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# *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.

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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

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of genes, which encode evolutionarily conserved zinc-finger transcription factors required for embryonic development.[11,12] In *Drosophila*, the odd skipped gene, *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.[11] *odd* was shown to be required for leg development and also for malpighian tubule development, the renal organs of *Drosophila*.[11,13] Homologs of *odd* have been cloned in mice and humans. The mice *osr* homolog *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.[10,12] Homozygous *Osr1* null mutants fail to form intermediate mesoderm and die *in utero* with cardiac defects and renal agenesis.[10] In humans, mutations in *OSR1* have yet to be associated with kidney agenesis; however, a variant *OSR1* allele, which disturbs *OSR1* mRNA expression in renal progenitor cells, has been associated with reduction of newborn kidney size and function.[14] The zebrafish *odd* homolog *osr1* is first expressed in the early mesendoderm and later in a broad domain of lateral plate/intermediate mesoderm.[15] Knockdown experiments in zebrafish have also revealed a role for *osr1* in pronephric development. *osr1*-deficient embryos display loss of proximal tubule and a stage-specific arrest of glomerular morphogenesis. *osr1* plays an indirect role in proximal tubule development by repressing endoderm differentiation, which, in turn, favors proximal tubule formation.[15] However, it is not known how *osr1* functions in podocyte differentiation.

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

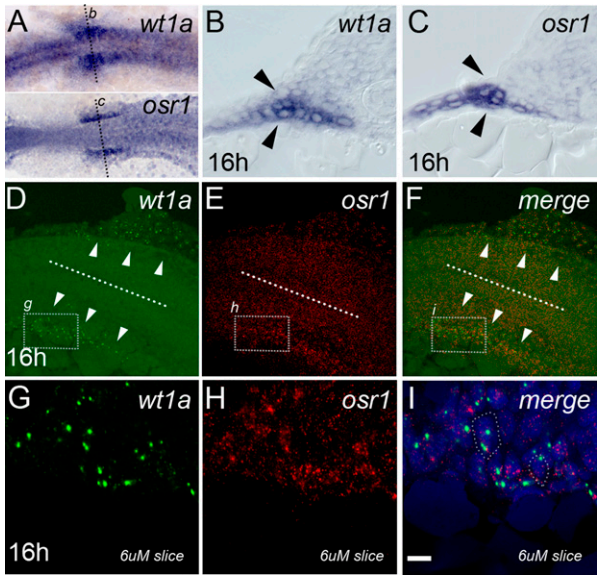


**Figure 1.** *osr1* and *wt1a* are coexpressed in podocyte progenitors. (A) Expression of *wt1a* and *osr1* in anterior intermediate mesoderm in a zebrafish embryo at 16 high-power field. Dashed lines (*b, c*) denote planes of section in B and C. (B and C) Cross-sections of embryos showing the expression domains of *wt1a* (B) and *osr1* (C) in anterior intermediate mesoderm (arrowheads). (D and E) *Wt1a* and *osr1* have overlapping expression domains in presumptive podocyte progenitors in the anterior intermediate mesoderm. Dual color fluorescent *in situ* hybridization of *wt1a* (green; D and G) and *osr1* (red; E and H) respectively at 16 high-power fields. *Wt1a* is expressed in intermediate mesoderm (dotted box g and white arrowheads in D) in the same cells as *osr1* (dotted box *h* in E). (F) Merge of E and F showing coexpression of *wt1a* and *osr1* (dotted box, white arrowheads) in anterior intermediate mesoderm. (G–I) Magnified views of single 6-$\mu$m confocal sections of boxed regions in D–F show *wt1a* expression (G), *osr1* expression (H), and coexpression of *wt1a* and *osr1* (I) in cells of anterior mesoderm. Dotted lines represent the scale of a single cell in G–I; scale bar in I=10 $\mu$m. Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue).

## RESULTS

### *osr1* and *wt1a* Are Coexpressed in Zebrafish Podocyte Progenitor Cells

We have shown previously that *osr1*-deficient embryos fail to form a functional pronephric glomerulus and do not express markers of the differentiated glomerular podocytes *podocin* and *nephrin*.[15] To assess how *osr1* might fit in the sequence of events leading to podocyte differentiation, we compared expression of *osr1* with expression of known podocyte markers. The Wilms' tumor suppressor *wt1a* marks podocyte differentiation and is first expressed in bilateral clusters of cells in the anterior intermediate mesoderm, adjacent to somites 1–3[16] (Figure 1A). At the same stage of development, whole-mount *in situ* hybridization revealed that *osr1* is expressed in similar cells as *wt1a* (Figure 1A). Histologic sections further suggested that *osr1* (Figure 1B) and *wt1a* (Figure 1C) were coexpressed in anterior intermediate mesoderm. Double fluorescent *in situ* hybridization in conjunction with confocal microscopy confirmed that *wt1a* and *osr1* were coexpressed in podocyte progenitors (Figure 1, D–I). These results suggest that defects in kidney glomerular differentiation in *osr1*-deficient embryos may be due to an early, cell-autonomous requirement for *osr1* in podocyte progenitors.

### *osr1* Is Required for Podocyte Development Downstream of *wt1a*

Coexpression of *wt1*a and *osr1* in podocyte progenitors and loss of podocyte marker expression in both *osr1*- and *wt1a*-deficient embryos[15,17] suggested that these two transcription factors could act together or sequentially in podocyte differentiation. *osr1* is not required for expression of *wt1a*,[15] but it is

not known whether expression of *wt1a* is required for *osr1* expression in podocyte progenitors. Morpholino knockdown of both *wt1a* and its paralog *wt1b* (Figure 2, A–C) caused loss of expression of podocyte markers, *podocin* (Figure 2, D and E) and *nephrin* (Figure 2, F and G), consistent with the known requirement for *wt1* in podocyte development.[18] Significantly, *wt1a/b* knockdown also eliminated *osr1* expression, specifically in podocyte progenitors, but not in endoderm (Figure 3, A–D). *wt1a* is a retinoic acid–regulated gene, and inhibition of local generation of retinoic acid, using retinoic acid–synthesizing (RALDH) enzymes inhibitor diethylaminobenzaldehyde

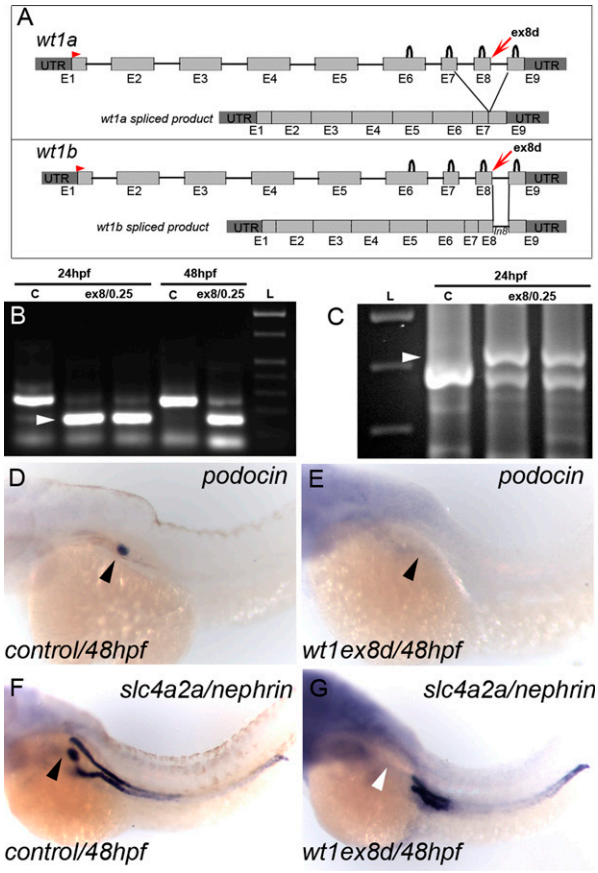


**Figure 2.** *wt1a/b* knockdown results in loss of podocyte nephrin and podocin expression. (A) *wt1a* and *wt1b* gene structure and position of a single morpholino oligo that targets exon 8 splice donor (MOexon8d) (red) in both *wt1* paralogs. (B and C) RT-PCR analysis of (MOexon8d) induced mis-splicing of both *wt1a* and *wt1b* reveals exon skipping in *wt1a* (B; arrowhead denotes PCR product lacking exon 8) and intron inclusion in *wt1b* (C; arrowhead denotes PCR product including intron 8). (D and E) Expression of *podocin* (arrowheads) in control and *wt1*ex8d morphants at 48 high-power fields (hpf). *podocin* expression is lost in *wt1*ex8d MO injected embryos. (F and G) Expression of *slc4a2a* (proximal tubules) and *nephrin* (podocytes) in control embryos (F) and *wt1*ex8d MO-injected embryos (G) at 48 hpf. *nephrin* expression (G) is lost (white arrowhead) in *wt1*ex8d MO-injected embryos. UTR, untranslated region.

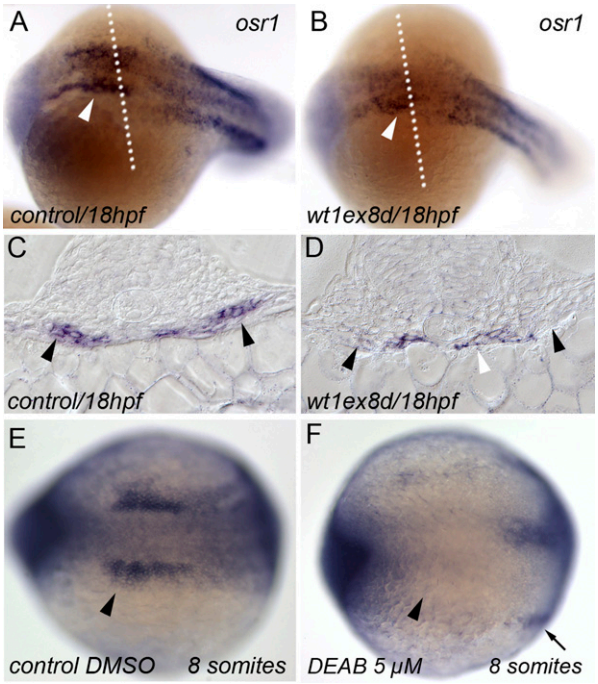


**Figure 3.** *wt1a/b* knockdown results in loss of *osr1* expression in podocyte progenitors. (A and B) Expression of *osr1* in intermediate mesoderm podocyte progenitors in control (A) and *wt1a/b* morphants (B) at 18 high-power fields. *osr1* expression is absent from podocyte progenitors (white arrowheads) but persists in endoderm of *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 *osr1* is absent in podocyte progenitors (black arrowheads) in *wt1a/b* morphants but persists in endoderm (white arrowhead). (E) *osr1* expression in podocyte progenitors in control embryos treated with DMSO. (F) Loss of *osr1* expression in podocyte progenitors of embryos treated with the differential expression of retinoic acid–synthesizing inhibitor 4-diethylaminobenzaldehyde.

(DEAB), blocks expression of *wt1a*.[19] Here we show that DEAB treatment also prevented expression of *osr1* (Figure 3, E and F), consistent with a requirement for *wt1a* in *osr1* expression. Together, these results suggest a sequential induction of *wt1a* by retinoic acid and subsequent *wt1a*-dependent induction of *osr1* in podocyte progenitors, ultimately leading to podocyte differentiation and expression of the structural genes *nephrin* and *podocin*.

### *osr1* Regulates Zebrafish Podocyte and Kidney Tubule Development by Distinct Mechanisms

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

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.

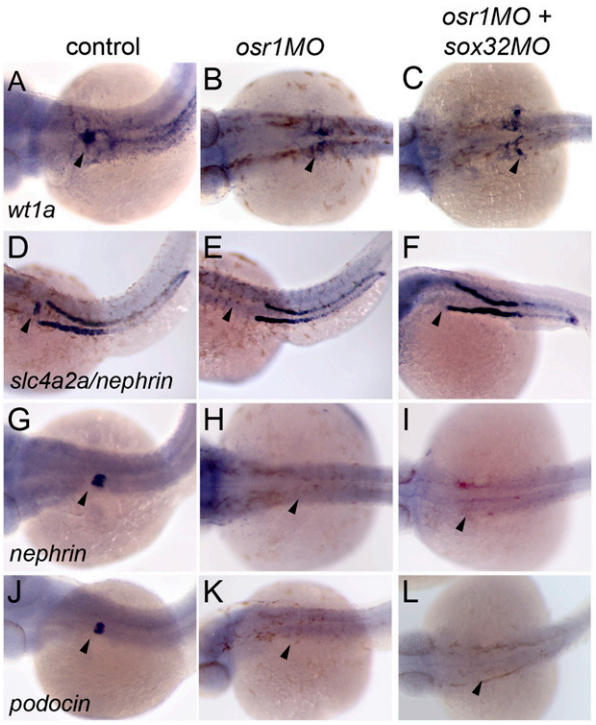


**Figure 4.** *osr1* is required downstream of *wt1* for glomerular development. (A–C) Expression of *wt1a* in control, *osr1* morphants, and *osr1/sox32* double morphants, respectively, at 48 high-power fields. *wt1a*-expressing podocyte progenitors coalesce at midline in control embryos (A) but not 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).

### *lhx1a* 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, *lhx1a* 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 *lhx1a* expression specifically in the presumptive anterior pronephric region of intermediate mesoderm in 15 high-power fields embryos (Figure 5B; $n$=15/15), while *lhx1a* expression in the tail bud of *osr1* morphants remained unaltered (data not shown). We therefore tested whether *lhx1a* 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 coinjected 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 *lhx1a* 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 GA-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*

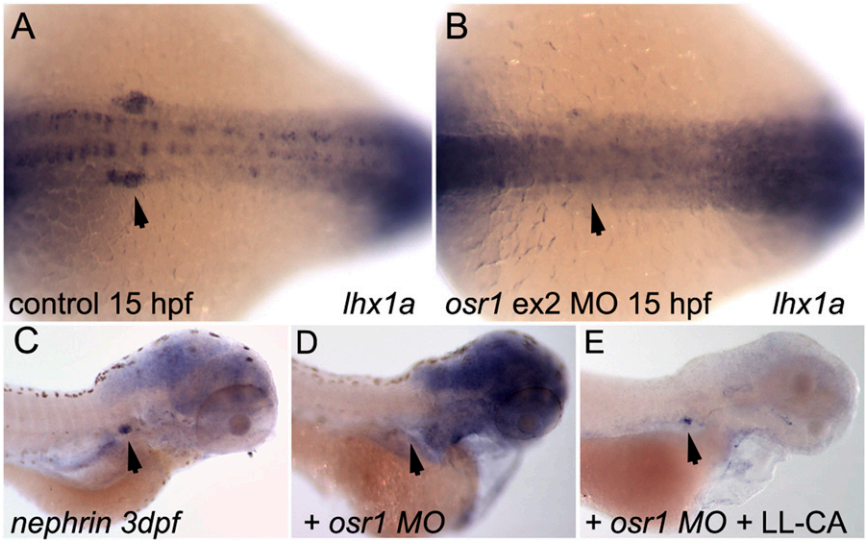


**Figure 5.** *osr1* acts *via lhx1a* to regulate *nephrin* expression. (A and B) Expression of *lhx1a* in podocyte progenitors in the anterior intermediate mesoderm in control (A) and *osr1* morphants (B; arrows). *Lhx1a* expression is lost in *osr1* morphants. (C) *nephrin* expression in control, (D) in *osr1* morphants alone, (E) in *osr1* morphants with forced expression of the activated *lhx1a* construct LL-CA. *nephrin* expression is lost in *osr1* morphants and restored upon forced expression of activated *lhx1a*.

knockout mice points to a role for *Osr1* in maintaining the self-renewing, proliferative metanephric nephron stem cell population.[10] 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,[29] 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 *wt1a* 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 *wt1a* expression is not dependent on *osr1*.[15] Conversely, here we show that *osr1* expression is absent in *wt1a* morphants, demonstrating that *osr1* is downstream of *wt1a* and regulated directly or indirectly by *wt1a* 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.[30–36] 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.[19,36,37] Regulatory elements in the *wt1a* locus contain retinoic acid responsive cis-elements, emphasizing a direct role for retinoic acid signaling in *wt1a* expression.[38] Additionally, it has been shown that RA-deficient embryos fail to express *wt1a* in the intermediate mesoderm, whereas RA-treated embryos show increased *wt1a* expression.[19] Given these findings, loss of *osr1* expression in DEAB treated embryos is probably due to loss of *wt1a* 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.[21,39,40] 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.[20] 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 *wt1a* as an effector of podocyte differentiation. Alternatively, it remains possible that *wt1a* 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[41] will provide more insight into molecular pathways required for glomerular morphogenesis.

## CONCISE METHODS

### Zebrafish Embryos

Wild-type zebrafish were maintained according to established protocols.[42] 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 *wt1a*,[34] *Osr1*,[15] *podocin*,[2] *nephrin*,[2] *slc4a2a*,[43] and *lhx1a*

have been previously described. For rescue experiments, synthetic capped active *lhx1a-ldb1* mRNAs was synthesized from Not1 linearized pCS2+_LLCA plasmid using SP6 polymerase mMessage Machine kit (Ambion).[22] 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.[44] 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 fluorescent *in situ* hybridization was performed as described previously (S. Holley, Yale University, New Haven, CT, personal communication).[45,46] 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.[15]

The following morpholino oligonucleotides were used in this study: (*1*) *osr1*ex2d: ATCTCATCCTTACCTGTGGTCTCTC and (*2*) *wt1*exon8d: 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 *wt1*exon8d morpholino and 0.2 mM for *Osr1*ex2d. Efficiency of morpholino splicing was confirmed by RT-PCR. In RA inhibitor experiments, DEAB was dissolved in DMSO and applied to embryos at 5 $\mu$M 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 $\mu$m using a LEICA RM 2165 rotary microtome.

## ACKNOWLEDGMENTS

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## DISCLOSURES

None.

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