Members of the Foxo family, Foxo1 (Fkhr), Foxo3 (Fkhrl1), and Foxo4 (Afj) are mammalian homologs of daf-16, which influences life span and energy metabolism in Caenorhabditis elegans. Mammalian FOXO proteins also play important roles in cell cycle arrest, apoptosis, stress resistance, and energy metabolism. In this study, we generated Foxo1-deficient mice to investigate the physiological role of FOXO1. The Foxo1-deficient mice died around embryonic day 11 because of defects in the branchial arches and remarkably impaired vascular development of embryos and yolk sacs. In vitro differentiation of embryonic stem cells demonstrated that endothelial cells derived from wild-type and Foxo1-deficient embryonic stem cells were able to produce comparable numbers of colonies supported by a layer of OP9 stromal cells. Although the morphology of the endothelial cell colonies was identical in both genotypes in the absence of exogenous vascular endothelial growth factor (VEGF), Foxo1-deficient endothelial cells showed a markedly different morphological response compared with wild-type endothelial cells in the presence of exogenous VEGF. These results suggest that Foxo1 is essential to the ability of endothelial cells to respond properly to a high dose of VEGF, thereby playing a critical role in normal vascular development.

The Foxo family is one of the forkhead-type transcription factor families and is unique in that its members are downstream components of the insulin signaling pathway involving phosphatidylinositol 3-kinase/Akt (1). They are also mammalian homologs of the daf-16 gene in Caenorhabditis elegans, which is essential for the extension of life span and dauer formation in C. elegans. DAF-16 confers resistance to stress such as heat and UV light, and dauer formation is observed under adverse conditions such as food deprivation (2, 3). Just as in C. elegans, in mammals, the Foxo family functions to regulate the transcription of genes involved in stress resistance and energy metabolism. Genes regulated by FOXO proteins are grouped into four categories. The first group includes the genes for proteins involved in cell cycle arrest and DNA repair such as p27 and GADD45 (4–7). The second group is made up of genes whose products are related to apoptosis such as FasL and Bim (1, 7). The third group comprises genes for proteins related to resistance to oxidative stress such as manganese-containing superoxide dismutase and catalase (8, 9). The last group is composed of genes whose products are involved in energy metabolism such as glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and insulin-like growth factor-1-binding protein-1 (10–13). We previously reported that each member of the Foxo family (Foxo1 (Fkhr), Foxo3 (Fkhrl1), and Foxo4 (Afj)) show a tissue-specific and developmentally specific expression pattern (14). Foxo4 mRNA is abundant in skeletal muscle throughout life, whereas Foxo1 mRNA is abundantly found in adipose tissues. Foxo3a mRNA is scant during ontogeny, but becomes abundant after birth (14). Moreover, recent studies showed that FOXO1 plays important roles in the differentiation of adipocytes and in the myotube fusion of myoblasts in vitro (15, 16). It is therefore suggested that each member plays a distinct role in a tissue-specific and developmentally specific manner in vivo. Indeed, studies using Foxo1 transgenic mice and heterozygous Foxo1-deficient mice showed that FOXO1 is involved in the differentiation of beta-cells in the pancreas and energy metabolism (15, 17).

In this study, we generated Foxo1-null mice to address the role of FOXO1 in vivo. Foxo1-null mice died at around embryonic day (E) 11. We found abnormal angiogenesis in the yolk sacs and embryos as well as underdevelopment of branchial arches at around E9.5. Endothelial cells differentiated from embryonic stem (ES) cells lacking Foxo1 showed a morphologically abnormal response to exogenous vascular endothelial growth factor (VEGF), viz. a flat polygonal morphology in contrast to the elongated spindle-like shape of wild-type endothelial cells. These results suggest that FOXO1 plays a critical role in qualitatively controlling the VEGF signaling pathway and is necessary for normal development of the vascular system early in life.

The abbreviations used are: E, embryonic day; ES, embryonic stem; VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; VE-cadherin, vascular endothelial cadherin; RT, reverse transcription; PFA, paraformaldehyde; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1.
Targeted Disruption of the Foxo1 Gene—A 625-bp probe starting from the ATG codon of the mouse Foxo1 gene was used to screen a 129SVJ genomic library (Stratagene). Analysis of several overlapping clones revealed that the first coding exon including the ATG codon ended 256 bp after the ATG codon. Further phage analysis revealed that the rest of the coding exons are not located within at least 15 kb downstream from the first coding exon. To construct a targeting vector, a 5-kb EcoRV-NotI fragment whose 3′-end terminated 330 bp upstream of the ATG codon was used as the 5′-arm. A 4.2-kb balP-POKneo-poly(A)-balP-FOXO1-3A-poly(A) cassette, when probed with a 300-bp SacI genomic fragment for 3′-probe detected the 2.1- and 7.4-kb restriction fragments corresponding to the wild-type and targeted alleles, respectively. The 5′-probe detected the 7.5- and 6.3-kb restriction fragments corresponding to the wild-type and targeted alleles, respectively. C, RT-PCR analysis of total RNA from a wild-type and homozygous embryo at E9.5 detected a specific band only in the wild-type embryo. The primers flanking the ATG codon gave a 333-bp PCR product. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. D, Western blot analysis of protein from wild-type embryos at E9.5 detected a specific band at ~75 kDa, but no band in homozygous Foxo1-deficient embryos.

In Vitro Differentiation of ES Cells—In vitro differentiation of ES cells was induced using higher concentrations of G418 (18). One cell line heterozygous for the targeted allele was cultivated in medium supplemented with 1.2, 1.8, 2.4, 3.0, or 3.6 mg/ml G418. Thirty-three colonies were picked up from the plate with 3.6 mg/ml G418 after selection for 11 days and expanded. The genomic DNA was extracted and analyzed by Southern blotting using the probe specific for Foxo1 described above. Only one was cloned.

Reverse Transcription (RT)-PCR—Total RNA was purified from embryos and the yolk sac of wild-type and Foxo1-null embryos at E9.5 (n = 3) using TRIzol (Invitrogen). Quantitative PCR analysis of several genes involved in vasculogenesis and angiogenesis was performed using a QuantiTect SYBR Green PCR kit (QiAGEN Inc.); oligonucleotides specific for angiopoietin-1, angiopoietin-2, VEGF, Flt-1, Flk-1, Tie-1, Tie-2, EphB3, EphB4, ephrin-B2, connexin-37, connexin-40, connexin-43, and smooth muscle α-actin (Progli Japan); and a Light Cycler system.
defects in B neural crest cells in the second branchial arch (arrows), and somites of G sense probe revealed the pathway of migrating crest cells populating hybridization technique, but RT-PCR confirmed the transcripts to be and E ISV (upper, G -GATTGGTGTGTGGCATTCCTGACCTG-3') under the following conditions: 94 °C for 2 min; 35 cycles at 94 °C for 10 s, 63 °C for 30 s, and 72 °C for 45 s; and 72 °C for 2 min. RNA loading was controlled by amplification of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. Negative controls were performed for each sample using non-reverse-transcribed RNA.

In Situ Hybridization—Whole mount in situ hybridization was carried out as described previously (21) with minor modifications. Embryos were collected in ice-cold phosphate-buffered saline (PBS), fixed in 4% PFA in PBS at 4 °C for 3 h, washed with PBS containing 0.1% Triton X-100 (PBST), dehydrated, and stored in methanol at −20 °C until used. After being bleached with 6% hydrogen peroxide in methanol for 2 h at room temperature, embryos were rehydrated though a 75, 50, 25, and 0% methanol and PBST series. Embryos were incubated with 20 μg/ml proteinase K in PBST for 6 min at E8.5–9.0 or for 2 min at E9.5 and refixed in 4% PFA and 0.2% glutaraldehyde in PBS for 20 min. After incubation in hybridization buffer at 63 °C for 4 h, embryos were incubated in the same buffer containing 0.5 μg/ml digoxigenin-labeled RNA probe (Roche Applied Science) at 63 °C for 18 h. The hybridization buffer used and the steps for probe washing, RNase reaction, and RNase inactivation were as described previously (21). Aminoalkylation with 10% heat-inactivated goat serum and 2 μm levamisole (Sigma) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), embryos were treated with 0.24 units/ml anti-digoxigenin Fab fragments (Roche Applied Science) in TBST for 2 h at 4 °C. After a thorough washing with 2×TBST, embryos were treated with 0.1 M NaCl, 0.1 M Tris-HCl (pH 9.5), 20 mM MgCl2, 0.1% Tween 20, and 2×TBST for 40 min, and hybridization products were visualized using BM Purple (Roche Applied Science) as a substrate. The Foxo1 and Crabp1 (cellular retinoic acid-binding protein-1) probe regions used in the in situ study were as described previously (14, 22).

Immunohistochemistry—Whole mount immunohistochemistry was performed with mAb ME13.3 (rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody, Pharmingen) as described previously (23, 24). Embryos were fixed in 2% PFA and PBS at 4 °C for 1 h and dehydrated in methanol. Embryos were bleached in 5% hydrogen peroxide in methanol for 30 min at 4 °C. This was followed by rehydration and blocking in PBS containing 3% skim milk and 2% Triton X-100 (PBSTM) at 4 °C for 2 min. Embryos were incubated overnight in 10 μg/ml anti-mouse PECAM-1 antibody in PBSTM at 4 °C, washed with PBSTM at 4 °C, and incubated overnight with horseradish peroxidase-conjugated antibody in PBSTM at 4 °C. Embryos were again washed with PBSTM at 4 °C and rinsed in PBST at room temperature for 20 min. For detecting signals, embryos were incubated in 0.3 mg/ml diaminobenzidine (Sigma) in PBSTM containing 0.5% NiCl2 for 20 min; hydrogen peroxide was added to a final concentration of 0.015%; and the embryos were incubated for 20–30 min. The staining reaction was terminated by rinsing the embryos in PBST. Embryos were post-fixed overnight in 4% PFA and PBS at 4 °C. To better observe the vasculature, embryos were dehydrated through graded solutions up to 100% methanol.

For the immunohistochemistry of endothelial cell colonies, 6-well culture plates were fixed in 2% PFA and stained first with purified anti-VE-cadherin mAb (Zymed Laboratories Inc.) and then with horseradish peroxidase-labeled goat anti-rat IgG antibody (BIOSOURCE). VE-cadherin and VE-cadherin mAb (Zymed Laboratories Inc.) and then with horseradish peroxidase-labeled goat anti-rat IgG antibody (BIOSOURCE).

Histology—For histological analysis, embryos were embedded in paraffin after fixation with 4% paraformaldehyde (PFA), 300 μm in J.

Histology—For histological analysis, embryos were embedded in paraffin after fixation with 4% paraformaldehyde (PFA), 300 μm in J. A, whole mount in situ hybridization revealed Foxo1 expression in the first branchial arch, migrating neural crest cells in the second branchial arch (arrows), and somites of an E8.75 embryo. B and C, expression localized to the first and second branchial arches (arrows) and somites at E9.0 and E9.5, respectively. D and E, Foxo1 transcript expression localized in somites (S) and inter-somitic vessels (ISV) at E9.0 and 9.5, respectively. F, the expression of Foxo1 in the yolk sac was below the detection limit of the in situ hybridization technique, but RT-PCR confirmed the transcripts to be present in the yolk sac at E9.5. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. G and H, comparison of the appearance of wild-type and Foxo1-deficient embryos, respectively, showed apparent growth retardation and a small first branchial arch, but no second branchial arch, and often remarkable pericardial swelling (arrow) in E9.5 mutants. I and J, whole mount in situ hybridization with a Crabp1 antisense probe revealed the pathway of migrating crest cells populating branchial arches at E9.5. Specific staining in wild-type embryos can be seen in streams of cells migrating ventrally into the arches (arrows). The staining pattern in the mutant embryos was very similar to that in the wild-type embryos. K and L, shown is the hematoxylin/eosin staining of transverse sections at the level of the aortic arch. E9.5 Foxo1-deficient embryos had dorsal aortas that were severely underdeveloped and irregularly formed in addition to hypoplasia of branchial arches and unclear aortic arch arteries in contrast to wild-type embryos (arrows). The arrow shows the dorsal aorta. Scale bars = 200 μm in A–E, 300 μm in G and H, and 150 μm in J–L.

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Fig. 2. Expression of Foxo1 in embryos and morphological defects in Foxo1-deficient mice. A, whole mount in situ hybridization revealed Foxo1 expression in the first branchial arch, migrating neural crest cells in the second branchial arch (arrows), and somites of an E8.75 embryo. B and C, expression localized to the first and second branchial arches (arrows) and somites at E9.0 and E9.5, respectively. D and E, Foxo1 transcript expression localized in somites (S) and inter-somitic vessels (ISV) at E9.0 and 9.5, respectively. F, the expression of Foxo1 in the yolk sac was below the detection limit of the in situ hybridization technique, but RT-PCR confirmed the transcripts to be present in the yolk sac at E9.5. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. G and H, comparison of the appearance of wild-type and Foxo1-deficient embryos, respectively, showed apparent growth retardation and a small first branchial arch, but no second branchial arch, and often remarkable pericardial swelling (arrow) in E9.5 mutants. I and J, whole mount in situ hybridization with a Crabp1 antisense probe revealed the pathway of migrating crest cells populating branchial arches at E9.5. Specific staining in wild-type embryos can be seen in streams of cells migrating ventrally into the arches (arrows). The staining pattern in the mutant embryos was very similar to that in the wild-type embryos. K and L, shown is the hematoxylin/eosin staining of transverse sections at the level of the aortic arch. E9.5 Foxo1-deficient embryos had dorsal aortas that were severely underdeveloped and irregularly formed in addition to hypoplasia of branchial arches and unclear aortic arch arteries in contrast to wild-type embryos (arrows). The arrow shows the dorsal aorta. Scale bars = 200 μm in A–E, 300 μm in G and H, and 150 μm in J–L.
Table I

| Genotypy of mice resulting from matings of heterozygous FKHR b−/− mice | 9.5 days postcoitus | 10.5 days postcoitus |
|---------------------------------------------------------------|----------------|----------------|
| Genotype                                      | FKHR b+/− | FKHR b+/− | FKHR b−/− | FKHR b+/− | FKHR b+/− | FKHR b−/− |
| Expected | 22 | 44 | 22 | 8 | 16 | 8 |
| Observed | 29 | 39 | 16 | 6 | 16 | 9 |
| Live births | 12 | 17 | 0 | 7 | 14 | 7 |

* Genotyping of three embryos in the 9.5-day postcoitus group was inconclusive.
* Genotype of one embryo was inconclusive.
* Eight of nine FKHR b−/− embryos were abnormal and underwent absorption.

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Results

Expression of Foxo1 in Embryos—Whole mount in situ hybridization at E8.5 revealed remarkable expression of Foxo1 in the neural crest cells migrating toward branchial arches and in the putative somites (Fig. 2A). The expression of Foxo1 was subsequently down-regulated and localized to the first and second branchial arches and putative somites at E9.0 and E9.5 (Fig. 2, B–E). The expression of Foxo1 was also observed in the intersomitic vessel, although it could not be determined whether the expression was localized to the endothelial cells (Fig. 2, D and E). The expression of Foxo1 in the yolk sac was below the detection limit of the in situ hybridization technique. However, RT-PCR confirmed the expression in the yolk sac at E9.5 (Fig. 2F).

Targeted Inactivation of the Foxo1 Gene—The replacement vector disrupts the coding sequence by inserting a lox-PGK-neo-poly(A)-loxP-FOXO1-3A-poly(A) cassette into the locus (Fig. 1, A and B). This deletes a portion of the first intron (including the splice donor) and the first 208 codons of the first coding exon, which encodes the N-terminal half of the forkhead embryonic phenotype (Table I). The first Foxo1−/− embryos were identified at E11.5, having been reabsorbed. Viable embryos were indistinguishable in size; but after E9.5, Foxo1−/− embryos exhibited apparent growth retardation (Fig. 2, G and H). By E9.5, mutant embryos had developed a small first branchial arch, but no second branchial arch, and often exhibited marked pericardial swelling (Fig. 2, H and J). Since cranial neural crest cells significantly contribute to the formation of the branchial arches, we followed the pathway of crest cells using whole mount in situ hybridization with a Crabp1 probe as a specific marker for neural crest cells at E9.5. Specific staining in three well defined streams of representing cells migrating ventrally toward the arches could be seen in both wild-type and mutant embryos (Fig. 2, I and J).

Histological analysis of transverse sections of E9.5 Foxo1−/− embryos revealed that the dorsal aorta was severely underdeveloped and irregularly formed. Hypoplasia of branchial arches and aortic arch arteries was also observed (Fig. 2, K and L). To examine the extent of vascularization of Foxo1−/− embryos, we performed whole mount immunohistochemical staining at E8.5–9.5 using a mAb against the endothelium-specific marker VE-cadherin (including the splice donor) and the first 208 codons of the first coding exon, which encodes the N-terminal half of the forkhead domain of FOXO1. Mice heterozygous for the targeted allele and apparently arrested at the primary plexus stage (F), G–N show abnormal vascular remodeling in the yolk sacs of Foxo1−/− embryos. The wild-type yolk sac appeared to have a vasculature (G), but the Foxo1-deficient yolk sac was pale and had no clear vasculature (H). I–L show whole mount immunohistochemical staining of E8.75 and E9.5 embryos using a mAb against the endothelium-specific marker PECAM-1. A honeycomb-like vascular plexus was evident by E8.75 (I), and subsequent remodeling of the vasculature resulted in defined vessels by E9.5 (K). Foxo1-deficient yolk sacs showed a honeycomb-like vascular plexus similar to that of wild-type yolk sacs at E8.75 (J), but had not developed a normal vasculature at E9.5 (L). Histological analysis by hematoxylin/eosin staining revealed that whereas capillary-like vessels containing blood cells with an endothelial cell lining were present in wild-type yolk sacs (M), no distinct blood vessels were evident in Foxo1-deficient yolk sacs (N). Scale bars = 160 μm in A and B, 200 μm in C–L, and 50 μm in M and N.
Abnormal Vascular Remodeling in the Yolk Sacs of Foxo1 mutant embryos (Fig. 3, E and F).

**Abnormal Vascular Remodeling in the Yolk Sacs of Foxo1 mutant embryos**—The vitelline circulation in the embryonic yolk sac represents the earliest circulatory system and is the first site of vasculogenesis and angiogenesis in the embryo. As shown in Fig. 3 (G and H), the wild-type yolk sacs appeared to have a vasculature, but the Foxo1-deficient yolk sacs were pale and had no clear vasculature. The visceral endoderm and mesoderm forming the yolk sacs were not fused except at discrete foci, giving the yolk sacs of Foxo1 mutants a characteristic appearance (Fig. 3 H), with large cavities present in the mutant yolk sacs that were lined by endothelial cells and that contained blood cells. PECAM-1 labeling revealed that endothelial cells were present in both wild-type and Foxo1-deficient yolk sacs at E8.75 (Fig. 3, I and J). Normally, a honeycomb-like vascular plexus was evident by E8.75 (Fig. 3J), and subsequent remodeling of the vasculature (angiogenesis) resulted in the formation of large vitelline vessels and a fine network of smaller vessels by E9.5 (Fig. 3K). Foxo1-deficient yolk sacs showed a honeycomb-like vascular plexus similar to that of wild-type yolk sacs at E8.75 (Fig. 3J), but failed to develop a normal vasculature at E9.5 (Fig. 3L). Histological analysis revealed that no distinct blood vessels were evident in the Foxo1-deficient yolk sacs (Fig. 3N), whereas endothelial cell-lined capillary-like vessels containing blood cells were seen in the wild-type yolk sacs (Fig. 3M). All together, the above evidence suggests that vasculogenesis (but not angiogenesis) proceeds without functional FOXO1.

To determine whether other molecules contributed to the impaired angiogenesis in the mutants, we examined the expression of several factors and receptors involved in vasculogenesis and angiogenesis. By quantitative RT-PCR analysis, transcripts of VEGF, Flt-1, Flk-1, angiopoietin-1, angiopoietin-2, Tie-1, Tie-2, EphB2, EphB3, EphB4, connexin-37 (Cx37), connexin-40 (Cx40), connexin-43 (Cx43), connexin-45 (Cx45), and smooth muscle α-actin (Sm actin) were examined in the yolk sacs of wild-type (WT) and Foxo1-deficient (knockout (KO)) embryos at E9.5. Data are means ± S.E. of three independent experiments, where the expression level in the yolk sacs of wild-type embryos was regarded as 1.

**Fig. 4. Expression of several factors involved in vasculogenesis and angiogenesis.** By quantitative RT-PCR, the expression levels of transcripts for angiopoietin-1 (ang1), angiopoietin-2 (ang2), VEGF, Flt-1, Flk-1, Tie-1, Tie-2, EphB2, EphB3, EphB4, ephrin-B2, connexin-37 (Cx37), connexin-40 (Cx40), connexin-43 (Cx43), connexin-45 (Cx45), and smooth muscle α-actin (Sm actin) were examined in the yolk sacs of wild-type (WT) and Foxo1-deficient (knockout (KO)) embryos at E9.5. Data are means ± S.E. of three independent experiments, where the expression level in the yolk sacs of wild-type embryos was regarded as 1.

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![Expression of several factors involved in vasculogenesis and angiogenesis.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
Foxo1-deficient yolk sacs were significantly reduced to −10, 1, and 35% of the wild-type levels, respectively (p < 0.01). It is possible that the vascular defects in the Foxo1-deficient yolk sacs are due in part to the reduction of expression of the specific connexins and ephrin among the molecules examined. However, we cannot eliminate the possibility of changes in unidentified gene expression in specific regions of mutant embryos.

Expression of Foxo1 Transcripts in Endothelial Cells Derived from ES Cells—We demonstrated the presence of Foxo1 transcripts in the yolk sac (Fig. 2F), although the localization could not be determined by in situ hybridization. We examined the expression of the Foxo1 gene in the endothelial cell lineage using an in vitro differentiation system composed of ES cells. Foxo1+/+ and Foxo1−/− ES cells were co-cultured with OP9 stromal cells to induce differentiation of endothelial cells. Under the culture conditions used in this study, the frequency of endothelial cells among the differentiating ES cells was almost comparable between cultures initiated from Foxo1+/+ and Foxo1−/− ES cells (data not shown). CD31+ and VE-cadherin+ endothelial cells were purified using a fluorescence-activated cell sorter and subjected to RT-PCR analysis for the expression of several genes. Foxo1 transcripts were detected in the endothelial cells derived from Foxo1+/+ (but not Foxo1−/−) ES cells (Fig. 5A, panel a), suggesting that Foxo1 has some functional role in the endothelial cell lineage. Consistent with the observations in the yolk sac described above, transcripts of Flt-1, Flk-1, Flt-4, Tie-1, and Tie-2 were detected at similar levels in Foxo1+/+ and Foxo1−/− endothelial cells (Fig. 5A, panel b), indicating that the expression of these molecules does not depend upon Foxo1.

Abnormal Behavior of Foxo1−/− Endothelial Cells in Response to VEGF—Endothelial cells derived from Foxo1+/+ and Foxo1−/− ES cells by co-cultivation with OP9 stromal cells were purified using a fluorescence-activated cell sorter and re-cultured on OP9 stromal cell layers to allow colonies to form. The colony formation analysis of endothelial cells derived from ES cells has proved a useful means of revealing the behavior of developing endothelial cells in response to various angiogenic stimuli (19, 25). We took advantage of this culture system to determine whether Foxo1 has any functional role in endothelial cells. Endothelial cells of the two genotypes gave rise to comparable numbers of colonies (Fig. 5B). The addition of 50 ng/ml VEGF-A165 to the cultures did not influence the number of colonies formed. Therefore, the results suggest that a lack of
FOXO1 does not influence the proliferation and survival of endothelial cells.

We next examined the morphology of the endothelial cell colonies. In the absence of exogenous VEGF-A165, there was no morphological difference between colonies generated from Foxo1+/− and Foxo1−/− endothelial cells. Most of the colonies were packed round clusters composed of flat polygonal endothelial cells (Fig. 5C, panels a–f). A small fraction of colonies consisted of a dispersed assemblage of flat endothelial cells (Fig. 5C, panels c and f). When 50 ng/ml VEGF-A165 was added to the cultures, however, Foxo1+/− and Foxo1−/− endothelial cell colonies exhibited quite distinct morphological changes. As reported previously (25), VEGF-A165 induced a morphological change in wild-type endothelial cells from a polygonal to a spindle-like shape (Fig. 5C, panels g-i). The elongated endothelial cells formed either scattered colonies or densely packed bundles. In contrast, ~30% of the Foxo1−/− endothelial cell colonies remained round even in the presence of exogenous VEGF-A165 (Fig. 5C, panels j and k). They consisted of packed flat polygonal endothelial cell clusters surrounded by fibrous endothelial cells. Most of the other colonies were also made up of flat endothelial cells, but exhibited a holly leaf-like shape (Fig. 5C, panel l).

Finally, confocal microscopic analyses of endothelial cell colonies stained with anti-VE-cadherin antibody revealed that endothelial cells exhibited a similar flat polygonal shape regardless of Foxo1 expression in the absence of exogenous VEGF-A165 (Fig. 6, A–C). Stimulation with exogenous VEGF-A165 induced elongation of Foxo1+/− endothelial cells, whereas Foxo1−/− endothelial cells remained flatten (Fig. 6, D and E). Endothelial cells of the latter genotype exhibited rather straight adherens junctions and appeared to overlap each other. Cell overlaps were also observed on elongated (VEGF-A165-stimulated) wild-type endothelial cells, but were rarely found on unstimulated colonies. Despite repeated trials, we could isolate only one ES clone homozygous for the Foxo1-null allele. To exclude the possibility that the abnormal phenotype of Foxo1−/− endothelial cells is attributed to some genetic lesions irrelevant to the Foxo1 gene, we restored expression of Foxo1 in the differentiating Foxo1−/− ES cells by means of an endothelial cell-specific promoter/enhancer cassette. The coding sequence of Foxo1 cDNA was inserted between the 2.5-kilobase pair 5′-flanking (26) and the 4-kilobase pair 3′-flank-

![Fig. 6. Immunolocalization of VE-cadherin on the endothelial cell colonies.](http://www.jbc.org/Downloaded_from)
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(27–29). These studies imply that activation of the phosphatidylinositol 3-kinase/Akt signaling system leads to promoted angiogenesis. Recently, it was reported that VEGF promotes survival and growth of endothelial cells via inhibition of FOXO family members through the phosphatidylinositol 3-kinase/Akt signaling system (30). However, Foxo1-deficient endothelial cells exhibited normal colony formation in response to a low dose of VEGF and an abnormal morphological response to a high dose of VEGF. Taken together, these findings imply that FOXO family members other than Foxo1 are involved in the responses to a low dose of VEGF such as growth and survival of Foxo1-deficient endothelial cells.

In contrast, the Foxo1-deficient endothelial cells showed markedly different responses compared with the wild-type endothelial cells in the presence of exogenous VEGF (50 ng/ml). It has been reported that the formation of blood vessels is impaired not only in homozygous VEGF-deficient embryos, but also in heterozygous VEGF-deficient embryos (31, 32). Therefore, the function of VEGF is clearly dependent upon its expression levels. The abnormal phenotype for blood vessel formation in Foxo1-deficient mice is similar to that in heterozygous VEGF-deficient mice in terms of the presence of endothelial cells, enlarged yolk sac vessels, and hypoplasia of the dorsal aorta (31, 32). Taken together, these results suggest that a dose of VEGF above a certain threshold and the ability of endothelial cells to properly respond to it are essential for normal vascular development and that products transcriptionally regulated by FOXO1 are required for the latter. Although it is not well understood how the behavior of individual endothelial cells accounts for the processes of vascular development, undoubtedly the morphological response of endothelial cells serves as a driving force for vascular remodeling in vivo. Thus, it is more likely that the impaired angiogenesis observed in the mutants was due to the abnormal response of endothelial cells to VEGF and/or other angiogenic factors. However, we cannot formally exclude the possibility that the defects in branchial arches secondarily influenced vascular remodeling. It is necessary to elucidate whether endothelium-specific inactivation of Foxo1 would lead to the same phenotype.

In this study, we found that the transcripts of connexin-37 and connexin-40 were remarkably decreased in Foxo1-deficient yolk sacs. It is known that these connexins are rich in endothelial cells and that double connexin-37/connexin-40-deficient mice have vascular abnormalities such as blood vessel dilation and congestion (33–35). Moreover, the transcript of ephrin-B2 was also reduced to 35% of the wild-type yolk sac. Ephrin-B2-null mice die in utero before E11.5 because of defects in the remodeling of the embryonic vascular system and display defects in angiogenesis by both arteries and veins in the capillary networks of heads and yolk sacs (35–37). The phenotypes are very similar to that of Foxo1-deficient mice. Since Foxo1-deficient mice have at least 35% of the ephrin-B2 transcripts of the wild-type mice and since heterozygous ephrin-B2-deficient mice show a normal phenotype (35–37), the ephrin-B2 reduction alone can hardly cause the vascular defects in Foxo1-deficient mice. A synergistic effect of some factors, including connexin-37, connexin-40, and ephrin-B2, may induce the abnormal phenotype. It is not clear whether the connexins and ephrin-B2 are direct target genes of Foxo1 or whether the impairment of endothelial cells by Foxo1 deficiency secondarily induces the reduced expression of the genes. Mukoyama et al. (38) showed similar losses of connexin-40 and ephrin-B2 in blood vessels of limb skin of mice lacking neuron-1 and neuronin-2. They attributed the loss of arterial markers to loss of VEGF produced by peripheral nerves and showed that VEGF could induce connexin-40 and ephrin-B2 in endothelial cells in vitro (38). The Foxo1-deficient embryos showed the same loss of arterial markers and of connexin-37, a third known arterial marker. The data in Figs. 4 and 5 show that VEGF receptors were retained, but Fig. 6 shows the endothelial cells to be resistant to VEGF. Because restoring Foxo1 restores sensitivity to VEGF, FOXO1 seems to be required for responsiveness to VEGF. Thus, the ability of the primary vascular plexus to respond to an angiogenic signal mediated by VEGF may be blocked in Foxo1-deficient embryos.

Finally, defects in vascular remodeling and pericardial fluid accumulation similar to those described here have also been reported in embryos that bear mutations in components of the transforming growth factor-β signaling pathway, including the type I receptor (ALK5), ALK1, endothin, Smad5, and so on (39–42). Remarkably, genetic analyses in C. elegans imply that the transforming growth factor-β signaling pathway and the insulin/DAF-2 signaling pathway converge on a common target, daf-9, which encodes a cytochrome P-450 related to vertebrate steroidogenic hydroxylases (43–45). Thus, it is possible that a component of the transforming growth factor-β signaling pathway directly or indirectly interacts with a component of the insulin/insulin-like growth factor-1 signaling pathway during angiogenesis in mammals. It remains to be examined whether or not the target genes controlled by the transforming growth factor-β signaling pathway are properly expressed in the Foxo1-deficient mice.

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