WNK1 Protein Kinase Regulates Embryonic Cardiovascular Development through the OSR1 Signaling Cascade*

Received for publication, January 8, 2013, and in revised form, January 29, 2013. Published, JBC Papers in Press, February 5, 2013, DOI 10.1074/jbc.M113.451575

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Background: WNK1 is critical for embryonic cardiovascular development.

Results: A global deletion of Osr1 in mice phenocopies the Wnk1 deletion; expression of the constitutively active mutant OSR1 rescues developmental defects in Wnk1-null embryos.

Conclusion: WNK1 activation of OSR1 is essential for mouse embryo development.

Significance: The WNK1-OSR1 signaling cascade is a novel pathway that regulates embryonic angiogenesis and cardiac formation.

WNK1 is a widely expressed serine/threonine protein kinase that regulates multiple cellular and organ functions via diverse mechanisms. We previously reported that endothelial-specific deletion of Wnk1 in mice results in embryonic lethality, with angiogenesis and cardiac defects beginning at embryonic day ~10.5. Here, we further investigated the signaling mechanism by which WNK1 regulates embryonic cardiovascular development. We found that mice with a global deletion of Osr1, which encodes oxidative stress-responsive kinase-1, a protein kinase activated by WNK1, died in utero beginning at embryonic day ~11. The defects in Osr1-null yolk sacs and embryos were virtually identical to those observed in Wnk1-knock-out mice: no mature large vessels in yolk sacs, defective angiogenesis in the brain and intersomitic vessels, and smaller chambers and reduced myocardial trabeculation in mutant hearts. Endothelial-specific deletion of Osr1 generated by crossing Osrlflox/fox mice with Tie2-Cre mice phenocopied defects caused by global Osr1 deletion. To investigate whether OSR1 acts downstream of WNK1 in embryonic angiogenesis, we generated a mouse line with specific expression of the constitutively active mutant OSR1, generated by Tie2-Cre-mediated excision of floxed stop codons in the mutated ROSA26 locus under the control of a cassette of floxed transcription stop codons. We found that endothelial-specific expression of the constitutively active mutant OSR1, generated by Tie2-Cre-mediated excision of floxed stop codons in the mutated ROSA26 locus, rescued angiogenesis and cardiac defects in global Wnk1-null embryos. These results indicate that WNK1 activation of the OSR1 signaling cascade is an essential pathway that regulates angiogenesis and cardiac formation during mouse embryo development.

WNK1 (with no lysine (K) 1) is a member of the family of serine/threonine protein kinases characterized by the unique placement of the catalytic lysine (1). There are four mammalian members of the WNK kinase family, WNK1–4, each encoded by a separate gene (1–3). The WNK1 protein is >2100 amino acids long; WNK2–4 are between 1200 and 1800 amino acids in length. The four WNK kinases share a conserved kinase domain, an autoinhibitory domain, one to two coiled-coil domains, and multiple proline-rich motifs for potential protein-protein interactions. Outside these recognizable motifs, the amino acid sequences of WNK kinases are not well conserved. Despite the atypical kinase domain structure, WNKs are bona fide protein kinases and catalyze phosphorylation of endogenous substrates (4).

The functions of WNK kinases were first revealed by the discovery that mutations in WNK1 and WNK4 in humans cause the autosomal dominant disease pseudohypoaldosteronism type 2, which is characterized by hypertension and hyperkalemia (2). Subsequent studies have shown that WNK1 and WNK4 play important roles in the regulation of ion transport proteins involved in Na⁺ and K⁺ homeostasis (4). Regulation involves kinase activity-dependent as well as activity-independent mechanisms. With respect to kinase-dependent mechanisms, WNK1 and WNK4 activate SPAK (Ste20-related proline/glutamine-rich kinase) and OSR1 (oxidative stress-responsive kinase-1) by conferring phosphorylation on a critical threonine residue in the catalytic loop and probably also on a serine in the regulatory domain (5–8). In turn, activated OSR1 and SPAK can phosphorylate and activate cation-chloride cotransporters NCC and NKCC1/2 to regulate renal Na⁺ reabsorption and vascular smooth muscle contractility (5–8). Through kinase-independent events, WNK1 binds and somehow activates SGK kinase, causing it to increase surface expression of the epithelial Na⁺ channel (9); and WNK1 and WNK4 interact with the endocytic scaffold protein intersectin to enhance endocytosis of the renal K⁺ channel ROMK (10). Dysregulation of Na⁺ and K⁺ transport in the kidney contributes to the hypertension and hyperkalemia of pseudohypoaldosteronism type 2.

WNK1 also regulates intracellular signaling. WNK1 activates ERK5, and knockdown of WNK1 decreases activation of ERK5 by epidermal growth factor receptors (11). More recently, it has
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been shown that WNK1 plays an important role in G protein-coupled receptor and phospholipase C signaling (12). WNK1 stimulates phospholipase Cβ signaling by promoting the synthesis of phosphatidylinositol 4,5-bisphosphate via stimulation of phosphatidylinositol 4-kinase IIIα, and this regulation is amplified when WNK1 is phosphorylated by Akt kinase. This new signaling mechanism allows crosstalk between G protein-coupled receptors and Akt-activating growth factors and provides a way to independently control phospholipase C signaling and membrane phosphatidylinositol 4,5-bisphosphate availability.

Another important function of WNK1 is in embryonic development. Mice homozygous for Wnk1 inactivation die during embryonic development (13). We have recently shown that Wnk1-ablated mice die in utero from cardiac developmental and angiogenesis defects (14). The defects in Wnk1-null embryos are distinct from abnormalities observed in embryos with disruption of known angiogenesis pathways, e.g. VEGF and Notch signaling pathways, and are not associated with alterations of expression of genes in these pathways. In this study, we demonstrate that deletion of Osr1 in endothelial cells virtually phenocopied defects of Wnk1-deleted embryos and that endothelial-specific expression of constitutively active OSR1 rescued cardiovascular defects in Wnk1-deleted embryos. These findings indicate that WNK1 activation of OSR1 is a novel pathway essential for embryonic cardiovascular development in mice.

EXPERIMENTAL PROCEDURES

Mouse Strains and Genotyping—Wnk1+/−, Wnk1floxed/+, Tie2-Cre transgenic, Sox2-Cre transgenic, Osr1+/−, and Osr1floxed/+ mice have been described (14, 15). All animal maintenance and experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center at Dallas. For timed breeding, the appearance of a vaginal plug was checked twice a day at 9 a.m. and 6 p.m., and 1 a.m. or 1 p.m. of the day in which a plug was found was designated as embryonic day 0, respectively. The embryonic stage was further confirmed by the number of somites at the time of embryo dissection. For genotyping, mouse tail tips, ear punches, toe clips, or portions of yolk sacs of embryos were digested overnight in Viagen DirectPCR® reagents with 0.2 mg/ml proteinase K at 55 °C; heat-inactivated at 85 °C for 45 min; and analyzed by PCR as described (14). All genotyping PCRs were performed with 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The primers for genotyping of Wnk1−/−, Wnk1loxP/loxP mice were as described (14, 15). The primer sequences used for Osr1floxed knock-out genotyping (see Fig. 1B) were AAACCTGCTGG-GCTTCTATG (forward) and TGGGGTTAGTGGGGGATAA (reverse); those used for Osr1floxed conditional knock-out genotyping (see Fig. 4B) were TGTTTCAGCATCTCAGTGTA (forward) and TGGTGAATGCGAAATGTGT (reverse); and those used for genotyping the targeted ROSA26-hOSR1loxP allele (see Fig. 5B) were CCCCTGAAACTCAGCGA (F1), GAGT-GCTTCTGCTGCTCTCG (F2), and AAGTCTGCTGGACTACAGCATGTC (R1).
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TABLE 1

Distribution of E9.5–13.5 embryos and pups from heterozygous Osr1 crossing

| Age   | Total | +/+ 50% | +/-30% | +/-10% | +/- 0% |
|-------|-------|--------|--------|--------|--------|
|       |       |        |        |        |        |
| E9.5  | 24    | 6 (25%)| 11 (46%)| 7 (29%)|
| E10   | 17    | 4 (24%)| 8 (47%)| 5 (29%)|
| E10.5 | 20    | 3 (15%)| 9 (45%)| 8 (40%)|
| E11.5 | 23    | 9 (39%)| 13 (57%)| 4 (15%)|
| E12.5 | 15    | 4 (27%)| 8 (53%)| 3 (20%)|
| E13.5 | 19    | 3 (21%)| 10 (53%)| 6 (32%)|
| Pups  | 67    | 21 (31%)| 46 (69%)| 0 (0%)|

RESULTS

Osr1−/− Embryos Die between Embryonic Days 10.5 and 11.5 with Cardiovascular Defects—Deletion of Osr1 in mice causes embryonic lethality (15). To examine the cause of death of Osr1-deleted embryos, we examined phenotypes of timed embryos by crossing heterozygous Osr1-knock-out mice and collected embryos at various embryonic stages. Viable homozygous Osr1-deleted embryos (Osr1−/−) were obtained with the expected Mendelian ratios at embryonic days (E)2 9.5–10.5 (Table 1; see Fig. 1B for genotyping). At E9.5, the homozygous embryos were normal and phenotypically indistinguishable from their wild-type and heterozygous littermates. However, Osr1−/− embryos started to show growth retardation beginning at E10 (five of five abnormal at E10 and eight of eight abnormal at E10.5) (Table 1). All Osr1−/− embryos at the embryonic stage between E11.5 and E13.5 were dead, and no Osr1−/− embryos were recovered beyond E13.5.

Compared with wild-type embryos, Osr1−/− embryos at E10.5 exhibited growth retardation of varying severity, with pericardial edema (Fig. 1A, black arrows) and hemorrhage in various locations, including around the heart (yellow arrows), common cardinal veins, and the head. Despite a smaller overall size, the body axis and somite number of living Osr1−/− mutant embryos at E10.5 were similar to those of wild-type embryos (Fig. 1A). Osr1+/− embryos were phenotypically indistinguishable from wild-type embryos (data not shown). Wild-type yolk sacs at E10.5 showed a network of large and small vessels filled with circulating blood (Fig. 1A, white arrow). In contrast, yolk sacs of E10.5 mutants were noticeably thinner and paler and lacked larger branching vitelline vessels and blood circulation (Fig. 1A).

The death of embryos at midgestation, lack of detectable blood circulation and large vessels in the mutant yolk sac, hemorrhage, and pericardial edema are all indicative of cardiovascular developmental defects. To analyze cardiovascular defects, we sectioned the embryos and examined the histology of hearts and blood vessels. In Fig. 2 (A and B), the transverse sections through the hearts of E10.5 wild-type and Osr1−/− embryos revealed hypoplasia of atrial (a) and ventricular (common ventricle (cv) and bulbus cordis (bc)) chambers in mutant embryos compared with wild-type embryos. The myocardial trabeculations in the developing ventricular chambers were extensive in wild-type embryos but were significantly reduced in mutants. Pericardial edema was evident from the dilatation of the pericardial sac (Fig. 2B, arrowhead). Thus, Osr1-ablated embryos exhibited defective heart development, including reduced heart chamber size and reduced myocardial trabeculation. In addition, in Fig. 2 (C and D), the cross-sections of wild-type and mutant embryos revealed collapsed head veins (hv) and carotid arteries (ca) and much smaller dorsal aortas (da) and anterior cardinal veins (acv) in the mutants.

PECAM1 Immunostaining Reveals Angiogenesis Defects in Osr1−/− Embryos—We further examined embryonic vascular development using whole-mount immunostaining of embryos using an antibody against a pan-endothelial cell marker, PECAM1. Formation of the vasculature framework was evident by PECAM1 immunostaining in both wild-type and Osr1-null embryos.
mutant embryos at E10.5 (Fig. 3A). However, the density of blood vessels was lower, and their organization was defective in mutant embryos compared with wild-type embryos (Fig. 3, B and C). In wild-type embryos, intersomitic vessels displayed a distinct pattern and complexity with multiple branches (Fig. 3B, arrows). In contrast, the intersomitic vessels of mutant embryos were disorganized and reduced in number, with irregular patterning and poor branching. Similarly, the density of vessels and sprouting and branching of vessels in the head region were markedly reduced in mutant embryos compared with wild-type embryos (Fig. 3C, box).

Endothelial-specific Conditional Knock-out of Osr1 Phenocopies Developmental Defects Caused by Global Osr1 Ablation—Non-endothelial cell types surrounding developing vasculature also play critical roles in angiogenesis (17). To define the cell lineages in which OSR1 function is essential for cardiovascular development, we generated conditional knock-out of Osr1 in endothelial cells by crossing Osr1-flox/flox mice with a Tie2-Cre transgenic mouse line that expresses Cre recombinase specifically in endothelial cell lineages (18). We found that some Osr1^{flox/flox}; Tie2-Cre embryos started to show growth defects and abnormality similar to the global Osr1-null embryos from E10.5 (Table 2; see also Fig. 4). The percentage of abnormal Osr1^{flox/flox}; Tie2-Cre embryos increased at E11.5–12.5, with the presence of dead mutant embryos. At E13.5, Osr1^{flox/flox}; Tie2-Cre embryos were either dead or severely defective (Table 2). No live Osr1^{flox/flox}; Tie2-Cre embryos were recovered beyond E13.5. Also, as in global Osr1^{-/-} mutants, the yolk sac vasculature failed to remodel in E10.5 Osr1^{flox/flox}; Tie2-Cre mutants (Fig. 4A; see Fig. 4B for genotyping). Osr1^{flox/flox}; Tie2-Cre embryos were growth-retarded and displayed hemorrhage in multiple regions and pericardial edema compared with Osr1^{flox/flox} embryos. Osr1^{flox/flox} embryos were phenotypically indistinguishable from wild-type embryos.

**FIGURE 3.** Whole-mount PECAM staining of E10.5 embryos reveals angiogenesis defects in Osr1^{+/-} mutants. A, a major framework of blood vessels formed in mutant embryos but was less organized, and the density of vessels was lower compared with wild-type embryos. B, enlarged view showing irregular patterning and reduced branching of intersomitic vessels (arrows) in a mutant embryo compared with a wild-type embryo. C, enlarged view showing disorganized vessels in the head region of a mutant embryo compared with a wild-type embryo.

### TABLE 2

| Age     | Total | Osr1^{flox/flox}; Tie2-Cre (25% expected) | Osr1^{flox/flox}; Tie2-Cre (25% expected) |
|---------|-------|-------------------------------------------|-------------------------------------------|
| E9.5    | 36    | 10 (29%)                                  | 7 (19%)                                   |
| E10.5   | 42    | 12 (29%)                                  | 10 (24%, 4^{th})                          |
| E11.5   | 23    | 7 (30%)                                   | 5 (22%, 3^{th} + 1^{st})                  |
| E12.5   | 51    | 14 (27%)                                  | 9 (18%, 3^{th} + 4^{th})                  |
| E13.5   | 37    | 12 (32%)                                  | 6 (16%, 2^{nd} + 4^{th})                  |
| E14.5–16.5 | 67 | 25 (37%)                                  | 4^{th} (6%)                               |
| Pups    | 107   | 34 (32%)                                  | 0 (0%)                                    |

Conditional Expression of a Constitutively Active hOSR1 Transgene—To study the function of OSR1 kinase in different tissues and cell lineages independently of its upstream activator(s), we generated a mouse line that allows for tissue-specific expression of activated OSR1 under the control of Cre recombinase. Phosphorylation by WNK kinases at Thr-185 and Ser-325 activates OSR1 kinase (5–8, 19). We mutated both threonine and serine residues to glutamate render OSR1 constitutively active independently of WNK kinases (5–8, 19). We mutated both threonine and serine residues to glutamate and placed the mutant human OSRI transgene (hOSRI^{ca}) in the transcriptionally active mouse ROSA26 locus behind a cassette of floxed transcription terminator codons (Fig. 5A). Mice carrying the ROSA26-hOSR1^{ca} allele will express constitutively active OSR1 in tissues of interest when crossed with transgenic mice expressing tissue-specific Cre recombinase. As shown, when ROSA26-hOSR1^{ca} mice were crossed with a ubiquitous Sox2-Cre mouse line, only the cleaved form of ROSA26-hOSR1^{ca} was detected in the genotyping PCR of mouse tail DNA, indicating that the targeted allele was activated globally (Fig. 5B, second lane). In contrast, when ROSA26-hOSR1^{ca} mice were crossed with Tie2-Cre mice, both cleaved and uncleaved forms of ROSA26-hOSR1^{ca} were detected in the genotyping PCR analy-
sis, consistent with the notion that the transgene was cleaved and activated in the endothelial cells and the uncleaved form was present in other tissues (Fig. 5B, third lane). The expression of exogenous human OSR1α cDNA in the kidneys of ROSA26-hOSR1α;Sox2-Cre mice but not of wild-type mice was confirmed by RT-PCR using primers specific for human OSR1, which detected the cDNA of the transgene (Fig. 5C, lanes 1 and 2). For comparison, primers that recognize sequence conserved between hOSR1α cDNA and endogenous mouse Osr1 cDNA detected signals in both ROSA26-hOSR1α;Sox2-Cre and wild-type mice (Fig. 5C, lanes 3 and 4).

We next tested the ability of Cre recombinase-mediated excised hOSR1α cDNA to substitute for the function of Osr1 in Osr1-knock-out mice by intercrossing of ROSA26-hOSR1α; Sox2-Cre;Osr1<sup>1</sup>/<sup>−</sup> mice with Osr1<sup>1</sup>/<sup>−</sup> mice. The intercrossing generated ROSA26-hOSR1α; Sox2-Cre;Osr1<sup>1</sup>/<sup>−</sup> offspring with the expected Mendelian ratios (data not shown), indicating that transgenic expression of hOSR1α rescued the embryonic lethality caused by Osr1 deletion. The rescued mice were fertile and grossly normal, with no apparent morphological difference compared with wild-type littermates (Fig. 5D). Quantitative real-time PCR of adult kidney mRNAs (using primers targeting the exons that are deleted in Osr1-knock-out mice but that are conserved between mouse Osr1 and human OSR1 sequences) confirmed the expression of hOSR1α in rescued mice, and the level was ~2-fold relative to the level of endogenous Osr1 mRNA in wild-type mice (Fig. 5E). As expected, the Osr1 mRNA was reduced in the Osr1 heterozygotes.

Rescue of Developmental Defects in Wnk1<sup>−/−</sup> Embryos by hOSR1α Transgene Expression—Having validated the expression of hOSR1α by Cre recombinase, we next tested whether expression of activated OSR1 can rescue the developmental defects in Wnk1<sup>−/−</sup> mice. Wnk1 and ROSA26 alleles are both located on mouse chromosome 6, only ~6.9 megabases apart. To increase the frequency of obtaining the desired genotypes from breeding, we first intercrossed Wnk1<sup>−/−</sup> and ROSA26-hOSR1α mice to create Wnk1<sup>−/−</sup>; ROSA26-hOSR1α double heterozygous mice in which the Wnk1 mutant locus and the ROSA26-hOSR1α transgene locus were initially on different chromatids (Fig. 6A). These mice were then repeatedly bred with wild-type mice to generate [Wnk1<sup>−/−</sup>; ROSA26-hOSR1α] mice, in which the two loci became “linked” on the same chromatid through chromosomal crossover. Further crossing of these mice with Wnk1<sup>−/−</sup>; Cre<sup>+</sup> animals generated a population of Wnk1<sup>−/−</sup>; ROSA26-hOSR1α mice that were 12.5% frequency-positive and 12.5% frequency-negative for Cre (Fig. 6A). Fig. 6B shows the results from genotyping PCR analysis of the Wnk1 allele.

Table 3 shows the results of intercrossing of [Wnk1<sup>−/−</sup>; ROSA26-hOSR1α] mice with Wnk1<sup>−/−</sup>; Tie2-Cre mice analyzed at different embryonic stages. Note that the distribution of Wnk1<sup>−/−</sup>; ROSA26-hOSR1α embryos with either Tie2-Cre<sup>+</sup> or Tie2-Cre<sup>−</sup> negative at E10.5–11.5 follows the expected Mendelian ratios (i.e. ~12.5% each). Overall, this intercrossing produced a total of 22 Wnk1<sup>−/−</sup>; ROSA26-hOSR1α (Cre<sup>+</sup> or Cre<sup>−</sup>) embryos from a total of 169 embryos collected between E10.5 and E18.5. Among these, five embryos were Tie2-Cre<sup>+</sup> and either dead or defective by E13.5; 17 embryos were Tie2-Cre<sup>−</sup>, 7 of 17 Wnk1<sup>−/−</sup>; ROSA26-hOSR1α; Tie2-Cre embryos were recovered at E12.5–13.5, and among these, two were normal phenocopies of wild-type embryos at E13.5 (Table 3 and Fig. 6C). In separate experiments in which we crossed unlinked
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**FIGURE 5. Conditional expression of catalytically and constitutively active OSR1 in mice.**

A. A targeting scheme. A mutant human OSR1 cDNA encoding the constitutively active form of OSR1 kinase (hOSR1ca) was made by site-directed mutagenesis of Thr-185 and Ser-325 to a phosphomimicking glutamate (T185E and S325E). The cDNA was inserted downstream of a loxP-flanking (triangle) triple-poly(A) site (tpA). This fragment was targeted into a constitutively transcribing ROSA26 locus to generate ROSA26-hOSR1ca mice. When crossed with transgenic Cre-expressing mice, Cre-mediated excision would delete the triple-poly(A) site so that hOSR1ca was expressed tissue-specifically in the Cre progeny. B. Genotyping PCR analysis of different genotypes of mice with ROSA26-hOSR1ca, ROSA26-hOSR1ca; Sox2-Cre, and ROSA26-hOSR1ca; Tie2-Cre. C. Human OSR1 PCR analysis reveals the expression of human OSR1 in ROSA26-hOSR1ca mice (lane 2) but not in wild-type mice (lane 1). D. PCR analysis reveals the expression of hOSR1 in kidney RNAs with primers targeting the deleted exon 10 in Osr1 knock-out mice. As expected, the Osr1 mRNA level in Osr1+/− mice was about half that in wild-type mice. Expression of hOSR1 in Osr1+/−; ROSA26-hOSR1ca; Sox2-Cre mice was ~2-fold that of Osr1 mRNA in wild-type mice. The forward and reverse PCR primers used were aatgatttcattgtgccttca and tcactcactcccagcctcc, respectively.

Wnk1+/−; ROSA26-hOSR1ca mice with Wnk1+/−; Tie2-Cre mice, we found that Wnk1+/−; ROSA26-hOSR1ca; Tie2-Cre embryos were phenotypically indistinguishable from wild-type embryos (data not shown). We have found that, without genetic rescue, all Wnk1+/− embryos are dead by E12.5 (14). These results indicate that transgenic expression of hOSR1ca in endothelial cells could rescue cardiovascular defects of Wnk1 knock-out. Because the expression Osr1 is not altered in Wnk1 knock-out embryos (Fig. 6D), the effect of rescue is likely due to constitutive activation of OSR1 kinase activity. The developing hearts and other organs (including the lung, liver, kidney, and gastrointestinal tract) of the E13.5 rescued embryos were largely similar to those of wild-type embryos (data not shown). However, most of the Wnk1+/−; ROSA26-hOSR1ca; Tie2-Cre embryos between E10.5 and E18.5 were dead or defective, and no Wnk1-null pups were found (Table 3).

To determine whether the incomplete rescue of developmental defects of Wnk1-null mice by Tie2-Cre-mediated activation of hOSR1ca may be due to a requirement of WNK1-OSR1 signaling in tissues outside endothelial cells, we crossed [Wnk1+/−; ROSA26-hOSR1ca] mice with Wnk1+/−; Sox2-Cre mice to see whether the lethality of Wnk1 knock-out can be rescued by globally expressed active hOSR1ca. Of 215 new born pups (days 0−7), we found 19 Wnk1+/−; Sox2-Cre mice, and all of them were positive for Sox2-Cre (Table 4). The frequency of Wnk1+/− pups rescued by Sox2-Cre-mediated activated OSR1 was ~9%, not markedly different from that expected. However, a large percentage of live born pups died within the first few
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FIGURE 6. Expression of hOSR1<sup>ca</sup> rescues Wnk1 knockout mice. A, breeding scheme for Wnk1 knockout rescue. The initial breeding between Wnk1<sup>+/−</sup> and ROSA26-hOSR1<sup>ca</sup> mice (not illustrated here) produced mice carrying both alleles in two separate chromatids. The Wnk1<sup>+</sup> and ROSA26 loci in mice are both located on chromosome 6, only ~6.9 megabases apart. To increase the frequency of progeny with both alleles, we bred mice carrying both Wnk1<sup>+/−</sup> and ROSA26-hOSR1<sup>ca</sup> alleles in separate chromatids with wild-type mice to generate mice in which the two loci were linked on the same chromosome, i.e. [Wnk1<sup>+/−</sup>; ROSA26-hOSR1<sup>ca</sup>]. Crossing these mice with Sox2-Cre<sup>−/−</sup>Cre<sup>+</sup> mice produced Wnk1<sup>−/−</sup> progeny carrying ROSA26-hOSR1<sup>ca</sup> with 25% frequency. Among them, half were Cre<sup>+</sup> (12.5% each), and half were Cre<sup>−</sup> (12.5% each). B, genotyping PCR analysis for wild-type, Wnk1<sup>−/−</sup> and Wnk1<sup>+/−</sup>. C, a live and normal Wnk1<sup>−/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Tie2-Cre embryo at E13.5 appeared to be identical to its wild-type littermate. D, Osr1 mRNA levels in E9.0 Wnk1-null embryos (relative to wild-type embryos) analyzed by quantitative real-time PCR (n = four each). E, a Wnk1<sup>−/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Sox2-Cre mouse at 3 months of age was smaller than its wild-type littermate. F, growth curve of Wnk1<sup>−/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Sox2-Cre mice compared with wild-type controls.

TABLE 3
Distribution of Wnk1<sup>−/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Tie2-Cre embryos and pups from [Wnk1<sup>+/−</sup>; Tie2-Cre × [Wnk1<sup>+/−</sup>; ROSA26-hOSR1<sup>ca</sup>]] intercrossing

| Age      | Total | Tie2-Cre<sup>−/−</sup> (12.5% expected) | Tie2-Cre<sup>+/+</sup> (12.5% expected) |
|----------|-------|----------------------------------------|----------------------------------------|
| E10.5–11.5 | 26    | 3 (11.5%, 1<sup>st</sup> + 2<sup>nd</sup>) | 4 (14.4%, 2<sup>nd</sup>)               |
| E12.5–13.5 | 65    | 2 (3.1%, 2<sup>nd</sup>)               | 7 (10.8%, 2<sup>nd</sup>)              |
| E14.5–16.5 | 47    | 0 (0%)                                 | 4 (8.5%, 1<sup>st</sup> + 3<sup>rd</sup>)|
| E17.5–18.5 | 31    | 0 (0%)                                 | 2 (6.5%, 2<sup>nd</sup>)              |
| Pups      | 84    | 0 (0%)                                 | 0 (0%)                                 |

Note that Wnk1 and ROSA26-hOSR1<sup>ca</sup> alleles are linked on the same chromosome. ab, live abnormal (defective) embryos; d, dead embryos.

TABLE 4
Distribution of Wnk1<sup>−/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Sox2-Cre pups from [Wnk1<sup>+/−</sup>; Sox2-Cre × Wnk1<sup>+/−</sup>; ROSA26-hOSR1<sup>ca</sup>]] intercrossing

| Age          | Total | Sox2-Cre<sup>−/−</sup> (12.5% expected) | Sox2-Cre<sup>−/−</sup> (12.5% expected) |
|--------------|-------|----------------------------------------|----------------------------------------|
| Pups         |       |                                        |                                        |
| Days 0–1     | 126   | 0 (0%)                                 | 12 (9.5%, 7<sup>th</sup>)             |
| Days 2–7     | 89    | 0 (0%)                                 | 7 (7.9%, 4<sup>th</sup>)              |
| Adults (>30 days) | 184 | 0 (0%)                                 | 6 (3.3%, runted)                       |

Note that Wnk1 and ROSA26-hOSR1<sup>ca</sup> alleles are linked on the same chromosome. ab, live abnormal (defective) embryos; d, dead embryos.

days after birth, and only ~3% of adult mice were Wnk1<sup>−/−</sup> and Sox2-Cre<sup>+</sup>. Those that survived to adulthood were growth retarded compared with wild-type littermates of the same gender (Fig. 6, E and F). Thus, activation of OSR1 by WNK1 in other tissues besides the endothelium may be required for embryonic development. Alternatively, the recombination frequency of Sox2-Cre may be higher compared with Tie2-Cre in endothelial cells. The perinatal death and growth retardation suggest that a function(s) of WNK1 independent of OSR1 is necessary for postnatal growth and organ function(s) of adult mice. Of note, the gross morphology and body weight of Wnk1<sup>+/−</sup>; ROSA26-hOSR1<sup>ca</sup>; Sox2-Cre pups and adult mice up to 8 weeks old (from intercrossing between unlinked Wnk1<sup>+/−</sup>; ROSA26-hOSR1<sup>ca</sup> and Wnk1<sup>−/−</sup>; Sox2-Cre mice) were indistinguishable from those of wild-type littermates (data not shown). Whether transgenic expression of constitutively active OSR1 alters organ function later in life awaits future studies.

DISCUSSION

Embryonic vascular development occurs via two separate processes (20, 21). Vasculogenesis results in the formation of the primordial heart tube and major axial vessels as well as the primary vessels in the embryo proper and extraembryonic structures such as the yolk sac. The second process, angiogenesis, is responsible for the formation of blood vessels in organs such as the brain and kidney through further growth, sprouting, branching, and remodeling of the primary vessels. Angiogenesis defects can also lead to cardiac developmental defects (22, 23). We have previously reported that WNK1 function in endothelial cells is critical for embryonic angiogenesis and cardiac development (14). The signaling pathways for the regulation were unknown. Here, we have demonstrated that endothelial-specific deletion of OSR1, a downstream kinase activated by WNK1, phenocopies Wnk1-deleted embryos. Expression of a constitutively active OSR1 transgene in endothelial cells res-
cued cardiovascular defects of Wnk1-deleted embryos. These results provide compelling evidence to support the hypothesis that WNK1 regulates embryonic cardiovascular development in mice by activation of OSR1 signaling in endothelial cells.

Two previous studies also reported that OSR1 is important for embryogenesis. Rafiqi et al. (24) generated knock-in mice carrying a T185A mutation in the T-loop of OSR1. Delpire and Gagnon (8) described another loss-of-function OSR1 mouse model created by gene-trap insertion in exon 15. In both models, the mice died before term. Detailed analysis of timing of death was not performed in either study, but some embryos were recovered at E17.5. This apparent timing of death is later than what we observed in Osr1-deleted mice (approximately E10.5–11.5). Studies have show that T185A mutant OSR1 does not phosphorylate and activate NKCC1 in response to WNK1 in Xenopus oocytes and in vitro assay (5, 6), yet WNK1 kinase is capable of phosphorylating T185A mutant OSR1 at Ser-325 in the S-motif (5). Gene-trap insertion in exon 15 resulted in partial loss of amino acids in the conserved C terminus of OSR1 known to be important for interacting with its substrates and other proteins (8). Our knock-out mice were generated by targeted deletion of exons 9 and 10 of Osr1, which results in truncation of the kinase domain and loss of kinase activity (15). The expression of endogenous Osr1 is not altered in Wnk1-deleted mice. Our finding that catalytically and constitutively active OSR1 rescues phenotypes of Wnk1 deletion argues against the idea that OSR1 regulates angiogenesis independently of its kinase activity. Thus, the apparent delayed lethality in the knock-in and gene-trap mice may be due to residual kinase activity of OSR1 in vivo. Alternatively, OSR1 may also function as a scaffold and thereby contribute to the regulation of embryogenesis.

Many molecules have been identified to play critical roles in regulating vascular development. These include growth factors and their receptors (VEGF, Flt1, Flk1, etc.), factors that activate the Notch signaling pathway, angiopoietins, ephrins, neuroepilins, transcription factor COUP-TFII, integrins, extracellular matrices, and proteases (20–23). We found that expression of many of these known factors is not altered in Wnk1−/− embryos (14). Consistent with the notion of a non-transcriptional mechanism for WNK1 regulation of angiogenesis, our current findings suggest that WNK1-regulated embryonic cardiovascular development involves activation of the downstream kinase OSR1. Members of the SLC family of ion transporters such as the sodium-potassium–chloride cotransporters NKCC1 and NKCC2 and the sodium-chloride cotransporter NCC are substrates for OSR1 (4–8). These sodium transporters are unlikely to be the downstream mediators of the WNK1-OSR1 signaling pathway in embryonic cardiovascular development because deletion of these genes does not cause embryonic developmental defects (25–27). Conceivably, OSR1 may phosphorylate and regulate function of the abovementioned angiogenesis factors such as VEGF, angiopoietins, ephrins, and COUP-TFII.

Alternatively, OSR1 may regulate angiogenesis through PAKs (p21-activated kinases). PAKs are effector proteins for the Rho family of small GTPases and important regulators of cellular processes involving actin cytoskeletal dynamics (28). Studies have found that PAKs modulate multiple endothelial cell processes critical for angiogenesis, including proliferation, sprouting, migration, and lumen formation and maturation (29–31). Deletion of Pak4 and functional inactivation of Pak2 in mice both cause embryonic lethality at approximately E10.5, and these mutant embryos exhibit embryonic and extraembryonic vascular formation defects resembling Wnk1 and Osr1 knock-out (32, 33), as we have observed. OSR1 phosphorylates PAK1 and alters its activation by Cdc42 (34). Future studies will need to investigate whether OSR1 activates PAK2 and/or PAK4 to regulate embryonic vascular development.

Acknowledgments—We thank Dr. M. Cobb for the human OSR1 cDNA; Dr. T. Carroll for the pBgtT and pROSA plasmids; and Drs. Cobb, O. Cleaver, and A. Rodan for discussions and comments.

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