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FoxO1-Mediated Activation of Akt Plays a Critical Role in Vascular Homeostasis

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Abstract

Rationale—Forkhead box-O transcription factors (FoxOs) transduce a wide range of extracellular signals, resulting in changes in cell survival, cell cycle progression, and a number of cell type-specific responses. FoxO1 is expressed in many cell types, including endothelial cells. Previous studies have shown that FoxO1 knockout in mice results in embryonic lethality at E11 due to impaired vascular development. In contrast, somatic deletion of FoxO1 is associated with hyperproliferation of endothelial cells. 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 endothelial cell (EC)-specific disruption of FoxO1 in mice phenocopies the full knockout. While endothelial expression of FoxO1 rescued otherwise FoxO-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 VEGF-induced Akt-mTOR1 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.

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DISCLOSURES

None.

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Keywords

FoxO1; endothelial cells; angiogenesis; endothelial function; angiogenesis; transgenic mice; transcription factors

INTRODUCTION

The FoxO family of transcription factors belongs to the winged helix or forkhead box class of transcription factors [1]. Invertebrates possess one FoxO gene, termed daf-16 in the worm and dFOXO in the fly. Mice and humans possess four 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, mono-ubiquitination, and polyubiquitination [2, 3]. 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 three conserved amino acids that are targets for phosphorylation by Akt or 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 [4-6]. Unrestrained FoxO activity can result in cellular senescence, autophagy and atrophy and can promote a catabolic state [7, 8].

Both FoxO3- and FoxO4-null mice are viable [9, 10]. In contrast, mice that are null for FoxO1 are embryonic lethal at E11 due to impaired vasculogenesis [10, 11]. 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, cardiomycoytes, adipose tissue, somites, branchial arches, and trigeminal ganglia [10-13].

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/or FoxO4 [14] 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 [15]. However, another study reported that endothelial-specific deletion of FoxO1 is embryonic lethal [16]. Conditional deletion of all three FoxO factors in the endothelium is compatible with survival and protects against atherosclerosis in low-density lipoprotein receptor knockout mice [17]. Mice with lineage-specific knockouts of FoxO1 in other tissues, including bone [18], liver [19], heart [7], T cells [20], and teeth enamel [21], 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 [14]. Moreover, the expression profile differed between

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FoxO1/O3/O4-deficient liver and lung endothelial cells, as did the proliferative capacity of the cells in response to VEGF [14].

In the current study, we have generated a number of genetic mouse models to more definitively address the role of endothelial FoxO1 in homeostasis. 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-only Data Supplement.

Generation of gene-targeted and transgenic mice

The generation of $FoxO1^{-/-}$, $FoxO1_{EC}{}^{-/-}$, TET-TM-FoxO1, TET-LacZ and FoxO1-rescue mice is detailed in the online-only Data Supplement. VE-cadherin-tTA mice were a generous gift from Dr. Laura Benjamin. 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 non-endothelial cells used in this study are described in the online-only Data Supplement. All experiments with primary cell lines were carried out between passage three and six. Mouse endothelial cells were harvested and grown as previously described [22]. Cells were either taken off or maintained on 10 mg/L of tetracycline. For VEGF treatment, cells were serum-starved in 0.5% FBS for 16 h and treated with human VEGF-165 (50 ng/mL; Peprotech) for the times indicated.

Adenoviruses

HUVEC were infected with Ad-CMV-β-galactosidase, Ad-WT-FoxO1, Ad-TM-FoxO1 or Ad-CMV-Akt1 (Vector Biolabs) as previously described [23].

Transfection of endothelial cells with siRNA and shRNA

HUVEC were plated at a density 5×10^5 cells per 6-cm plate and transfected with one of six siRNAs against FoxO1 (FoxO1-#1 through FoxO1-#6) or three siRNAs against FoxO3 (FoxO3-#1, FoxO-#2 and FoxO1-#3) in Opti-MEM (Invitrogen) with lipofectin (10 ectinEM (Invitrogen) with fected with AAuthor><Year[23]. siRNA sequences are shown in the online-only Data Supplement. FoxO1-#2, FoxO1-#3 and FoxO3-#2, FoxO3-#3 are identical to those used by Potente et al [24]. For shRNA experiments, HUVEC were incubated with 20yPoteor>Abid</Author><Year>2006</Year><RecNum>4464</RecNum><refor 6 h in

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the presence of 8 μg/mL polybrene. Forty-eight hours after infection, cells were selected with puromycin (10 μg/ml) for one week before they were expanded for experiments.

Quantitative real-time polymerase chain reaction (qPCR) assays

qPCR assays were carried out as previously described [25]. Primer sequences are available upon request.

Cardiac morphometric and pressure-volume (PV) 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 *s*agittal plane at 6-μm thickness. Paraffin embedded hearts were examined using a Wild Heerbrugg M400 Photomakroskop (Wild Heerbrugg Ltd., Switzerland). The ventricles and atria volumes were quantified with ImageJ software (National Institutes of Health). PV loop analysis was performed as previously reported [26].

Westerns blots

Western blots were performed as previously described [27]. Blots were analyzed using antibodies detailed in the online-only Data Supplement. The bands were visualized with a chemiluminescence detection kit (ECl, Amersham Biosciences) and were quantified with ImageJ software (National Institutes of Health).

Blood chemistry

Blood was collected via cardiac puncture. Serum creatinine levels in VE-cadherin-tTA;TET-FoxO1 mice were measured using the i-STAT Chem8+ cartridge and analyzed using i-STAT analyzer (Abbot Point of Care, Inc.). Urine samples were collected directly from the urinary bladder. Urine albumin levels were assayed for using an ELISA kit protocol (Exocell) provided by the supplier. Rescue mice blood samples were assayed by the Diagnostic Laboratory of MIT.

Transmission electron microscopy

Transmission electron microscopy was carried out as previously described [28].

LacZ staining

LacZ staining was carried out as previously described [22].

Aortic ring assay

Detailed procedures for this assay is provided in the online-only 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-only Data Supplement.

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Statistical analysis

Data were expressed as means ± standard deviations of three independent experiments. The mean statistical difference was determined using a t-test or one-way analysis of variance 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 FoxO1-null mice (FoxO1$^{-/-}$) as well as mice in which FoxO1 was conditionally deleted in the endothelium (FoxO1$_{EC}^{-/-}$) (Figs. 1A-B). FoxO1 protein was not detected in Western blot analyses of E10.5 FoxO1$^{-/-}$ embryos (see Fig. 6E). Consistent with what has been previously reported, targeted disruption of the FoxO1 gene in mice resulted in embryonic lethality around E11 (Supplemental 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 (Figs. 2A-C). In whole mount E9.5-E10.5 embryos stained for platelet-endothelial cell adhesion molecule 1 (PECAM-1), the dorsal aorta appeared thin, under-developed and irregularly formed in FoxO1$^{-/-}$ embryos (Supplemental Fig. IA). This was confirmed in H&E-stained tissue sections (Supplemental Fig. IIA). Intersomitic vessels were similarly poorly developed in FoxO1$^{-/-}$ embryos (Fig. 2D and Supplemental Fig. IA). The heart was smaller compared with that of wild type controls (Supplemental Fig. IIB). The head vasculature of FoxO1$^{-/-}$ embryos was arrested in the primary plexus stage (Supplemental Figs. IB-C). Yolk sacs were pale and had poorly formed vasculature (Figs. 2A, 2E).

FoxO1$_{EC}^{-/-}$ mice were generated by crossing FoxO1-LoxP-targeted mice (FoxO1$^{fl/fl}$) with the Tie2-Cre line. Similar to the full knockout, FoxO1$_{EC}^{-/-}$ embryos died at approximately E11.0 (Supplemental Table 2II. Cre-mediated excision of FoxO1 in the endothelium of mice resulted in a virtually identical phenotype to that observed with the complete knockout, with the exception that the second branchial arch was preserved (Figs. 2F-J, Supplemental Figs. ID-K, and Supplemental Figs. IIC-E). Electron microscopy of aortas and intersomitic vessels from E9.5 FoxO1$^{-/-}$ and FoxO1$_{EC}^{-/-}$ mouse aortas did not demonstrate any ultrastructural abnormalities (not shown). Although the Tie2-Cre line is the most commonly used driver strain in endothelial-specific knockout studies, it has been shown to yield Cre-mediated recombination in hematopoietic cells [29]. To confirm our data, we bred transgenic mice with Cre recombinase expressed from VE-cadherin (VEC) regulatory sequences (VEC-Cre) with FoxO1$^{fl/fl}$. The resulting offspring (FoxO1$_{EC\text{-}VE}^{-/-}$) demonstrated an identical phenotype to that of the FoxO1$_{EC}^{-/-}$ mice (Supplemental Figs. IL-M show whole mounts at E10). Together, these findings indicate that endothelial expression of FoxO1 is necessary for normal development and that its absence accounts for the majority of the phenotypic manifestations of the complete FoxO1 knockout.

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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 *FoxO1* cDNA (Fig. 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. 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 (Supplemental Table II), those that did survive were grossly indistinguishable from wild-type littermates (Fig. 3A). There was no difference in body weight or weight of individual organs (with the exception of fat) between the rescue mice and wild type littermates (Fig. 3B). qPCR analysis of various mouse organs demonstrated near normal mRNA levels of FoxO1 in most tissues as well as expression of endothelial-restricted FoxO1 target genes including endothelial nitric oxide synthase (eNOS, *Nos3*), angiopoietin 2 (*Ang2*), and endothelial-specific molecule 1 (*Esm1*) (Fig. 3C). Similarly, qPCR assays of primary mouse lung endothelial cells revealed detectable levels of FoxO1 and selected target genes (Fig. 3D). Tunel staining of various tissues did not reveal any difference in the number of apoptotic cells between rescue mice and wild-type littermates (Fig. 3E shows liver, heart and lung). Moreover, vascular density was similar in rescue mice and wild-type controls, as assayed by CD31 staining (Fig. 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 compared with wild-type littermates (Supplemental 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 (Supplemental Fig. IIIA). However, hepatic expression of phosphoenolpyruvate carboxykinase 1 (*Pck1*), another gluconeogenic enzyme whose expression was reduced in liver-specific knockout mice, was unaffected in the FoxO1-res mice (Supplemental Fig. IIIA). 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 compared with wild-type littermates (Supplemental Fig. 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 G1 cell cycle arrest and inhibition of VEGF-mediated migration and proliferation

We next wished to study the mechanism by which endothelial FoxO1 silencing interferes with vascular development. To that end, we transfected HUVEC with siRNA against FoxO1 (si-FoxO1-#1) or control siRNA (si-CTR). As shown in Fig. 4A, si-FoxO1 transfection resulted in an 80% decrease in FoxO1 mRNA expression. In FACS analyses, FoxO1 knockdown was associated with an increased number of cells in G1 and a corresponding decrease in the numbers in S and G2/M, indicating an arrest in G1 (Figs. 4B). In a modified Boyden chamber assay, si-FoxO1 transfection resulted in a significant reduction in VEGF-

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induced cell migration (54% reduction) (Fig. 4C). In a scratch wound assay, FoxO1-siRNA-transfected cells also showed a significant decrease in percentage of area covered under both under basal and VEGF induced conditions (63% and 75% reduction, respectively) (Figs. 4D). Finally, in proliferation assays, basal and VEGF-mediated thymidine incorporation was reduced 7-fold and 16-fold, respectively, in si-FoxO1-treated HUVEC (Fig. 4E). Similar results were in observed in cell cycle, migration, and proliferation assays using two other siRNA against FoxO1 (FoxO1-#2 and FoxO1-#3, Supplemental Fig. IV). Thus, FoxO1 plays a role in cell cycle progression and in VEGF-mediated endothelial cell migration and proliferation.

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/or 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, expression of *ESM1*, *ANG2*, *IRS2*, *BMP2, SOD2 and CITED2* (established FoxO1 targets in endothelial cells) was downregulated (Fig. 4F and Supplemental Figs. V and VI). Similar results were observed in HUVEC grown to different degrees of confluence (Supplemental Figs. 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* (Fig. 4F and Supplemental Figs. V and VI). Of particular note were changes 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*. Neuropilin 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 (Fig. 4F and Supplemental Figs. V and VI). si-FoxO1 increased *VEGF* mRNA expression, but absolute levels remained very 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 $p27^{kip1}$, $p21^{WAFI/CIP1}$, decreased expression of *MYC* and *CDK4*, decreased expression of cyclins specific to the S and G2 phase of cell cycle, CyclinE1 (*CCNE1*), CyclinB1 (*CCNB1*), CyclinB2 (*CCNB1*), as well as decreased expression of proliferating cell nuclear antigen (*PCNA*) (Fig. 4G and Supplemental Figs. V-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-only Data Supplement (and shown in Supplemental Figs. VIII), eNOS expression was increased in HUVEC with prolonged lentivirus shRNA-mediated knockdown of FoxO1 as well as in E9.5 $FoxO1_{EC}^{-/-}$ 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.

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Endothelial overexpression 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 FoxO3/4) leads to the development of hemangiomas [14]. Together with a plethora of published data implicating a role for FoxO1 as an antiproliferative and proapoptotic gene, these findings suggested that increased activity of FoxO1 in the intact endothelium might lead to widespread endothelial death. To test this hypothesis, we employed homologous recombination in the *Hprt* locus to generate transgenic mice that carry a tetracycline-responsive promoter (TET) coupled to a constitutively active human FoxO1 in which the three Akt phosphorylation sites were mutated to alanines (triple mutant [TM]-FoxO1). These mice (TET-TM-FoxO1) were crossed with endothelial-specific tTA mice (VE-cadherin-tTA) to generate inducible (Tet-Off) binary transgenic mice that express TM-FoxO1 in the endothelium upon withdrawal of tetracycline from the drinking water (Fig. 1D). Since commercially available FoxO1 antibodies perform poorly in immunohistochemistry, we decided to monitor efficacy of our binary system by generating a separate line of mice in which the Hprt locus was targeted with the TET promoter coupled to LacZ (TET-LacZ). Since both TET-TM-FoxO1 and TET-LacZ are inserted as single copies into an identical locus, LacZ expression should serve as a surrogate marker for TM-FoxO1 expression. Analysis of VE-cadherin-tTA;TET-LacZ mice revealed minimal leakage of expression in mice on tetracycline and endothelial-restricted inducible expression off tetracycline (Supplemental Fig. IX). VE-cadherin-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) (Supplemental Fig. 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, $p21^{WAFI/CIP1}$ (but not $p27^{kip1}$), cyclin G2 (Ccng2), and Bcl6b (Supplemental Fig. 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 (Supplemental Fig. XA shows qPCR data). Nor did TM-FoxO expression affect the expression of the cell adhesion molecules, Vcam-1 and Icam-1 (data not shown). Endothelial cells were isolated from the lungs and hearts of VE-cadherin-tTA;TET-TM-FoxO1 mice and grown in the absence of presence of tetracycline. Upon removal of tetracycline from the culture medium, there was significant induction of FoxO1 and FoxO1 target genes, including Esm1, Ang2, $p21^{Cip1}$, cyclin G2 (Ccng2), Gadd45a and Sod2 (Fig. 5A shows Esm1 and Ang2). Induction of TM-FoxO1 had no effect on eNOS mRNA levels in heart ECs and actually increased eNOS expression in lung ECs (Fig. 5A). There was no change in Vcam-1 or Icam-1 expression (data not shown).

Inducible expression of TM-FoxO1 in the endothelium resulted in lethality after seven days. The dry weight of the lung, but not other organs was increased (not shown). Morphometric analyses of the heart revealed left ventricular enlargement (Figs. 5B-5C). Pressure-volume loop experiments demonstrated increased peripheral vascular resistance, lower mean arterial pressure, and decreased cardiac output (Fig. 5D). There was no evidence of endothelial cell hyper-proliferation or increased apoptosis, as measured by Tunel staining and BrdU

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incorporation/CD31 staining, respectively (Supplemental Figs. XB and XC). A previous study implicated a role for endothelial FoxO proteins in monocyte recruitment [17]. However, CD45 of various mouse tissues staining did not reveal any difference in the number of CD45+ cells between Tet-ON and Tet-OFF mice (Supplemental Fig. XIA shows heart, kidney and liver). Moreover, H&E stains did not demonstrate any evidence of inflammation (Supplemental Fig. XIB).

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

To determine whether TM-FoxO1 expression in endothelial cells impaired sprouting angiogenesis, we carried out aortic ring assays using samples from VE-cadherin-tTA;TET-TM-FoxO1 mice. As demonstrated in Fig. 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 anti-proliferative role of FoxO1 in the endothelium.

Endothelial 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 VE-cadherin-tTA;TET-TM-FoxO1 mice demonstrated increased cell volume. This lack of reproducibility may reflect a loss of larger cells during isolation process. However, infection of HUVEC with adenovirus (Ad) expressing-TM-FoxO1 (Ad-TM-FoxO1) resulted in a significant increase in cell volume and size (Fig. 6A). Cell volume is controlled primarily by the Akt-mTORC1 signaling pathway [32]. Previous studies in non-endothelial cells have shown FoxO1 may feed back to activate Akt, while inhibiting mTORC1, thus uncoupling Akt and mTOR signaling [33-35]. In Western blot analyses of HUVEC, phospho (p)-Akt was elevated in cells infected with adenovirus expressing wild type (WT) FoxO1 (in which the phosphorylation sites are intact) compared with Ad-β-galactosidase-infected controls and was further increased in cells expressing the phosphorylation-resistant TM-FoxO1 (Fig. 6B). The phosphorylation status of S6 and S6K is commonly used to evaluate mTORC1 activity [36]. In Western blots, Ad-WT-FoxO1- and Ad-TM-FoxO1-infected HUVEC demonstrated progressively increased levels of p-S6 and p-S6K (Figs. 6B). Interestingly, total levels of S6 were increased in TM-FoxO1-expressing cells, while total levels of S6K were decreased. To determine whether the effect of TM-

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FoxO1 (i.e., activation of Akt and mTROC1) was specific to HUVEC, we repeated these experiments using other types of endothelial cells as well as non-endothelial cells. As shown in Supplemental Fig. XIII, TM-FoxO1 induced both p-Akt and p-S6K in human coronary artery endothelial cells (HCAEC), human dermal microvascular endothelial cells (HDMVEC) and human coronary artery vascular smooth muscle cells (CAVSMC). 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.

The Akt-mTORC1 pathway is attenuated in FoxO1-deficient endothelial cells

Based on 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 (Fig. 6C and Supplemental Figs. XIVA-C). By contrast, siRNA-mediated knockdown of FoxO3 had no effect on Akt levels (Supplemental Fig. XIVD). Similar results with si-FoxO1 were observed with HCAEC and HDMVEC (and Supplemental Figs. XIVE and XIVF). FoxO1 knockdown in HUVEC did not affect VEGF-mediated phosphorylation of VEGFR2 (Supplemental Fig. XIVG), 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-tranfected cells with constitutively active Akt. In these experiments, siRNA against FoxO1 resulted in a marked diminution in thymidine uptake in the absence, but not presence of CA-Akt (Fig. 6D). We harvested E10.5 $FoxO1^{-/-}$ embryos for protein and carried out Western blot analyses for Akt and mTORC1 activation. As shown in Fig. 6E and Supplemental Fig XV, homozygous knockout embryos demonstrated reduced p-Akt and p-S6, despite an increased total level of S6. Western blots of $FoxO1_{EC}{}^{-/-}$ embryos demonstrated a trend towards reduced p-Akt (some blots, such as the one shown in Fig. 6F demonstrated marked reduction) and significantly reduced levels of p-S6 (Fig. 6F and Supplemental Fig XV). Together, these data suggest that FoxO1 deficiency is associated with a loss of feedback activation of Akt-mTORC1.

Previous studies have implicated a number of FoxO-responsive genes in mediating feedback activation of Akt, including Sestrin 3 (Sesn3) [33], Rictor [33], and the pseudokinase, *TRB3* [35]. In qPCR assays, si-RNA-mediated knockdown of FoxO1 resulted in downregulation of *SESN3* (Fig. 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 (Fig. 6G). Finally, there was an increase in both *Rictor* and *Sesn3* mRNA levels in endothelial cells isolated from the hearts of VE-cadherin-tTA;TET-TM-FoxO1 mice (Fig. 6G).

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DISCUSSION

Previous studies support an important role for FoxO1 in vascular homeostasis. Most importantly, two 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 only 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 [18], 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$^{-/-}$. 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 knock out 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 towards 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 FoxO1 deletion in neural crest cells in the FoxO1$^{-/-}$ mice interferes with chemoattraction-mediated invasion of branchial arch 2.

FoxO transcription factors are widely considered to have anti-proliferative and pro-apoptotic functions. According to the canonical pathway, growth factors such as insulin or insulin-like growth factor activate PI3K and Akt. Akt, in turn phosphorylates FoxO proteins, leading to their nuclear exclusion and downregulation of FoxO-dependent death genes. Consistent with this model, Paik et al reported that the somatic deletion of three FoxO genes (FoxO1, FoxO3 and FoxO4) resulted in a proliferative endothelial phenotype in some (but interestingly not all) organs [14]. Endothelial cells from the liver of FoxO1/3/4-null mice (but not the lung) demonstrated enhanced VEGF- and fibroblast growth factor (FGF)-stimulated proliferation [14]. In another study, conditional knockout of all three FoxO factors in the endothelium resulted in increased proliferation, reduced cellular senescence and decreased apoptosis in aortic endothelial cells [17]. Together, these data support the notion that FoxO proteins are anti-proliferative and pro-apoptotic 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 FoxO proteins exhibit vascular bed-

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specific properties in adult mice (see [14]) 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/or 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, 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 [24]. However, in our hands, the si-FoxO1s used in the latter study had inhibitory effect on endothelial cell migration, cell cycle and proliferation (see Supplemental Fig. IV). Moreover, lentivirus shRNA-mediated knockdown of FoxO1 also inhibited VEGF-mediated proliferation of endothelial cells (see Supplemental Fig. VIII). At this time, we cannot explain the reason for the discrepant findings. However, as we discuss below, we believe that our *in vitro* and *in vivo* data strongly support a model in which FoxO1 is necessary for Akt-mTOR1 signaling and growth/proliferation in endothelial cells.

Some [24, 39], but not all [14, 15, 40], 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 (>4,000 copies/cell) led to marked reduction in eNOS mRNA levels (see Supplemental Fig. VIIIH), while mice over-expressing 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 FoxO levels, to vascular bed type and/or to *in vitro* vs. *in vivo* settings.

The finding that mice with an endothelial-deletion of all three FoxO proteins are viable [17], whereas those with a single deletion of FoxO1 die at E11 indicates that FoxO3 and/or FoxO4 deficiency rescues the FoxO1 defect. It is possible that the hyperproliferative effect of the combined FoxO1/O3/O4 knockout compensates for the vascular phenotype in $FoxO1^{-/-}$ mice. Indeed, we showed that FoxO3 knockdown 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 (see Supplemental Results and Supplemental Fig. VI, online-only Data Supplement). The data indicate that the difference between single knockout of FoxO1 and triple knockout of FoxO1/O3/O4 in the endothelium of mice is explained, at least in part, by the non-redundancy of FoxO 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

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resistance. Instead, electron microcopy 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 Aktm-TORC1, which is the principle signaling pathway for cell growth.

Recent studies have demonstrated that in non-endothelial 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/PP2A-dependent mechanism [34]. Moreover, Ad-TM-FoxO1 delivery to the liver of mice resulted in a paradoxical increase in p-Akt [35]. 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, while 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 [17].

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 [33]. mTORC1 normally feeds back to inhibit Akt [41]. Thus, the net effect of FoxO1-stimulated Sestrin 3 is to induce p-Akt levels. Second, FoxO1 may increase the expression of Rictor [33]. 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 also been shown to suppress the expression of Trb3 [35]. Trb3, in turn, has been shown to inhibit Akt activity (without affecting mTORC1) [42]. Here, we have shown that in human endothelial cells, TM-FoxO1 increases *RICTOR* expression and dramatically inhibits *TRB3* expression, while in mouse endothelial cells, TM-FoxO1 expression increases both *Rictor* and Sestrin 3 (*Sesn3*) mRNA expression.

FoxO1-mediated induction of Sestrin 3 and Rictor are associated not only with increased p-Akt but also with reduced mTOR1 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 [43], 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 (reviewed in [36, 44]). Thus, FoxO1-mediated activation of Akt and secondary

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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 $FoxO1_{EC}{}^{-/-}$ 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 $FoxO1^{-/-}$ mice.

In summary, we have provided evidence that FoxO1 in endothelial cells feeds back to activate Akt-mTORC1 (Fig. 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 (Fig. 7B), G1 arrest, inhibition of proliferation and reduced mTOR1-mediated metabolism, even in the presence of growth factors (Fig. 7C shows proliferation). When FoxO1 levels exceed the normal range, accentuated feedback leads to increased p-Akt-mTORC1 signaling (Fig. 7B), with a resulting increase in cell size. Excessively high levels of FoxO1 (especially if uncoupled from inhibition by p-AKT) may override the pro-proliferative effect of p-Akt and induce G2 arrest and apoptosis (as occurs in Ad-TM-FoxO1-infected HUVEC) (Fig. 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 (see Supplemental Fig. XVI).

Our findings raise interesting questions that have important mechanistic and therapeutic implications. What are the paracrine effects of Akt-mTORC1 activity in endothelial cells on other cell types and how do these alter organ function? Does FoxO1 activate Akt in every cell type? In those cells in which FoxO1 does feedback to activate Akt, is p-Akt associated with increased or reduced mTORC1 activity? Cells in which mTORC1 activity is concomitantly induced (e.g., endothelial cells) are more likely to grow in response to overactivation of FoxO1, while those in which mTORC1 activity is inhibited are more likely to undergo autophagy and cell cycle arrest. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

ANG2	angiopoietin 2
CCNB1	cyclinB1
CCNB2	cyclinB2
CCNE2	cyclinE2
EFNB2	ephrin B2
eNOS	endothelial nitric oxide synthase
ESM1	endothelial-specific molecule 1
HUVEC	human umbilical vein endothelial cells
IRS2	insulin receptor substrate 2
JAG1	jagged 1
NRP1	neuropilin 1
NRARP	NOTCH-regulated ankyrin repeat protein
NR2F2	nuclear receptor subfamily 2
PCK1	phosphoenolpyruvate carboxykinase 1
PCNA	proliferating cell nuclear antigen
PECAM-1	platelet-endothelial cell adhesion molecule 1
SES3	sestrin 3
TRB3	tribbles 3

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Novelty and Significance

What Is Known?

- FoxO1 promotes cell death and inhibits proliferation in many cell types.
- Foxo1–null mice die at E11 due to 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 endothelium-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 non-vascular 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.

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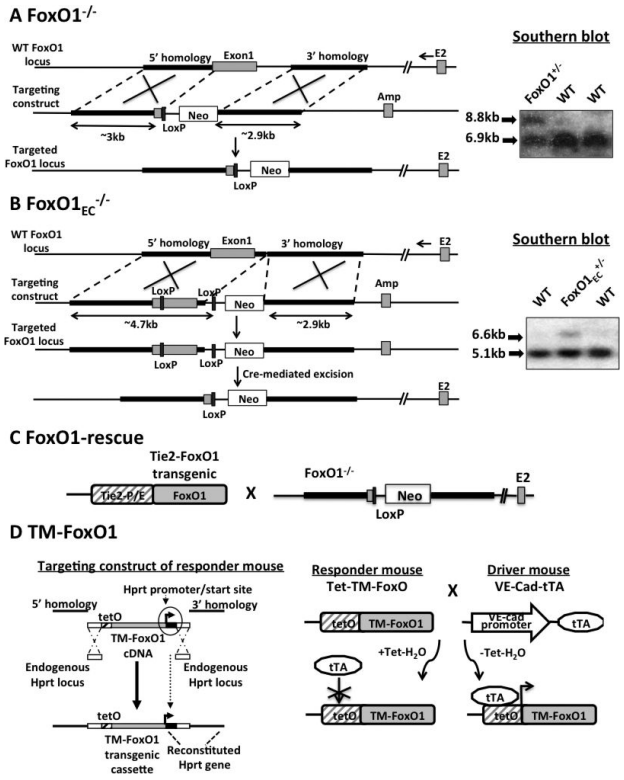

Figure 1. Schematics of targeting constructs

A-B, *Left*, Targeting construct to generate full FoxO1 knockout mice ($FoxO1^{-/-}$; A) and FoxO1 endothelial-specific knockout mice ($FoxO1_{EC}^{-/-}$; B). Bold lines indicate the homology arms. *Right*, Recombinant ES clones were identified by Southern blot analysis. **C,** Transgenic construct and breeding scheme to generate FoxO1 rescue mice. **D,** *Left*, Targeting construct to generate Tet-FoxO1-TM *Hprt*-targeted mice. *Right*, Breeding scheme to generate inducible endothelial-specific overexpression of constitutively active FoxO1 (VE-cadherin-tTA;TET-TM-FoxO1). WT, wild type; E, exon; LoxP, LoxP site; Neo, neomycin resistance cassette; Amp, ampicillin resistance gene; FoxO-TM, human FoxO1 cDNA with triple mutations (T24A, S256A, S319A); tetO, tetracycline operator; tTA, tetracycline transactivator; Tet-H_2O, drinking water containing tetracycline; Tie2P/E, Tie2 promoter/enhancer.

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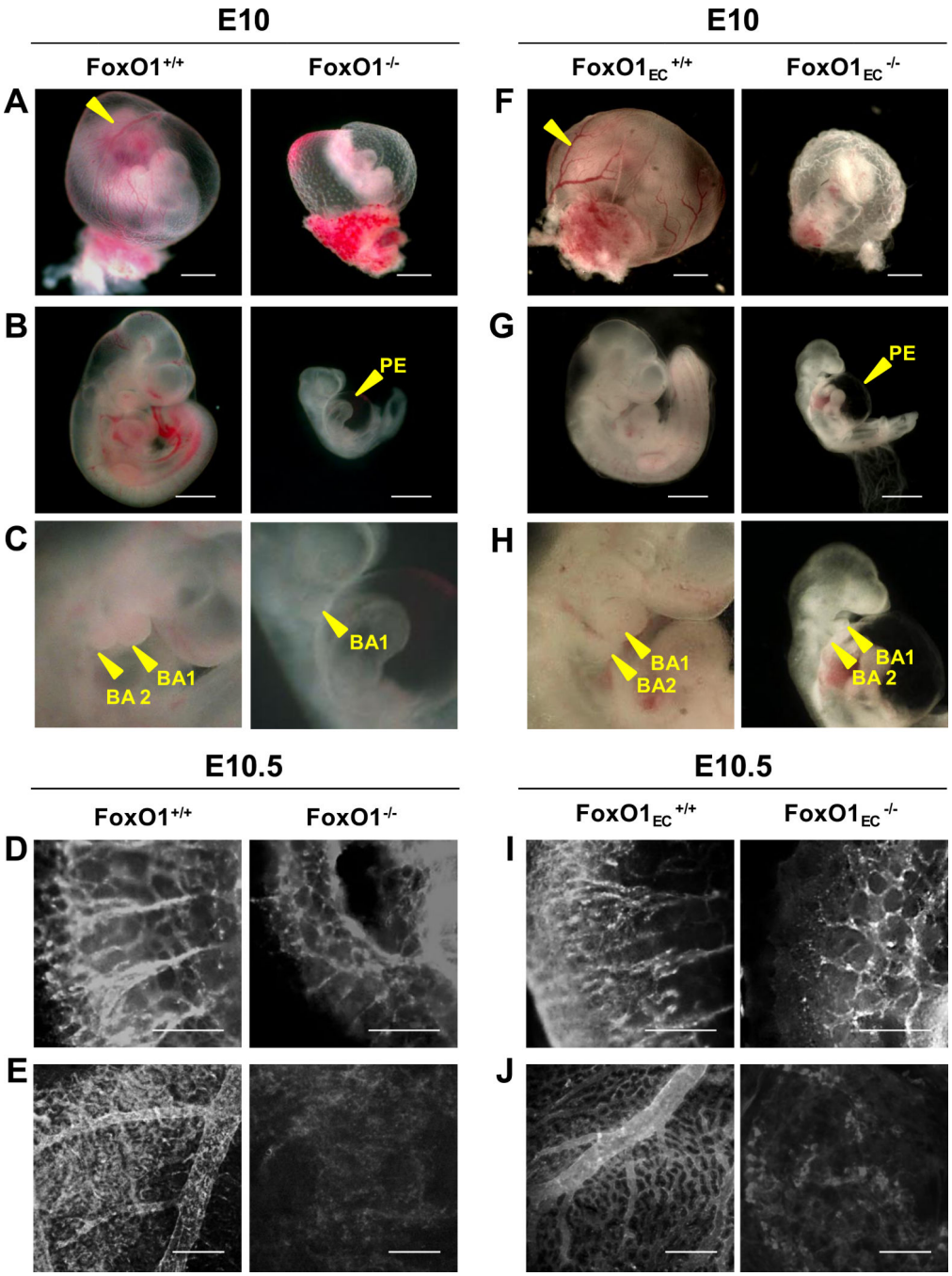

Figure 2. U. Global FoxO1 knockout and endothelial-specific deletion of FoxO1 result in similar phenotypes with embryonic lethality and impaired vascular development

Representative whole mount images of $FoxO1^{-/-}$, $FoxO1_{EC}{}^{-/-}$ and wild type ($FoxO1^{+/+}$, $FoxO1_{EC}{}^{+/+}$, respectively) embryos collected at E10-E10.5. **A and F,** Compared with wild type yolk sacs, $FoxO1^{-/-}$ and $FoxO1_{EC}{}^{-/-}$ yolk sacs are pale, wrinkled and have no distinct vessels (arrow indicates blood vessel in wild type yolk sac). **B and G,** $FoxO1^{-/-}$ and $FoxO1_{EC}{}^{-/-}$ 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). **H,** $FoxO1_{EC}{}^{-/-}$ embryos develop smaller than normal first and second

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branchial arches (BA1, BA2). **D and I,** Whole-mount PECAM-1 immunostaining of E10.5 FoxO1$^{-/-}$ and FoxO1$_{EC}{}^{-/-}$ embryos show poorly developed intersomitic vessels. **E and J,** Whole-mount PECAM-1 immunostaining of E10.5 FoxO1$^{-/-}$ and FoxO1$_{EC}{}^{-/-}$ yolk sacs lack a distinct vasculature relative to wild type yolk sacs. Scale bar, 200 μm.

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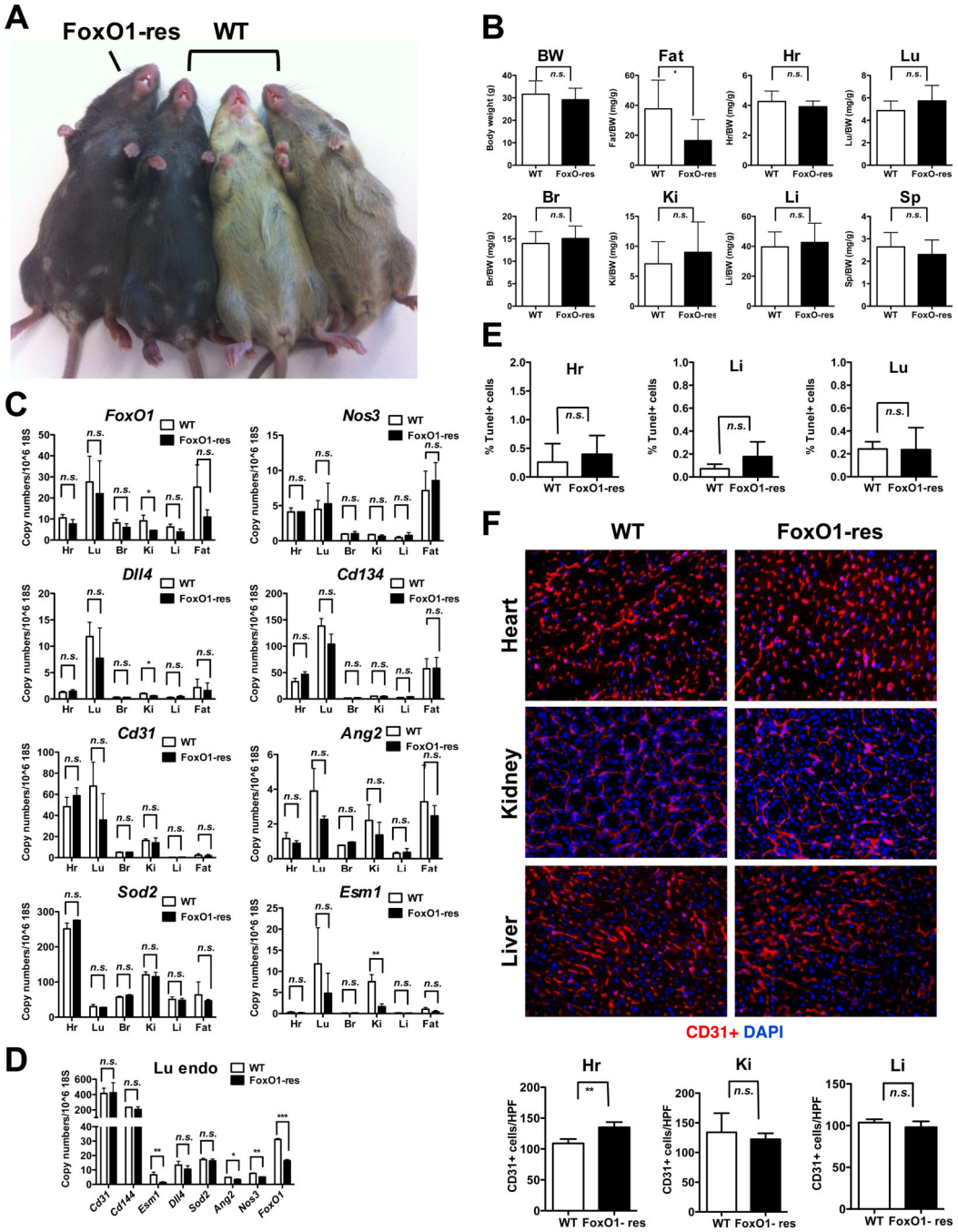

Figure 3. FoxO1 expression in the endothelium rescues FoxO1$^{-/-}$ lethality

A, Representative images of 4-week old Tie2-FoxO1;FoxO1$^{-/-}$ (FoxO1-res) mice and wild type (WT) littermates. **B,** Tissue weights from FoxO1-res mice and their WT littermates. Data are presented as organ-weight-to-body-weight ratio (mg/g) in comparison to WT littermates. **C,** qPCR analysis of FoxO1 and FoxO1 target gene expression in whole organs from FoxO1-res mice and WT littermates. Data are normalized to 18S copy number (n=3). **D,** qPCR analysis of FoxO1 and FoxO1 target gene expression in endothelial cells isolated from the lungs of FoxO1-res mice and WT littermates. Data are normalized to 18S copy

number (n=3). **E,** Apoptosis as measured by Tunel staining of tissue sections from FoxO1-res mice and WT littermates. Data are presented as % of total cells that are Tunel +. **F,** CD31 staining of tissue sections from the heart, kidney and liver of FoxO1-res mice and WT littermates (*top*). Quantitation of CD31+ cells per 40X field (*bottom*).

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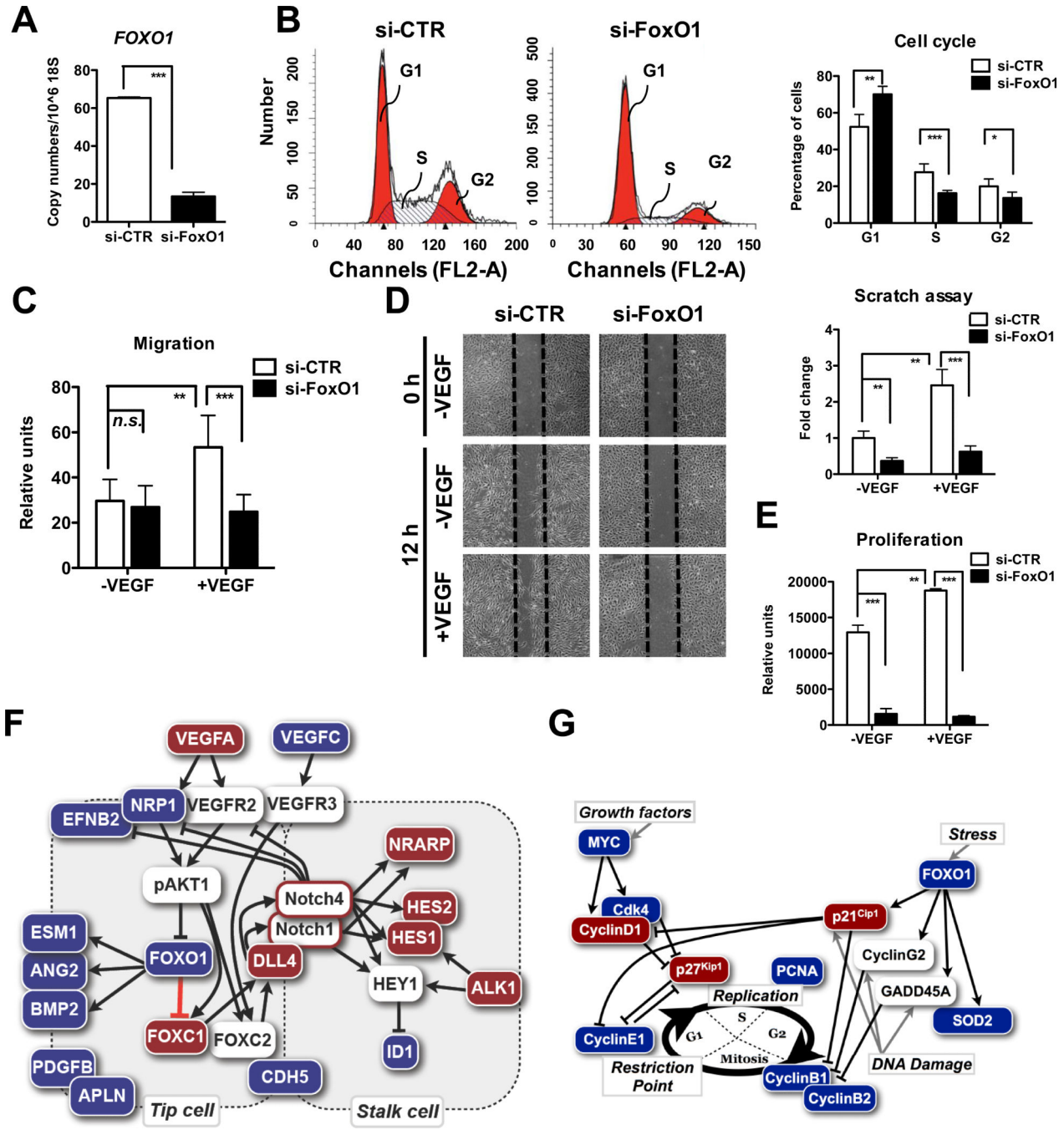

Figure 4. FoxO1 knockdown leads to G1 growth arrest in cultured endothelial cells

HUVEC were transfected with si-FoxO1 FoxO1-#1 (si-FoxO1) or control siRNA (si-CTR). **A,** FoxO1 mRNA expression was evaluated by qPCR and normalized to 18S (n=3). **B,** Cell cycle was assessed by propidium iodide staining followed by FACS analysis (n=3). Migration was assessed by modified Boyden chamber (n=3) (**C**) and scratch wound assay (**D**) in HUVEC treated in the absence or presence of 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

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based on qPCR data (shown in Supplemental Fig. 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, *NRP1*), while expression of Notch target genes is increased (*HES1*, *HES2*, *DLL4*, *NRARP*). 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 in si-FoxO1 treated vs. si-CTR treated HUVEC; red border: increased Notch activity). **G,** Regulatory map of genes involved in cell cycle based on qPCR data (shown in Supplemental Fig. VI). siRNA against FoxO1 blocks HUVEC from entering the cell cycle: expression of $p27^{Kip1}$ and $p21^{Cip1}$ is increased; *MYC* and *CDK4* expression is decreased. Although repression of Cyclin D1 (*CCND1*) by FoxO1 is relieved, Cyclin E1 (*CCNE1*), proliferating cell nuclear antigen (*PCNA*), and Cyclin B1/2 (*CCNB1/2*) are significantly decreased, indicating that cells do not cycle through division (red / blue / white background: significant increase / significant decrease / no significant change). A-E, Data are presented as mean+/−SD. n.s., non significant; *p<0.05; **p<0.01, ***p<0.001.

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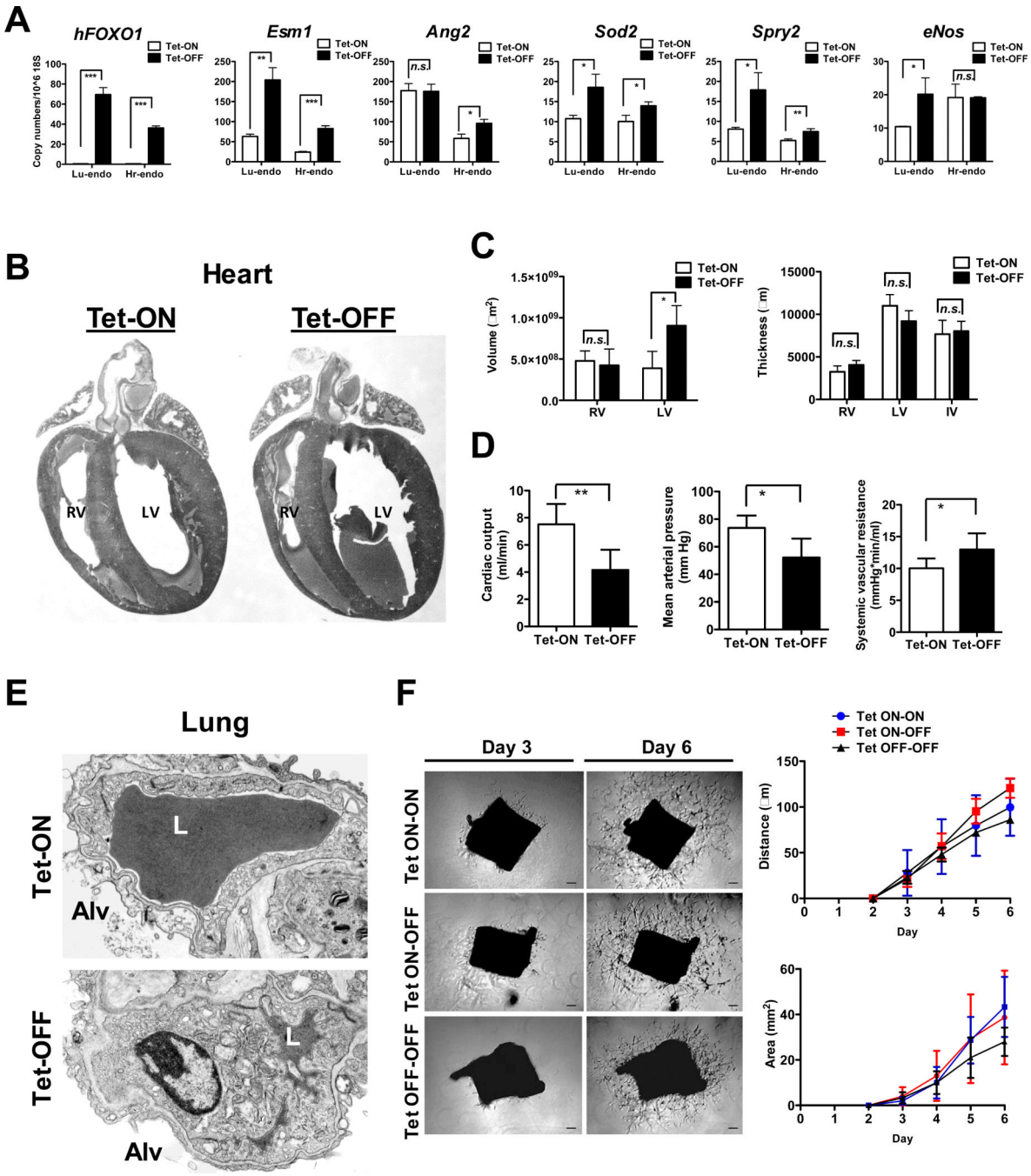

Figure 5. Overexpression of TM-FoxO1 leads to cardiac dysfunction

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) 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

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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 lumen (L). A large immature nucleus is seen in the Tet-OFF endothelial cell. Alv, alveolar air space. **F,** Aortic ring assay. Shown are the sprouting distance and sprouting area over indicated time points following 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, D, Data are presented as mean +/−SD. n.s., non significant; *p<0.05; **p<0.01; ***p<0.001. Scale bar, 500 μm (B) and 200 μm (F). IV, interventricular.

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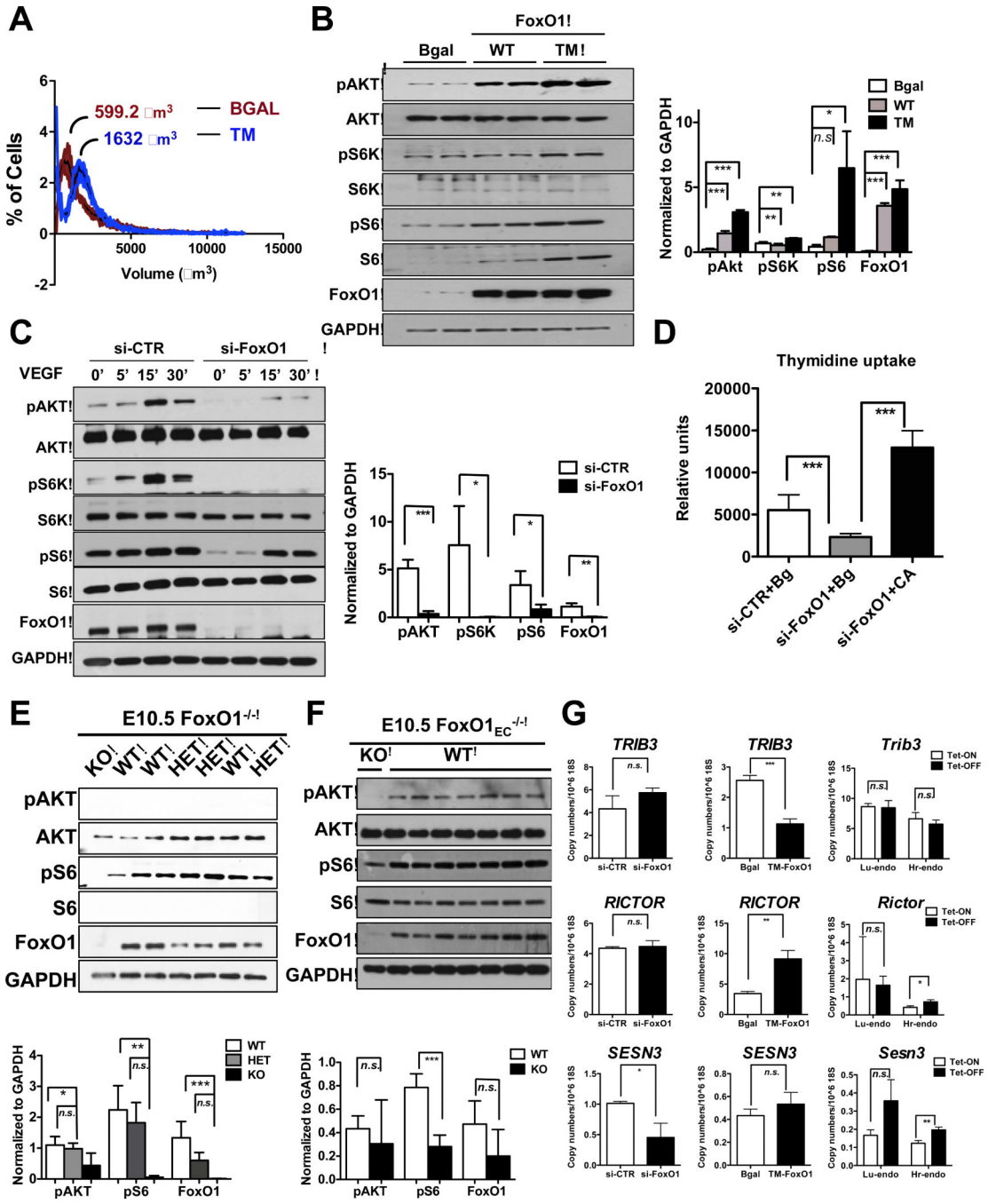

Figure 6. FoxO1 regulates the Akt-mTOR pathway

A, Cell size of HUVEC infected with Ad-FoxO1-TM (TM) or Ad-b-gal (Bgal) as a control (n=3). **B,** Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6K (pS6K), total S6K, phospho-S6 (pS6), total S6, FoxO1 and GAPDH (as loading control) in HUVEC infected with Ad-b-gal (Bgal), Ad-FoxO1-WT (WT) or Ad-TM-FoxO1 (TM). Shown is a representative Western blot (*left*) and quantitation of 3 independent experiments (*right*). **C,** Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6K (pS6K), total S6K, phospho-S6 (pS6), total S6, FoxO1 and GAPDH (as loading control) in HUVEC transfected

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with si-CTR or si-FoxO1 siRNA and treated with 50 ng/ml VEGF for the times indicated. Shown is a representative Western blot (*left*) and quantitation of si-CTR vs. si-FoxO1 at 15 min of VEGF treatment (n=3) (*right*). Additional quantitation is shown in Supplemental Fig. XIVB. **D,** Thymidine uptake in HUVEC transfected with si-CTR or si-FoxO1 siRNA and infected with Ad-b-gal (Bg) or constitutively active Akt (CA). **E,** Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6 (pS6), total S6, FoxO1 and GAPDH (as loading control) 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 (*top*) and quantitation from 3 independent litters (*bottom*). **F,** Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-S6 (pS6), total S6, FoxO1 and GAPDH (as loading control) in E10.5 $FoxO1_{EC}^{-/-}$ (KO) and $FoxO1_{EC}^{+/+}$ (wild type, WT) littermate embryos. Shown is a representative Western blot of a single litter (*top*) and quantitation from 3 independent litters (*bottom*). **G,** qPCR analysis of *TRIB3*, *RICTOR* and *SESN3* in HUVEC transfected with si-CTR or si-FoxO1 or infected with Ad-b-gal (Bg) or TM-FoxO1 (normalized to 18S, n=3). Data are presented as mean +/−SD. n.s., non significant; *p<0.05; **p<0.01; ***p<0.001.

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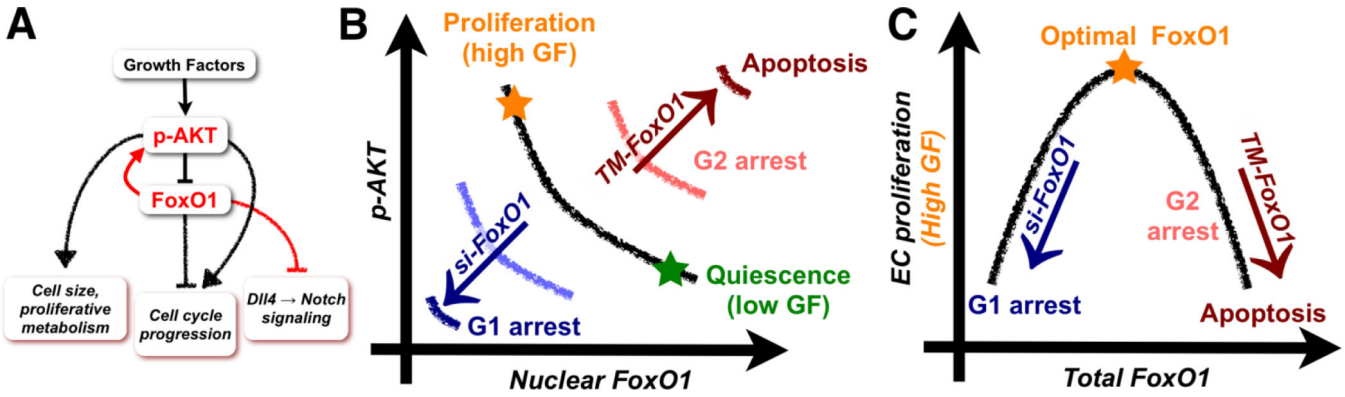

Figure 7. Model of homeostatic feedback between FoxO1 and p-Akt

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. For a comparison with FoxO3, which does not feed back negatively on Akt, see Fig. XVI, online-only Data Supplement.