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Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization

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Forkhead box O (Foxo) transcription factors are emerging as critical transcriptional integrators among pathways regulating differentiation, proliferation, and survival, yet the role of the distinct Foxo family members in angiogenic activity of endothelial cells and postnatal vessel formation has not been studied. Here, we show that Foxo1 and Foxo3a are the most abundant Foxo isoforms in mature endothelial cells and that overexpression of constitutively active Foxo1 or Foxo3a, but not Foxo4, significantly inhibits endothelial cell migration and tube formation in vitro. Silencing of either Foxo1 or Foxo3a gene expression led to a profound increase in the migratory and sprout-forming capacity of endothelial cells. Gene expression profiling showed that Foxo1 and Foxo3a specifically regulate a nonredundant but overlapping set of angiogenesis- and vascular remodeling–related genes. Whereas angiotropin 2 (Ang2) was exclusively regulated by Foxo1, eNOS, which is essential for postnatal neovascularization, was regulated by Foxo1 and Foxo3a. Consistent with these findings, constitutively active Foxo1 and Foxo3a repressed eNOS protein expression and bound to the eNOS promoter. In vivo, Foxo3a deficiency increased eNOS expression and enhanced postnatal vessel formation and maturation. Thus, our data suggest an important role for Foxo transcription factors in the regulation of vessel formation in the adult.

Introduction

The forkhead box O (Foxo) subclass of transcription factors is evolutionary conserved and plays an important role in the control of cell and organismal growth, development, metabolism and longevity. These forkhead or winged helix proteins are structurally related transcriptional regulators, of which the mammalian Foxo members Foxo1 (Fkhrl), Foxo3a (Fkhrl-1), and Foxo4 (Afx) represent a subfamily that is regulated by growth factor–dependent activation of the PI3K pathway (1–3). Foxo proteins are involved in various cellular processes ranging from programmed cell death and cell cycle progression to stress detoxification. At the organismal level, Foxo transcription factors play a crucial role in the regulation of tissue homeostasis in organs such as the pancreas and the ovaries and complex diseases such as diabetes and cancer (4–8). Activation of the PI3K pathway blocks the function of all 3 Foxo members by Akt-dependent phosphorylation of 3 conserved residues, which leads to inhibition of DNA binding, nuclear exclusion, and subsequent sequestration in the cytoplasm (9–11). Dephosphorylation of Foxo factors in turn stimulates nuclear entry, leading to the activation or repression of apoptosis- and cell cycle–related genes such as Bim, p27 Kip1, Mnsod, or Gadd45 (12–15).

Nonstandard abbreviations used: Ang1, angiopoietin 1; ChIP, chromatin immunoprecipitation; DBE, DNA-binding element; EBM, endothelial basal medium; ELK-3, ETS domain protein Elk-3, Foxo, forkhead box O; HUVEC, human umbilical vein endothelial cell; siRNA, small interfering RNA.

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Despite redundant functions of Foxo proteins in vitro, their in vivo roles in development and physiology are diverse, and genetic loss of the distinct Foxo isoforms results in different phenotypes. For example, mice homozygous for a Foxo1+/− allele, but not Foxo3a+/− or Foxo4+/− mice, die during embryogenesis from defects in vascular development (16, 17). Although these studies suggest an essential role of Foxo1 in the formation and maturation of the nascent vasculature, relatively little is known about the function and significance of the distinct Foxo family members for the angiogenic activity of endothelial cells and postnatal vessel formation. In mature endothelial cells, inhibition of Foxo1 activity has been shown to be an important mechanism through which angiotropin 2 (Ang1) modulates endothelial function (18). In addition, overexpression of Foxo3a enhances apoptosis via downregulation of the antiapoptotic protein flice-like inhibitory protein (19) and blocks growth factor–induced proliferation of endothelial cells (20). In endothelial progenitor cells, Foxo4 has been recently shown to play a pivotal role in the regulation of apoptosis by upregulating the proapoptotic gene Bim (21).

Here, we demonstrate that the Foxo transcription factors Foxo1 and Foxo3a, but not Foxo4, are critical regulators of endothelial sprout formation and migration in vitro. Gene expression profiling showed that endogenous Foxo1 and Foxo3a specifically regulate a nonredundant but overlapping set of angiogenesis- and vascular remodeling–related genes, including eNOS, which is essential for postnatal neovascularization. In vivo, Foxo3a deficiency increased eNOS expression and enhanced vessel formation and maturation in 2 models of postnatal neovascularization. Thus, our data suggest an important role for Foxo transcription factors in the regulation of vessel formation in the adult.
Results

Foxo1 and Foxo3a are the predominant Foxo factors in endothelial cells. To investigate the role of Foxo transcription factors in the regulation of endothelial cell function, we first assessed the expression profile of the Foxo isoforms Foxo1, Foxo3a, and Foxo4 in endothelial cells. Microarray analysis of total RNA isolated from human umbilical vein endothelial cells (HUVECs) showed that Foxo1 and Foxo3a are the most abundant Foxo isoforms expressed, whereas Foxo4 expression was comparably low (Figure 1A). Western blotting of cell extracts confirmed the presence of Foxo1 and Foxo3a in endothelial cells (Figure 1B). Moreover, treatment of HUVECs with VEGF led to time-dependent phosphorylation of Foxo1 and Foxo3a; this was prevented by preincubation with the PI3K inhibitor LY294002, which suggests that Foxos are not only expressed but also regulated by physiologically important stimuli (Figure 1, C, and D).

Foxo1 and Foxo3a inhibit endothelial tube formation and migration. To characterize the importance of Foxo1 and Foxo3a for endothelial cell function, we examined whether transfection of HUVECs with constitutively active constructs of Foxo1 and Foxo3a influences endothelial tube formation and migration. Therefore, we used the constitutively active Foxo constructs lacking the 3 Akt-dependent phosphorylation residues (Foxo A3), which are rendered resistant toward Akt-dependent inhibition (Figure 2A). Overexpression of constitutively active Foxo1 (Foxo1 A3) and Foxo3a (Foxo3a A3) led to a significant impairment of tube-forming activity (Figure 2, C–E) and VEGF-induced endothelial cell migration (Figure 2E). In contrast, transfection of constitutively active Foxo4 had no effect on either tube formation or endothelial cell migration (Figure 2, C and D), despite efficient expression (Figure 2A) and comparable activity on a forkhead responsive element–driven luciferase construct with 6 repeated binding sites (6xDBE) (Figure 2B).

Conversely, silencing of endogenous Foxo1 or Foxo3a gene expression using small interfering RNA (siRNA) significantly increased endothelial migration and tube formation in the Matrigel assay (Figure 3, A–D). Western blotting confirmed the efficient and specific suppression of Foxo1 and Foxo3a by the respective siRNA oligonucleotides (Figure 3A). As a control for the specificity of this approach, a second Foxo1- and Foxo3a-specific siRNA was generated and gave identical results in terms of migration and tube formation in the Matrigel assay (Figure 3, A–C and data not shown).

To further substantiate a role of Foxo1 and Foxo3a in angiogenic activity of endothelial cells, we employed a 3-dimensional spheroid system of endothelial differentiation and in vitro angiogenesis. Therefore, Foxo1 or Foxo3a siRNA–transfected endothelial cell spheroids were embedded in collagen gels, and outgrowth of capillary-like structures was assessed. Consistent with the enhanced tube formation in the Matrigel assay, silencing of Foxo1 or Foxo3a enhanced the sprouting activity of the endothelial spheroids (Figure 3E), which indicates that endogenous Foxo1 and Foxo3a are functionally important for the angiogenic activity of mature endothelial cells.

Transcriptome analysis of Foxo-regulated genes in endothelial cells. In order to identify Foxo-regulated genes that might mediate the effect of endogenous Foxos on angiogenesis, we analyzed the expression profile of isolated mRNA in Foxo1- and Foxo3a-silenced endothelial cells by microarray technology. HUVECs were transfected with Foxo1- and Foxo3a-specific siRNAs or the respective scrambled control, and total RNA was isolated after 24 hours. In this set of experiments, knockdown of Foxo1 and Foxo3a expression resulted in changes in expression of known Foxo target genes that were in accordance with previously described functions of Foxo (Table 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI3216DS1).

In addition to those of known Foxo target genes, we observed changes in expression of several angiogenesis- and vascular remodeling–related genes. In particular, Foxo1 and Foxo3a shared a subset of target genes with important vascular functions. Among these, we identified eNOS and the proangiogenic transcription factor ETS domain protein Elk-3 (ELK-3, also known as Net) as Foxo-regulated genes that were upregulated in Foxo-silenced endothelial cells (Table 1 and Figure 4, A and B). Silencing of Foxo1 or Foxo3a additionally downregulated signaling molecules, including bone morphogenic protein 4 (BMP4), chemokine (C-X-C motif) receptor 4 (CXCR4), stabilin-1, and PDGF, β polypeptide (PDGF-B) (Table 1).
Evidently, there were also apparent differences in the respective transcriptional profiles. Thus, Ang2 was significantly downregulated in Foxo1-silenced endothelial cells (18) but was unaffected in Foxo3a siRNA–transfected endothelial cells (Table 1). Likewise, several other angiogenesis- and vascular remodeling–related genes such as MMP10 or the transcription factor inhibitor of DNA binding 2 (ID2) were regulated by Foxo1 siRNA transfection but not in Foxo3a-silenced endothelial cells. This differential regulation also involved known Foxo target genes implicated in the well-established proapoptotic function of Foxo factors (22–25), which suggests a specific and unique role of each Foxo isoform in the endothelium. For example, knockdown of Foxo1 resulted in enhanced transcription of the proapoptotic genes Bim (0.44-fold reduction) and TNF-related apoptosis-inducing ligand (TRAIL) (0.6-fold reduction), whereas silencing of Foxo3a was associated with reduced expression of the proapoptotic genes Bim (0.44-fold reduction) and TNF-related apoptosis-inducing ligand (TRAIL) (0.6-fold reduction).

The changes in gene expression in response to Foxo siRNA transfection of the target genes eNOS, Ang2, and ELK-3, which might contribute to the antiangiogenic activity of Foxos in endothelial cells, was additionally confirmed by Western blotting (Figure 4B). Taken together, our data show that both Foxo transcription factors Foxo1 and Foxo3a regulate distinct but overlapping sets of angiogenesis- and vascular remodeling–related genes, which supports an important and unique role of Foxo factors in the vascular endothelium.

**Foxo-dependent regulation of eNOS expression.** Since eNOS is a novel Foxo target that is essential for endothelial function and postnatal neovascularization (26–28), we further elucidated Foxo-dependent regulation of eNOS expression.

Overexpression of both Foxo1 A3 and Foxo3a A3 significantly decreased eNOS protein expression (Figure 5A). Consistent with these results, eNOS protein expression was significantly upregulated in the Foxo1 and Foxo3a siRNA–transfected endothelial cells (Figure 5B). Moreover, compared with that in wild-type mice, eNOS protein expression was enhanced in explanted aortas of Foxo3a Δ/Δ mice (Figure 5C), which suggests that eNOS is a physiologically relevant Foxo target gene in vivo.

Promoter analysis revealed that the eNOS promoter contains a conserved optional forkhead-responsive element (FHRE) at position –2,753 relative to the start codon (TTGTTTAC) (29). Chromatin immunoprecipitation (ChIP) assays showed that Foxo1 and Foxo3a bind to the eNOS promoter region encompassing this DNA binding element (Figure 5D). However, no binding was detected when exon-specific primers were used (Figure 5D), which confirmed the specificity of the Foxo/eNOS promoter interaction. Additionally, overexpression of Foxo1 A3 inhibited
the basal transcriptional activation of the eNOS promoter as assessed by a reporter gene assay using a 3,500-bp fragment of the human eNOS gene (Figure 5E).

Foxo3a is a modulator of postnatal neovascularization in vivo. In order to determine the in vivo significance of these findings, we assessed the neovascularization capacity after inducing hind limb ischemia in genetically deficient mice. Since Foxo1−/− mice die during embryogenesis (16, 17), we used Foxo3a−/− mice. As shown in Figure 6, Foxo3a−/− mice had significantly increased limb perfusion 14 days after the induction of ischemia compared with wild-type Foxo3a (Foxo3a+/+) mice (Figure 6A). The enhanced recovery of blood flow was associated with an increased capillary density in Foxo3a−/− mice after hind limb ischemia (Figure 6B and Supplemental Figure 2). Consistent with these findings, the number of small-sized (<50 µm) actin-positive vessels was significantly increased in Foxo3a−/− versus wild-type mice (Figure 6C), which suggests that Foxo factors are involved not only in the formation but also in the maturation and stabilization of the nascent vasculature. To further validate the in vivo relevance of Foxo3a for postnatal neovascularization, we investigated the effect of Foxo3a deficiency in a Matrigel plug assay. Matrigel implants were administered subcutaneously to wild-type and Foxo3a−/− mice, and blood vessel infiltration of the

Figure 3
Silencing Foxo1 or Foxo3a activity enhances the angiogenic activity of endothelial cells. (A) HUVECs were transfected with 2 different siRNAs targeted against either Foxo1 (Foxo1 I and Foxo1 II) or Foxo3a (Foxo3a I and Foxo3a II). A nonrelated scrambled siRNA was used a control. Cell lysates were subjected to Western blotting using antibodies against Foxo1 and Foxo3a. Tubulin was used a loading control. (B and C) HUVECs were transfected with 2 different siRNAs targeted against Foxo1, Foxo3a, or a scrambled oligonucleotide. After 18 hours, cells were seeded on a Growth Factor Reduced Matrigel in the presence of VEGF (50 ng/ml). Cumulative sprout length of capillary-like structures was measured by light microscopy after 24 hours. Representative micrographs and statistical summary are shown. Data are presented as mean ± SEM; n = 4 (Foxo1), n = 6 (Foxo3a). *P < 0.001 versus control. (D) Representative micrographs and statistical summary of endothelial cells transfected with siRNAs targeted against Foxo1, Foxo3a, or scrambled control. After 18 hours, cells were seeded in the upper chamber of modified Boyden chamber. Endothelial cell migration was assessed using VEGF (50 ng/ml) as chemoattractant. After 24 hours, nonmigrating cells on the upper side of the chamber were mechanically removed, and the remaining cells on the lower side were fixed and stained with DAPI. Data are presented as mean ± SEM; n = 5. *P < 0.001 versus control. (E) Three-dimensional in vitro angiogenesis with collagen gel–embedded spheroids of Foxo1, Foxo3a, or scrambled siRNA–transfected endothelial cells. Cumulative length of all sprouts originating from an individual spheroid was quantified after 24 hours. Statistical summary represents the mean ± SEM; n = 3. **P < 0.05 versus control. Magnification: ×50 (B); ×200 (D); ×100 (E).
implants was quantified. Compared with wild-type mice, Foxo3a−/− mice had a significantly elevated number of vessels infiltrating the Matrigel (Figure 6D). In summary, these data indicate that Foxo factors are important negative regulators of postnatal vessel formation and maturation in vivo and that a loss of Foxo function augments postnatal neovascularization capacity.

**Discussion**

Forkhead transcription factors of the Foxo family function as important transcriptional integrators of several signaling cascades, thereby serving as a transcriptional endpoint for pathways regulating differentiation, cell growth, and survival. However, the role of Foxo family members in the regulation of postnatal neovascularization has not been defined. This study identifies Foxo transcription factors as important modulators of vessel formation and maturation in the adult, thus providing evidence for a central function of Foxo factors in vascular homeostasis.

We show that Foxo1 and Foxo3a are the predominant Foxo isoforms expressed in mature endothelial cells and that overexpression of a gain-of-function mutant of Foxo1 or Foxo3a significantly inhibits endothelial cell migration and tube formation in vitro. Accordingly, knockdown of either Foxo1 or Foxo3a gene expression using siRNA led to a profound increase in endothelial sprout formation and migration. Notably, overexpression of a constitutively active Foxo4 had no effect on either migration or sprout formation, which indicated a specific regulatory effect of Foxo1 and Foxo3a as opposed to Foxo4.

The formation and maturation of the vasculature is a complex process involving multiple gene products all contributing to an integrated sequence of events. Consistent with an important role of Foxo transcription factors in these processes, our study identified a number of novel Foxo target genes involved at different stages of angiogenesis. Moreover, direct comparison of gene expression changes in Foxo1−/− and Foxo3a−/− endothelial cells revealed that both transcription factors appear to regulate a common transcriptional program important for the angiogenic activity and maintenance of vascular homeostasis, thereby providing a mechanistic basis for the functional overlapping roles of Foxo1 and Foxo3a in this study. Among these genes, we identified eNOS as a novel Foxo target gene that was significantly upregulated in Foxo1−/− and Foxo3a−/− silenced endothelial cells. Several other genes with important vascular functions, such as PDGF-B, BMP4, and ELK-3 (Net), were also regulated by Foxo1 or Foxo3a gene silencing. However, there were also gene expression changes that do not sup-

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HUVECs were transfected with Foxo1- or Foxo3a-specific siRNA and a scrambled oligonucleotide siRNA (each n = 3). Total RNA was isolated after 24 hours and the gene expression profile was assessed with the Affymetrix gene chip. The table lists a selection of angiogenesis- and vascular remodeling–related genes as well as known Foxo target genes whose RNA level changed by more than 1.5 fold up or down.

| Table 1 |
| Genes regulated by Foxo1 or Foxo3a in endothelial cells |
port the proangiogenic phenotype of the Foxo-silenced endothelial cells, such as a reduction in mRNA levels of kinase insert domain receptor (KDR, or FLK-1) and CXCR4.

Transcriptional repression of eNOS by Foxo1 and Foxo3a, as shown by our data, might particularly contribute to the antiangiogenic effects of Foxo factors in endothelial cells. Moreover, that eNOS expression is enhanced in Foxo3a−/− mice indicates that eNOS is a Foxo-regulated gene in vivo, which adds to the proangiogenic phenotype of the Foxo3a−/− mice. Indeed, NO synthesized by the eNOS is essential for endothelial cell survival, migration (30, 31) and postnatal neovascularization (26–28). Although eNOS was initially defined as a constitutively expressed enzyme, recent studies highlight the contribution of both transcription and RNA stabilization to the expression level of eNOS mRNA (32, 33). Evidence for a direct effect of Foxo1 and Foxo3a on eNOS transcription was obtained by ChIP, which revealed that both Foxo factors bind to a conserved FHRE in the eNOS promoter located at −2,765 bp upstream of the start codon (29). Likewise, constitutively active Foxo1 significantly inhibited basal promoter activity of a reporter gene driven by the human eNOS promoter. Interestingly, Foxo4 neither bound to the eNOS promoter nor affected eNOS promoter activity (Figure 5D and data not shown), which supports the functional diversification of Foxo proteins in endothelial cells.

Our data show that, in addition to regulating eNOS, Foxo1 and Foxo3a also share several other target genes implicated in vessel formation and stabilization. For instance, ELK-3, which is an ETS ternary complex transcription factor, was significantly increased in Foxo1 and Foxo3a siRNA–treated endothelial cells. ELK-3 has recently been described to be expressed at sites of angiogenesis and vasculogenesis (34) and has been demonstrated to promote the angiogenic switch (35). Although further studies are necessary to confirm the direct transcriptional suppression of ELK-3 by Foxo factors, ELK-3 contains a conserved, inverse Foxo binding site (GTAAACAA) in the proximal promoter region that might be targeted by Foxos.

Despite these overlaps, there were also apparent differences in the respective transcriptional profiles of Foxo1 and Foxo3a. These included known apoptosis-related Foxo target genes such as Bim, TRAIL, and survivin as well as angiogenesis- and vascular remodeling–related genes such as Ang2 and MMP10. For example, Ang2,
which has recently been described to be an important target gene of Foxo1 in endothelial cells (18), was significantly downregulated in Foxo1-silenced cells but remained essentially unchanged in Foxo3a siRNA–transfected endothelial cells. By binding but not activating Tie-2, Ang2 is thought to block a stabilizing signal provided by Ang1, thus contributing to vessel instability and postnatal remodeling (36–38). The downregulation of Ang2 in Foxo1 siRNA–treated endothelial cells might therefore affect vessel stabilization. Likewise, the changes in expression of several extracellular matrix proteins such as collagen and MMPs indicate that Foxos might be involved in regulating vessel remodeling as well.

A similar role for Foxo1 as a critical regulator of vascular maturation and remodeling has recently been shown by Daly and colleagues, who investigated the role of Foxo1 as transcriptional...
Foxo3a modulates neovascularization capacity in vivo. (A) Foxo3a+/+ and Foxo3a−/− mice were subjected to hind limb ischemia, and perfusion was assessed 14 days after onset of ischemia using laser Doppler imaging. Low or no perfusion is shown as dark blue, whereas the highest perfusion level is shown as red. Arrows indicate the ischemic leg. Quantitative results are presented as mean ± SEM; n = 8. *P = 0.002. (B) Capillary density (ratio of the number of capillaries to the number of myocytes) was determined in 8-µm frozen sections of the adductor and semimembranous muscles. Quantitative results are presented as mean ± SEM; n = 8 (Foxo3a+/+), n = 7 (Foxo3a−/−). (C) Conductance vessels in the adductor and semimembranous muscles were identified by size (>20 µm) and smooth muscle actin staining using a Cy3-labeled mouse monoclonal antibody for smooth muscle actin. The number of small (<50 µm), medium (50–100 µm), and large vessels was determined separately. Data are presented as mean ± SEM; n = 6 (Foxo3a+/+), n = 5 (Foxo3a−/−). *P = 0.01. (D) Statistical summary and representative micrographs of blood vessel infiltration in Matrigel sections stained with a smooth muscle actin antibody in wild-type and Foxo3a−/− mice. Quantitative results are presented as mean ± SEM; n = 7 (Foxo3a+/+), n = 8 (Foxo3a−/−). Scale bars in C and D, 100 µm.

In view of the fact that vascular development of the Foxo1−/− mice is severely impaired, which indicates that disruption of Foxo1 leads to defective embryonic angiogenesis, the finding that Foxo1 has an entirely opposite effect on mature endothelial cells is intriguing (16, 17). Although the reasons underlying this seemingly opposing role of Foxo1 in embryonic versus adult vessel formation are unclear, one could presume that the molecular signals and mechanisms that govern the activity and specific transcriptional output of the Ang1/Akt axis in endothelial cells (18). Likewise, some of our transcriptional profiling data are consistent with this study. Nevertheless, there were also significant differences in the respective data sets, which might be related to the different experimental strategies used in the 2 studies to identify Foxo1-regulated genes in endothelial cells. In fact, Daly and colleagues used a constitutively active Foxo1 adenovirus to analyze the transcriptional changes in endothelial cells, whereas our study used a gene-silencing approach. Indeed, many of the Foxo1-regulated genes described by Daly et al. are expressed at very low levels under basal cell culture conditions, which makes it difficult to detect any decrease in expression that results from siRNA-mediated transient downregulation. Likewise, knockdown of Foxo1 would be expected to primarily affect expression of target genes, whose basal expression level critically depends on Foxo1 activity. We found several Foxo1-regulated genes described by Daly and coworkers, including Ang2, ID2, and survivin, which suggests that functional Foxo activity is important for the basal expression of these genes (Supplemental Figure 1).

The physiological significance of Foxo factors in the vasculature is underscored by recent work showing that Foxo1 deficiency is embroyonically lethal due to defects in vascular development (16, 17). In contrast, Foxo3a−/− mice are viable, which suggests that this isoform is not essential for embryonic vascular development (5). Nevertheless, Foxo3a−/− mice showed a significantly augmented neovascularization capacity in 2 models of postnatal vessel formation. This phenotype is reminiscent of that of the eNOS−/− mice, which do not show an embryonal developmental defect but have profoundly impaired adult neovascularization, which indicates that embryonic vascular development and postnatal vascularization are not necessarily mediated by the same signals (26–28). The question remains why Foxo1−/− mice exhibit a phenotype distinct from that of Foxo3a−/− mice, although both transcription factors show similar effects in mature endothelial cells. There may be a variety of reasons for the apparent differences in phenotype, such as spatiotemporal changes in Foxo isoform expression, which has been shown to occur during adipocyte progenitor differentiation (39). Additionally, given that transcriptional profiling experiments revealed important nonredundant functions of Foxos in regulation of target genes, it is tempting to speculate that such differential regulation of genes with crucial vascular functions might contribute to the differences in the phenotype of the knockout mice. Since all Foxo factors utilize the same forkhead responsive element (29), it will be therefore of interest to identify factors that determine the specific biological output of Foxo signaling in endothelial cells.
of Foxo1 in embryonic vascular development are not necessarily functional during postnatal vessel formation. For instance, Ang2 is an important Foxo1 target gene in mature endothelial cells but was essentially unchanged in Foxo1−/− embryonic stem cell–derived endothelial cells (17, 18).

Taken together, these findings suggest that Foxo transcription factors play important and unique roles in the vascular endothelium by functioning as important transcriptional integrators of multiple downstream signaling molecules important for vessel formation and maturation. Since downregulation of Foxo3a enhanced neovascularization in vivo, the regulation of the expression and activity of Foxo transcription might evolve as a potential target for modulating therapeutic angiogenesis and revascularization after critical ischemia in the adult. In contrast, forced expression or activation of Foxos might limit unwanted neovascularization—as in the case of tumor angiogenesis—thereby revealing another aspect of the well-known antitumorigenic activity of Foxos (3).

Methods

Cell culture. Pooled HUVECs were purchased from CellSystems and cultured in endothelial basal medium (EBM; Cambrex) supplemented with hydrocortisone (1 µg/ml), bovine brain extract (12 µg/ml), gentamicin (50 µg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% FCS (Invitrogen Corp.) until the third passage. After detachment with trypsin, cells were grown in 6-cm culture dishes for at least 18 hours.

Plasmid constructs and transfection. HUVECs (3.5 × 10⁶ cells/6-cm well) were grown to 60–70% confluence and then transfected with 4 µg of plasmids. Plasmids included constitutive active Foxo1 A3 (pDNA3-Flag Foxo1 A3), constitutively active Foxo3a A3 (pECE-HA Foxo3a A3), and constitutively active Foxo4 A3 (pMT2-HA Foxo4 A3). The Foxo1 construct was described previously (12) and was kindly provided by William R. Sellers (Dana-Farber Cancer Institute). The Foxo3a plasmid and the pGL3-6xDBE reporter construct were from Paul Coffer (University Medical Center, Utrecht, Netherlands), and the Foxo4 construct was a gift from Boudewijn M.T. Burgering (University Medical Center, Utrecht, Netherlands) (10).

Transfection was performed using the GeneTrans II reagent (MoBiTec) according to the manufacturer's protocol. Cells were incubated with the DNA transfection reagent complexes at 37°C for 4 hours, and this was followed by recovery in the presence of 10% FCS. Transfection efficiency was approximately 40% as determined using green fluorescent protein, and maximal transfection efficiency was achieved overnight, after which they were embedded into collagen gels. The cell transfection efficiency was quantified after 24 hours by measuring the cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program KS300 3.0 (Zeiss).

Tube formation assays. HUVECs (1 × 10⁵) were cultured in a 12-well plate (Greiner Bio-One) coated with 200 µl Matrigel Basement Membrane Matrix or Growth Factor Reduced Matrigel (BD Biosciences). Tube length was quantified after 24 hours by measuring the cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program KS300 3.0 (Zeiss).

Spheroid-based angiogenesis assay. Endothelial cell spheroids of defined cell number were generated as described previously (41). In brief, 12 hours after transfection, HUVECs were suspended in culture medium containing 0.20% (wt/vol) carboxymethylcellulose (Sigma-Aldrich) and seeded in nonadherent round-bottom 96-well plates (Greiner Bio-One). Under these conditions, all suspended cells contribute to the formation of a single spheroid per well of defined size and cell number (400 cells/spheroid). Spheroids were generated overnight, after which they were embedded into collagen gels. The spheroid-containing gel was rapidly transferred into prewarmed 24-well plates and allowed to polymerize (30 minutes), after which 100 µl EB M was added on top of the gel. After 24 hours, in vitro angiogenesis was quantified by measuring the cumulative length of the sprouts that had grown out of each spheroid using digital imaging software (AxioVision 3.1; Zeiss), with 10 spheroids analyzed per experimental group and experiment.

ChIP assay. We cross-linked HUVECs (1 × 10⁵) for 10 minutes by directly adding 1% formaldehyde to the culture medium. The fixed cells were lysed with lysis buffer (1% Triton X-100, 0.32 mol/l sucrose, 5 mmol/l EDTA, 1 mmol/l PMSF, 1 µg/ml leupeptin, 10 mmol/l Tris/HCl, pH 8.0) and sonified 5 times for 10 seconds with output 5 (Branson Sonifier 450; Branson). For ChIP, cell lysates were incubated with an antibody against Flag (M2; Sigma-Aldrich) or HA (12CA5; Roche Diagnostics Corp.), Ang2 (1:100; R&D Systems), Elk-3 (Net A-20, 1:200; Santa Cruz Biotechnology Inc.), and tubulin (1:1,000; Labvision/Neomarkers). Enhanced chemiluminescence was performed according to the manufacturer's instructions (Amersham Biosciences).

Migration assays. To determine the migration of endothelial cells, HUVECs were detached with trypsin, harvested by centrifugation, resuspended in 500 µl EB M without supplements, counted, and placed in the upper chamber of a modified Boyden chamber (5 × 10⁶ cells per chamber; pore size, 8 µm; BD Biosciences). The chamber was placed in a 24-well culture dish containing EB M with 10% FCS and growth factors. After incubation at 37°C, the nonmigrating cells on the upper side of the chamber were mechanically removed, and the remaining cells on the lower side fixed with 4% paraformaldehyde. For quantification, cell nuclei were stained with DAPI. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields.

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sequence (forward, 5′-CCACGTAGCAGGAAAAGTCACCAT-3′ and reverse, 5′-GTCCTGGAGCCATACAGGATT-3′).

Luciferase assay.Reporter gene constructs were generated using the vector plasmid pGL3-Basic (Promega), which contains the luciferase gene, as described previously (42). pGL3-eNOS-3500 contained a 3.5-kb human eNOS promoter fragment. HUVECs were transiently transfected with 2 µg of reporter gene and 2 µg of the indicated plasmid using the TransGen II transfection reagent (MobiTec). After 24 hours of incubation, cells were lysed in passive lysis buffer (Promega), and luciferase activity was measured using the Luciferase Assay System (Promega) with a luminometer (Lumat LB 9501; Berthold).

Marine model of hind limb ischemia. The role of Foxo3a in ischemia-induced neovascularization was investigated in a murine model of hind limb ischemia using Foxo3a−/− and Foxo3a+/− mice (FVB/n), which have been described in ref. 5. The present study was performed with permission of the State of Hesse, Regierungspräsidium Darmstadt, according to section 8 of the German Law for the Protection of Animals, and conforms to the German Guide for the Care and Use of Laboratory Animals. The proximal portion of the femoral artery, including the superficial and the deep branch, and the distal portion of the saphenous artery as well as all arterial branches between the ligations were obliterated using an electrical coagulator. The overlying skin was closed using 3 surgical staples. Fourteen days after surgery, the ratio of ischemic (right) to normal (left) limb blood flow was measured using a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, moorLDI-Mark 2; Moor Instruments). Before scanning was initiated, mice were placed on a heating pad at 37°C to minimize variations in temperature. After laser Doppler color images were recorded, the perfusions of the ischemic and nonischemic limb were calculated on the basis of color-ed histogram pixels. To minimize variables, including ambient light and temperature, calculated perfusion was expressed as the ratio of ischemic to nonischemic hind limb perfusion.

Vascular histology. Capillary density was determined in 8-µm frozen sections of the adductor and semimembranosus muscles. Endothelial cells were stained for CD31 (PE-labeled; BD Biosciences). Myocyte membranes were stained using an antibody to laminin (rabbit) followed by anti-rabbit Alexa 488. Conductance vessels in the adductor and semimembranous muscles were identified by size (>20 µm) and staining using a Cy3-labeled mouse monoclonal antibody specific for smooth muscle actin (Sigma-Aldrich). The number of small (<50 µm), medium (50–100 µm), and large vessels was determined separately.

In vivo Matrigel plug assay. This assay was carried out as described (43) in wild-type and Foxo3a−/− mice using 0.5 ml of Matrigel Basement Membrane Matrix (BD Biosciences) containing 40 units/ml of heparin (Sigma-Aldrich). At 14 days, blood vessel infiltration in Matrigel pellets was quantified by analysis of smooth muscle actin–stained (Cy3-labeled; Sigma-Aldrich) sections using a Zeiss confocal microscope (LSM 510).

Statistical analysis. Data are expressed as mean ± SEM. Two treatment groups were compared by the unpaired Student’s t test; 1-way ANOVA was performed for serial analysis. P values less than 0.05 were considered statistically significant.

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