RESEARCH ARTICLE

Osr1 Interacts Synergistically with Wt1 to Regulate Kidney Organogenesis

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Abstract

Renal hypoplasia is a common cause of pediatric renal failure and several adult-onset diseases. Recent studies have associated a variant of the \textit{OSR1} gene with reduction of newborn kidney size and function in heterozygotes and neonatal lethality with kidney defects in homozygotes. How OSR1 regulates kidney development and nephron endowment is not well understood, however. In this study, by using the recently developed CRISPR genome editing technology, we genetically labeled the endogenous Osr1 protein and show that Osr1 interacts with Wt1 in the developing kidney. Whereas mice heterozygous for either an \textit{Osr1} or \textit{Wt1} null allele have normal kidneys at birth, most mice heterozygous for both \textit{Osr1} and \textit{Wt1} exhibit defects in metanephric kidney development, including unilateral or bilateral kidney agenesis or hypoplasia. The developmental defects in the \textit{Osr1+/-Wt1+/+} mouse embryos were detected as early as E10.5, during specification of the metanephric mesenchyme, with the \textit{Osr1+/-Wt1+/+} mouse embryos exhibiting significantly reduced Pax2-positive and Six2-positive nephron progenitor cells. Moreover, expression of \textit{Gdnf}, the major nephrogenic signal for inducing ureteric bud outgrowth, was significantly reduced in the metanephric mesenchyme in \textit{Osr1+/-Wt1+/+} embryos in comparison with the \textit{Osr1+/+} or \textit{Wt1+/+} littermates. By E11.5, as the ureteric buds invade the metanephric mesenchyme and initiate branching morphogenesis, kidney morphogenesis was significantly impaired in the \textit{Osr1+/-Wt1+/+} embryos in comparison with the \textit{Osr1+/+} or \textit{Wt1+/+} embryos. These results indicate that Osr1 and Wt1 act synergistically to regulate nephron endowment by controlling metanephric mesenchyme specification during early nephrogenesis.

Introduction

Renal hypoplasia, defined as abnormally small kidney with normal morphology and reduced nephron number, is a common cause of congenital kidney failure and a significant risk factor for hypertension or chronic renal failure in adults [1–3]. The molecular mechanisms that determine nephron number are still not well understood, however. In mammals, three distinct types of kidney structures develop bilaterally during embryogenesis along the anterior-posterior...
body axis: the pronephroi, which form in the anterior intermediate mesoderm (IM) and regress quickly but the nephric duct continues to extend posteriorly to induce subsequent kidney development; the mesonephroi, which are structurally more complex but are also transient during midgestation; and the metanephroi, which continue morphogenesis from midgestation through perinatal stages and function as the blood filters throughout postnatal life. In mice, metanephric kidney development initiates around embryonic day 10 (E10) with the establishment of a unique population of nephrogenic cells, called metanephric mesenchyme (MM), in the posterior IM. The MM induces outgrowth of the ureteric bud (UB) from the nephric duct at the level of hindlimb buds. The UB invades into MM and induces MM cells to condense around the UB tip, forming the cap mesenchyme (CM). As development proceeds, the CM induces UB to branch repeatedly and a subset of CM cells in the armpit of each new branch undergo mesenchymal-epithelial transformation to form a renal vesicle, which subsequently differentiates into a nephron. All nephrogenic progenitor cells in the metanephric kidney are depleted by the final wave of nephrogenesis in the perinatal period and no new nephron formation initiates thereafter [4].

Prior to UB outgrowth, the MM expresses a unique combination of signaling molecules and transcription factors, including the glial derived neurotrophic factor (Gdnf) and the transcription factors Eya1, Pax2, Six1, and Six2 [5]. Gdnf is the major signal for UB induction, acting through its receptors Ret and Gfra1 in the nephric duct epithelium. Mice lacking Gdnf, Ret, or Gfra1, fail to form the UB and die perinatally with bilateral renal agenesis [6–11]. Genetic down-regulation of the Gdnf/Ret signaling pathway results in significantly reduced ureteric bud branching, nephron number and kidney size, which are observed in the Gdnf +/- mice and RetY1062F knockin homozygous mice [12–14]. The Eya1, Pax2, and Six1 transcription factors are each required for activation and/or maintenance of Gdnf expression in the metanephric mesenchyme and mice lacking any one of them die perinatally with bilateral renal agenesis [15–17]. Mutations in EYA1, PAX2, SIX1, or SIX2, have been found in a subset of human patients with renal agenesis or hypoplasia [18–22]. A frameshift mutation in Pax2 also resulted in renal hypoplasia in heterozygous mice, which correlated with elevated apoptosis in the UB epithelium [23]. Mice lacking Six2 function exhibited severe renal hypoplasia due to premature differentiation and rapid depletion of nephron progenitor cells following initial UB branching [24]. These results indicate that MM or UB cell survival, the reciprocal interactions between the MM and UB epithelium, and the balance between progenitor maintenance and differentiation, all play important roles in controlling the nephron number.

The odd-skipped related 1 (Osr1) gene encodes a homolog of the Drosophila odd-skipped zinc finger protein [25, 26]. Osr1 expression is first activated in the nascent IM at the late gastrula stage (E7.5) during mouse embryogenesis [27]. Strong Osr1 expression persists in the nephrogenic mesenchyme but is completely down-regulated upon mesenchymal-epithelial transition into the nephric duct or renal vesicles during kidney development [27, 28]. Genetic lineage tracing studies demonstrated that Osr1-expressing IM cells give rise to the majority of cell types in the kidney but Osr1 expression itself undergoes progressive restriction to the CM cells during metanephric kidney organogenesis [29]. In Osr1 +/- mutant mouse embryos, the nephric duct formed and extended to the posterior IM, but no morphologically distinguishable MM was detected and the nephrogenic mesenchyme cells exhibited aberrant apoptosis from E9.5 to E10.5, indicating that Osr1 function is required for early nephrogenic mesenchyme cell survival [27, 28]. Through tissue-specific genetic analysis, we showed that Osr1 also plays a critical role in maintaining the balance between progenitor cell renewal and nephron differentiation during metanephric kidney organogenesis [30]. Recently, a single nucleotide variant in an exonic splice enhancer in the OSR1 gene was associated with reduction in newborn kidney size and function in humans [31]. In addition, homozygosity of the same variant OSR1 allele
was associated with neonatal lethality with congenital kidney defects [32]. In this report, we demonstrate that Osr1 interacts synergistically with another important kidney developmental regulator Wt1 [33] to control nephron endowment through regulation of MM specification.

Results

Osr1 and Wt1 are co-expressed in the metanephric mesenchyme and form protein interaction complexes

We first co-expressed Myc or Flag-tagged Osr1 with each of several transcription factors, including Wt1, Lhx1, Six2 and Pax2, which are all co-expressed with Osr1 at some point in the developing metanephric mesenchyme [24, 30, 33–37], in HEK293T cells and performed co-immunoprecipitation assays (Fig 1). We showed previously that Osr1 and Six2 interact with each other in maintaining nephron progenitor cells during metanephric kidney development, thus the Osr1-Six2 interaction provides a positive control [30]. Immunoprecipitation of Myc-Osr1 pulled down Flag-Wt1, but not Flag-Lhx1, from the co-transfected cells (Fig 1A). Immunoprecipitation of Flag-Osr1 did not pull down myc-Pax2 from the co-transfected cells (Fig 1B). These results indicate that Osr1 could form interactive protein complexes with Wt1 when co-expressed.

To validate that Osr1 interacts with Wt1 during kidney development in vivo, we used the recently developed CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated) genome editing system [38, 39] to insert a 2xTY1 epitope tag at the N-terminus of the endogenous Osr1 protein (Fig 2A). The Osr1TY1/+ CRISPR founder mice were crossed to wildtype C57BL/6J mice and the G1 Osr1TY1/+ hemizygous mice were identified by PCR genotyping and verified by Sanger sequencing. Intercrossing of the Osr1TY1/+ hemizygous mice produced Osr1TY1/TY1 homozygous mice, which were born at Mendelian ratio and did not display any phenotypic difference from wildtype littermates. We further crossed Osr1TY1/TY1 homozygous mice to Osr1+- mice heterozygous for a targeted null Osr1 allele [27] and found that the Osr1TY1/- mice survive and breed normally, indicating that the inframe fused TY1 tag did not affect the Osr1 protein function.

We compared the expression pattern of TY1-Osr1 from Osr1TY1 allele and that of the endogenous Wt1 protein in kidney development in Osr1TY1/TY1 embryos at E10.5, E11.5, and

![Fig 1. Osr1 and Wt1 form protein interaction complex when co-expressed.](https://example.com/image1.png)

**A** Myc-Osr1: + + + - Flag-Wt1 Lhx1 Six2 vector

**B** IP: Flag 2%input

IP: Myc WB: Flag

2%input WB: Flag

IP: Myc WB: Myc

Flag-Osr1

Flag-Osr1: + + - - + -

Myc-Pax2: + - - + + +

Myc-Osr1: + + + + + +

Flag-Wt1

Flag-Six2

IP: Flag 2%input

Myc-Osr1: + + + + + +

Myc-Pax2: + - - + + +

Flag-Osr1: + + + + + +

Flag-Wt1

Flag-Six2

IP: Flag 2%input

Myc-Osr1: + + + + + +

Myc-Pax2: + - - + + +

Flag-Osr1: + + + + + +

Flag-Wt1

Flag-Six2

**Fig 1. Osr1 and Wt1 form protein interaction complex when co-expressed.** (A) HEK293T cells were co-transfected with plasmids expressing Myc-tagged Osr1 and Flag-tagged Wt1, Lhx1, Six2, or a Flag-tag vector, respectively. Immunoprecipitation was carried out with an anti-Myc antibody, and the resulting protein complexes resolved by western blotting and detected with an anti-Flag antibody. Note that Flag-Wt1 and Flag-Six2 were co-immunoprecipitated with Myc-Osr1. (B) HEK293T cells were co-transfected with plasmids expressing Flag-tagged Osr1 and Myc-tagged Pax2. Immunoprecipitation was carried out with an anti-Flag antibody, and the resulting protein complexes resolved by western blotting and detected with an anti-Myc antibody.

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We found that TY1-Osr1 fusion protein was co-expressed with Wt1 in the metanephric mesenchyme cells at E10.5 and E11.5 (Fig 2C and 2D). At E13.5, TY1-Osr1 was expressed at high levels in the undifferentiated cap mesenchyme but is absent from the renal vesicles (Fig 2E). In contrast, Wt1 expression was down-regulated in the cap mesenchyme but strongly up-regulated in more differentiated structures (Fig 2E). To further confirm these results, we also took advantage of Osr1GCE knockin mice [29], which expressed an eGFP-Cre fusion protein from Osr1 locus and examine the expression pattern of Osr1-GFP and endogenous Wt1 protein (in sections of E10.5 (C), E11.5 (D), and E13.5 (E) Osr1GCE/embryos. The ureteric bud is outlined with white dashed circle in (C-H)). E16.5 kidneys were collected from Osr1TY1/ and wildtype embryos. Immunoprecipitation was carried out with an anti-TY1 antibody, and the resulting protein complexes resolved by western blotting and detected with anti-Six2 and anti-Wt1 antibodies. Scale bar, 50 μm.

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E13.5, respectively (Fig 2C–2E). We found that TY1-Osr1 fusion protein was co-expressed with Wt1 in the metanephric mesenchyme cells at E10.5 and E11.5 (Fig 2C and 2D). At E13.5, TY1-Osr1 was expressed at high levels in the undifferentiated cap mesenchyme but is absent from the renal vesicles (Fig 2E). In contrast, Wt1 expression was down-regulated in the cap mesenchyme but strongly up-regulated in more differentiated structures (Fig 2E). To further confirm these results, we also took advantage of Osr1GCE knockin mice [29], which expressed an eGFP-Cre fusion protein from Osr1 locus and examine the expression pattern of Osr1-GFP and endogenous Wt1 protein in Osr1GCE/embryos similarly (Fig 2F–2H). While TY1-Osr1 fusion protein displays a nuclear subcellular localization, whereas the Osr1-GFP is expressed in the cytoplasm, TY1-Osr1 and Osr1-GFP exhibited very similar expression pattern during kidney development (Fig 2C–2H).

To test the interaction of endogenous Osr1 and Wt1 in vivo, we dissected E16.5 kidneys from Osr1TY1/ and wildtype embryos, respectively, and performed co-immunoprecipitation assays (Fig 2I). TY1-Osr1 was co-precipitated with endogenous Wt1 and Six2 (Fig 2I). Together, these results suggest that Osr1 and Wt1 interact in the nephrogenic mesenchyme to regulate kidney formation.

**Osr1 interacts genetically with Wt1 to regulate metanephric kidney development**

We crossed Osr1+/− mice with Wt1GFPCre/− mice (abbreviated as Wt1+/− mice in the rest of the article), in which a GFP-Cre fusion construct is inserted in the Wt1 locus and disrupts the

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**Fig 2. Osr1 and Wt1 are co-expressed in the early metanephric mesenchyme during kidney development and physically interact in vivo.** (A) Schematic diagram of the Osr1 gene structure and strategy for CRISPR/Cas9-mediated insertion of the 2xTY1 epitope tag at the N-terminus of the endogenous Osr1 protein. The gRNA target sequence is shown in red font. The sequence of the mid-portion of the oligonucleotide donor template for homology directed repair contains the 2xTY1 tag-coding sequence (shown in green font). The endogenous Osr1 ATG codon is shown in blue font. The tag sequence contains its own ATG initiation codon at the 5' end (indicated by M* underneath the sequence). (B) Osr1TY/ and Osr1TY1/ mice were identified by PCR genotyping. (C–E) Immunofluorescent staining for the TY1 epitope (red) and the Wt1 protein (green) on sections of E10.5 (C), E11.5 (D), and E13.5 (E) Osr1TY1/embryos. (F–H) Immunofluorescent staining for eGFP (red), Wt1 (green) and Jag1 (blue) on sections of E10.5 (F), E11.5 (G), and E13.5 (H) Osr1GCE/embryos. The ureteric bud is outlined with white dashed circle in (C–H). (I) E16.5 kidneys were collected from Osr1TY1/ and wildtype embryos. Immunoprecipitation was carried out with an anti-TY1 antibody, and the resulting protein complexes resolved by western blotting and detected with anti-Six2 and anti-Wt1 antibodies. Scale bar, 50 μm.

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endogenous Wt1 gene [40], and found that Osr1+/− Wt1+/− double heterozygous pups exhibited variable kidney defects, including bilateral agenesis (5/44), unilateral agenesis (15/44) and bilateral hypoplasia (22/44) (Fig 3A–3F), while neither Osr1+/− nor Wt1+/− mice had any obvious kidney defects at birth. The surviving Osr1+/− Wt1+/− mice with unilateral kidney agenesis had significant variation in kidney size and nephron number, but the mean nephron number per kidney was not statistically significantly different from the controls at P21 (Fig 3G). The number of nephrons per kidney of surviving Osr1+/− Wt1+/− mice that had two kidneys was about 72% of that of their control littermates at P21, with the differences between these groups being highly significant (p < 0.005) (Fig 3G). Together, these results indicate that Osr1 and Wt1 act cooperatively to control metanephric kidney development.

Osr1 interacts with Wt1 to regulate metanephric mesenchyme specification

We investigated whether the developmental abnormalities of the Osr1+/− Wt1+/− mice could be due to impairment of maintenance of the nephron progenitor cells during metanephric kidney organogenesis. By P0, the kidneys in the Osr1+/− Wt1+/− mice were obviously smaller than in the Osr1+/− and Wt1+/− littermates (Fig 4A–4C). Histologically, however, the nephrogenic zone in the cortical region of kidney was present in the Osr1+/− Wt1+/− mutants as well as in the Osr1+/− and Wt1+/− littermates (Fig 4A–4C). Immunofluorescent staining of the Six2 protein, a marker of nephron progenitor cells, on serial sections of the P0 kidneys showed the presence of Six2+ nephron progenitor cells in similar proportions in the Osr1+/− Wt1+/− mice that had two kidneys was about 72% of that of their control littermates at P21, with the differences between these groups being highly significant (p < 0.005) (Fig 3G). Together, these results indicate that Osr1 and Wt1 act cooperatively to control metanephric kidney development.

To investigate further the kidney developmental processes affected in the Osr1+/− Wt1+/− embryos, we analyzed kidney morphogenesis using organ cultures. In 48 hours of organ
culture, metanephric explants from E11.5 Osr1+/− and Wt1+/− embryos underwent 5 to 6 rounds of UB branching, with Jag1+ renal vesicles forming on the medullary side of each new ureteric bud branch (Fig 4G and 4H). In explants from Osr1+/-Wt1+/− embryos, there were significantly fewer ureteric bud branches after 48 hours of culture (Fig 3I). After 96 hours of culture, Osr1+/−Wt1+/− explants displayed fewer ureteric bud branches and obviously fewer Wt1-positive differentiating nephrons than in the Osr1+/− or Wt1+/− explants (Fig 4J–4L). We further examined UB branching in vivo by whole mount immunofluorescent staining for the Pax2 protein, which is expressed in both the UB and MM cells. At E12.5, compared with the kidneys in the Osr1+/− and Wt1+/− embryos, which had gone through 4 to 5 rounds of UB branching (Fig 4M and 4N), the kidneys of Osr1+/-Wt1+/− mutant littermates showed apparently reduced UB branching (Fig 4O). These results indicate that ureteric bud growth and branching morphogenesis are impaired in Osr1+/-Wt1+/− embryos compared with the Osr1+/− and Wt1+/− embryos.

To define the onset and progression of kidney developmental defects in the Osr1+/−Wt1+/− mice, we compared the formation of MM and UB in the Osr1+/−Wt1+/− embryos with that of the Osr1+/− and Wt1+/− littermates at E10.5 and E11.5, respectively. By E10.5, the MM is clearly demarcated by Pax2 protein expression in the control embryos. Whole mount immunofluorescent detection of Pax2 protein showed that the domain of MM is significantly smaller in the Osr1+/−Wt1+/− embryos than that in the Osr1+/− and Wt1+/− embryos at E10.5 (Fig 5A–5C and 5I). In 2 out of 12 Osr1+/−Wt1+/− embryos, no condensed Pax2+ MM domain was detected at E10.5 (Fig 5D). We also performed whole mount immunofluorescent staining for Six2, a more
specific marker for the MM cells, and found that the Six2+ MM domain was apparently smaller in the Osr1+/-Wt1+/- embryos than that in the Osr1+/+ and Wt1+/+ embryos at E10.5 (S1 Fig). By E11.5, the ureteric bud had undergone the first round of branching morphogenesis, forming a “T” shape and the MM cells were condensed around the UB tips, in wildtype as well as in the Osr1+/+ and Wt1+/+ embryos (Fig 5E, 5F, 5J and 5K). In E11.5 Osr1+/-Wt1+/+ embryos, however, the UBs were smaller and fewer MM cells surrounded the UB tips (Fig 5G and 5L). In 2 out of 10 Osr1+/-Wt1+/+ embryos, the UB failed to invade into the MM by E11.5 (Fig 5H and 5M). In 1 out of 10 Osr1+/-Wt1+/+ embryos, the UB reached the MM but had not branched by E11.5.

A number of genes expressed in the MM, including Gdnf, Eya1, Sall1, and Pax2 are each essential for UB induction and early kidney morphogenesis [7–9, 16, 17, 41, 42]. We analyzed the expression patterns of Gdnf, Eya1 and Sall1 in Osr1+/-Wt1+/+ embryos and their littermates at E10.5 by whole mount in situ hybridization and found that the expression domain of Gdnf, Eya1 and Sall1 were reduced, and the levels of Gdnf were decreased in the nephrogenic mesenchyme in Osr1+/-Wt1+/+ embryos compared with the Osr1+/+ and Wt1+/+ embryos (Fig 6A–6I). To further quantify the levels of expression of these marker genes in the nephrogenic mesenchyme, we took advantage of the GFP expression from the Wt1GFPCre allele [40] to isolate the Wt1-GFP+ cells from Osr1+/-Wt1+/+ and Wt1+/+ embryos, respectively, and performed quantitative real-time RT-PCR assays. We found that the level of Gdnf mRNAs was significantly reduced, but the levels of Eya1 and Sall1 were not significantly changed, in Osr1+/-Wt1+/+ embryos compared with Wt1+/+ embryos at E9.5 (Fig 6J). These results indicate a critical role for the synergistic actions of Osr1 and Wt1 in the specification of the MM during early nephrogenesis.

The Osr1+/-Wt1+/+ embryos had no aberrant apoptosis or cell proliferation during early nephrogenesis

Both Osr1+/+ and Wt1+/+ mutant mouse embryos had aberrant apoptosis of the nephrogenic mesenchyme at the early stages of kidney development [27, 28, 33, 43]. Therefore, we examined cell proliferation and apoptosis in the Osr1+/-Wt1+/+ embryos and their littermates at E10.5 and
E11.5 (Fig 7). Very few apoptotic cells and no significant differences in the proportion of cells undergoing apoptosis were detected in the metanephric mesenchyme in the Osr1+/−, Wt1+/−, and Osr1+/−Wt1+/− embryos at both stages (Fig 7A–7H). To determine if there was any difference in the proliferation of MM cells, we used immunofluorescent staining of Six2 to mark the MM cell nuclei and compared the percentage of BrdU-labeled MM cells in the Osr1+/−, Wt1+/−, and Osr1+/−Wt1+/− embryos at E10.5 and E11.5. We didn’t detect a significant difference in the cell proliferation index in the MM of Osr1+/−Wt1+/− embryos and the Osr1+/− littermate at either stage (Fig 7I–7P). These results indicate that the renal agenesis or hypoplasia in the Osr1+/−Wt1+/− mutant embryos resulted primarily from an impairment of MM specification rather than from increased MM apoptosis or reduced MM cell proliferation.

Discussion

In humans, the number of nephrons per kidney varies greatly, ranging from 200,000 to >2.5 million, among individuals [1]. Renal hypoplasia, with abnormally low number of nephrons, underlies more than one third of pediatric chronic or end-stage kidney diseases and is a significant risk factor for hypertension in adulthood [1, 2]. Whereas the mechanisms that determine the number of nephrons are incompletely understood, genetic studies in mice in recent years have provided significant insights into the molecular regulation of nephrogenesis and the developmental basis of renal hypoplasia [44]. Since Osr1 is expressed in nephrogenic progenitor cells throughout kidney development and since mice lacking Osr1 function exhibit...
complete kidney agenesis [27, 28], it was hypothesized that genetic variation in OSR1 might contribute to the variation in nephron number in humans [32]. Indeed, a single nucleotide polymorphism, rs12329305 (C/T), residing in an exonic splice enhancer in the human OSR1 gene, has been associated with reduction in newborn kidney size and function [31]. However, how Osr1 functions to regulate nephron number is not known. Previous studies showed that nephrogenic mesenchyme underwent aberrant apoptosis prior to UB induction in the Osr1-/- embryos [27, 28]. Recently, we demonstrated that Osr1 interacts with Six2 to maintain nephron progenitor cells in the CM against Wnt/β-catenin driven nephron differentiation [30]. In this study we found that, although the Osr1+/− and Wt1+/− mice did not have obvious kidney developmental defects, the majority of Osr1+/−Wt1+/− had kidney agenesis or hypoplasia. Our data demonstrate that, in addition to the previously determined roles in nephrogenic mesenchyme survival and CM maintenance, Osr1 interacts with Wt1 to regulate specification of the MM.

The Wt1 gene encodes a zinc finger protein that acts both as a transcription factor and RNA-binding protein [45–47]. Wt1 expression is activated in the nephrogenic mesenchyme as early as E9.0 during mouse embryogenesis and Wt1−/− mouse embryos showed complete lack of metanephric kidneys [33]. However, in contrast to the lack of morphologically distinguishable MM in the E10.5 Osr1−/− embryos [27, 28], Wt1−/− embryos not only had morphologically distinguishable MM by E11.5 (n = 7) (M-P) embryos. BrdU index is calculated by the ratio of BrdU-positive cells (green) versus Six2-positive cells (red). Scale bar, 100 μm.

**Fig 7. Analysis of cell apoptosis and proliferation during early kidney development in Osr1+/−Wt1+/− embryos and littermates.** (A-H) Cell apoptosis is analyzed by TUNEL (green) and counterstained with DAPI (blue). TUNEL assay detected no obvious change in cell apoptosis in Osr1+/−Wt1+/− metanephric mesenchyme (C, D, G, H) compared with Osr1−/− (A, E) and Wt1−/− (B, F) metanephric mesenchyme at E10.5 (A-D) and E11.5 (E-H). (I-P) Analysis of cell proliferation by BrdU incorporation at E10.5 (I-L) (n = 18) and E11.5 (n = 7) (M-P) embryos. BrdU index is calculated by the ratio of BrdU-positive cells (green) versus Six2-positive cells (red). Scale bar, 100 μm.

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The expression of Gdnf in the MM is also reduced in the Osr1+/−/Wt1+/− embryos in comparison with the Osr1+/− and Wt1+/− littermates. Together with our data demonstrating overlapping expression and protein-protein interaction between Osr1 and Wt1, these results indicate that both Osr1 and Wt1 play critical roles in MM specification.

We previously showed that Osr1−/− embryos failed to activate expression of MM markers, including Eya1, Pax2, and Six2, at E10.5 [28]. Since the Osr1−/− embryos exhibit aberrant apoptosis of the nephrogenic mesenchyme starting at E9.5 [27], however, it had not been clear whether Osr1 plays a direct role in MM formation and whether the lack of MM in Osr1−/− embryos was secondary to the requirement of Osr1 for nephrogenic mesenchyme survival. On the other hand, the Wt1−/− mutant embryos showed morphologically distinguishable MM at E10.5 and E11.5 but the Wt1−/− MM did not respond to wildtype UB while UB outgrowth was induced from Wt1−/− mutant nephric duct by wildtype MM in recombinant explant cultures [48], indicating that Wt1 is required cell autonomously in the MM for production and/or processing of UB-inducing signals and for competence to respond to UB-derived nephrogenic signals. In this study, we found that the Osr1+/−/Wt1+/− embryos had reduced MM in the absence of any detectable defects in cell proliferation or apoptosis. These results clearly demonstrate that synergistic interactions of Osr1 and Wt1 play critical roles in MM formation and function.

We recently demonstrated that tissue-specific inactivation of Osr1 in the cap mesenchyme resulted in premature differentiation and depletion of nephron progenitor cells [30]. In this study, we found that the Osr1+/−/Wt1+/− mutant embryos had normal maintenance of the CM nephron progenitor cells even though the kidneys were reduced in size. A recent study showed that genetically ablating 40% of the nephron progenitor cells (cap mesenchymal cells) caused decreased ureteric bud branching with normal CM condensation around the UB tips and resulted in hypoplastic kidneys [49]. Thus, the number of nephron progenitor cells at the beginning of metanephric kidney organogenesis is an important determinant of nephron endowment [49]. Our results indicate that impairment in MM specification is one of the mechanisms underlying renal hypoplasia.

Zhang et al. (2011) showed that kidney volume was more significantly reduced in infants bearing both the OSR1rs12329305(T) allele and the RETrs1800860(A) allele, suggesting that the effects of impaired OSR1 function on nephrogenesis was additive with other regulators of UB branching [31, 32]. We found that the Osr1+/−/Wt1+/− mouse embryos display reduced MM volume and significantly decreased Gdnf expression. Since Gdnf is the major signal and acts primarily through the Ret receptor for UB induction [6–10], the reduced Gdnf expression likely contributes to the reduction in UB branching in the Osr1+/−/Wt1+/− mouse embryos and is likely part of the mechanism underlying renal hypoplasia in infants heterozygous for the OSR1rs12329305(T) allele. The synergistic interaction of Osr1 and Wt1 in the regulation of MM specification, together with the finding that about 6% of the Caucasian population carries the functionally impaired OSR1rs12329305(T) allele [31, 32], suggest that OSR1 has a significant contribution to renal hypoplasia and other renal conditions in humans. Further studies of the molecular mechanisms involving Osr1 in kidney development, in particular identification of direct downstream target genes and its interaction with other transcription factors and molecular pathways, will significantly improve the understanding of renal disease pathogenesis.

Materials and Methods

Mouse strains
This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Cincinnati.
Children’s Hospital Medical Center (Permit Number IACUC2013-0036). The mice were housed in standard microisolater cages with ventilation, standard mouse food, and automated water supply. Breeder male mice are housed individually and adult female mice are housed at 4 mice/cage. To harvest mouse embryos for the experimental studies, timed pregnant female mice were euthanized by asphyxiation with carbon dioxide gas generated from a pressurized cylinder followed by cervical dislocation in accordance with the Panel on Euthanasia of the American Veterinary Medical Association. Embryos were fixed in 4% paraformaldehyde immediately upon dissection. All methods and procedures are reviewed for humaneness by the Institutional Animal Care and Use Committee. When necessary, consultation and training in the application of analgesic and anesthetics for laboratory animals are provided through an annual training program supplemented by individualized training in consultation with the veterinarian.

The Osrl+/– (Osrltm1Jian), OsrlGCE/+ and Wt1+/– (Wt1GFPcre/) mice have been described previously [27, 29, 40]. The Osrl+/– (Osrltm1Jian) and OsrlGCE/+ were maintained by crossing to C57BL/6J mice. Osrl+/– Wt1+/– male mice were crossed with CD1 (Charles river) female mice to generate Osrl+/–, Wt1+/– single heterozygous and Osrl+/– Wt1+/– double heterozygous mice. Noon of the day a vaginal plug was identified was designated as embryonic day (E) 0.5.

**Generation of mice carrying the OsrlTY1 allele using CRISPR/Cas9-mediated genome editing**

A guide RNA (gRNA) targeting sequence near the translation start site in Exon2 of the Osrl gene (gRNA target sequence 5’- GCGGTTCCCAGCGACAGAAA-3’) was selected using the CRISPR Design Tool (http://crispr.mit.edu). Guide RNAs were synthesized in vitro and co-injected with a synthesized single-stranded oligonucleotide donor (5’- ttgggtctgccccacaggctctctctgttcgttgcagatgtttcagctcccacagctgcggtgggttcccagcgacagCCATGGAGGTGCACACCAACCAGGCCGCCGAAGTCCATACAAATCAGGATCCTCTGGATGCCGCAatgGgcagcaaaaccttgc cagcaggctcacattcctacacgccagggttccagcagctt -3’) as template for homology-directed repair to insert the 2xTY1 epitope tag immediately 5’ to the translation start site, and humanized Cas9 mRNAs into zygotes from B6D2F1 (C57BL/6 X DBA2) mice to generate gene-targeted mice in the CCHMC Transgenic Animal and Genome Editing Facility (the concentrations for the gRNA, oligonucleotide donor template, and Cas9 mRNAs were 50 ng/μl, 100 ng/μl, and 100 ng/μl, respectively). Transgenic founder mice were identified by PCR genotyping of genomic DNA isolated from tail biopsies using the primers OsrlnF 5’-GGCAGTAGGTTCATGGGTGG-3’ and OsrlnR 5’-CATACAGGTTGGGCAGGTGG-3’, which produces PCR products of 311 bp and 377 bp, respectively from the wildtype and OsrlTY1 alleles. Out of 12 G0 founder mice, 4 carried the in-frame 2xTY1 insertion. All 4 positive G0 mice were bred to C57BL/6J inbred mice to test for germline transmission and 3 (one male and 2 female G0 founders) transmitted the correct in-frame 2xTY1 tag insertion. Genotypically verified G1 OsrlTY1/+ hemizygous mice were intercrossed to generate OsrlTY1/TY1 homozygotes.

**TUNEL and cell proliferation assays**

Apoptotic cells were detected on 7μm paraffin sections using DeadEnd™ Fluoro-metric TUNEL System (Promega, G3250) according to manufacturer’s instructions. Six kidney sections from each of two embryos of each genotype at each developmental stage were analyzed.

To determine proliferative activity of metanephric mesenchyme, timed-mated pregnant mice were injected intraperitoneally with 10 μl/g BrdU (5 mg/ml stock) (Sigma-Aldrich, B5002). Embryos were harvested 1 hour after injection. The BrdU-labeling index was defined...
as the number of BrdU-positive nuclei relative to Six2-positive nuclei, which was detected by immunostaining.

**Kidney explant culture**

The metanephric rudiments were dissected from E11.5 mouse embryos and positioned on top of a culture plate insert (1.0 μm pore size, BD Falcon, 353102) within an individual well of a 6-well tissue culture plate and cultured in DMEM/F12 media plus 10% fetal bovine serum (Invitrogen). The explant cultures were maintained at 37°C at an atmosphere of 5% CO2 and 100% humidity.

**Nephron Number Quantification, Histology, and Immunofluorescent staining**

The number of nephrons per kidney was measured using a previously established protocol [50]. For histological analysis, embryos were dissected at desired stages from timed pregnant mice, fixed in 4% paraformaldehyde (PFA), dehydrated through an ethanol series, embedded in paraffin, sectioned at 7μm thickness, and stained with hematoxylin and eosin.

Immunofluorescent staining of paraffin sections was performed following standard protocols. Antibodies and reagents used are: rabbit anti-Six2 (ProteinTech, 11562-1-AP), mouse anti-Pan cytokeratin (Sigma), rabbit anti-Jagged1 (Santa Cruz, sc-8303), rabbit anti-Wt1 (Santa Cruz, sc-192), rabbit anti-Pax2 (Invitrogen, 71–6000), chicken anti-GFP (AVES labs, GFP-1010).

For whole mount immunostaining staining, E10.5 lower trunk or E11.5 urogenital regions were dissected and fixed in 1% PFA at 4°C over night. After fixation, embryos were washed, and transferred to 100% methanol. The embryos were bleached in a solution of methanol/H2O2/DMSO (4:1:1) for 2 hours at room temperature. After rehydration, embryos were blocked with 2.5% goat serum and 5% BSA in PBST for 2 hours, incubated in primary antibody diluted in blocking buffer overnight at 4°C. Embryos were washed, and then incubated with secondary antibody overnight at 4°C. Specimens were examined and photographed using a Nikon inverted confocal microscope. The volume of MM was analyzed by Imaris software after confocal imaging.

**In situ hybridization**

Whole mount in situ hybridization was performed as previously described. At least three embryos of each genotype were hybridized to each probe and only probes that detected consistent patterns of expression in all samples were considered as valid results [26].

**Co-immunoprecipitation**

The Osr1 coding sequence was subcloned into the pCS2 vector to express Osr1 with Myc-epitope tag. Osr1, Wt1, Lhx1 and Six2 coding sequences were subcloned into pCMV7.1 (Sigma) vectors with a Flag-tag. Pax2 coding sequence was subcloned into pcDNA3 (invitrogen). Flag-Six2, Flag-Lhx1 and Myc-Pax2 plasmids were kindly provided by Dr. Joo-Seop Park (Cincinnati Children’s Hospital, Medical Center).

For immunoprecipitation assays, HEK293T cells were co-transfected with plasmids as indicated. After transfection, cells were cultured in DMEM supplemented with 10% fetal bovine serum for 48 hours. The cells were lysed in RIPA buffer containing proteinase inhibitors (Santa Cruz, SC-24948). Whole cell lysate was incubated with anti-c-Myc antibody conjugated to protein-G agarose beads (4A6, Millipore, 16–219), or anti-Flag (M2, sigma, F1804) coupled with
protein-G dynabeads (Thermo Fisher scientific, 10003D) and rotated at 4°C over night. The beads were washed five times with RIPA buffer. Western blot was performed using anti-Flag (M2, Sigma F3165), anti-\(\beta\)-Myc (4A6, Millipore 05-724) antibodies. E16.5 kidney samples from Osr1\(TY1/TY1\) or wildtype embryos were harvested and lysed in lysis buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.25% TX-100, and 1x complete mini protease inhibitor [Roche, 0469315001]). Lysates were incubated with anti-TY1 antibody (Diagenode, C15200054) coupled dynabeads at 4 C for overnight. The beads were washed five times with lysis buffer. The western blot was performed with anti-Six2 anti-body (Proteintech, 11562-1-AP), anti-Flag antibody (M2, Sigma, F1804), or anti-Wt1 antibody (Santa Cruz, SC-192).

**FACS sorting and real-time RT-PCR**

The trunk tissues of E9.5 embryos from wildtype females crossed with Osr1\(^{+/+}\)/Wt1\(^{+/+}\) double heterozygous males were manually microdissected and digested with trypsin-EDTA (Invitrogen) at 37°C for 4 minutes. After inactivation of trypsin with DMEM containing 10% FBS, cells were dissociated by pipetting. The dissociated cells were resuspended in PBS with 2% FBS and 10 mM EDTA, and filtered through a 40 \(\mu\)m nylon cell strainer (BD Falcon, 352340). GFP + cells were isolated using BD FACSaria II. FACS-isolated GFP+ cells from Osr1\(^{+/+}\)/Wt1\(^{+/+}\) double heterozygous embryos and Wt1\(^{+/+}\) heterozygous littermates were used for RNA extraction. First-strand cDNAs were synthesized by the Gene Expression Core at Cincinnati Children’s Hospital Medical Center. Real-time PCR was performed using a Bio-Rad CFX96 Real-Time System using conditions recommended by the manufacturer. Each reaction was performed in triplicates. The quantity of each mRNA was first determined using a standard curve method and normalized to the internal control (\(\beta\)-Actin).

**Statistical analysis**

All results were presented as mean ± SEM. All statistical analyses were done using Excel software. Two-tailed Student’s t tests were used for comparisons between two groups. P value less than 0.05 was considered significant.

**Supporting Information**

**S1 Fig. Osr1\(^{+/+}\)/Wt1\(^{+/+}\) embryos exhibit defects in Six2-positive metanephric mesenchyme.** (A-C) Whole mount immunofluorescent staining for Six2 protein (red) in E10.5 Osr1\(^{+/+}\) (A), Wt\(^{+/+}\) (B), and Osr1\(^{+/+}\)/Wt1\(^{+/+}\) (C) embryos. The embryos were counterstained with DAPI (Blue). The white dotted line outlines the metanephric mesenchyme. Scale bar, 100 \(\mu\)m. (TIF)

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**Author Contributions**

Conceived and designed the experiments: JX HL YL RJ. Performed the experiments: JX HL OHC YL. Analyzed the data: JX HL OHC YL RJ. Contributed reagents/materials/analysis tools: JX HL YL RJ. Wrote the paper: JX HL YL RJ.
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