osr1 Is Required for Podocyte Development Downstream of wt1a

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ABSTRACT

Odd-skipped related 1 (Osr1) encodes a zinc finger transcription factor required for kidney development. Osr1 deficiency in mice results in metanephric kidney agenesis, whereas knockdown or mutation studies in zebrafish revealed that pronephric nephrons require osr1 for proximal tubule and podocyte development. osr1-deficient pronephric podocyte progenitors express the Wilms’ tumor suppressor wt1a but do not undergo glomerular morphogenesis or express the foot process junctional markers nephrin and podocin. The function of osr1 in podocyte differentiation remains unclear, however. Here, we found by double fluorescence in situ hybridization that podocyte progenitors coexpress osr1 and wt1a. Knockdown of wt1a disrupted podocyte differentiation and prevented expression of osr1. Blocking retinoic acid signaling, which regulates wt1a, also prevented osr1 expression in podocyte progenitors. Furthermore, unlike the osr1-deficient proximal tubule phenotype, which can be rescued by manipulation of endoderm development, podocyte differentiation was not affected by altered endoderm development, as assessed by nephrin and podocin expression in double osr1/sox32-deficient embryos. These results suggest a different, possibly cell-autonomous requirement for osr1 in podocyte differentiation downstream of wt1a. Indeed, osr1-deficient embryos did not exhibit podocyte progenitor expression of the transcription factor lhx1a, and forced expression of activated forms of the lhx1a gene product rescued nephrin expression in osr1-deficient podocytes. Our results place osr1 in a framework of transcriptional regulators that control the expression of podocin and nephrin and thereby mediate podocyte differentiation.


The major function of the kidney is to filter blood and maintain tissue fluid homeostasis. Podocytes are terminally differentiated cells of the glomerulus that surround blood capillaries with interdigitating foot processes to form specialized intercellular junctions called slit diaphragms that prevent loss of macromolecules from blood into the urinary space.1,2 Mutations in genes encoding podocyte slit diaphragm proteins, such as nephrin, podocin, CD2AP, protocadherin FAT, and P-cadherin, result in effacement of foot processes, proteinuria, and kidney failure.3–9 Thus, differentiated podocytes have an essential role in maintaining the integrity of the glomerular blood filtration barrier, and loss of podocyte differentiation by injury or congenital defect results in kidney disease. However, the pathways leading to podocyte differentiation are not well understood.

Odd skipped related 1 (osr1) is one of the earliest genes expressed in the intermediate mesoderm and plays an essential role in kidney and heart organogenesis.10 Osr1 belongs to the Odd skipped family
of genes, which encode evolutionarily conserved zinc-finger transcription factors required for embryonic development.\textsuperscript{11,12} In\emph{Drosophila}, the odd skipped gene, \textit{odd}, was first identified as a pair rule gene on the basis of the finding that mutations at this locus cause loss of portions of the odd-numbered segments in the embryo.\textsuperscript{11} \textit{Odd} was shown to be required for leg development and also for malpighian tubule development, the renal organs of \emph{Drosophila}.\textsuperscript{11,13} Homologs of \textit{odd} have been cloned in mice and humans. The mice \textit{osr} homolog \textit{Osr1} is first expressed in the early intermediate mesoderm, which gives rise to all renal structures, and later is expressed in developing limb and branchial arches of embryo.\textsuperscript{10,12} Homozygous \textit{Osr1} null mutants fail to form intermediate mesoderm and die in\emph{u}tero with cardiac defects and renal agenesis.\textsuperscript{10} In humans, mutations in \textit{OSR1} have yet to be associated with kidney agenesis; however, a variant \textit{OSR1} allele, which disturbs \textit{OSR1} mRNA expression in renal progenitor cells, has been associated with reduction of newborn kidney size and function.\textsuperscript{14} The zebrafish \textit{odd} homolog \textit{osr1} is first expressed in the early mesendoderm and later in a broad domain of lateral plate/intermediate mesoderm.\textsuperscript{15} Knockdown experiments in zebrafish have also revealed a role for \textit{osr1} in pronephric development. \textit{osr1}-deficient embryos display loss of proximal tubule and a stage-specific arrest of glomerular morphogenesis. \textit{osr1} plays an indirect role in proximal tubule development by repressing endoderm differentiation, which, in turn, favors proximal tubule formation.\textsuperscript{15} However, it is not known how \textit{osr1} functions in podocyte differentiation.

In the current work we have investigated the role of \textit{osr1} in podocyte differentiation and provide evidence that \textit{osr1} regulates podocyte differentiation by acting downstream of \textit{wt1a}, most likely in a cell autonomous manner. Significantly, we find that the transcription factor \textit{lhx1a} is required downstream of \textit{osr1} for \textit{nephrin} expression and podocyte differentiation. Our results place \textit{osr1} in a network of transcriptional regulators (RA → \textit{wt1a} → \textit{osr1} → \textit{lhx1a} → [\textit{podocin}/\textit{nephrin}]) that mediate podocyte differentiation.

**RESULTS**

\textit{osr1} and \textit{wt1a} Are Coexpressed in Zebrafish Podocyte Progenitor Cells

We have shown previously that \textit{osr1}-deficient embryos fail to form a functional pronephric glomerulus and do not express markers of the differentiated glomerular podocytes \textit{podocin} and \textit{nephrin}.\textsuperscript{15} To assess how \textit{osr1} might fit in the sequence of events leading to podocyte differentiation, we compared expression of \textit{osr1} with expression of known podocyte markers. The Wilms’ tumor suppressor \textit{wt1a} marks podocyte differentiation and is first expressed in bilateral clusters of cells in the anterior intermediate mesoderm, adjacent to somites 1–3\textsuperscript{16} (Figure 1A). At the same stage of development, whole-mount \textit{in situ} hybridization revealed that \textit{osr1} is expressed in similar cells as \textit{wt1a} (Figure 1A). Histologic sections further

![Figure 1.](image)

\textit{osr1} and \textit{wt1a} are coexpressed in podocyte progenitors. (A) Expression of \textit{wt1a} and \textit{osr1} in anterior intermediate mesoderm in a zebrafish embryo at 16 high-power field. Dashed lines (b, c) denote planes of section in B and C. (B and C) Cross-sections of embryos showing the expression domains of \textit{wt1a} (B) and \textit{osr1} (C) in anterior intermediate mesoderm (arrowheads). (D–I) Magnified views of single 6-μm confocal sections of boxed regions in D–F show \textit{wt1a} expression (G), \textit{osr1} expression (H), and coexpression of \textit{wt1a} and \textit{osr1} (I) in cells of anterior mesoderm. Dotted lines represent the scale of a single cell in G–I; scale bar in I=10 μm. Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue).

suggested that \textit{osr1} (Figure 1B) and \textit{wt1a} (Figure 1C) were coexpressed in anterior intermediate mesoderm. Double fluorescent \textit{in situ} hybridization in conjunction with confocal microscopy confirmed that \textit{wt1a} and \textit{osr1} were coexpressed in podocyte progenitors (Figure 1, D–I). These results suggest that defects in kidney glomerular differentiation in \textit{osr1}-deficient embryos may be due to an early, cell-autonomous requirement for \textit{osr1} in podocyte progenitors.

\textit{osr1} Is Required for Podocyte Development Downstream of \textit{wt1a}

Coexpression of \textit{wt1a} and \textit{osr1} in podocyte progenitors and loss of podocyte marker expression in both \textit{osr1}- and \textit{wt1a}-deficient embryos\textsuperscript{15,17} suggested that these two transcription factors could act together or sequentially in podocyte differentiation. \textit{osr1} is not required for expression of \textit{wt1a},\textsuperscript{15} but it is
not known whether expression of \textit{wt1a} is required for \textit{osr1} expression in podocyte progenitors. Morpholino knockdown of both \textit{wt1a} and its paralog \textit{wt1b} (Figure 2, A–C) caused loss of expression of podocyte markers, \textit{podocin} (Figure 2, D and E) and \textit{nephrin} (Figure 2, F and G), consistent with the known requirement for \textit{wt1} in podocyte development.\textsuperscript{18} Significantly, \textit{wt1a/b} knockdown also eliminated \textit{osr1} expression, specifically in podocyte progenitors, but not in endoderm (Figure 3, A–D). \textit{wt1a} is a retinoic acid–regulated gene, and inhibition of local generation of retinoic acid, using retinoic acid–synthesizing (RALDH) enzymes inhibitor diethylaminobenzaldehyde (DEAB), blocks expression of \textit{wt1a}.\textsuperscript{19} Here we show that DEAB treatment also prevented expression of \textit{osr1} (Figure 3, E and F), consistent with a requirement for \textit{wt1a} in \textit{osr1} expression. Together, these results suggest a sequential induction of \textit{wt1a} by retinoic acid and subsequent \textit{wt1a}-dependent induction of \textit{osr1} in podocyte progenitors, ultimately leading to podocyte differentiation and expression of the structural genes \textit{nephrin} and \textit{podocin}.

\textbf{osr1 \textsuperscript{Regulates Zebrafish Podocyte and Kidney Tubule Development by Distinct Mechanisms}}

Previously we found that \textit{osr1}-deficient embryos lack kidney proximal tubules and show expanded vascular development. Loss of proximal tubules is caused indirectly by an early effect of \textit{osr1} deficiency that expands endoderm development.\textsuperscript{15} We therefore tested whether podocyte differentiation and glomerular morphogenesis might similarly be inhibited by endoderm signals, using \textit{sox32} morpholino knockdown to prevent

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{wt1a/b knockdown results in loss of podocyte nephrin and podocin expression. (A and B) Expression of \textit{osr1} in intermediate mesoderm podocyte progenitors in control (A) and \textit{wt1a/b} morphants (B) at 18 high-power fields. \textit{osr1} expression is absent from podocyte progenitors (white arrowheads) but persists in endoderm of \textit{wt1a/b} morphants. (C and D) Cross-sections of A and B at the level indicated (dotted lines in A and B) show that expression of \textit{osr1} is absent in podocyte progenitors (black arrowheads) in \textit{wt1a/b} morphants but persists in endoderm (white arrowhead). (E) \textit{osr1} expression in podocyte progenitors in control embryos treated with DMSO. (F) Loss of \textit{osr1} expression in podocyte progenitors of embryos treated with the differential expression of retinoic acid–synthesizing inhibitor 4-diethylaminobenzaldehyde.}
\end{figure}
endoderm expansion in osr1-deficient embryos. At 48 high-power fields, wt1a marks podocytes that form a compact glomerular structure at the embryo midline in wild-type embryos (Figure 4A). Glomerular morphogenesis is blocked in osr1 morphants where wt1a-positive podocytes persist as scattered, lateral groups of cells (Figure 4B). In contrast to its effect of restoring proximal tubule development in osr1-deficient embryos (Figure 4, D–F),15 sox32 knockdown did not restore midline glomerular morphogenesis (Figure 4C). Similarly, sox32 knockdown did not restore nephrin (Figure 4, G–I) or podocin (Figure 4, J–L) expression in osr1-deficient embryos, indicating that the function of osr1 in podocytes is distinct from its role in proximal tubule development. These results suggest that osr1 may regulate podocyte development cell autonomously, as part of a transcriptional cascade.

**lhxl a Acts Downstream of osr1 to Regulate Podocyte Cell Differentiation**

The LIM homeodomain transcription factor Lhx1a is required for podocyte differentiation during mouse kidney development.20 In zebrafish, lhxl a is expressed transiently in early podocyte progenitors, subsequent to wt1a and osr1 expression and before expression of podocin and nephrin (Figure 5A).21 Interestingly, we found that osr1 knockdown completely eliminated lhxl a expression specifically in the presumptive anterior pronephric region of intermediate mesoderm in 15 high-power fields embryos (Figure 5B; n=15/15), while lhxl a expression in the tail bud of osr1 morphants remained unaltered (data not shown). We therefore tested whether lhxl a might act downstream of osr1 specifically in podocyte development by assaying whether expression of an activated form of Lhx1a (Ldb1-Lhx1; denoted LL-CA)22 could rescue podocyte differentiation in osr1 morphants. The Lim homeodomain of Lhx1a interacts with the LIM binding protein, Ldb, and this interaction triggers Lhx1a activation.22 We expressed a constitutively active Ldb1-Lhx1 protein (LL-CA), which is a fusion of the Ldb1 dimerization domain with the linker, C-terminal and homeodomain of zebrafish Lhx1a.22 High doses of LL-CA mRNA (320 pg) caused early embryonic lethality associated with failed gastrulation cell movements, consistent with the known role for Lhx1a in gastrulation.23 An optimized injection dose (100 pg) allowed for 20% survival of embryos to 3 days post fertilization (n=11/50), allowing us to test whether activated Lhx1a could restore nephrin expression in osr1 morphants. Compared with osr1 morphants alone that never showed differentiated podocytes marked by nephrin expression (Figure 5D; 0%, n=0/30), 40% of surviving osr1 morphants co-injected with activated LL-CA mRNA showed rescue of nephrin expression in podocytes (Figure 5E; n=4/10). This partial rescue of podocyte differentiation (nephrin expression) suggests that lhxl a is required downstream of osr1 in podocyte development. Taken together, our results indicate that osr1 is part of an essential transcriptional cascade driving podocyte differentiation and glomerular morphogenesis.

**DISCUSSION**

Podocytes are complex cells whose differentiation depends on the interplay of multiple transcription factors, including Wt1a, Foxc1/2, Hey1, Notch, Lmx1b, MafB, and Gα-binding protein.24–27 However, the network of the transcription factors that govern podocyte differentiation remains incomplete. Our findings show that osr1 is coexpressed with wt1a in podocyte progenitors, pointing to a novel role for osr1 in early podocyte differentiation. In the mouse, Osr1 is expressed early in the intermediate mesoderm and later in metanephric cap mesenchyme.28 The kidney agenesis phenotype observed in Osr1

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**Figure 4.** osr1 is required downstream of wt1a for glomerular development. (A–C) Expression of wt1a in control, osr1 morphants, and osr1/sox32 double morphants, respectively, at 48 high-power fields. wt1a-expressing podocyte progenitors coalesce at midline in control embryos (A) but not in osr1 morphants (B) or osr1/sox32 double morphants (C). (D–F) Expression of slc4a2a and nephrin in control (D), osr1 morphants (E), and osr1/sox32 double morphants (F) at 48 high-power fields. osr1 knockdown resulted in loss of nephrin expression and absence of proximal tubules (E). Double knockdown of osr1/sox32 rescued proximal tubules but not nephrin expression (F). (G–I) nephrin expression in control (G), osr1 morphants (H), and osr1/sox32 double morphants (I). nephrin expression is lost in osr1 morphants (H) and not restored in osr1/sox32 double morphants (I). (J–L) podocin expression in control (J), osr1 morphants (K), and osr1/sox32 double morphants (L). podocin expression is lost in osr1 morphants (K) and not restored in osr1/sox32 double morphants (L).

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knockout mice points to a role for Osr1 in maintaining the self-renewing, proliferative metanephric nephron stem cell population. In the absence of data on Osr1 conditional knockouts, it is not known whether Osr1 may have additional roles later in kidney morphogenesis and cell type differentiation. In zebrafish, the pronephros develops directly from intermediate mesoderm, bypassing a requirement for a self-renewing kidney mesenchyme and allowing for direct examination of the roles of kidney transcription factors in renal cell type differentiation.

Coexpression of osr1 with wti1a in the zebrafish intermediate mesoderm raises the possibility that these two transcription factors could be working synergistically or sequentially for podocyte differentiation. Our previous studies showed that wti1a expression is not dependent on osr1. Conversely, here we show that osr1 expression is absent in wti1a morphants, demonstrating that osr1 is downstream of wti1a and regulated directly or indirectly by wti1a in podocyte progenitors. osr1 expression was also absent in DEAB-treated embryos, suggesting a role for retinoic acid (RA) in osr1 expression. Early specification of renal progenitor cells is known to require RA signals from the axial/paraxial mesoderm. Consistent with this, the kidney progenitor cell field is expanded upon ectopic application of RA, and renal progenitor specification is inhibited by blocking the RA pathway. Regulatory elements in the wti1a locus contain retinoic acid responsive cis-elements, emphasizing a direct role for retinoic acid signaling in wti1a expression. Additionally, it has been shown that RA-deficient embryos fail to express wti1a in the intermediate mesoderm, whereas RA-treated embryos show increased wti1a expression. Given these findings, loss of osr1 expression in DEAB treated embryos is probably due to loss of wti1a expression, although it remains possible that RA signaling could also have a direct role in osr1 expression.

Loss of osr1 function blocked expression of the podocyte terminal differentiation markers nephrin and podocin. In screening candidate transcription factors that might act as mediators of osr1 in podocyte terminal differentiation, we found that lhx1a expression was also lost in osr1 morphants. Lhx1a (lim1) is a LIM homeo-domain-containing transcription factor involved in protein-protein interactions as opposed to DNA binding. In mice, Lhx1a acts cell autonomously at multiple stages of metanephric kidney development. In chimeric Lhx1a-deficient embryos, it was shown that Lhx1a null cells could not contribute to tissue that later formed glomerular podocytes. Our finding that lhx1a expression is lost in the intermediate mesoderm of osr1-deficient embryos places lhx1a downstream of osr1. Further, our finding that ectopic expression of an activated lhx1a fusion protein at least partially rescued podocyte differentiation in osr1 morphants indicates that lhx1a is a downstream mediator of osr1 in podocyte differentiation.

In summary, our results demonstrate that osr1 is not required for initial specification of podocytes but rather acts after wti1a as an effector of podocyte differentiation. Alternatively, it remains possible that wti1a may be required for activation of osr1 and then both work synergistically to activate downstream target genes required for podocyte differentiation. Further studies examining direct binding of transcription factors to osr1 regulatory elements will be required to establish the hierarchy of regulators responsible for podocyte differentiation. Moreover, further studies on deletion of Osr1 at later stages of mice kidney development using Osr1 conditional knockout mice will provide more insight into molecular pathways required for glomerular morphogenesis.

**CONCISE METHODS**

**Zebrafish Embryos**
Wild-type zebrafish were maintained according to established protocols. 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.

**Whole-Mount In Situ Hybridization**
The plasmid constructs used in this work to synthesize antisense RNA probes for wti1a, Osr1, podocin, nephrin, slc4a2a, and lhx1a
have been previously described. For rescue experiments, synthetic capped active lhx1a-lldb1 mRNAs was synthesized from Not1 linearized pCS2+_LLCA plasmid using SP6 polymerase mMessage Machine kit (Ambion). Whole-mount in situ hybridization was performed on embryos of different stages using antisense RNA probes labeled with digoxigenin or fluorescein (Boehringer Ingelheim, Mannheim, Germany) as described previously. Stained embryos were fixed, cleared with dimethylformamide transferred into PBS:glycerol (1:1), and photographed on a Leica MZ12 or Nikon E800 microscope equipped with Spot Image digital camera. Double graphed on a Leica MZ12 or Nikon E800 microscope equipped with Spot Image digital camera. Double fluorescent in situ hybridization was performed as described previously (S. Holley, Yale University, New Haven, CT, personal communication). Stained embryos were dehydrated in methanol, cleared with 2:1 benzyl benzoate:benzyl alcohol, and examined with a Zeiss LSM5 Pascal-confocal microscope. All images were processed using Adobe Photoshop software.

**Morpholino Design, Microinjections, and Molecular Analysis**

Morpholino oligonucleotides were designed to target the splice donor site of exon8 of the wt1a gene, which targeted both zebrafish wt1 paralogs and resulted in skipping of exon8 in wt1a mRNA and inclusion of intron8 in wt1b mRNA. osr1 morpholinos oligonucleotides targeted the splice donor site of exon2 as described previously.

The following morpholino oligonucleotides were used in this study: (1) osr1ex2d: ATCTCATCCTTACCTTGTTCTCTC and (2) wt1exon8d: TTACGCACTTGTTTTACCTGTATGT

Morpholino oligonucleotides were diluted in 100 mM KCl, 10 mM HEPES, and 0.1% phenol red (Sigma-Aldrich), and 4.6 nl was injected to each embryo using a Nanoject2000 Microinjector (World Precision Instruments, Sarasota, FL). Injection concentration used in the study is 0.5 mM for wt1exon8d morpholino and 0.2 mM for Osr1ex2d. Efficiency of morpholino splicing was confirmed by RT-PCR. In RA inhibitor experiments, DEAB was dissolved in DMSO and applied to embryos at 5 μM at the shield stage.

**Histology**

For histologic analysis after in situ hybridization of embryos, embryos were fixed in 4% P paraformaldehyde FA in PBS at 4°C overnight followed by dehydration in an ethanol:PBS series. Dehydrated embryos were embedded in JB-4 glycolmethacrylate resin (Polysciences, Warrington, PA) and sectioned to a thickness of 5 μm using a LEICA RM 2165 rotary microtome.

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**DISCLOSURES**

None.

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