FOXO1-Mediated Activation of Akt Plays a Critical Role in Vascular Homeostasis


Rationale: Forkhead box-O transcription factors (FOXOs) transduce a wide range of extracellular signals, resulting in changes in cell survival, cell cycle progression, and several cell type-specific responses. FOXO1 is expressed in many cell types, including endothelial cells (ECs). Previous studies have shown that Foxo1 knockout in mice results in embryonic lethality at E11 because of impaired vascular development. In contrast, somatic deletion of Foxo1 is associated with hyperproliferation of ECs. Thus, the precise role of FOXO1 in the endothelium remains enigmatic.

Objective: To determine the effect of endothelial-specific knockout and overexpression of FOXO1 on vascular homeostasis.

Methods and Results: We show that EC-specific disruption of Foxo1 in mice phenocopies the full knockout. Although endothelial expression of FOXO1 rescued otherwise Foxo1-null animals, overexpression of constitutively active Foxo1 resulted in increased EC size, occlusion of capillaries, elevated peripheral resistance, heart failure, and death. Knockdown of FOXO1 in ECs resulted in marked inhibition of basal and vascular endothelial growth factor–induced Akt-mammalian target of rapamycin complex 1 (mTORC1) signaling.

Conclusions: Our findings suggest that in mice, endothelial expression of FOXO1 is both necessary and sufficient for embryonic development. Moreover, FOXO1-mediated feedback activation of Akt maintains growth factor responsive Akt/mTORC1 activity within a homeostatic range. (Circ Res. 2014;115:238-251.)

Key Words: angiogenesis ■ endothelial cells ■ FOXO1 protein, human ■ mice, transgenic ■ transcription factors

The FOXO family of transcription factors belongs to the winged helix or forhead box class of transcription factors. Invertebrates possess 1 FOXO gene, termed daf-16 in the worm and dFoxO in the fly. Mice and humans possess 4 FOXO members: FOXO1, FOXO3, FOXO4, and FOXO6. FOXO1, FOXO3, and FOXO4 are highly related homologs with overlapping patterns of expression and transcriptional activities. The FOXO protein family is regulated primarily by post-translational modifications, including phosphorylation, acetylation, monoubiquitination, and polyubiquitination. These various modifications control subcellular localization and protein levels, as well as efficacy of DNA binding and transcriptional activity. Most notably, FOXO1, FOXO3, and FOXO4 have 3 conserved amino acids that are targets for phosphorylation by Akt or serum/glucocorticoid-regulated kinase (SGK). Phosphorylation at these sites leads to nuclear exclusion of the transcription factor. FOXOs have been shown to play a role in many physiological processes, including the control of cell proliferation and survival, cell cycle progression, DNA repair, oxidative stress resistance, energy metabolism, and cell differentiation. Unrestrained FOXO activity can result in cellular senescence, autophagy, and atrophy and can promote a catabolic state.

Both Foxo3- and Foxo4-null mice are viable. In contrast, mice that are null for Foxo1 are embryonic lethal at E11 because of impaired vasculoogenesis. Thus, FOXO1 has a specific role in vascular development, which cannot be compensated for by other FOXO family members. FOXO1 is expressed in multiple cell types and tissues during development, including endothelial cells, smooth muscle cells, neural crest cells, cardiomyocytes, adipose tissue, somites, branchial arches, and trigeminal ganglia.

In contrast to the embryonic knockout, widespread somatic deletion of Foxo1 in adult tissues predisposes to the development of vascular bed–specific hemangiomas, an effect that is accentuated by combined deletion of Foxo3 and Foxo4.

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Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>Ang2</td>
<td>angiopoietin 2</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>ESM1</td>
<td>endothelial-specific molecule 1</td>
</tr>
<tr>
<td>Hprt</td>
<td>hypoxanthine guanine phosphoribosyl transferase</td>
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<tr>
<td>HVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>mTORC1</td>
<td>Akt-mammalian target of rapamycin complex 1</td>
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<tr>
<td>PCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>TM</td>
<td>triple mutant</td>
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<tr>
<td>tTA</td>
<td>tetracycline transactivator</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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One previous study showed that mice with endothelial cell–specific knockout of Foxo1 are born at term in normal Mendelian ratios and show no gross or metabolic abnormalities. However, another study reported that endothelial-specific deletion of Foxo1 is embryonic lethal. Conditional deletion of all 3 FOXO factors in the endothelium is compatible with survival and protects against atherosclerosis in low-density lipoprotein receptor knockout mice. Mice with lineage-specific knockouts of Foxo1 in other tissues, including bone, liver, heart, T cells, and teeth enamel, are viable but have wide-ranging phenotypes, implying an important role for this transcription factor in homeostasis.

The effect of FOXO activity on gene expression and cell function is highly cell-type-specific. For example, combined deletion of Foxo1, Foxo3, and Foxo4 in postnatal mice resulted in altered expression of 608 genes in liver endothelial cells and 610 genes in thymocytes, only 11 of which overlapped. Moreover, the expression profile differed between Foxo1/03/O4-deficient liver and lung endothelial cells, as did the proliferative capacity of the cells in response to vascular endothelial growth factor (VEGF).

In the current study, we have generated several genetic mouse models to address the role of endothelial FOXO1 in homeostasis more definitively. Using a combination of global knockout, endothelial cell–specific knockout, endothelial rescue, and endothelial overexpression mouse models, we provide evidence that endothelial FOXO1 is both necessary and sufficient for viability, and that a balanced level of FOXO1 activity is required for survival. Moreover, we show that a primary role of FOXO1 in the endothelium is to feed back and activate Akt-mTORC1, thus sensitizing cells to the effect of VEGF.

**Methods**

A detailed description of the Methods used in this study is provided in the Online Data Supplement.

**Generation of Gene-Targeted and Transgenic Mice**

The generation of Foxo1−/−, Foxo1−/+−, tetracycline (Tet)-triple mutant (TM)-FOXO1, Tet-LacZ, and Foxo1-rescue (Foxo1-res) mice is detailed in the Online Data Supplement. VE-cadherin (VEC)-tetracycline transactivator (tTA) mice were a generous gift from Dr Laura Benjamin. \(\text{Tie2-Cre} (B6.Cg-Tg(Tek-cre)1Ywa/J)\) and VEC-Cre (B6;129-Tg(Cdh5-cre)1Spe/J) were obtained from Jackson Laboratory. All animal studies were approved by the Animal Care and Use Committee at the Beth Israel Deaconess Medical Center.

**Cell Culture**

Endothelial and nonendothelial cells used in this study are described in the Online Data Supplement. All experiments with primary cell lines were performed between passage 3 and 6. Mouse endothelial cells were harvested and grown as previously described. Cells were either taken off or maintained on 10 mg/mL of tetracycline. For VEGF treatment, cells were serum-starved in 0.5% FBS for 16 hours and treated with human VEGF-165 (50 ng/mL; Peprotech) for the times indicated.

**Adenoviruses**

Human umbilical vein endothelial cells (HVEC) were infected with adenovirus (Ad)-cytomegalovirus (CMV)-β-galactosidase (Ad-bgal), Ad-wild-type (WT)-FOXO1, Ad-TM-FOXO1, or Ad-CMV-Akt1 (Vector Bioslabs) as previously described.

**Transfection of Endothelial Cells With siRNA and shRNA**

HVEC were plated at a density 5x10⁴ cells per 6-cm plate and transfected with 1 of 6 siRNAs against FOXO1 (FOXO1-1 through FOXO1-6) or 3 siRNAs against FOXO3 (FOXO3-1, FOXO3-2, and FOXO3-3) in Opti-MEM (Invitrogen) with lipofectin. siRNA sequences are shown in the Online Data Supplement (Online Table I). FOXO1-2, FOXO1-3, FOXO3-2, and FOXO3-3 are identical to those used by Potente et al. For shRNA experiments, HVEC were incubated with lentiviral shRNA against FOXO1 or control shRNA at 20 MOI for 6 hours in the presence of 8 μg/mL polybrene. Forty-eight hours after infection, cells were selected with puromycin (10 μg/mL) for 1 week before they were expanded for experiments.

**Quantitative Real-Time Polymerase Chain Reaction Assays**

Quantitative polymerase chain reaction (qPCR) assays were performed as previously described. Primer sequences are available on request.

**Cardiac Morphometric and Pressure–Volume Loop Studies**

Heart tissues were harvested and fixed overnight at 4°C in 4% paraformaldehyde and sterile PBS. Fixed hearts were paraffin-embedded and sectioned on the sagittal plane at 6-μm thickness. Paraffin-embedded hearts were examined using a Wild Heerbrugg M400 Photomakroskop (Wild Heerbrugg Ltd). Ventricular volumes were quantified with ImageJ software (National Institutes of Health). Pressure–volume loop analysis was performed as previously reported.

**Westerns Blots**

Western blots were performed as previously described. Blots were analyzed using antibodies detailed in the Online Data Supplement. The bands were visualized with a chemiluminescence detection kit (ECI; Amersham Biosciences) and were quantified with ImageJ software (National Institutes of Health).

**Blood Chemistry**

Blood was collected via cardiac puncture. Serum creatinine levels in VEC-tTA; Tet-FOXO1 mice were measured using the i-STAT Chem8+ cartridge and analyzed using i-STAT analyzer (Abbott Point of Care, Inc). Urine samples were collected directly from the urinary bladder. Urine albumin levels were assayed using an ELISA kit from Exocell. Rescue mice blood samples were assayed by the Division of the Comparative Medicine, Massachusetts Institute of Technology.

**Transmission Electron Microscopy**

Transmission electron microscopy was performed as previously described.

**LacZ Staining**

LacZ staining was performed as previously described.

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Aortic Ring Assay
Detailed procedures for this assay are provided in the Online Data Supplement.

Analysis of Cell Cycle, Thymidine Uptake, Modified Boyden Chamber, and Scratch Wound Assays
Detailed procedures for these assays are provided in the Online Data Supplement.

Statistical Analysis
Data were expressed as mean±SD of 3 or more independent experiments. The mean statistical difference was determined using a t test with P<0.05 as statistically significant. Data analysis and generation of all graphs were performed in PRISM5 software (GraphPad).

Results
Endothelial-Specific Deletion of Foxo1 Phenocopies the Full Foxo1 Knockout
We wished to determine the extent to which endothelium is responsible for mediating the vascular phenotype in Foxo1−/− mice. To that end, we generated both Foxo1-null mice (Foxo1−/−) and mice in which Foxo1 was conditionally deleted in the endothelium (Foxo1EC−/−; Figure 1A and 1B). FOXO1

Figure 1. Schematics of targeting constructs. A and B, Left, Targeting construct to generate mice with global knockout of Forkhead box O1 transcription factor (Foxo1) (Foxo1−/−; A) and mice with endothelial-specific knockout of Foxo1 (Foxo1EC−/−; B). Bold lines indicate the homology arms. Right, Recombinant embryonic stem (ES) cell clones were identified by Southern blot analysis. C, Transgenic construct and breeding scheme to generate Foxo1-rescue (Foxo1-res) mice. D, Left, Targeting construct to generate tetracycline (Tet)-triple mutant (TM)-Foxo1 Hprt-targeted mice. Right, Breeding scheme to generate inducible endothelial-specific overexpression of constitutively active FOXO1 (VEC-tetracycline transactivator (tTA); Tet-TM-FOXO1). Amp indicates ampicillin resistance gene; E, exon; TM-FOXO1, human FOXO1 cDNA encoding FOXO1 with triple mutation (T24A, S256A, and S319A); LoxP, LoxP site; Neo, neomycin resistance cassette; tetO, tetracycline operator; Tet-H2O, drinking water containing tetracycline; Tie2-P/E, Tie2 promoter/enhancer; tTA, tetracycline transactivator; and WT, wild-type.
protein was not detected in Western blot analyses of E10.5 Foxo1−/− embryos (Figure 6E). Consistent with what has been previously reported, targeted disruption of the Foxo1 gene in mice resulted in embryonic lethality around E11 (Online Table II). Foxo1+/+ and Foxo1−/− embryos were indistinguishable until E9.5 at which time mutant embryos were smaller, lacked a second branchial arch, and often exhibited marked pericardial swelling (Figure 2A–2C). In whole-mount E9.5 to E10.5 embryos stained for CD31, the dorsal aorta appeared thin, underdeveloped, and irregularly formed in Foxo1−/− embryos (Online Figure 1A). This was confirmed in Hematoxylin and eosin–stained tissue sections (Online Figure II). Intersomitic vessels were similarly poorly developed in Foxo1−/− embryos (Figure 2D; Online Figure 1A). The heart was smaller when compared with that of WT controls (Online Figure IIB). The head vasculature of Foxo1−/− embryos was arrested in the primary plexus stage (Online Figure IB and IC). Yolk sacs were pale and had poorly formed vasculature (Figure 2A and 2E).

Foxo1−/− mice were generated by crossing Foxo1−/− mice with the Tie2-Cre targeted mice (Foxo1fl/fl) with the Full Knockout. Cre-mediated excision of Foxo1 in the endothelium resulted in embryonic lethality around E11 (previously reported, targeted disruption of the gene in Foxo1−/− and Foxo1−/− embryos resulted in embryonic lethality around E11). Cre-mediated excision of Foxo1 in the endothelium (Online Figure IA). This was confirmed in Hematoxylin and eosin–stained tissue sections (Online Figure IA). The heart was smaller when compared with that of WT controls (Online Figure IIB). The head vasculature of Foxo1−/− embryos was arrested in the primary plexus stage (Online Figure IB and IC). Yolk sacs were pale and had poorly formed vasculature (Figure 2A and 2E).

Endothelial Expression of FOXO1 Partially Rescues the Full Foxo1 Knockout

Our next goal was to establish whether endothelial expression of FOXO1 could rescue the Foxo1−/− phenotype. To that end, we generated transgenic mice with a DNA cassette containing the Tie2 promoter/enhancer coupled to murine

Figure 2. Global Forkhead box-O transcription factor (Foxo1) knockout and endothelial-specific deletion of Foxo1 result in similar phenotypes with embryonic lethality and impaired vascular development. Representative whole-mount images of Foxo1−/−, Foxo1−/−, and wild-type (Foxo1+/+ and Foxo1−/−, respectively) embryos collected at E10 to E10.5. A and F. When compared with wild-type yolk sacs, Foxo1−/− and Foxo1−/− yolk sacs are pale, wrinkled, and have no distinct vessels (arrow indicates blood vessel in wild-type yolk sac). B and G. Foxo1−/− and Foxo1−/− embryos display growth retardation and pericardial swelling (PE). C, Foxo1−/− embryos develop a smaller than normal first branchial arch (BA1) but not the second branchial arch (BA2). D and I, Whole-mount CD31 immunostaining of E10.5 Foxo1−/− and Foxo1−/− embryos show poorly developed intersomitic vessels. E and J, Whole-mount CD31 immunostaining of E10.5 Foxo1−/− and Foxo1−/− yolk sacs lack a distinct vasculature relative to wild-type yolk sacs. Scale bar, 200 μm.
Foxo1 cDNA (Figure 1C). The Tie2 promoter/enhancer that we used has been shown to direct integration-independent endothelial-specific expression throughout the vasculature of transgenic mice.30 Two independent Tie2-Foxo1 lines were crossed with Foxo1−/− mice. Foxo1−/− mice hemizygous for the transgene were then crossed with Foxo1+/− mice. In both cases, offspring were obtained that expressed the Foxo1 transgene on a Foxo1-null background. Although the number of rescue mice (Foxo1-res) born was less than the expected Mendelian ratio (Online Table II), those that did survive were grossly indistinguishable from WT littersmates (Figure 3A). There was no difference in body weight or weight of individual organs (with the exception of fat) between the rescue mice and WT littersmates (Figure 3B). qPCR analysis of various mouse organs demonstrated near-normal mRNA levels of Foxo1 and endothelial-restricted Foxo1 target genes, including endothelial nitric oxide synthase (eNOS; Nos3), angiopoietin 2 (Ang2), and endothelial-specific molecule 1 (Esm1) (Figure 3C). Similarly, qPCR assays of primary mouse lung endothelial cells revealed detectable mRNA levels of Foxo1 and selected target genes (Figure 3D). Tunel staining of various tissues did not reveal any difference in the number of apoptotic cells between rescue mice and WT littersmates (Figure 3E shows liver, heart, and lung). Moreover, vascular density was similar in rescue mice and WT controls, as assayed by CD31 staining (Figure 3F shows heart, kidney, and liver). It was previously reported that liver-specific knockout of Foxo1 results in fasting hypoglycemia.19,31 Consistent with these data, blood glucose levels were significantly reduced in overnight-fasted Foxo1-res mice when compared with WT littersmates (Online Table III). Also consistent with the phenotype of liver-specific knockout mice, fasting Foxo1-res mice demonstrated reduced mRNA expression of G6pc (a rate-limiting enzyme for gluconeogenesis) and insulin receptor substrate 2 (Irs2) in the liver (Online Figure IIIA). However, hepatic expression of phosphoenoiphosphorylase carboxykinase 1 (Pck1), another gluconeogenic enzyme whose expression was reduced in liver-specific knockout mice, was unaffected in the Foxo1-res mice (Online Figure IIIB). In a previous study, the conditional knockout of Foxo1 in ameloblasts of the teeth was shown to result in a chalky, white tooth phenotype consistent with enamel hypomaturation and attrition.21 Foxo1-res mice also demonstrated abnormally white, chalky incisors when compared with WT littersmates (Online Figure IIIB). Taken together, the data suggest that endothelial FOXO1 can rescue embryonic lethality in otherwise Foxo1-null mice.

siRNA-Mediated Knockdown of FOXO1 in Endothelial Cells Results in Altered Expression of Many Genes Involved in Vascular Health

Next, we wished to determine whether FOXO1 deficiency results in altered expression of established FOXO1 target genes and other genes that are known to be important for vascular development. To that end, we transfected primary human endothelial cells with si-CTR or si-FOXO1 and assayed the cells for mRNA expression using qPCR. As expected, the expression of ESM1, ANG2, IRS2, BMP2, SOD2, and CITED2 (established FOXO1 targets in endothelial cells) was downregulated (Figure 4F; Online Figures V and VI). Similar results were observed in HUVEC grown to different degrees of confluence (Online Figure VII). Importantly, FOXO1 knockdown also affected the expression of genes implicated in angiogenesis and vessel maturation, including VE-cadherin (CDH5), ephrin B2 (EFNB2), and ALK1 (Figure 4F; Online Figures V and VI). Of particular note were changes in the expression of genes involved in the Notch signaling pathway. For example, FOXO1-deficient endothelial cells demonstrated elevated expression of DLL4 and the downstream Notch target genes, NRARP (NOTCH-regulated ankyrin repeat protein), HES1 and HES2. Neuruplin 1 (NRP1), which is repressed by NOTCH1, was downregulated. Expression of FOXC1, which has been previously shown to induce expression of DLL4, was increased. These data are consistent with overactive Notch signaling (Figure 4F; Online Figures V and VI). si-FOXO1 increased VEGF mRNA expression, but absolute levels remained low (<2 copies mRNA per cell). Finally, consistent with cell cycle arrest at the G1/S boundary, siRNA against FOXO1 resulted in increased expression of p27Kip1, p21WAF1/CIP1, decreased expression of MYC and CDK4; decreased expression of cyclins specific to the S and G2 phase of cell cycle, including CyclinE1 (CCNE1), CyclinB1 (CCNB1), CyclinB2 (CCNB2); and decreased expression of proliferating cell nuclear antigen (PCNA; Figure 4G; Online Figures V and VI). In contrast to a previous study showing that FOXO1 inhibits eNOS expression,24 si-FOXO1 did not result in increased eNOS mRNA levels. However, as discussed in the Online Data Supplement (Online Figure VIII), eNOS expression was increased in HUVEC with prolonged...
lentivirus shRNA-mediated knockdown of FOXO1, as well as in E9.5 Foxo1<sup>−/−</sup> mice. Together, these data suggest that FOXO1 deficiency in endothelial cells leads to altered expression of many genes implicated in vascular development and cell cycle control.

**Endothelial Expression of Constitutively Active FOXO1 Leads to Endothelial Overgrowth, Vascular Occlusion, Cardiac Failure, and Death**

Previous studies have shown that somatic deletion of Foxo1 (particularly when associated with the loss of an Foxo3/04)
Figure 4. Forkhead box-O transcription factor (FOXO1) knockdown leads to G1 growth arrest in cultured endothelial cells. Human umbilical vein endothelial cells (HUVEC) were transfected with siRNA against FOXO1 (FOXO1-1; si-FOXO1) or control siRNA (si-CTR). A, FOXO1 mRNA expression was evaluated by quantitative polymerase chain reaction (qPCR) and normalized to 18S (n=3). B, Cell cycle was assessed by propidium iodide staining followed by fluorescence-activated cell sorting (FACS) analysis (n=3). Migration was assessed by modified Boyden chamber (n=3) and scratch wound assay (D) in HUVEC treated in the absence or presence of vascular endothelial growth factor (VEGF; n=3). E, Proliferation was assayed by thymidine uptake in si-CTR or si-FOXO1–transfected cells in the presence or absence of VEGF (n=3). F, Regulatory map of genes involved in sprouting angiogenesis based on qPCR data (Online Figure VI). siRNA against FOXO1 alters mRNA expression of angiogenic patterning genes characteristic of tip or stalk cell phenotypes. Most tip cell markers are significantly decreased (ESM1, ANG2, PDGFB, Apelin, and NRARP), whereas expression of Notch target genes is increased (HES1, HES2, DLL4, and NRP1). Expression of FOXC1 (driver of tip-cell–specific DLL4 induction and subsequent activation of Notch) and ALK1 (also known to promote Notch target gene expression) are also increased. (Red/blue/white background: significant increase/significant decrease/no significant expression change). G, Regulatory map of genes involved in cell cycle based on qPCR data (Online Figure VI). siRNA against FOXO1 blocks HUVEC proliferation. C and D: Relative units. A–E, Data are presented as mean±SD. ns indicates nonsignificant; *P<0.05, **P<0.01, ***P<0.001.
serve as a surrogate marker for TM-FOXO1 expression. Analysis of VEC-tTA; Tet-LacZ mice revealed minimal leakage of expression in mice on tetracycline and endothelium-restricted inducible expression off tetracycline (Online Figure IX). VEC-tTA; Tet-TM-FOXO1 mice that were maintained on tetracycline demonstrated low-level expression of human FOXO1 in various organs (<5 copies per 1×10^6 18S copies; Online Figure XA). Seven days after withdrawal of tetracycline from the drinking water, FOXO1 mRNA levels were significantly induced. TM-FOXO1 expression also resulted in increased expression of established endothelial cell–restricted FOXO1 target genes in several organs, including Esm1, Ang2, p21cip1 (but not p27kip1), cyclin G2 (Ccng2), and Bcl6b (Online Figure XA shows Esm1 and Ang2). Despite the putative role of FOXO1 as a repressor of eNOS, inducible expression of TM-FOXO1 in the endothelium did not alter eNOS mRNA or protein levels in any tissue examined (Online Figure XA shows qPCR data). Nor did TM-FOXO1 expression affect the expression of the cell adhesion molecules, vascular cell adhesion molecule 1 (Vcam1) and intercellular molecule 1 (Icam1) (data not shown). Endothelial cells were isolated from the lungs and hearts of VEC-tTA; Tet-TM-FOXO1 mice and grown in the absence or presence of tetracycline. On removal of tetracycline from the culture medium, there was significant induction of FOXO1 and FOXO1 target genes, including Esm1, Ang2, p21cip1, cyclin G2 (Ccng2), Gadd45a, and Sod2 (Figure 5A shows Esm1 and Ang2). Induction of TM-FOXO1 had no effect on eNOS mRNA levels in heart ECs and

Figure 5. Overexpression of triple mutant (TM)-Forkhead box-O transcription factors (FOXO1) leads to cardiac dysfunction. VEC-tetracycline transactivator (tTA); Tet-TM-FOXO1 mice were maintained on tetracycline from birth. At 6 to 8 weeks, tetracycline was removed from the drinking water (Tet-Off) or was continued (control, Tet-On) for 7 days. A, qPCR analysis of FOXO1 and FOXO1 target genes in endothelial cells isolated from the lung (Lu-endo) or heart (hr-endo; normalized to 18S; n=3). B, Representative photomicrographs of longitudinal sections of heart. C, Morphometric analysis of right ventricular (RV) and left ventricular (LV) volume and thickness (n=3). D, Pressure–volume loop analysis (n=5–7). E, Electron microscopy (EM) of lung capillaries. EM of the Tet-On capillary shows a thin attenuated endothelium with well-defined lateral borders and many caveolae. The lumen (L) is filled with a red blood cell. EM of the Tet-Off capillary shows endothelial cells with abundant caveolae- and ribosome-rich cytoplasm, which is impinging on a narrow L. A large immature nucleus is seen in the Tet-Off endothelial cell. F, Aortic ring assay. Shown are the sprouting distance and sprouting area over indicated time points after the addition (On) or removal (Off) of tetracycline to the culture medium to cells that were harvested from Tet-Off or Tet-On mice (n=4). A, C, and D, Data are presented as mean±SD. *P<0.05, **P<0.01, ***P<0.001. Scale bar, 200 μm (F). Alv indicates alveolar air space; IV, interventricular; and ns, nonsignificant.
actually increased eNOS expression in lung ECs (Figure 5A).

There was no change in Vcam1 or Icam1 expression (data not shown).

Inducible expression of TM-FOXO1 in the endothelium resulted in lethality after 7 days. The dry weight of the lung, but not other organs, was increased (not shown). Morphometric analyses of the heart revealed left ventricular enlargement (Figure 5B and 5C). Pressure–volume loop experiments demonstrated increased peripheral vascular resistance, lower mean arterial pressure, and decreased cardiac output (Figure 5D). There was no evidence of endothelial cell hyperproliferation or increased apoptosis, as measured by Tunel staining and bromodeoxyuridine (BrdU) incorporation/CD31 staining, respectively (Online Figure XB and XC). A previous study implicated a role of endothelial FOXO proteins in monocyte recruitment. However, CD45 staining of various mouse tissues did not reveal any difference in the number of CD45+ cells between Tet-On and Tet-Off mice (Online Figure XIA shows heart, kidney, and liver). Moreover, Hematoxylin and eosin stains did not demonstrate any evidence of inflammation (Online Figure XIB).

Electron microscopy revealed enlarged endothelial cells with large nuclei, abundant rough endoplasmic reticulum, and occasional multilaminar basement membrane in capillaries, resulting in a narrowed capillary lumen with trapped red blood cells (Figure 5E; Online Figure XII). There was focal loss in the fenestrae of the renal glomerular endothelium. The latter finding was associated with albuminuria and increased blood creatinine (Online Figure XD). Electron microscopy of organs from an unrelated Hprt-targeted mouse line VEC-tTA; Tet-placental growth factor (PIGF) mice, in which PIGF is inducibly expressed in the endothelium on withdrawal of tetracycline, did not reveal a similar phenotype, arguing against nonspecific effects of transgenic expression in the Hprt locus (data not shown).

To determine whether TM-FOXO1 expression in endothelial cells impaired sprouting angiogenesis, we performed aortic ring assays using samples from VEC-tTA; Tet-TM-FOXO1 mice. As demonstrated in Figure 5F, the inducible expression of TM-FOXO1 had no effect on sprouting distance or area. Together with the BrdU incorporation/CD31 staining of adult tissues, these findings argue against a significant antiproliferative role of FOXO1 in the endothelium.

Overexpression of Constitutively Active FOXO1 in Endothelial Cells Results in Increased Cell Size and Activation of Akt-mTORC1

The electron microscopy results suggested that the endothelial cells of mice overexpressing constitutively active FOXO1 in the endothelium were enlarged (thus compromising the lumen of small vessels). In some, but not all experiments, endothelial cells isolated from the heart and lung of VEC-tTA; Tet-TM-FOXO1 mice demonstrated increased cell volume. This lack of reproducibility may reflect a loss of larger cells during the isolation process. However, infection of HUVEC with adenovirus expressing TM-FOXO1 (Ad-TM-FOXO1) resulted in a significant increase in cell volume and size (Figure 6A). Cell volume is controlled primarily by the Akt-mTORC1 signaling pathway. Previous studies in nonendothelial cells have shown that FOXO1 may feed back to activate Akt, while inhibiting mTORC1, thus uncoupling Akt and mTORC1 signaling. In Western blot analyses of HUVEC, phospho (p)-Akt was elevated in cells infected with adenovirus expressing WT FOXO1 (in which the phosphorylation sites are intact) when compared with Ad-bgal–infected controls and was further increased in cells expressing the phosphorylation-resistant TM-FOXO1 (Figure 6B). The phosphorylation status of S6 and S6K is commonly used to evaluate mTORC1 activity. In Western blots, Ad-WT-FOXO1– and Ad-TM-FOXO1–infected HUVEC demonstrated progressively increased levels of p-S6 and p-S6K (Figure 6B). Interestingly, total levels of S6 were increased in TM-FOXO1–expressing cells, whereas total levels of S6K were decreased. To determine whether the effect of TM-FOXO1 (ie, activation of Akt and mTORC1) was specific to HUVEC, we repeated these experiments using other types of endothelial cells, as well as nonendothelial cells. As shown in Online Figure XIII, TM-FOXO1 induced both p-Akt and p-S6K in human coronary artery endothelial cells, human dermal microvascular endothelial cells, and human coronary artery vascular smooth muscle cells. In contrast, TM-FOXO1 inhibited p-S6K in HEK cells. Taken together, these results suggest that FOXO1 feeds back to activate Akt. However, in contrast to what has been reported in other cell types (and what we observed in HEK cells), Akt is free to activate the mTORC1 pathway. Thus, overexpression of constitutively active, nuclear FOXO1 may result in Akt-mTORC1–mediated cell growth.

Akt-mTORC1 Pathway Is Attenuated in FOXO1-Deficient Endothelial Cells

On the basis of the results of the TM-FOXO1–overexpressing cells, we hypothesized that FOXO1 deficiency may be associated with blunted Akt signaling in endothelial cells. Indeed, in Western blot analyses of HUVEC, siRNA against FOXO1 resulted in a significant reduction in basal and VEGF-inducible levels of p-Akt, p-S6, and p-S6K (Figure 6C; Online Figure XIV–XIVC). By contrast, siRNA-mediated knockdown of FOXO3 had no effect on Akt levels (Online Figure XIVD). Similar results with si-FOXO1 were observed with human coronary artery endothelial cells and human dermal microvascular endothelial cells (Online Figure XVE and XVF). FOXO1 knockdown in HUVEC did not affect VEGF-mediated phosphorylation of VEGF receptor 2 (Online Figure XVG), suggesting that FOXO1 exerts its effect on Akt signaling distal to the VEGF receptor. To determine whether overexpression of Akt could rescue the phenotype of FOXO1-deficient cells, we infected si-CTR– and si-FOXO1–transfected cells with constitutively active Akt. In these experiments, siRNA against FOXO1 resulted in a marked diminution in thymidine uptake in the absence but not in the presence of CA-Akt (Figure 6D). We harvested E10.5 Foxo1−/− embryos for protein and performed Western blot analyses for Akt and mTORC1 activation. As shown in Figure 6E and Online Figure XVA, homozygous knockout embryos demonstrated reduced p-Akt and p-S6, despite an increased total level of S6. Western blots of Foxo1−/− embryos demonstrated a trend toward reduced p-Akt (some blots, such as the one shown in Figure 6F demonstrated marked reduction) and significantly reduced levels
of p-S6 (Figure 6F; Online Figure XVB). Together, these data suggest that FOXO1 deficiency is associated with a loss of feedback activation of Akt-mTORC1.

Previous studies have implicated several FOXO1-responsive genes in mediating feedback activation of Akt, including Sestrin 3 (Sesn3), Rictor, and the pseudokinase, TRB3. In qPCR assays, siRNA-mediated knockdown of FOXO1 resulted in downregulation of SESN3 (Figure 6G). Expression of TM-FOXO1 in endothelial cells resulted in increased expression of RICTOR and reduced expression of TRB3 but no change in SESN3 mRNA levels (Figure 6G). Finally, there was an increase in both Rictor and Sesn3 mRNA levels in endothelial
cells isolated from the hearts of VEC-tTA; Tet-TM-Foxo1 mice (Figure 6G).

Discussion

Previous studies support an important role for Foxo1 in vascular homeostasis. Most importantly, 2 independent groups have shown that Foxo1+/− embryos die at E11 as a consequence of incomplete vascular development.10,11 We have confirmed these findings in the present study. The vascular phenotype of Foxo1−/− mice raises the important question as to whether endothelial Foxo1 is primarily responsible for the embryonic lethality of the knockout mouse. We and others have previously shown that Foxo1 is expressed in endothelial cells and is functionally relevant. Here, we show that endothelial-specific deletion of Foxo1 essentially phenocopies the full knockout, the one obvious difference being in the formation of the branchial arches. By contrast, embryonic development is not compromised in mice with lineage-specific deletions of Foxo1 in bone,14 liver,19 heart,7 T cells,20 and teeth enamel.21 Collectively, these findings suggest that embryonic lethality in the full knockout is attributed primarily to the loss of Foxo1 in the endothelium. Finally, we were able to show that endothelial expression of Foxo1 can rescue Foxo1−/− mice. Although the number of rescue mice born was less than the expected Mendelian ratio, the findings indicate that Foxo1 is also sufficient for embryonic survival. Taken together, our study supports a central role for endothelial-derived Foxo1 in vascular homeostasis.

The only detectable difference in phenotype between the full knockout and the endothelial-specific knockout of Foxo1 was the absence of the second branchial arch in the Foxo1+/− mice. It was previously shown that Foxo1 is expressed in neural crest cells migrating toward branchial arches at E8.5 and becomes localized to the first and second branchial arches between E8.5 and E9.5.11 Interestingly, other studies have demonstrated that neural crest cell invasion of branchial arch 2 involves an interaction between NRP1-expressing neural crest cells and VEGF-expressing ectoderm in the second arch.37,38 Given our findings that Foxo1 is required for VEGF signaling, it is tempting to speculate that loss of Foxo1 in neural crest cells in Foxo1+/− mice interferes with chemotaxis-mediated invasion of branchial arch 2.

Foxo1 transcription factors are widely considered to have antiproliferative and proapoptotic functions. According to the canonical pathway, growth factors, such as insulin or insulin-like growth factor, activate PI3K and Akt. Akt, in turn, phosphorylates Foxo1 proteins, leading to their nuclear exclusion and downregulation of Foxo1-dependent death genes. Consistent with this model, Paik et al14 reported that the somatic deletion of 3 Foxo genes (Foxo1, Foxo3, and Foxo4) resulted in a proliferative endothelial phenotype in some (but interestingly not all) organs. Endothelial cells from the liver of Foxo1/foxo3/foxo4-null mice (but not the lung) demonstrated enhanced VEGF- and fibroblast growth factor–stimulated proliferation.14 In another study, conditional knockout of all 3 Foxo factors in the endothelium resulted in increased proliferation, reduced cellular senescence, and decreased apoptosis in aortic endothelial cells.15 Together, these data support the notion that Foxo1 proteins are antiproliferative and proapoptotic in the endothelium.

How, then, do we reconcile these published findings with the observation that whole-body or endothelial-specific deletion of Foxo1 leads to impaired vascular development and not overgrowth of endothelial cells? The observation that Foxo1 proteins exhibit vascular bed–specific properties in adult mice14 raises the possibility that Foxo1 functions differently in embryonic and postnatal endothelium. An alternative explanation is that Foxo1 alone is not antiproliferative, but rather exerts this function only when Foxo3 and Foxo4 are absent. In fact, our data suggest that endothelial Foxo1 has the opposite effect of promoting basal and VEGF-stimulated migration and proliferation, as well as cell cycle progression through G1. These results are at odds with those of Potente et al,25 who demonstrated that siRNA-mediated Foxo1 knockdown in HUVEC significantly increased endothelial migration, tube formation in the Matrigel assay and sprouting activity of endothelial spheroids. However, in our hands, the si-Foxos used in the latter study had an inhibitory effect on endothelial cell migration, cell cycle, and proliferation (Online Figure IV). Moreover, lentivirus shRNA-mediated knockdown of Foxo1 also inhibited VEGF-mediated proliferation of endothelial cells (Online Figure VIIIE and VIIIIF). At this time, we cannot explain the reason for the discrepant findings. However, as we discuss below, we think that our in vitro and in vivo data strongly support a model in which Foxo1 is necessary for Akt-mTORC1 signaling and growth/proliferation in endothelial cells.

Some24,30 but not all,14,15,41 studies have shown that eNOS is negatively regulated by Foxo1. Lentiviral shRNA-mediated knockdown of Foxo1 in HUVEC increased the expression of eNOS, as did endothelial cell–specific knockout of Foxo1 at E10.5. Interestingly, overexpression of constitutively active Foxo1 also yielded variable results. For example, adenovirus-mediated high-level expression of constitutively active TM-Foxo1 in HUVEC (>4000 copies/cell) led to marked reduction in eNOS mRNA levels (Online Figure VIIIHI), whereas mice overexpressing TM-Foxo1 in the endothelium (<70 copies/cell) demonstrated normal tissue levels of eNOS mRNA and protein. Together, these data suggest that the effect of Foxo1 levels on eNOS expression is highly context dependent and may vary according to absolute Foxo1 levels, to vascular bed type and to in vitro versus in vivo settings.

The finding that mice with an endothelial deletion of all 3 Foxo proteins are viable,17 whereas those with a single deletion of Foxo1 die at E11 indicates that Foxo3 and Foxo4 deficiency rescues the Foxo1 defect. It is possible that the hyperproliferative effect of the combined Foxo1/03/04 knockout compensates for the vascular phenotype in Foxo1+/− mice. Indeed, we showed that Foxo3 knockout in endothelial cells partially reverses the inhibitory effect of Foxo1 deficiency on VEGF-mediated proliferation and rescues the expression of several genes that are critical for sprouting angiogenesis (Results in the Online Data Supplement; Online Figure VI). The data indicate that the difference between single knockout of Foxo1 and triple knockout of Foxo1/03/04 in the endothelium of mice is explained, at least in part, by the nonredundancy of Foxo1 factors.
The phenotype of mice expressing a constitutively active form of FOXO1 also argues against an antiproliferative function of FOXO1 in endothelial cells, at least in the postnatal period. These animals demonstrated normal CD31 counts and BrdU uptake in multiple vascular beds. Moreover, there was no evidence of increased apoptosis, as measured by Tunel assay. Tissues from the TM-FOXO1-expressing mice revealed normal eNOS mRNA and protein levels, arguing against a role of NO deficiency in mediating the increased peripheral resistance. Instead, electron microscopy of various organs suggested that endothelial cells were enlarged to such an extent that they were impinging on the blood vessel lumen, in some cases occluding small capillaries. This effect likely accounts for the increased vascular resistance and reduced cardiac output observed in pressure-volume loop studies. Consistent with the in vivo data, TM-FOXO1 expression in cultured endothelial cells resulted in increased cell size and activation of Akt-mTORC1, which is the principle signaling pathway for cell growth.

Recent studies have demonstrated that in nonendothelial cells, transcriptionally active FOXO1 activates Akt, creating a negative feedback loop. For example, in cardiomyocytes, forced expression of FOXO1 triggers Akt phosphorylation via a calcineurin/protein phosphatase 2A (PP2A)-dependent mechanism. Moreover, Ad-TM-FOXO1 delivery to the liver of mice resulted in a paradoxical increase in p-Akt. These findings, together with ours, indicate that FOXO1 engages in a feedback loop whereby in nutrient- or growth factor–depleted states, nuclear (unphosphorylated) FOXO1 activates Akt, thus preventing the complete extinction of Akt signaling and sensitizing the cell to subsequent growth factor signals. At the same time, the increased p-Akt will lead to phosphorylation and inactivation of FOXO1 (in other words, although the FOXO1-Akt arc is positive, the FOXO1-Akt-FOXO1 feedback loop is negative). Thus, it has been hypothesized that FOXO1 provides acute but not long-term reprieve from metabolic stress/starvation.

Additional studies have provided insights into the mechanisms that underlie FOXO1-mediated activation of Akt. First, FOXO1 has been shown to induce Sestrin 3 expression, which in turn inhibits mTORC1. Thus, the net effect of FOXO1-stimulated Sestrin 3 is to induce p-Akt levels. Second, FOXO1 may increase the expression of Rictor. Rictor forms part of the mTORC2 complex. mTORC2 activates Akt (both directly and by reducing the pool of mTORC1 by competing for mTOR). Therefore, the net effect of FOXO1-stimulated Rictor is to induce p-Akt levels. Finally, FOXO1 has been shown to suppress the expression of Trb3. Trb3, in turn, has been shown to inhibit Akt activity (without affecting mTORC1). Here, we have shown that in human endothelial cells, TM-FOXO1 increases RICTOR expression and dramatically inhibits TRB3 expression, whereas in mouse endothelial cells, TM-FOXO1 expression increases both Rictor and Sestrin 3 (Sestrin3) mRNA expression.

FOXO1-mediated induction of Sestrin 3 and Rictor are associated not only with increased p-Akt but also with reduced mTORC1 activity. However, in endothelial cells (as well as vascular smooth muscle cells), FOXO1 promotes activation of both Akt and mTORC1, as evidenced by increased phosphorylation of S6 and S6K. Thus, a more likely explanation is the profound repression of Trb3, which activates Akt independently of any effects on mTORC1. Alternatively, mutual repression between mTORC1 and mTORC2 may be cell-type-specific, and absent in endothelial cells. In this case, FOXO1-mediated induction of Rictor may result in upregulation of both Akt and mTORC1. Regardless of the underlying mechanism, our data suggest that in contrast to certain other cell types, FOXO1 expression in endothelial cells does not uncouple Akt and mTORC1 activities. mTORC1 plays an important role in regulating cell growth by activating protein synthesis and suppressing autophagy. Thus, FOXO1-mediated activation of Akt and secondary activation of mTORC1 may account for the increase in endothelial cell size in TM-FOXO1–expressing endothelial cells.
Our finding that TM-FOXO1 activates Akt and mTORC1 raised the distinct possibility that FOXO1 deficiency inhibits p-Akt-mTORC1 signaling. To test this possibility, we examined FOXO1-deficient HUVEC, as well as Foxo1−/− and Foxo1−/− embryos, and found that indeed p-Akt and mTORC1 activity are significantly reduced. These data may explain why siRNA-mediated knockdown of FOXO1 attenuates VEGF signaling and proliferation. More importantly, they may provide an explanation for the lethal vascular phenotype of Foxo1−/− mice.

In summary, we have provided evidence that FOXO1 in endothelial cells feeds back to activate Akt-mTORC1 (Figure 7A). According to this model, there exists an optimal FOXO1 range in which endothelial cells are most responsive to growth factor signaling. When FOXO1 levels fall below that range, there is a loss of feedback, which results in reduced Akt-mTORC1 signaling (Figure 7B), G1 arrest, inhibition of proliferation, and reduced mTORC1-mediated metabolism, even in the presence of growth factors (Figure 7C shows proliferation). When FOXO1 levels exceed the normal range, accentuated feedback leads to increased p-Akt-mTORC1 signaling (Figure 7B), with a resulting increase in cell size. Excessively high levels of FOXO1 (especially if uncoupled from inhibition by p-AKT) may override the proporative effect of p-Akt and induce G2 arrest and apoptosis (as occurs in Ad-TM-FOXO1–infected HUVEC; Figure 7C). A comparison with FOXO3, which does not activate Akt, shows that the presence or absence of FOXO-Akt feedback has a profound effect on the phenotypic response of endothelial cells to altered FOXO levels (Online Figure XVI).

Our findings raise interesting questions that have important mechanistic and therapeutic implications. What are the paracrine growth factors (Figure 3C)? A comparison with FOXO1−/− and FOXO1−/− HUVEC, as well as 1-Akt–infected HUVEC; FOXO1 does regulate vascular endothelial cells. Finally, does the FOXO1-Akt-mTORC1 feedback circuit behave differently in endothelial cells from different vascular beds? If so, therapeutic manipulation of FOXO1 activity in the endothelium may yield vascular bed–specific effects. These and related questions are ripe for further study.

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Disclosures
None.

References
27. Abid MR, Nadeau RJ, Spokes KC, Minami T, Li D, Shih SC, Aird WC. Hepatocyte growth factor inhibits VEGF-forkhead-dependent...

**Novelty and Significance**

What is Known?

- FoxO1 promotes cell death and inhibits proliferation in many cell types.
- FoxO1-null mice die at E11 because of impaired vasculogenesis.
- Endothelium-specific deletion of FoxO1 was shown to be embryonic lethal in one study but embryonic viable in another.

What New Information Does This Article Contribute?

- Endothelial FoxO1 is necessary for embryonic development.
- Overexpression of constitutively active FoxO1 in the endothelium results in increased size of endothelial cells (ECs) and occlusion of capillaries.
- FoxO1 feeds back to activate Akt-mTORC1 in ECs.

FoxO1 affects multiple facets of cellular function in several organs, but its therapeutic potential is currently limited by a need to untangle its occasionally paradoxical, cell-type-specific effects. To define the role of FoxO1 in the endothelium, we generated several mouse models with altered FoxO1 activity in the endothelium. We found that endothelial-specific deletion of FoxO1 phenocopied the full knockout of FoxO1 (with the exception of branchial arch development), and endothelial expression of FoxO1 rescued FoxO1-null mice. Expression of a constitutively active form of FoxO1 in the endothelium of mice resulted in increased cell size, occlusion of capillaries, increased peripheral vascular resistance, and heart failure. Finally, we found that FoxO1 activates Akt and mTORC1 in ECs and that knockdown of FoxO1 in ECs results in marked inhibition of basal and vascular endothelial growth factor–induced Akt-mTORC1 signaling. In nonvascular cells, FoxO1 activated Akt but not mTORC1. These findings may explain why mice that are null for FoxO1 develop a lethal vascular phenotype. Moreover, the data support a model in which FoxO1-mediated feedback activation of Akt in ECs maintains growth factor responsive Akt-mTORC1 activity within a homeostatic range.
FOXO1-Mediated Activation of Akt Plays a Critical Role in Vascular Homeostasis

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The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/2/238

An erratum has been published regarding this article. Please see the attached page for:
/content/115/4/e9.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/05/29/CIRCRESAHA.115.303227.DC1
In the *Circulation Research* article by Dharaneeswaran et al (FOXO1-Mediated Activation of Akt Plays a Critical Role in Vascular Homeostasis. *Circ Res*. 2014;115:238–251. DOI: 10.1161/CIRCRESAHA.115.303227), a correction was needed.

Figure 7 is incorrect and should only have panels A, B, and C. The correct figure appears below.

The error has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/115/2/238.
SUPPLEMENTAL MATERIAL

DETAILED METHODS

Targeting constructs

*pFTF*: This vector was constructed for the conditional knockout of mouse *Foxo1*. The structure of the *Foxo1* gene, two coding exons containing 621 bp of N-terminal coding and 1335 bp of C-terminal coding separated by an intron of more than 75 kb, offered limited options for the targeting. Deletion of the second exon carried the risk that the intact promoter and exon 1 could produce a cryptically spliced transcript and a partially functional translation product. Deletion of the first exon via loxP sites in the promoter and intron 1 ran the risk of altering promoter function in unpredictable fashion. Deletion of the first exon via loxP sites in the 5'-UTR and intron 1 could affect mRNA lifetime and/or translation efficiency, but was chosen as the strategy with the lowest potential for problems. The direction of loxP insertion was chosen to avoid the introduction of spurious start codons.

Three BAC clones that hybridized with a 600 bp DNA probe from the 5'-end of *Foxo1* intron 1 (amplified from ES cell genomic DNA) were isolated from a mouse 129 strain library by Invitrogen. However, all sequence used to design PCR primers for this probe and products used in the following construction steps was from the publicly funded sequencing of the C57/black mouse genome.
A 405 bp PCR product, amplified from BAC DNA with a sense primer that created a SacI site and a kinased anti-sense primer and digested with SacI, and a 417 bp PCR product, amplified from BAC DNA with a kinased sense primer that contained a native NdeI site and an anti-sense primer that created an XbaI site and digested with XbaI, were inserted head-to-tail into pL253 vector digested with XbaI and partially digested with SacI. The resulting capture vector was designated pFTGRB and was digested exhaustively with NdeI for use.

Two of the BAC clones, designated A and B, were transformed into EL350 cells. These were grown to mid-log phase, heat-shocked to induce recombination activities, and electroporated with linearized pFTGRB. Ampicillin resistant clones that had captured ~7.6 kb of the Foxo1 locus around exon 1 were designated pFTZA and pFTZB, to differentiate the parental BACs.

Recombination proficient cells were co-electroporated with pFTZA or pFTZB and the floxed neo' cassette from pBBX96 with ligated homology arms to direct its insertion between nucleotides 254 and 264 of the 5'-UTR. Kanamycin resistant clones that had inserted floxed neo' into the Foxo1 5'-UTR were designated pFTKA and pFTKB. These constructs were electroporated into cells induced to express Cre recombinase, resulting in the excision of neo' and the retention of a loxP sequence in the 5'-UTR. Ampicillin resistant clones were dotted in replicate
on ampicillin and kanamycin plates to confirm loss of kanamycin resistance. Confirmed constructs were named pFTLA and pFTLB.

Recombination proficient cells were co-electroporated with pFTLA and the fritted neo’ cassette from pBneoB with ligated homology arms to direct its insertion between nucleotides 490 and 491 of the intron. Kanamycin resistant clones with this insertion were named pFTF. This construct was never used to target ES cells. It was instead used to create a targeting vector with diphtheria toxin negative selection.

\textit{pIFTD}: pFTF was digested with EcoRV and XhoI and products of 4845 and 2765 bp were isolated. A 1676 bp SacI to XhoI restriction fragment gel-purified from a partial SacI digestion of the former and a 2164 bp XhoI to XbaI restriction fragment gel-purified from an XbaI digestion of the latter were inserted between SacI and SpeI of pM253x (pM253 from which the unique XhoI site had been deleted by blunting). This construct contains the 5’- and 3’-ends of targeting homology from pFTF, with a unique XhoI site at their junction.

\textit{pFTD}: A 6163 bp product from XhoI digestion of pFTF was inserted into the XhoI site of pIFTD. One direction of insertion reconstituted the conditional Foxo1 targeting region in a vector with diphtheria toxin negative selection.
pFKO: A construct for conventional knock-out of the Foxo1 gene was made by transforming cells induced to express cre recombinase with pFTD. Clones that had lost the region between nucleotide 264 of the 5'-UTR and nucleotide 490 of the intron were characterized by the size of a PCR product generated between a primer in the residual 5'-UTR and one in neo' coding.

pT2FKHRN: A construct containing mouse Foxo1 cDNA was obtained from the mouse ORFeome collaboration (clone # 100016178). A sense primer designed to append an Nhel site internal to a PspOMI and a kinased anti-sense primer were employed to amplify 229 bp from the 5'-end of the cDNA. A kinased anti-sense primer and a sense primer designed to append a stop codon and a Pvu site internal to an Apal site were used to amplify 184 bp from the 3'-end of the cDNA. These products included unique Nael and HindIII sites of the cDNA. They were digested with PspOMI and Apal, respectively, and inserted between Nael and Apal of pBluKSP. The resulting construct, pIMFKHR1, was digested with Nael and HindIII and the Nael to HindIII fragment of the cDNA was inserted to create pIMFKHR2. Complete mouse Foxo1 cDNA was excised from this as an Nhel to PvuI fragment and inserted between the unique Nhel and PacI sites of pT2XN.

pMP-TRE2-LacZ: pTRE2(mod), with a Nael site inserted after the β-globin polyA signal to permit excision of promoter-reporter-pA from the construct, was partially digested with BgII (two sites). Linearized plasmid was gel-purified, blunted with
Klenow polymerase, digested with XbaI, and gel-purified to recover the largest product (from which the vector MCS was deleted). Gel-purified cassette from BsaI digestion of pBXZneoB was blunted, digested with XbaI, and the largest product, containing all of lacZ, was isolated. It was inserted into the pTRE2(mod) vector to produce pTRE2-LACZ. The promoter-lacZ-β-globin pA was excised as an XhoI (blunted) to NotI fragment an inserted into pMP8II between Pmel and NotI.

**pMPP-TRFK:** The construction of a vector for targeting a tet-responsive promoter driving human Foxo1 cDNA (with three phosphorylation sites mutated to alanine) to the Hprt locus started with a modified pBluKSP. Re-circularizing blunted BamHI and ClaI ends removed sites for Smal, PstI, EcoRI, EcoRV, HindIII, and ClaI while retaining blue phenotype on X-gal plates. An XhoI to SalI restriction fragment of pTRE2, comprising the tet-responsive promoter and most of the adjacent MCS, was inserted into the XhoI site of this vector in the orientation that placed the promoter adjacent to the vector Apal site to produce pBTRE. An expression construct containing triple-mutant Foxo1 was digested with XbaI, blunted, and digested with SalI to excise Foxo1 coding sequence with 230 bp of 5'-UTR and 139 bp of 3'-UTR. This was inserted between EcoRI, blunted, and SalI of pBTRE, removing the MCS derived from pTRE2 and creating pTRE-tmFKHR. The promoter-reporter assembly was removed from this as an XhoI to SalI, blunted, restriction fragment and inserted between XhoI and Pmel of pMPXpA1 to create the shuttle construct pMPX-TRFK. This was linearized by
exhaustive digestion at the Ascl site adjacent to the upstream pMP8 homology. Recombination proficient EL350 cells were co-electroporated with this and pMP8III. Recombination generated the kanamycin-resistant construct pMPK-TRFK. This was electroporated into EL350 cells induced to express cre-recombinase. Removal of the floxed kan’ cassette yielded pMPP-TRFK, an Hprt targeting vector containing a single loxP site preceding the tet-responsive promoter.

**Adenoviruses.** Briefly, human umbilical vein endothelial cells (HUVEC) were plated at a density 1x10^5 cells per 6-cm plate. Adenoviruses (3 MOI) were added 24 h later. Cells were harvested 48 h post infection. Expression of adenovirus was confirmed using qPCR and Western blot.

**Cell culture.** HUVEC, human coronary artery endothelial cells (HCAEC), and human dermal microvascular endothelial cells (HDMVEC) were grown in endothelial basal medium-2 (Lonza) with growth supplements (EGM-2-MV Bullet Kit, Lonza). Human coronary artery vascular smooth muscle cells (CAVSMC) were grown in smooth muscle growth medium-2 (Lonza) with growth supplements (SmGM-2-MV BulletKit, Lonza). Human embryonic kidney cells (HEK 293) were grown in Dulbecco’s Modified Eagle Media (Cellgro) supplemented with 10% FBS, 1% Pen/Strep, 2mM L-Glutamine (Gibco), and 0.1mM non-essential amino acids (Gibco).
**Western blots.** Membranes were probed with the following antibodies: anti-phospho-Tyr1175-VEGFR2 (Cell Signaling, 2478), anti-phospho-Ser473-Akt (Cell Signaling, 9271), anti-phospho-Thr389-p70-S6kinase (Cell Signaling, 9205), anti-phospho-Ser235/236-S6 (Cell Signaling, 2211), anti-Akt (Cell Signaling, 9272), anti-p70-S6kinase (Cell Signaling, 2708), anti-S6 (Cell Signaling, 2217), anti-FOXO1 (Cell Signaling, 2880), anti-eNOS/NOS III (Upstate, 07520), anti-VEGFR2 (Cell Signaling, 2479), and anti-GAPDH (Millipore, MAB374).

**Analysis of cell cycle and cell size.** Cells were washed in cold sterile PBS and trypsinized for 5 min. Cells were then harvested and spun down at 800 rpm for 8 min at 4°C. Supernatant was discarded and the cells were washed in cold sterile PBS three times and then fixed in 70% cold ethanol overnight at 4°C. Cells were spun down at 800 rpm for 8 min at 4°C. The supernatant was discarded and the cells were washed in cold sterile PBS three times. Cells were incubated in propidium iodide solution (eBiosciences) with sterile PBS and RNAse for 30 min at 37°C and filtered with a 40 µm cell strainer. Cell cycle analysis was performed on BD-FACScan Analytic Flow Cytometer (Becton Dickinson) and analyzed using the ModFit LT software (Verity Software House). Cell size was measured using the Scepter 2.0 handheld automated cell counter with 60 µM sensors (Millipore, PHCC60050) and analyzed on PRISM5 software (Graphpad).

**Thymidine uptake, modified Boyden chamber and scratch wound assays.**

Thymidine uptake and modified Boyden chamber assays were carried out as
previously described. For scratch wound assays, HUVEC were transfected with si-CTR or si-FOXO1. After 36 h of incubation, cells were starved overnight and scratched with a plastic p200 tip. Images were collected at time 0 and 12 h later in the presence or absence of vascular endothelial growth factor (VEGF) (50 ng/ml) using Zeiss Axiovision microscope. The area covered was measured and analyzed by ImageJ software (NIH).

**Aortic ring assay.** Aortas were dissected from 7-9 week-old mice. The periaortic fibroadipose tissue was removed in cold sterile PBS and aortas were sectioned into 1 mm long pieces. Aortic rings were rinsed in 2% FBS endothelial growth medium-2-MV (EGM-2, Clonetics) medium for 5 min and transferred to a 24-well plate coated with Matrigel (250 μL/well, BD Biosciences). Aortic rings were covered with additional Matrigel (250 μl/well) and incubated at 37°C and 5% CO₂ for 30 min to polymerize the gel. Medium (500 μl of 2% FBS EGM-2/well) was added to each well and replaced every 48 h. The rings were incubated at 37°C and 5% CO₂ for a week and examined every 24 h using a Carl Zeiss microscope (Axio Image.A1). The branching area and distance of each ring was quantified with ImageJ software (National Institutes of Health).

**Histology and immunochemistry:** E8.5-E14.5 embryos and yolk sacs were harvested for histological analysis and fixed in 4% paraformaldehyde (PFA) and sterile PBS. Genotype was determined by PCR analysis of yolk sacs. Whole mount embryos were paraffin embedded and sectioned transversely at 6-μm
thickness. Embryos were stained with hematoxylin & eosin solution and examined using a Zeiss Imager.A1 (Carl Zeiss MicroImaging). For PECAM-1 staining, whole mount embryos and yolk sacs were washed in PBS containing 0.1% Triton X (PBS-T) for 30 min at room temperature and blocked in PBS-T containing 5% milk (PBSMT) overnight at 4°C. This was followed by overnight incubation at 4°C in anti-mouse PECAM-1 antibody (BD Pharmingen, 558068) in PBSMT. Embryos were washed with PBS-T containing 1% milk for 90 min (3 exchanges) at room temperature. Embryos were incubated in anti-rat Cy3 antibody in PBSMT for 2 h at room temperature. Embryos were rewashed with PBS-T containing 1% milk for 90 min (3 exchanges) at room temperature. Embryos were mounted on vectashield mounting medium (Vector Laboratories, H-1200) and examined using a Nikon TE300 microscope (Nikon Instruments).

**Tunel assay and Bromodeoxyuridine (BrdU) uptake assay.** Organ tissues were harvested and frozen in OCT compound. Frozen organ tissues were sectioned at 5-μm thickness and Tunel analysis was performed using the TUNEL Apoptosis Detection Kit protocol provided by the supplier (Genscript). For BrdU assays, mice were injected intraperitoneally with bromodeoxyuridine (BrdU, BD Pharmingen) at a dose of 100 mg/kg. Twenty-four h post injection mice were sacrificed. Organs were harvested and frozen in OCT compound and sectioned at 5-μm thickness. Sections were fixed in acetone for 2 min at -20°C and then permeabilized in 80% methanol for 10 min at 4°C. Sections were rinsed three times in PBS for 1 min and blocked in Protein Block Serum-Free (Dako) for 20
Sections were incubated with anti-mouse PECAM-1 (BD Pharmingen, 558068) and anti-mouse BrdU antibody (Abbiotec, 250563) in a humidified chamber overnight at 4°C. Sections were rinsed three times in PBS for 1 min, incubated with anti-rat FITC (Invitrogen) and anti-rabbit antibody (Invitrogen, A10520) and in Protein Block Serum-Free for 2 h, rinsed three times in PBS for 1 min and mounted on Vectashield mounting medium (Vector Laboratories). Sections were examined using a Zeiss Imager.A1 (Carl Zeiss MicroImaging).

**CD45 staining.** For CD45 staining, tissues were fixed in 4% paraformaldehyde and sectioned at 7-μm thickness. Sections were rehydrated, blocked in protein block serum-free for 20 min, and processed for antigen retrieval in heated 10 mM sodium citrate buffer solution, pH 6 for 15 min. Tissue sections were incubated with anti-mouse CD45 antibody (BD Pharmingen, 553079) overnight at 4°C, followed by biotinylated anti-mouse antibody (Vector, BA2000) at room temperature for 30 min. CD45 staining was visualized using VECTASTAIN universal elite ABC kit (Vector Laboratories) coupled with 3, 3’-diaminobenzidine (DAB) substrate (Vector Laboratories).

**Pathway maps.** Fig. 4F is based on information from 2-6. Fig. 4G is based on information from 7. Supplemental Figure VI is based on information from 2-6,7,8.
SUPPLEMENTAL RESULTS

Effect of FoxO1 knockdown on eNOS expression

It was previously reported that FOXO1 inhibits expression of eNOS in HUVEC. As shown in Supplemental Figs. VI and VIIIA, si-RNA-mediated knockdown of FOXO1 (si-FOXO1-#1) did not result in a significant increase in eNOS mRNA levels (iNOS levels were undetectable in HUVEC). Similar results were observed at a protein level using Western blot analyses (Supplemental Fig. VIIIB). We repeated these assays using an additional 5 siRNAs against FOXO1, including two from the original paper by Potente et al. Of these, only two (si-FOXO1-#3 and si-FOXO1-#4) led to an upregulation of eNOS mRNA and/or protein expression (Supplemental Figs. VIIIA and VIIIB). Interestingly, these two si-FOXO1s had relatively poor knockdown efficiency compared with the other si-FoxOs (Supplemental Figs. IVA and VIIIB). Previous studies have demonstrated that eNOS mRNA expression is sensitive to the growth state of endothelial cells. However, si-FOXO1 had no effect on eNOS expression in pre-confluent and post-confluent cells (Supplemental Fig. VIIIC). We wondered whether derepression of eNOS might require longer-term depletion of FOXO1 in endothelial cells. To address this question, we infected HUVEC with lentivirus expressing shRNA against FOXO1 and harvested the cells for qPCR and Western blot analyses. Lentiviral RNA-mediated knockdown of FOXO1 resulted in a significant reduction in FOXO1 mRNA levels and a concomitant induction of eNOS.
expression compared with control shRNA (Supplemental Fig. VIIID). Consistent with the siRNA data, the FOXO1-deficient endothelial cells demonstrated decreased basal and VEGF-induced proliferation (Supplemental Fig. VIII E) as well as decreased migration in a scratch wound assay (Supplemental Fig. V I I I F). Finally, we examined eNOS protein expression from E9.5 of Foxo1EC-/ mice. As shown in Supplemental Fig. VIII G, these embryos consistently demonstrated increased eNOS protein levels compared with wild-type littermates. Together, these data suggest that prolonged inhibition of FOXO1 is associated with eNOS induction.

FoxO1 and FoxO3 have overlapping but distinct effects on endothelial cell function and gene expression

In contrast to the embryonic lethality of Foxo1EC-/ mice, a previous study showed that mice with endothelial-specific deletion of Foxo1, Foxo3 and Foxo4 survived embryonic development 17. To examine the question of non-redundancy of FOXO factors, we transfected HUVEC with siRNA against FOXO1 and/or FOXO3 (FOXO3-#1). si-FOXO1 resulted in >80% inhibition of FOXO1 expression, but had no effect on FOXO3 mRNA levels (Supplemental Fig. VIA). Conversely, si-mediated knockdown of FOXO3 resulted in >70% reduction in FOXO3 mRNA levels, with no change in FOXO1 expression (Supplemental Fig. VIA). Unlike FOXO1, siRNA against FOXO3 did not inhibit cell proliferation, as measured by thymidine uptake (Supplemental Fig. VIB). However, siRNA against FOXO3
partially rescued the effect of FOXO1 knockdown on endothelial cell proliferation (Supplemental Fig. VIB).

In qPCR assays, FOXO1 and FOXO3 demonstrated overlapping but distinct effects on the expression of genes related to vascular health (Supplemental Figs. V and VI). Importantly, FOXO3 knockdown resulted in the partial or complete rescue of several genes affected by FOXO1 knockdown, including those involved in vessel maturation (ANG2, VE-cadherin [CDH5]), Notch signaling (DLL4, NRARP, ID1), cell cycle control (CDK4), growth-promoting metabolism (GLUT1, IGF2) and cellular stress (SOD2, CITED2). In still other cases, double knockdown, but not single knockdown, influenced the expression of genes, including jagged 1 (JAG1), claudin 5 (CLD5), and nuclear receptor subfamily 2 (NR2F2, also known as COUP-TFII). Moreover, FOXO1 and FOXO3 knockdown had opposing effects on NRP1, ALK1 and cyclin D1. Collectively, these data suggest that FOXO1 and FOXO3 are non-redundant in their activities.


Supplemental Figure I. Global FoxO1 knockout and endothelial-specific deletion of FoxO1 result in similar phenotypes with embryonic lethality and impaired vascular development. A-F, Representative whole-mount PECAM-1 immunostaining of FoxO1<sup>+/+</sup>, FoxO1<sub>EC</sub>-/- and wild type (FoxO1<sup>+/+</sup>, FoxO1<sub>EC</sub>+/+, respectively) embryos collected at E10.5. Both FoxO1<sup>+/+</sup> and FoxO1<sub>EC</sub>-/- embryos display irregularly developed intersomitic vessels (A and D, respectively) and cranial vasculature (B-C and E-F, respectively). G-K, Representative whole mount PECAM-1 immunostaining of wild type (FoxO1<sub>EC</sub>+/+) and FoxO1<sub>EC</sub>-/- embryos/yolk sacs collected at E9.5. G, Low power view of embryos. H, Head vasculature of FoxO1<sub>EC</sub>-/- is arrested in the primary plexus stage and lacks properly formed branches of the carotid artery (arrow). FoxO1<sub>EC</sub>-/- embryos display a thin and disorganized dorsal aorta (I, arrow) and irregularly developed intersomitic vessels (J, arrow). K, FoxO1<sub>EC</sub>-/- yolk sac shows improperly formed vasculature compared with wild type yolk sac. L and M, Representative whole mount images of wild type (FoxO1<sub>EC-VE</sub>+/+) and FoxO1<sub>EC-VE</sub>-/- embryos collected at E10. L, FoxO1<sub>EC-VE</sub>-/- yolk sac is pale, wrinkled and has no distinct vessels relative to the wild type yolk sac (arrow). M, FoxO1<sub>EC-VE</sub>-/- embryo displays growth retardation and pericardial swelling (PE). Both the first and second branchial arches are visible. Scale bar, 200 µm (A-F, L-M), 250 µm (G), 125 µm (H-I), and 62.5 µm (J-K).
Supplemental Figure II. FoxO1\(^{-/-}\) and FoxO1EC\(^{-/-}\) embryos display impaired cardiovascular development. A, Representative images of H&E-stained transverse sections of FoxO1\(^{-/-}\) embryos collected at E9.5 show underdeveloped or non-existent dorsal aortas (open arrowheads), hypoplasia of branchial arches and unclear aortic arch arteries (closed arrowheads) compared with wild type FoxO1\(^{+/+}\) embryos. B, Representative whole mount PECAM-1 immunostaining of wild type (FoxO1\(^{+/+}\)) and FoxO1\(^{-/-}\) embryos collected at E10.5. FoxO1\(^{-/-}\) embryos show retarded cardiac looping with small left ventricles. C-D, Representative images of H&E-stained transverse sections of FoxO1EC\(^{-/-}\) embryos collected at E9.5 (C) and E10.5 (D) show underdeveloped or non-existent dorsal aortas (open arrowheads), hypoplasia of branchial arches and unclear aortic arch arteries (closed arrowheads) compared with wild type (FoxO1EC\(^{+/+}\)) embryos. E, Representative whole mount PECAM-1 immunostaining of wild type (FoxO1EC\(^{+/+}\)) and FoxO1EC\(^{-/-}\) embryos collected at E10.5. FoxO1\(^{-/-}\) embryos show retarded cardiac looping with small left ventricles. LA, left atrium; LV, left ventricle; OFT, outflow tract. Scale bar, 200 µm (A-C, E), 100 µm (D).
Supplemental Figure III. Liver and teeth phenotype in FoxO1−/− mice rescued with endothelial expression of FoxO1. A, qPCR analysis of selected hepatic genes in liver tissue from FoxO1-res mice and WT littermates. Data are normalized to 18S copy number (n=3). B, At 4 weeks, FoxO1-res mice displayed discolored and brittle white teeth. Top, low magnification; bottom, high magnification. A, Data are presented as mean +/-SD. n.s., non significant; *p<0.05.
Supplemental Figure IV. Effect of FoxO1 knockdown on endothelial cell cycle, migration and proliferation.
HUVEC were transfected with several independent siRNAs against FoxO1 (si-FoxO1), FoxO3a (si-FoxO3a) or control siRNA (si-CTR). A, FoxO1, FoxO3a and ESM1 mRNA expression was evaluated by qPCR and normalized to 18S (n=3). Note the variable extent of knockdown with different siRNAs. Also note that si-FoxO3-#2 had a non-specific effect on FoxO1 mRNA expression. B, Western blot analysis of FoxO1 and GAPDH in HUVEC transfected with different concentrations of siFoxO1-#1 or with siFoxO1-#2 and #3. C, Cell cycle was assessed by propidium iodide staining followed by FACS analysis (n=3). D, Boyden chamber was used to assess migration of siRNA-transfected HUVEC treated in the absence or presence of VEGF (n=3). E, Proliferation was assayed by thymidine uptake in HUVEC transfected with different concentrations of siFoxO1-#1 or with siFoxO1-#2 and #3 and treated in the presence or absence of VEGF (n=3). Data are presented as mean +/- SD. n.s., non significant; *p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure V. Silencing of FoxO3 partially rescues the effects of FoxO1 deficiency (regulatory maps). Regulatory map of genes driving patterning in sprouting angiogenesis (top) and cell cycle and its arrest (bottom). si-FoxO1- vs. si-CTR-transfected HUVEC exposed to 5% serum (1st column; qPCR data in Suppl. Fig. VI) or VEGF (2nd column; qPCR data not shown) demonstrate altered expression of key angiogenic patterning genes. FoxO3 silencing only alters expression of a few genes in the same group, many of them in opposite direction (3rd column; qPCR data in Suppl. Fig. VI). Double knockdown of FoxO3 and FoxO1 reverses the effect of si-FoxO1 on approximately half of the angiogenic genes altered by si-FoxO1 (4th column; qPCR data in Suppl. Fig. VI), as indicated by green gene background. Red / blue / white background: significant increase / significant decrease / no significant expression change or below ~ 1 copy/cell; green background on 4th column: significant increase / significant decrease with si-FoxO1 single knockdown, followed by significant reversal in FoxO1/FoxO3 double knockdown; pink / light blue background on 1st column) paired with light green background on 4th column: non-significant trend toward increase / decrease with si-FoxO1 single knockdown, followed by significant reversal in FoxO1/FoxO3 double knockdown; red / blue gene label (4th column): significant increase/decrease from si-FoxO1 single knockdown to FoxO1/FoxO3 double knockdown; opaque grey gene: not assayed; red/blue border: increased Akt or Notch activity.
Supplemental Figure VI. Silencing of FoxO3 partially rescues the effects of FoxO1 deficiency (qPCR). A, qPCR analysis of FoxO1, FoxO3 and a panel of angiogenic genes in HUVEC transfected with si-CTR, si-FoxO1, si-FoxO3 or si-FoxO1 + si-FoxO3. B, Thymidine uptake in HUVEC transfected with si-CTR-, si-FoxO1, si-FoxO3 or si-FoxO1 and si-FoxO3 (n=3); qPCR analysis of cell cycle genes in HUVEC transfected with si-CTR, si-FoxO1, si-FoxO3 or si-FoxO1 + si-FoxO3. Data are presented as mean+/-SD. *p<0.05; ***p<0.001. Regulatory pathway maps for both gene panels are shown in Supplemental Fig. V.
Supplemental Figure VII. Effect of endothelial cell confluency on FoxO1 target gene expression. HUVEC were transfected with si-FoxO1-#1 (si-FoxO1), or control siRNA (si-CTR) at three different confluences and processed for qPCR analysis of FoxO1 and FoxO1 target genes, ANG2 and ESM1. Data normalized to 18S (n=3) and are presented as mean +/-SD. n.s., non significant; *p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure VIII. Effect of FoxO1 knockdown and Ad-TM-FoxO1 on eNOS expression. A, qPCR analysis of eNOS in HUVEC transfected with si-CTR, si-FoxO1 or si-FoxO3. B, Western blot analysis of eNOS, FoxO1 and GAPDH in HUVEC transfected with si-CTR or si-FoxO1 siRNA. C, qPCR analysis of eNOS in HUVEC transfected with siFoxO1-#1 (si-FoxO1) or control siRNA (si-CTR) at three different confluences. D, qPCR analysis of eNOS in HUVEC infected with lentiviral shRNA against FoxO1 (sh-FoxO1) or control (sh-CTR). E, Migration was assessed by modified scratch assay of HUVEC infected with lentiviral shRNA FoxO1 (sh-FoxO1) or control (sh-CTR) in presence or absence of VEGF. F, Proliferation was assayed by thymidine uptake in HUVEC infected with lentiviral shRNA against FoxO1 (sh-FoxO1) or control (sh-CTR) in the presence or absence of VEGF. G, Western blot analysis of eNOS, FoxO1 and GAPDH in E10.5 FoxO1EC−/− (KO) and FoxO1EC+/+ (wild type, WT) littermate embryos. Shown is a representative Western blot of a single litter. H, qPCR analysis of FoxO1 and eNOS in HUVEC infected with Ad-β-gal (Bgal) or Ad-TM-FoxO1 (TM-FOXO1). All qPCR data were normalized to 18S (n=3). Data are presented as mean +/-SD. n.s.= non significant; *p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure IX. Inducible expression of Hprt-targeted LacZ in the endothelium of mice. VE-cadherin-tTA;TET-LacZ mice were maintained on tetracycline from birth. At 6-8 weeks, tetracycline was removed from the drinking water (Tet-OFF) or was continued (control, Tet-ON), organs were harvested, and tissue sections were processed for LacZ staining.
Supplemental Figure X. Inducible expression of TM-FoxO1 in the endothelium of mice alters target genes expression. A, qPCR analysis of FoxO1 and FoxO1 target gene expression in VE-cadherin-tTA;TET-FoxO1 mice (normalized to 18S, n=3). B, Tunel immunostaining of tissue sections from VE-cadherin-tTA;TET-FoxO1 mice (n=5). Data are shown as % of total cells that are Tunel-positive. C, BrdU incorporation in VE-cadherin-tTA;TET-FoxO1 mice (n=4). Data are shown as fold-change in Tet-OFF vs. Tet-ON. D, Urine albumin (n=11-14) and serum creatinine (n=4-9) in VE-cadherin-tTA;TET-FoxO1 mice. Ki, kidney; Hr, heart; and Li, liver. Data are presented as mean +/-SD. n.s. = non significant; *p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure XI. Inducible expression of TM-FoxO1 in the endothelium of mice does not promote inflammation. A, CD45 immunostaining of tissue sections from VE-cadherin-tTA;TET-FoxO1 mice. Spleen and thymus are shown as positive controls. Quantitation on the right is from n=3 mice. B, Hematoxylin and eosin immunostaining of tissue sections from VE-cadherin-tTA;TET-FoxO1 mice. Hr, heart; Ki, kidney; Li, liver. A, Data are presented as mean +/-SD. n.s.= non significant.
Supplemental Figure XII. Electron microscopy of endothelium in TM-FoxO1 expressing mice. VE-cadherin-tTA;TET-FoxO1 mice were maintained on tetracycline from birth. At 6-8 weeks, tetracycline was removed from the drinking water (Tet-OFF) or was continued (control, Tet-ON). Organs were then harvested and processed for EM. Shown are capillaries from brain and heart. Compared with the Tet-ON control, the Tet-OFF brain capillary shows endothelial cells with far more abundant cytoplasm, which impinges on the blood vessel lumen. The cytoplasm is rich in polyribosomes, rough endoplasmic reticulum and Golgi. In addition, the capillary of the Tet-OFF brain demonstrates a multi-lamellar basement membrane, consistent with layering of endothelial cells. The Tet-OFF heart capillary also shows increased cytoplasm (compared with the Tet-ON control) as well as a multi-lamellar basement membrane.
Supplemental Figure XIII. Effect of TM-FoxO1 on Akt-mTORC1 signaling in various cell types. Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6K (pS6K), total S6K, phospho-S6 (pS6), total S6, FoxO1 and GAPDH in human coronary artery endothelial cells (HCAEC), human dermal microvascular endothelial cells (HDMVEC), human embryonic kidney (HEK) cells and human coronary artery vascular smooth muscle cells (CAVSMC) infected with Ad-β-gal (-), or Ad-TM-FoxO1 (+).
Supplemental Figure XIV. Effect of FoxO1 knockdown on Akt-mTORC1 signaling in endothelial cells. **A**, Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6K, total S6K, phospho-S6 (pS6), total S6, FoxO1 and GAPDH in HUVEC grown in normal serum transfected with si-CTR or si-FoxO1-#1 (si-FoxO1). Shown is a representative Western blot (top) and quantitation of 3 independent experiments (below). **B**, Expanded quantitation of Western blots shown in Fig. 6C. **C**, Western blot analysis of phospho-Akt (pAkt), FoxO1 and GAPDH in HUVEC transfected with si-CTR or multiple independent siRNAs against FoxO1. **D**, Western blot analysis of phospho-Akt (pAkt) and GAPDH in HUVEC transfected with si-CTR or si-FoxO3-#1 (si-FoxO3a) siRNA (left). qPCR of Foxo3 in a parallel plate of cells (right). **E-F**, Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6K, total S6K, phospho-S6 (pS6), total S6, FoxO1 and GAPDH in HCAEC (E) and HDMVEC (F). Cells were transfected with si-CTR or si-FoxO1-#1 (si-FoxO1) and treated with 50 ng/ml VEGF for the times indicated. **G**, Western blot analysis of p-VEGFR2, VEGFR2, FoxO1 and GAPDH in HUVEC transfected with si-CTR or si-FoxO1-#1 (si-FoxO1) and treated with 50 ng/ml VEGF for the times indicated. Data are presented as mean +/-SD. n.s.=p>0.05; *p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure XV. Downregulation of p-Akt-mTORC1 in FoxO1-deficient mice. A, Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6 (pS6), total S6, FoxO1 and GAPDH in E10.5 FoxO1^−/− (KO), FoxO1^+/− (heterozygote, HET) or FoxO1^+/+(wild type, WT) littermate embryos. Shown is a representative western blot of a single litter. B, Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6 (pS6), total S6, FoxO1 and GAPDH in E10.5 FoxO1EC^−/−(KO) and FoxO1EC^+/+(wild type, WT) littermate embryos. Shown is a representative Western blot of a single litter.
Supplemental Figure XVI. Comparison of endothelial cell phenotype with and without homeostatic feedback between FoxO and p-Akt. A-C, Negative feedback between FoxO1 and p-Akt (also shown in Figure 7 in main text). A, Negative feedback between FoxO1 and p-Akt. FoxO1 activates Akt, which in turn inactivates FoxO1, and promotes cell growth (size), cell cycle progression and Notch signaling. B, Negative feedback between FoxO1 and p-Akt restricts the joint activity of nuclear FoxO1 and p-Akt to the range marked by the black line (each point on the black line represents steady state nuclear FoxO1 and p-Akt in different environments). Growth factors push this circuit into the high p-Akt / low nuclear FoxO1 region (orange star). In their absence, endothelial cells settle into a low p-Akt / high nuclear FoxO1 state (green star). When FoxO1 is knocked out or silenced by si-RNA (blue lines), Akt activity is downregulated owing to the loss of feedback (irrespective of growth factors), leading to G1 arrest and reduced mTOR1-mediated metabolism/cell size. Overexpression of nuclear FoxO1 (pink/red line), on the other hand, increases nuclear FoxO1 and p-Akt (owing to accentuated feedback). While p-Akt/mTORC1 pushes cells past the G1/S boundary, the presence of high levels of p-Akt-insensitive nuclear FoxO1 (TM-FoxO1) eventually arrests cells at the G2/M boundary. Further increase in nuclear FoxO1 triggers apoptosis in a large fraction of ECs, G2 arrest in the rest. C, The overall effect of the negative feedback between FoxO1 and p-Akt is that both very low and very high levels of total FoxO1 block endothelial proliferation. There is an optimal FoxO1 range in which cells can best respond to growth factor signaling. In this range, there is enough nuclear FoxO1 to keep p-Akt high (even as p-AKT inhibits a significant fraction of FoxO1 factors), but not enough to induce G2 arrest. D-E, Lack of feedback between FoxO3 and p-Akt. D, Activated AKT inactivates FoxO3, relieving its repression on growth metabolism and cell cycle progression. In contrast to FoxO1, absence of FoxO3 weakens Notch signaling (see Supplemental Fig. VI). E, The lack of feedback between FoxO3 and p-Akt leaves the activity of p-Akt free to respond to growth factor stimulation. FoxO3 nuclear localization, in turn, is inhibited by p-Akt (all lines). The black line represents endogenous FoxO3 response to p-Akt different growth factor environments (high GF: high p-Akt / low nuclear FoxO3, orange star; low GF: low p-Akt / high nuclear FoxO3, green star). In contrast to FoxO1, when FoxO3 is knocked out or silenced by si-RNA, Akt activity is not downregulated. Moreover, FoxO3-mediated inhibition of cell growth and cell cycle progression are relieved, leading to increased proliferation in the presence of growth factors. Overexpression of FoxO3 (pink/red line) does not affect p-Akt. Consequently, in the presence of growth factors cells enter the cell cycle. Since FoxO3 is a strong inducer of cell cycle arrest and apoptotic genes, cell cycle arrests at the G2/M boundary (pink star), and at high levels of FoxO3 apoptosis is common (red star). As expected, in the absence of growth factors the apoptotic effect of FoxO3 manifests at weaker overexpression (pink line) where p-Akt levels are low (dark red star, bottom right). F, The lack of feedback between FoxO3 and p-Akt results in a simple inverse dose response between FoxO3 and proliferation: FoxO3 knockdown weakens cell cycle arrest and increases proliferation, while FoxO3 overexpression blocks the cell cycle and induces apoptosis.
## Supplemental Table I

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## Supplemental Table III

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<td><strong>Lipid profile</strong></td>
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<td>Cholesterol (mg/dL)</td>
<td>151.88 ± 28.36</td>
<td>162.25 ± 24.43</td>
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<tr>
<td>Triglyceride (mg/dL)</td>
<td>91.88 ± 28.50</td>
<td>86 ± 46.32</td>
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<td>HDL (mg/dL)</td>
<td>84.12 ± 16.24</td>
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<td>LDL (mg/dL)</td>
<td>49.38 ±15.53</td>
<td>71.25 ± 20.39</td>
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<td><strong>Liver function</strong></td>
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<td>ALK phosphatase (U/L)</td>
<td>64.88 ± 27.92</td>
<td>50.75 ± 32.26</td>
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<td>ALT (SGPT) (U/L)</td>
<td>23.75 ± 5.99</td>
<td>65.25 ± 35.05</td>
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<td>AST (SGOT) (U/L)</td>
<td>70.50 ± 9.55</td>
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<td>CK (U/L)</td>
<td>387 ± 185.8</td>
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<td>GGT (U/L)</td>
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<td>Amylase (U/L)</td>
<td>864.5 ± 100.36</td>
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<td>Lipase (U/L)</td>
<td>50.13 ± 10.47</td>
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<td>Albumin (g/dL)</td>
<td>3.03 ± 0.23</td>
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<td>Total protein (g/dL)</td>
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<td>Globulin (g/dL)</td>
<td>2.18 ± 0.17</td>
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<td>Total Bilirubin (mg/dL)</td>
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<td><strong>Renal function</strong></td>
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<td>Creatinine (mg/dL)</td>
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<td>BUN (mg/dL)</td>
<td>28.87 ± 2.79</td>
<td>39.75 ± 3.50</td>
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<td>Calcium (mg/dL)</td>
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<td>Phosphorus (mg/dL)</td>
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<td>TCO2 (mEq/L)</td>
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<td>Chloride (mEq/L)</td>
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<td>Potassium (mEq/L)</td>
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<td>Sodium (mEq/L)</td>
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<td>A/G ratio</td>
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<td>B/C ratio</td>
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<td>Anion Gap (mEq/L)</td>
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<td>Glucose (mg/dL)</td>
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