4 vector. Additional plasmid probes (lim1, pax2a, pax8, nbc1, nephrin, wtla, ae2, myoD, ret1, scl, gata1, fkl1, mxx2.5, etsrp1, pl.1 and trpm7) have been previously described. Synthetic capped mRNAs for rescue experiments were synthesized from linearized full-length plasmid constructs using mMessage Machine kit (Ambion, USA). Specifically, pax2a mRNA and osr1 mRNA was obtained by in vitro transcription with sp6 polymerase from a NorI-linearized and a KpnI-linearized full-length construct, respectively.

#### Zebrafish embryos

Wild-type zebrafish were maintained according to standard protocols (Westerdie, 1995). The embryos for experiments were collected from crosses of wild-type Tü/AB adults, grown at 28°C and fixed at the indicated developmental stages. 

### RESULTS

**osr1 expression during gastrulation and somitogenesis**

osr1 is expressed in the germ ring at 30% epiboly (Fig. 1A) (Tena et al., 2007) and, at 75% epiboly, in cells dispersed over the yolk in a pattern similar to endoderm progenitors (Kikuchi et al., 2000). Two-color in situ hybridization using osr1 and no tail (ntl) probes revealed that osr1 expression is restricted to vegetal tiers of mesendodermal cells closest to the margin and is not present in more-animal cells that express ntl. osr1 expression overlapped with sox32 expression in the most vegetal tiers of germ ring mesendoderm but not in cells of the yolk syncytial layer (YSL) (Fig. 1E,F). To discern whether osr1 was expressed in both mesoderm and endoderm progenitors, we used double-fluorescent in situ hybridization and confocal microscopy. Double-fluorescent in situ with osr1 and ntl probes reveal that osr1 and ntl expression overlapped in a subset of cells at 60% epiboly, whereas other more dispersed cells were positive for osr1 but not for ntl (Fig. 1G-I). To determine whether these cells were endodermal...
osr1: kidney versus vascular development

Previous studies reported that zebrafish osr1 was co-expressed with the nephric markers pax2a and lim1 in the intermediate mesendoderm during somitogenesis (Tena et al., 2007). To discern the osr1 expression pattern at higher resolution, we assayed its expression relative to known IM/LPM markers using single and two-color in situ hybridization. At the tailbud stage (10 hpf), lim1 is expressed in bilateral stripes of IM adjacent to presomitic mesoderm and in adaxial cells (Fig. 2A,B). By contrast, osr1 was expressed in cells displaced laterally and ventrally from the lim1 expression domain (Fig. 2C,D). During somitogenesis, this pattern persisted and osr1 was detected in bands of cells that superficially appear to be the intermediate mesoderm; however, histological sections of 18 hpf embryos show that these osr1-expressing cells lie ventral and lateral to the pronephros, and are excluded from the forming pronephros (Fig. 2E,F). This is also clear at 24 hpf (Fig. 2G,H) where sections of the trunk show osr1 expression in ventrolateral tissues, but not in the pronephros. Sections of 18 and 24 hpf embryos also revealed that osr1 was expressed in the endoderm at the midline. Endoderm expression was confirmed by examining older embryos where osr1 was shown to be expressed in the liver and gut at 48 hpf (see Fig. S1 in the supplementary material). Two color in situ revealed that osr1-expressing cells were lateral to pax2a (Fig. 2I,J), scl (Fig. 2L-M) and etsrp1 (Fig. 2O-Q) expressing cells. Sections of embryos double-stained for pax2a and osr1 confirmed that osr1 is expressed ventrally and laterally to the forming kidney (Fig. 2K). We conclude that osr1 is not expressed in the IM during somitogenesis, as previously reported. The lateral and ventral position of osr1-positive cells and the early expression osr1 in both mesoderm and endoderm suggests that this tissue is likely to be the zebrafish equivalent of the splanchnopleure (Funayama et al., 1999).

In light of previous reports on osr1 function in nephrogenesis and our results that osr1 is not expressed in kidney tissue, we re-examined the osr1 loss-of-function phenotype.

osr1 is specifically required for proximal pronephric nephron development

Previous studies suggested that osr1 loss of function resulted in the complete absence of kidney tissue and gross edema (Tena et al., 2007). By contrast, we found that osr1 loss of function by morpholino knockdown (Fig. 3A,B) resulted in a segment-specific defect in the proximal nephron, whereas the distal nephron was relatively unaffected (Fig. 3C-F). The chloride-bicarbonate exchanger ace2 is expressed highly in the proximal nephron and at a lower level in the distal nephron at 24 hpf (Fig. 3C) (Shmukler et al., 2005). In osr1 morphants, ace2 expression is specifically missing in the proximal nephron, whereas expression in the distal nephron is unchanged (Fig. 3D; 90% of injected embryos; n=10). Expression of the NaK ATPase α subunit marks the full length of the nephron at 72 hpf (Drummond et al., 1998) (Fig. 3E). In confocal images of osr1 morphants, NaK ATPase-positive tubules were truncated, with proximal tubule segments missing (100% of injected embryos, n=15; Fig. 3F). Both NaK ATPase staining and ace2 in situ often revealed an asymmetric loss of the proximal nephron (Fig. 3D). In one experiment, nine out of 17 embryos showed asymmetric loss of the proximal nephron, while the remaining eight showed a symmetric loss (as shown in Fig. 4H). Similar proximal segment-specific loss was observed using in situ probes for lim1, pax8, osr2 and nbc1 (data not shown). Expression of more distal nephron segment markers trpm7 (Elizondo et al., 2005; Liu et al., 2007; Wingert et al., 2007) and ret1 (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997) were unaffected by osr1 loss of function (see Fig. S2 in the supplementary material). Similar results were obtained with both an osr1 ATG

Fig. 1. osr1 expression during early gastrulation. (A,B) osr1 expression in the germ ring at 30% epiboly (A) and in the gastrulating cells at 75% epiboly (B). (C) Two-color in situ of osr1 (blue) and ntl (red) mRNA transcripts at 30% epiboly. (D) Cross-section of C at the level indicated shows that osr1 is not expressed in the ntl-positive mesodermal cells farther from the margin (red arrowhead) and is restricted to mesendoderm cells closest to the margin (blue arrowhead). (E) Colocalization of osr1 (blue) and sox32 (red) mRNA transcripts at 30% epiboly. (F) Cross-section of E at the level indicated shows osr1 expression in the mesendoderm cells with some overlap with sox32-positive endodermal cells (blue arrowhead) but the absence of osr1 expression in the sox32-positive YSL cells (red arrowhead). (G-O) Magnified lateral views of 60% epiboly embryos just above the blastoderm margin with dorsal on the right. (G-I) Double-fluorescent in situ hybridization of osr1 (red) and ntl (green) probes at 60% epiboly. The images represent a maximum intensity projection of a confocal z-series stack (four slices, 1.9 μm each). (I) Merge of G and H showing distinct expression of osr1 (G) in endodermal cells (red arrowhead, G,I) that do not express ntl (H). osr1 (G) is co-expressed with ntl (H) in mesendodermal cells (green arrowhead, H,I). (J-L) Double-fluorescent in situ hybridization of osr1 (red) and sox17 (green) at 60% epiboly. Images represent a single confocal slice of 1.9 μm. (L) Merge of J and L showing co-expression of osr1 and sox17 in endodermal cells. (M-O) Double-fluorescent in situ hybridization of osr1 (red) and sox32 (green) at 60% epiboly. Images represent a single confocal slice of 1.9 μm. (O) Merge of M and N showing co-expression of osr1 and sox32 in endodermal cells. Scale bars: in G,I,L, 10 μm for G-O.

progenitors, we assayed expression of the endodermal markers sox17 and sox32. Double-fluorescent in situ revealed that dispersed osr1-expressing cells also expressed sox17 (Fig. 1J-L) and sox32 (Fig. 1M-O). The data indicate that osr1 is expressed in mesendoderm closest to the YSL at 30% epiboly; later, as epiboly progresses, osr1 is expressed in endoderm progenitors as well as in mesodermal cells that express ntl.
initiation codon blocking morpholino and the exon 2 splice donor morpholino; all subsequent experiments were performed with the exon 2 donor morpholino as we could more rigorously determine the efficacy of osr1 knockdown using RT-PCR. Apoptosis assays also revealed that loss of the proximal nephron was not due to cell death (see Fig. S3 in the supplementary material). These results indicate that kidney defects in osr1 morphants are specific to the proximal nephron and also that osr1 loss of function does not result in a general re-patterning of nephron segments.

**osr1 is required for glomerular morphogenesis**

To determine whether pronephric glomerular development was affected by osr1 knockdown, we assayed expression of the Wilms tumor suppressor gene wt1a, a marker of podocyte specification (Bollig et al., 2006; Drummond et al., 1998; Majumdar and Drummond, 1999; Perner et al., 2007; Serluca and Fishman, 2001). In wild-type embryos, wt1a is expressed in anterior lateral mesoderm (Serluca and Fishman, 2001) and strongly in prospective pronephric podocytes at 24 hpf (Fig. 4A,C). At 48 hpf, wt1a-positive podocytes surround a compact glomerular vascular tuft derived from the aorta (Fig. 4E) (Drummond et al., 1998; Majumdar and Drummond, 1999). In osr1 morphants, wt1a was expressed at 26 hpf, although in a somewhat more dispersed pattern (Fig. 4B). Histological sections showed bilateral groups of podocyte progenitors ventral to the somites in all osr1 morphant embryos (Fig. 4D). However, these progenitors failed to coalesce into a compact structure at the midline, and a mature vascularized glomerulus is never formed. nephrin is an essential component of the pronephric glomerulus and is expressed in podocytes in wild-type embryos (Fig. 4G) (Kramer-Zucker et al., 2005). osr1 loss of function eliminated nephrin expression in podocytes (91% of injected embryos, n=12; Fig. 4H). Similarly, expression of podocin, another podocyte-specific marker, was absent from the glomerulus in osr1 morphants (data not shown). The data suggest that osr1 is not required for podocyte specification but rather functions at a later step in glomerular maturation associated with integration of blood vessels with podocytes and the expression of the podocyte cell adhesion molecules nephrin and podocin.

**osr1 loss of function expands the angioblast cell lineage**

As derivatives of the IM/LPM also include the vasculature, blood and the heart, we analyzed the expression of vascular and blood markers in wild-type and osr1 morphant embryos. The transcription factor scl is expressed in both vascular and hematopoietic progenitor cells (Gering et al., 1998). In zebrafish, scl is first expressed during somitogenesis in cells that occupy bilateral stripes in the trunk IM/LPM (Gering et al., 1998) (Fig. 5A,C). Strikingly, the number of scl-expressing cells in the IM/LPM was significantly expanded in osr1 morphants at the 12-somite stage (89% of injected embryos,
Similar to hemangioblasts and subsequently maintained in endothelial cell-specific receptor for VEGF is initially expressed in controls vascular development without affecting hematopoietic

...is significantly upregulated in the anterior trunk, similar to what we observed for...  

...is expressed during early somitogenesis in bilateral stripes in the head and trunk of wild-type embryos (Fig. 5A). Embryo staging was confirmed by double color in situ with...  

...is lost in osr1 morphants (D), whereas the distal pronephros is unaffected (black arrowhead).

Development (Sumanas and Lin, 2006). Similar to scl and flk1, etsrp1 is expressed in bilateral stripes in the head and trunk of wild-type embryos during somitogenesis (Sumanas and Lin, 2006) (Fig. 5E). In 12-somite osr1 morphants, etsrp1-expressing cells are significantly expanded in the anterior trunk IM/LPM (90% of injected embryos, n=11; arrows, Fig. 5F).

The effect of osr1 on hematopoietic lineages was evaluated with the markers pu.1 for the monocytic lineage (Lieschke et al., 2002) and gata1 for the erythropoietic lineage (Detrich et al., 1995). In wild-type embryos, pu.1-positive myeloid progenitors are expressed in both the rostral blood island and the posterior intermediate mesoderm or caudal blood island (Fig. 5I). Contrary to what we observe for scl, flk and etsrp, in osr1 morphants, pu.1 expression was not expanded and in fact was reduced in the anterior IM/LPM (87%...
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Fig. 5. osr1 knockdown expands vascular progenitor tissue. (A) scl (blue) and pax2a (red) expression in wild-type embryos labels adjacent bands (arrowheads) of intermediate mesoderm in 12-somite stage wild-type embryos. (B) osr1 knockdown results in expansion of scl-positive tissue, most prominently in anterior LPM (arrowheads) and loss of pax2a-expressing cells. (C) flik (blue) and scl (red) expression in 12-somite wild-type embryos (arrowhead). (D) osr1 knockdown increases the number of flik-expressing cells (arrowheads). (E) etsrp1 expression in control 12-somite embryos (arrowhead). (F) etsrp1 expression is expanded in 12-somite stage osr1 morphants (arrowheads). (G) At 26 hpf, scl is expressed in the blood islands and forming venous plexus (arrowhead). (H) 26 hpf osr1 morphants show an expansion of scl-positive tissue in the region of the forming venous plexus (arrowhead). (I,J) Expression of the monocyte lineage marker pu.1 in wild-type embryos (I) and osr1 morphants (J) shows a reduction of expression in the anterior aspect of its LPM expression domain (arrowheads). (K,L) Similarly, expression of the erythrocyte marker gata1 in wild-type embryos (K) and osr1 morphants (L) shows a reduction of expression in its most anterior expression domain (arrowheads).

osr1 overexpression expands kidney progenitors at the expense of angioblast cell number

To confirm results on osr1 loss of function, we tested whether osr1 gain of function would have opposite effects on patterning the anterior IM/LPM. By the 13-somite stage, pax2a is normally downregulated in the mid-portion of the IM in wild-type embryos (Fig. 6A). Ectopic expression of osr1 by synthetic osr1 mRNA injection at the one-cell stage resulted in enhanced expression of pax2a throughout the IM and specifically prevented the downregulation of pax2a in the mid-portion of the IM (78% of injected embryos, n=32; Fig. 6B). In a complementary fashion, scl expression in the anterior LPM (Fig. 6C) was specifically lost in osr1 morphants (70% of injected embryos, n=27) (Fig. 6D). No ectopic expression of either lineage marker was induced outside of their respective expression domains by osr1 overexpression. The results indicate that overexpression of osr1 is sufficient to re-pattern the anterior IM/LPM, adjacent to somites 1-8.

osr1 is required for pronephric epithelial differentiation and to limit the size of the axial vein

To assess whether a re-specification of mesoderm occurs in osr1 morphants, we sectioned control (Fig. 7A) and osr1 morphants (Fig. 7B) at 52 hpf, prior to the development of gross edema, to
examine the morphology of the glomerulus, pronephric ducts and vasculature. At the level of pectoral fin in control embryos (Fig. 7C, inset), the glomerulus was visible ventral to the aorta as a compact structure (arrowhead, Fig. 7C). However, in osr1 morphants, all sectioned embryos lacked a vascularized glomerulus and pronephric tubules at the level of the pectoral fin (Fig. 7D, inset; n=4). Instead, all sectioned embryos showed prominent profiles of the cardinal veins (Fig. 7D, n=4), which in serial sections, could be distinguished from the pronephros by the presence of red blood cells in the lumen. In more posterior sections of wild-type embryos (Fig. 7E), the pronephros was visible as bilateral epithelial tubules (arrowhead, Fig. 7E) that flank the medial aorta and vein, here filled with nucleated red blood cells. In osr1 morphants, the pronephric tubules in the trunk were present but appeared smaller (arrowhead, Fig. 7F) compared with controls.

Strikingly, the size of the medial vein was significantly enlarged in osr1 morphants (v in Fig. 7F) at all the A-P levels examined (v in Fig. 7D,F). To determine whether the increase in vein lumen size was associated with a corresponding increase in vein endothelial cell number and to rule out the possibility that vein expansion was secondary to edema, we counted DAPI stained endothelial cell nuclei in 15 μm sections (from the trunk region, as in Fig. 7E,F) of 36 hpf osr1 morphants that showed no pericardial expansion or other evidence of edema. osr1 morphants showed a significant increase in the number of vein endothelial cell nuclei [5.11±0.19/section (s.e.m.), n=26 compared with 3.04±0.09/section, n=25 in control]. Arterial size and endothelial cell number were not affected (2.6±0.1, n=25 in control versus 2.8±0.14, n=26 in morphants) in osr1 morphants. To further analyze the enlargement of veins in osr1 morphants, we performed microangiography on control and osr1 morphants at 48 hpf. Control embryos and osr1 morphants showed normal circulation in the dorsal aorta and intersomitic vessels. However, the venous plexus region distal to the yolk extension was dramatically expanded in all osr1 morphants examined (v in Fig. 7H; n=5) when compared with the control embryos (v in Fig. 7G). Taken together, the histology, cell counting and microangiography data strongly suggest that the venous cell fate is expanded in osr1 morphants.

**osr1 acts upstream of pax2a in kidney development**

Pax2 and Pax8 are known to function partially redundantly to control kidney development in the mouse (Bouchard et al., 2002). In zebrafish, pax2a is specifically required for proximal tubule cell differentiation in the pronephros (Majumdar et al., 2000). We therefore tested whether ectopic expression of pax2a in osr1 morphants would be sufficient to bypass a requirement for osr1 and revert the osr1 morphant phenotype to wild-type patterning of kidney and vascular tissues. We used the expression of ae2 as a measure of the proximal nephron length in control and experimental embryos. The length of ae2-positive proximal tubules in control embryos ranged from 300-500 μm (mean of 378 μm; 14/14, 100%) (Fig. 8A,G). In osr1 morphants, ae2 segment length was reduced (Fig. 8B) with 42% of embryos (11/26) in the 100-200 μm range, 50% of embryos in the 200-300 μm range and only 8% of the embryos (2/26) exhibiting wild-type segment length (Fig. 8G). The overall mean ae2-positive tubule length for osr1 morphants was 214 μm compared with 378 μm for controls. Injection of pax2a mRNA into osr1 morphants restored 60% of the embryos (18/28) to wild-type patterning of kidney and experimental embryos. The length of ae2-positive proximal tubules (mean of 378 μm) for controls. Injection of pax2a mRNA into osr1 morphants restored 60% of the embryos (18/28) to wild-type patterning of kidney and experimental embryos.

In a complementary fashion, pax2a expression reverted the expanded domain of scl expression (compare Fig. 8D with 8E) to a wild-type pattern (Fig. 8F). The data indicate that in the absence of osr1 function, ectopic expression of pax2a is sufficient to specify proximal pronephric tubule differentiation. In addition, ectopic expression of pax2a is sufficient to limit angioblast differentiation in the intermediate mesoderm.

**osr1 effects on mesoderm patterning are mediated by the endoderm**

The simplest interpretation of our results so far would be that osr1 functions early in the IM/LPM upstream of pax2a and scl to drive kidney development while repressing angioblast differentiation. However, several inconsistencies with this model were evident in our data. First, our finding that the anterior IM/LPM was preferentially affected in osr1 morphants was not consistent with the broad posterior expression of osr1 in the IM that extended to the
We were also surprised to find that the re-patterning of the IM/LPM by osr1 loss of function occurred progressively during somitogenesis and was not evident at the earliest stages of pax2a and scl expression. The early expression pattern of pax2a at the 5-somite stage in osr1 morphants (Fig. 9B) was, in fact, similar to the wild-type pattern (Fig. 9A). However the anterior IM pax2a expression domain at the 14-somite stage (Fig. 9C) was reduced in osr1 morphants (89% of injected embryos, n=37; Fig. 9D) and, by 24 hpf, completely absent (96% of injected embryos, n=53) (compare Fig. 9E with 9F). Similarly, scl expression was normal at the 8-somite stage in osr1 morphants (Fig. 9G,H) and only later, at the 14-somite stage, was found to be expanded in osr1 morphants (89% of injected embryos, n=19) (Fig. 9I,J). We could also rule out that the intermediate mesoderm was patterned by an antagonistic relationship between genes downstream of osr1 (pax2a and scl) as loss of function in these genes alone, or in combination with osr1 loss of function, did not result in expansion of the opposing lineage (see Fig. S5 in the supplementary material). These results, together with our finding that ectopic expression of osr1 only affected cell differentiation within the anterior IM/LPM, suggested that osr1 might act indirectly to pattern the mesoderm.
In addition to its expression in the IM/LPM, osr1 is expressed in the germ ring mesendoderm at the shield stage (Fig. 1) (Tena et al., 2007). We examined whether osr1 might function in mesendoderm patterning by assessing expression of the endoderm-specific markers, foxa2 and sox17. Strikingly, we found that endoderm differentiation was strongly enhanced in osr1 morphants. The number of sox17-positive cells at the shield stage (Fig. 10A-C) was significantly increased in osr1 morphants at the blastoderm margin (bar in E,F) and in the ventral region of the embryo (arrowhead, D) when compared with control (A,C; bar in B). (G-L) Expression of mesendoderm marker, foxa2 in control (G-I) and osr1 morphants (J-L) at the shield stage. (G,J) Dorsal views; (H,K) side views with dorsal facing; (I,L) magnified views of boxed regions in H,K, respectively. The number of tiers of foxa2-expressing cells were significantly increased in osr1 morphants with more layers of foxa2-expressing cells at the blastoderm margin (bar in K,L) and enhanced expression in the ventral region of the embryo (arrowhead, J) when compared with control (G,J; bar in H).

Development of foxa2-positive pharyngeal endoderm was enhanced in osr1 morphants (arrowheads, N) when compared with control embryos (arrowheads, M) and was completely blocked by sox32 knockdown (arrowheads, O).

Fig. 10. osr1 knockdown causes expansion of endoderm. (A-F) Expression of endoderm marker sox17 in control (A-C) and osr1 morphants (D-F) at the shield stage. (A,D) Dorsal views; (B,E) side views with dorsal facing; (C,F) magnified views of boxed regions in B,E, respectively. The number of tiers of sox17-expressing cells was significantly increased in osr1 morphants at the blastoderm margin (bar in E,F) and in the ventral region of the embryo (arrowhead, D) when compared with control (A,C; bar in B). (G-L) Expression of mesendoderm marker, foxa2 in control (G-I) and osr1 morphants (J-L) at the shield stage. (G,J) Dorsal views; (H,K) side views with dorsal facing; (I,L) magnified views of boxed regions in H,K, respectively. The number of tiers of foxa2-expressing cells were significantly increased in osr1 morphants with more layers of foxa2-expressing cells at the blastoderm margin (bar in K,L) and enhanced expression in the ventral region of the embryo (arrowhead, J) when compared with control (G,J; bar in H).

In addition to its expression in the IM/LPM, osr1 is expressed in the germ ring mesendoderm at the shield stage (Fig. 1) (Tena et al., 2007). We examined whether osr1 might function in mesendoderm patterning by assessing expression of the endoderm-specific markers, foxa2 and sox17. Strikingly, we found that endoderm differentiation was strongly enhanced in osr1 morphants. The number of sox17-positive cells at the shield stage (Fig. 10A-C) was significantly increased in osr1 morphants (100% of injected embryos, n=14; Fig. 10D-F). Similarly, the number of foxa2-positive cells (Fig. 10G-I) was increased in osr1 morphants (71% of injected embryos, n=21; Fig. 10J-L). Intensified expression of foxa2 at the 18-somite stage (86% of injected embryos, n=15; Fig. 10M,N) confirmed that development of the pharyngeal endoderm was enhanced by osr1 loss of function. sox32/casanova is a transcription factor that is required for all endodermal development (Alexander et al., 1999). As previously reported (Dickmeis et al., 2001), knockdown of sox32 using an antisense morpholino (Dickmeis et al., 2001) specifically eliminated foxa2-expressing endoderm (93% of injected embryos, n=15) but did not affect foxa2 expression in axial mesoderm (Fig. 10O).

The ability to block endoderm development by sox32/casanova knockdown allowed us to test whether expanded endoderm development was responsible for re-patterning the mesoderm in the context of osr1 loss of function. As expected, knockdown of osr1 alone resulted in a reduction in pax2a-positive cells (90% of injected embryos, n=14; Fig. 11B) compared with control (Fig. 11A) at 18...
somites. Knockdown of sox32 alone did not have noticeable effects on pax2a expression (98% of injected embryos, n=30; Fig. 11C). Remarkably, knockdown of sox32 and elimination of endoderm development in osr1 morphants restored pax2a expression to a normal wild-type pattern (74% of injected embryos, n=66). To confirm these results, we tested whether reduction in endoderm development in the mutant bonnie and clyde/mixer (bon) would rescue the osr1 loss-of-function phenotype. All embryos in an incross of bon heterozygotes showed a normal pattern of pax2a expression (Fig. 11E). Knockdown of osr1 in embryos of an incross of bon heterozygotes resulted in the expected osr1 phenotype in roughly three quarters of the embryos (73%; Fig. 11F), whereas the remaining quarter (27%) of the clutch showed a normal rescued pattern of pax2a expression (Fig. 11G). Rescue of pax2a expression by sox32 knockdown and the Mendelian ratio of pax2a rescued embryos in a bon+/− incross indicate that re-patterning of kidney versus vasculature in osr1 morphants can be accounted for by expanded development of endoderm. A primary function of osr1 may therefore be to pattern the mesendoderm during gastrulation.

**DISCUSSION**

The derivation of kidney and blood/vasculature from adjacent areas of mesoderm in developmental fate maps and the continued close association of progenitor cells during organogenesis (Crosier et al., 2002; Davidson and Zon, 2004; Fujimoto et al., 2001; Iraha et al., 2002; Kimmelman, 2006; Kimmelman and Griffin, 2000; Kimmel et al., 1990; Lane and Sheets, 2006; Vogeli et al., 2006; Walmsley et al., 2002) prompted us to examine whether genes acting during early development might affect the fate of both tissues. osr1 has been reported to be expressed in the intermediate mesoderm and required for mouse and zebrafish kidney development (James et al., 2006; So and Danielian, 1999; Wang et al., 2005). Our data indicate that osr1 is not simply required for kidney development but rather it acts early in development to pattern the mesendoderm that, in turn, has broader effects on development. Our results also show that in zebrafish, osr1 is not expressed in typical intermediate mesoderm but rather in more lateral and ventral cells that may represent the zebrafish splanchnopleure (Funayama et al., 1999). We find that osr1 expression in lateral cells (splanchnopleure) is not required for normal expression of pax2a as combined sox32/osr1 loss of function results in normal pax2a expression. Unexpectedly, the primary mechanism underlying osr1 loss-of-function phenotypes appears to be an increase in endoderm development that later acts to inhibit kidney and favor vascular cell differentiation in mesoderm.

**A role for osr1 in mesendoderm patterning**

Mesoderm and endoderm are derived from a mixed population of cells, the mesendoderm, that constitutes the germ ring in zebrafish embryos. Our results suggest that expression of osr1 in the germ ring plays an important role in mesendoderm patterning by acting as a repressor of endoderm formation. Both endoderm and mesoderm are induced by the Nodal-related factors cyclops and squint in zebrafish (Schier and Talbot, 2005). High levels of Nodal signals induce endoderm in the most marginal blastomeres, whereas in cells closer to the animal pole, induction of T-box factors and FGF promote mesoderm development and antagonize endoderm development (Schier and Talbot, 2005). Ventral expression of BMPs has also been shown to antagonize endoderm development (Poulain et al., 2006). osr1 expression is known to respond to BMP signaling in chick embryo mesoderm (James and Schultheiss, 2005) and we have confirmed that early expression of osr1 in zebrafish requires the activity of a functional bmp2b gene (data not shown). One model of osr1 activity would be that after induction by bmp2b signaling, osr1 acts as a transcriptional repressor in mesendoderm cells (Tena et al., 2007), antagonizing transcriptional responses downstream of Nodal signaling (Schier and Talbot, 2005). However, the expression pattern of osr1 throughout the germ ring suggests that, in addition to BMP signaling, osr1 might also be responsive to FGF or nodal signaling (Rodaway et al., 1999). Further experiments examining signals upstream of osr1 expression will be required to better define osr1 function in the context of mesendoderm patterning.

**Does altered mesendoderm patterning account for osr1 phenotypes?**

In mouse embryos, disruption of the Osr1 gene causes severe defects in urogenital development (Wang et al., 2005). Mutant mice show no evidence of ureteric bud or metanephric kidney development and cellular defects in the Wolffian duct are evident at a very early stage (E8.5) (James et al., 2006; Wang et al., 2005). The nephrogenic mesenchyme shows reduced expression of Wt1 (Wang et al., 2005) and also fails to express many other genes that define this tissue (James et al., 2006). The absence of properly specified nephrogenic mesenchyme in the mouse Osr1 mutants, taken together with our results in the zebrafish raise the possibility that Osr1 in the mouse may play additional roles outside of the nephrogenic mesoderm to ensure proper patterning of the intermediate mesoderm. Although Osr1 expression in endoderm has not been detected in the mouse by in situ hybridization, recent analysis of a mouse Osr1 (Osrl) bac transgenic shows that the Osr1 gene contains regulatory elements that drive reporter expression (Cre) in endodermal organs (Grieshammer et al., 2008). Although suggestive of a function for osr1 in endoderm, further experiments will be required to critically assess this possibility.

Our results differ from a previous study of osr1 expression and function in zebrafish kidney development (Tena et al., 2007) where it was concluded that ‘knockdown of osr1 and osr2 results in the loss of all pronephric structures including the glomerulus’. We find that osr1 morphant kidney defects are restricted to the proximal nephron, and that glomerular morphogenesis is arrested in a stage-specific fashion, subsequent to podocyte w14a expression. Our results are not due to a partial osr1 loss of function as we demonstrate that no wild-type osr1 mRNA can be detected by RT-PCR in osr1 morphants at 24 hpf. These discrepancies are most probably due to the fact that Tena et al. did not examine osr1 morphants with markers of the distal pronephros or the specification of glomerular podocytes by w14a expression. In addition, in contrast to Tena et al., we show that zebrafish osr1 is not expressed in pax2a-positive pronephric kidney cells during somitogenesis, nor in mature glomeruli. We observe osr1 expression in cells adjacent to the forming pronephros at the 18-somite stage, which could have been easily mis-identified as the pronephros by Tena et al. In addition, we observe strong osr1 expression in the liver, next to the glomerulus, at 48 hpf, which at low magnification may have been mistaken for glomerular expression by Tena et al. Our results agree with expression studies in the chick showing that Osr1 is not expressed in differentiated kidney cells. Ectopic expression studies in the chick also support the idea that osr1 expression may actually impede kidney epithelial differentiation (James et al., 2006).

Given the previous work on osr1 and its broad early expression in the intermediate mesoderm, the segment-specific loss of kidney tissue and the selective expansion of vascular tissue in the anterior trunk of osr1 morphants that we observed was unexpected. In addition, the fact that patterning defects in pax2a-positive kidney progenitors and scl-positive angioblasts were observed relatively...
late in development, during somitogenesis, argues that osr1 plays a role in maintenance, but not in specification, of mesodermal lineages. The simplest interpretation of our results is that signals that repress kidney and enhance angioblast development emanate from anterior endoderm and thus most strongly affect the anterior intermediate/lateral plate mesoderm. A central role for endoderm in the context of osr1 loss of function may also help explain other phenotypes of osr1 mutants/morphants. Both mouse and zebrafish embryos lacking osr1 often show asymmetric loss of the Wolffian duct/pronephric duct on the left side (Wang et al., 2005) (our results), which has been interpreted to suggest the existence of a latent left-right asymmetry in the normally bilaterally symmetric kidney. Our results raise the alternative possibility that asymmetric loss of kidney tissue could be due to underlying asymmetries in endodermal tissues that negatively affect Wolffian/pronephric duct formation. Asymmetric defects in Wolffian duct development have also been reported in Gata3 knockout mice (Grote et al., 2006), which might be due to cell-autonomous effects of Gata3 loss of function in the Wolffian ducts. Interestingly, however, Gata3 is also expressed in endodermal tissues (Caprioli et al., 2001; Debacker et al., 1999), which may indirectly affect kidney development.

Although we observed an increase in vascular tissue in osr1 morphants, we did not observe a corresponding increase in blood cell development. This could be due to the fact that the most strongly affected tissue in osr1 morphants, the anterior intermediate mesoderm, is known to be enriched for flk-and scl-positive angioblasts in zebrafish, whereas more posterior mesoderm contains both angioblasts and hematopoietic precursors that express gata1 (Dooley et al., 2005). Alternatively, signals from expanded endoderm in osr1 morphants may selectively favor angioblast development over erythropoiesis.

The role of the endoderm in mesodermal organogenesis

Our findings suggest that the effects of osr1 loss of function on kidney and vascular patterning are mediated by signals from the endoderm. The endoderm is known to regulate the development of other mesodermal derivatives such as the heart (Alexander et al., 1999; Dickmeis et al., 2001; Kikuchi et al., 2000; Reiter et al., 1999). In chick and frog, the anterior endoderm induces cardiogenesis by secreting a combination of BMPs and soluble inhibitors of Wnt signaling such as Crescent and Dkk-1 (Marvin et al., 2001; Schneider and Mercola, 2001). In addition to its potential role as an inducer of heart tissue, endoderm provides a matrix upon which cardiac progenitors and angioblasts migrate to form a fused heart tube (Jin et al., 2005; Trinh and Stainier, 2004). Interestingly, lack of endoderm in the one eyed pinhead zebrafish mutant has been associated with a specific loss of vein, but not of aorta, development (Brown et al., 2000), which would be consistent with our results that an early expansion of endoderm expands vein but not aorta development. In the chick and mouse, sonic hedgehog signaling from endoderm is important for vasculogenesis (Yokes et al., 2004); however, this is apparently not essential in zebrafish (Jin et al., 2005). A candidate signal for the effect of endoderm on kidney development might be sonic hedgehog, as it is expressed in the endoderm and when expressed ectopically, hedgehog proteins can inhibit nephrogenesis (Urban et al., 2006). However, we found that cyclopamine treatment did not reverse the effects of osr1 knockdown on kidney cell differentiation (Y.L. and I.D., unpublished), making it unlikely that hedgehog is the endoderm-derived signal. Thus, although these studies demonstrate that the endoderm is a rich source of soluble signaling molecules, it remains to be seen whether endoderm-derived soluble factors pattern kidney tissue in the IM.

In summary, our studies have uncovered a new role for osr1 in patterning mesendoderm. osr1 acts to inhibit endoderm differentiation during gastrulation. Our work has also uncovered a previously unknown role for endoderm in maintaining cell fate decisions in the intermediate mesoderm. Enhanced endoderm development favors the angioblast cell fate over kidney cell fate—presumably by non-cell-autonomous signals. Further identification of osr1 primary target genes and the signals emanating from endoderm are likely to reveal important aspects of kidney and vascular progenitor cell differentiation.

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Supplementary material

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References


