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Forkhead BoxO transcription factors restrain exercise-induced angiogenesis

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Key points

- The growth of new capillaries, angiogenesis, within skeletal muscle occurs only after weeks of repeated aerobic exercise. Paradoxically, large increases in pro-angiogenic factors such as vascular endothelial growth factor occur with a single exercise bout. The mechanisms underlying the substantial lag in the angiogenic response remain to be elucidated.
- We detected concomitant increases in the angiostatic Forkhead Box 'O' transcription factors FoxO1 and FoxO3a and the matrix protein thrombospondin-1 following a single bout of exercise, but these responses were repressed after 10 days of repeated exercise. This observation led us to hypothesize that FoxO proteins delay the initiation of exercise-induced angiogenesis.
- Endothelial cell-directed deletion of FoxO proteins abolished the increase in thrombospondin-1 following a single exercise bout, and resulted in a substantially accelerated angiogenic response.
- This study identifies an intrinsic endothelial-specific FoxO signalling pathway that opposes the onset of physiological angiogenesis within healthy exercising skeletal muscle and demonstrates that endothelial cell FoxO proteins are critical determinants of the angiogenic capacity within skeletal muscle.

Abstract The physiological process of exercise-induced angiogenesis involves the orchestrated upregulation of angiogenic factors together with repression of angiostatic factors. The Forkhead Box 'O' (FoxO) transcription factors promote an angiostatic environment in pathological contexts. We hypothesized that endothelial FoxO1 and FoxO3a also play an integral role in restricting the angiogenic response to aerobic exercise training. A single exercise bout significantly increased levels of FoxO1 and FoxO3a mRNA (5.5- and 1.7-fold, respectively) and protein (1.7- and 2.2-fold, respectively) within the muscles of mice 2 h post-exercise compared to sedentary. Training abolished the exercise-induced increases in both FoxO1 and FoxO3a mRNA and proteins, and resulted in significantly lower nuclear levels of FoxO1 and FoxO3a protein (0.5- and 0.4-fold, respectively, relative to sedentary). Thrombospondin 1 (THBS1) protein level closely mirrored the expression pattern of FoxO proteins. The 1.7-fold increase in THBS1 protein following acute exercise no longer occurred after 10 days of repeated exercise. Endothelial cell-directed conditional deletion of FoxO1/3a/4 in mice prevented the increase in THBS1 mRNA following a single exercise bout. Mice harbouring the endothelial FoxO deletion also demonstrated a significant 20% increase in capillary to muscle fibre ratio after only 7 days of training while 14 days of training was required to elicit a similar increase in wildtype littermates. Our results demonstrate that the downregulation of FoxO1 and FoxO3a proteins facilitates angiogenesis in response to repeated exercise. In conclusion, FoxO proteins can delay exercise-induced angiogenesis, and thus are critical regulators of the physiological angiogenic response in skeletal muscle.

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Abbreviations C/F, capillary to muscle fibre ratio; FoxO3a, Forkhead BoxO3a; FoxO1, Forkhead BoxO1; HPRT1, hypoxanthine-guanine phosphoribosyltransferase; Mx1, Myxovirus (influenza) resistance 1/interferon-inducible protein p78; qPCR, quantitative polymerase chain reaction; THBS1, thrombospondin-1; VEGFA, vascular endothelial growth factor A.

Introduction

The integrity and stability of the capillary network is tightly regulated, which is achieved by establishing a homeostatic balance between factors that promote and repress capillary growth. A disruption of this balance to favour a pro-angiogenic environment will drive the processes of endothelial cell proliferation, proteolysis and migration that underlie the formation of new capillaries. Exercise training provides one of the few situations in the adult that results in physiological angiogenesis. Exercise places an increased demand within the skeletal muscle for nutrients and oxygen. Repeated exposure to this stressor induces adaptations within the muscle, including expansion of the capillary network through the angiogenic process, which serves to increase the surface area available for exchange (Hudlicka *et al.* 1992; Bloor, 2005; Gustafsson, 2011). This compensatory adaptation is essential for maintaining an individual's optimal muscle function, but it may be most critical in people who have an insufficient lower limb blood supply. In peripheral artery disease patients, muscle capillary density has been shown to correlate closely with exercise tolerance; furthermore, a training programme can lead to increases in both capillary density and exercise capacity (Duscha *et al.* 2011; Robbins *et al.* 2011). Consequently, knowledge of the regulatory controls that dictate the progression of exercise-induced angiogenesis is critical for developing a thorough understanding of this physiological process as well as how it may be disrupted under pathological conditions.

Considerable emphasis has been placed on understanding the regulation and role of the pro-angiogenic factors in response to exercise training. A single exercise bout provokes a rapid increase in vascular endothelial growth factor A (VEGFA), with secretion of pre-packaged VEGFA from skeletal myocytes (Høier *et al.* 2010, 2013), the potential cleavage and release of heparin-bound isoforms of VEGFA (Rullman *et al.* 2007) and increases in VEGFA mRNA within skeletal muscle, which also remain elevated after repeated exercise bouts (Breen *et al.* 1996; Gustafsson *et al.* 1999, 2007; Richardson *et al.* 1999, 2000; Birot *et al.* 2003). The production and release of VEGFA from endothelial cells in response to muscle activity and shear stress (Milkiewicz *et al.* 2001; Gee *et al.* 2010) provides an additional avenue through which endothelial cells are exposed to elevated levels of this potent pro-angiogenic factor. In contrast, increased muscle capillarization is detectable in rodents only after 10–14 days of repeated exercise, thus considerably lagging behind the initial surge of VEGFA within the capillary microenvironment. This paradox may be rationalized by the speculation that this delayed response ensures that the initiation of the energy-intensive process of capillary growth does not occur until a repeated cycle of demand is placed on the muscle tissue. However, the mechanisms underlying this delayed angiogenic response remain to be established. A plausible hypothesis is that angiostatic factors act in opposition to the angiogenic factors and prevent the initiation of an angiogenic response. For example, the angiostatic matrix protein thrombospondin-1 (THBS1) is recognized to play a significant physiological role in repressing capillary growth within skeletal muscle, as mice deficient in THBS1 are characterized by increased muscle capillarity and a concomitant increase in exercise capacity (Malek & Olfert, 2009). THBS1 mRNA levels are elevated with an acute bout of exercise, and return to basal levels after 3 days of training (Olfert *et al.* 2006). To date, there remains a substantial lack of knowledge of the mechanisms underlying the regulation of angiostatic proteins in response to exercise training, despite a growing recognition that the modulation of these angiostatic factors is instrumental in the initiation of exercise-induced angiogenesis (Olfert & Birot, 2011).

The Forkhead Box 'O' (FoxO) family of transcription factors exert influences on a broad range of cellular processes, including metabolism, apoptosis and the cell cycle (Burgering & Medema, 2003; Huang & Tindall, 2007; Papanicolaou *et al.* 2008). Within this family, FoxO1 and FoxO3a are the most abundant in endothelial cells (Potente *et al.* 2005; Paik *et al.* 2007; Roudier *et al.* 2013). FoxO1 contributes significantly to stabilization of the vascular network during embryogenesis, as embryos deficient for FoxO1 fail to form a viable vascular network (Furuyama *et al.* 2004). In the adult, the actions of FoxO1 and, to a lesser extent, FoxO3a prevent excessive growth of endothelial cells (Paik *et al.* 2007). FoxO1 and FoxO3a regulate the transcription of several factors involved in the inhibition of angiogenesis (Potente *et al.* 2005; Paik *et al.* 2007; Oellerich & Potente, 2012). FoxO1 was shown to induce the transcription of Sprouty2, which

can inhibit endothelial cell proliferation, migration and tube formation *in vitro* (Paik *et al.* 2007). Recently, we demonstrated that FoxO1 acts to restrain angiogenesis within ischaemic skeletal muscle (Milkiewicz *et al.* 2011; Roudier *et al.* 2013) and that endothelial cell production of the matricellular protein THBS1 is transcriptionally regulated by FoxO1 (Roudier *et al.* 2013).

Based on these data, we hypothesize that the down-regulation of endothelial FoxO proteins in response to repeated exercise is a key determinant of the initiation of a physiological exercise-induced angiogenic response. We provide evidence that FoxO1 and FoxO3a increase in response to a single exercise bout, but that repeated exercise training causes a substantive reduction in total, as well as nuclear, FoxO protein levels. The endothelial-directed deletion of FoxO proteins results in an accelerated capillary growth in response to exercise training. Furthermore, the burst of THBS1 expression associated with an acute exercise challenge is abolished completely in animals with endothelial-directed deletion of FoxO proteins. Our study establishes that FoxO proteins are physiological regulators of THBS1 in skeletal muscle and, importantly, that down-regulation of endothelial FoxO proteins in response to exercise is a key determinant of the capacity of the capillary network to undergo angiogenesis.

Methods

Ethical approval

Animal studies were approved by York University Committee on Animal Care, and were performed in accordance with Animal Care Procedures at York University and the American Physiological Society's Guiding principles in the Care and Use of Animals.

Acute exercise bout

Female FVB/n mice, age 9 weeks, purchased from Charles River (Saint-Constant, QC, Canada), were housed on a 12/12 h light–dark cycle with water and food available ad libitum. All mice were acclimatized to the treadmill (#91447-3; Columbus Instruments, Columbus, OH, USA) for 3 days prior to the actual training. The mice were separated randomly into groups: sedentary ($n = 5$), 1 day of training ($n = 4$), 1 day of training plus 2 h rest ($n = 4$). The mice ran on the treadmill for 60 min at a speed of 25 m min^{-1}. Sedentary mice were placed on a treadmill daily to control for handling and environment. Mice were fasted 4 h prior to their exercise bout. Mice were anaesthetized using isoflurane/oxygen inhalation either immediately after or 2 h after the acute bout of exercise. The gastrocnemius, plantaris and soleus muscles were removed, weighed and frozen in liquid nitrogen. The mice were killed by exsanguination.

Mouse model of FoxO deletion

Breeding pairs of *MxCre*$^{+}$*;FoxO1,3,4*$^{flox/flox}$ or *MxCre*$^{-}$*;FoxO1,3,4*$^{flox/flox}$ on an FVB/n background (Paik *et al.* 2007) were generously provided by Dr Ronald DePinho (MD Anderson Cancer Center, TX, USA). The colony was initiated and maintained in pathogen-free conditions at the York University Vivarium. All experimental mice received a series of three injections of polyinosine–cytidine (400 μg per injection; Invivogen, San Diego, CA, USA) at 4 weeks of age. Polyinosine–cytidine binds toll-like receptor 3 to induce the production of interferon-α/β (IF-α/β), resulting in transient activation of the Myxovirus (influenza) resistance 1/interferon-inducible protein p78 (*Mx1*) promoter, inducing *Cre* expression and resulting in the deletion of floxed *FoxO* alleles (Kuhn *et al.* 1995). We and others have shown that this causes an almost complete deletion of FoxO within endothelial cells (Paik *et al.* 2007; Roudier *et al.* 2013) and no apparent deletion of FoxO in skeletal myocytes (Hayashi *et al.* 2004; Paik *et al.* 2007; Roudier *et al.* 2013). In all experiments, *MxCre+* (*FoxO*$^{\Delta}$) mice were compared to control age-matched littermate *MxCre-* (*FoxO*$^{L/L}$).

Endothelial cell isolation

Endothelial cells were isolated from epididymal fat excised from *FoxO*$^{\Delta}$ ($n = 4$) and *FoxO*$^{L/L}$ ($n = 4$) mice, according to our previously described protocol (Han *et al.* 2003). Following isolation, cells were lysed for 20 min using RIPA lysis buffer supplemented with 1 mg ml^{-1} PMSF, 1 mM Na_3VO_4, 1 mM NaF (Sigma-Aldrich, Mississauga, ON, Canada), 1× protease inhibitor cocktail (Complete mini; Roche Diagnostics, Laval, QC, Canada) and 1× phosphatase inhibitor cocktail (PhosSTOP; Roche Diagnostics, QC, Canada). Lysates were centrifuged and supernatant was stored at −80°C.

Exercise training

Two weeks following polyinosine–cytidine injection, mice were divided into two groups, *FoxO*$^{\Delta}$ ($n = 30$; 15 female, 15 male) or *FoxO*$^{L/L}$ ($n = 30$; 15 female, 15 male). The two groups of mice were further divided into the following subgroups: sedentary, or trained for 1, 7, 10 or 14 days. Mice were trained daily, using the same conditions as described above for acute exercise. Mice rested for 2 h following their respective final bout of training, were anaesthetized (isoflurane/oxygen inhalation), and hindlimb muscles were extracted, weighed and snap-frozen in liquid nitrogen.

The plantaris was embedded in cryogel and frozen in liquid nitrogen-cooled isopentane for histochemistry.

Morphometric measurements

Mice were weighed prior to and following their final bout of exercise. The wet masses of the soleus, plantaris and gastrocnemius were obtained following training. Wet muscle mass to body mass ratios were calculated for each group (Table 1).

Protein extraction from muscle

Protein was extracted from plantaris or gastrocnemius muscle samples using RIPA lysis buffer and a Retsch MM400 tissue lyser, as previously described (Milkiewicz *et al.* 2011), and stored at −80°C.

Nuclear and cytoplasmic protein extraction

Protein extracts of freshly isolated gastrocnemius muscle from sedentary, 1 day and 10 day trained $FoxO^{L/L}$ mice were generated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Fisher Thermo Scientific, Whitby, ON, Canada) as per the manufacturer's instructions.

Western blot

In total, 20–30 μg of protein per sample, as determined by BCA assay (Pierce, Fisher Thermo Scientific), was denatured and equivalent amounts of protein were separated using SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Immobilon P; Fisher Thermo Scientific). Membranes were probed using antibodies as follows: FoxO1, FoxO3a, p-Thr24 FoxO1/p-Thr32 FoxO3a, total AKT1/2/3, p-Ser473 Akt, total JNK1, pThr183/Tyr185 JNK, α/β-Tubulin (1:1000; #9454, 2497, 9464, 9272, 4058, 9258, 9255, 2148, respectively; Cell Signalling, Pickering, ON, Canada), THBS1 (1:200; #G3685605 Cedarlane Laboratories, Burlington, ON, Canada), Histone 2B (1:1000; #ab64165 Abcam, Cambridge, MA, USA) and β-actin (1:5000; #47778 Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, signals were generated using Super West Pico (Pierce; Fisher Thermo Scientific) or Immobilon Western ECL (Millipore; Fisher Thermo Scientific) and detected using an imaging station (Kodak 4000MM Pro; Carestream Molecular Imaging, Woodbridge, CT, USA). Bands were quantified using Carestream software. The relative value for the protein of interest was normalized to the loading control and expressed as a ratio to sedentary.

RNA extraction from muscle

RNA was extracted from ∼10 mg of plantaris muscle using the tissue lyser followed by the RNeasy Fibrous Tissue Mini Kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's instructions. RNA was reverse transcribed using standard protocols.

qPCR

cDNA samples were analysed by qPCR with an ABI 7500 Fast PCR System (Invitrogen Canada, Burlington, ON, Canada) using qPCR mastermix (P/N 11743; Invitrogen Canada) and Taqman probe/primer sets (Invitrogen Canada) for FoxO1 (Mm00490672_m1), FoxO3a (Mm01185722_m1), THBS1 (Mm01335418_m1) and HPRT1 (Mm00446968_m1). The comparative C_t method was used to determine relative quantification of mRNA expression, using HPRT-1 to normalize for the amount of RNA per sample.

Immunohistochemistry

Capillary to muscle fibre ratio, an indicator of angiogenesis, was assessed on 10 μm cross-sections of plantaris muscle, using fluorescein isothiocyanate-conjugated Griffonia Simplicifolia Lectin-1 (1:100; Vector Laboratories, Burlingame, CA, USA) to detect capillaries. Sections were examined at 200× magnification using an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) and digital images of four independent fields of view (encompassing 90–95% of the plantaris cross-section) were captured using Metamorph software. The numbers of capillaries and muscle fibres were counted by a blinded observer, averaging the values of all fields of view per mouse.

Statistical significance

All data are presented as mean ± standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* tests, two-way ANOVA followed by Bonferroni *post hoc* tests or Student's *t* tests, as appropriate (Prism4; Graphpad Software Inc., La Jolla, CA, USA). In all cases, *P* values of less than 0.05 were considered statistically significant.

Results

Acute running exercise modulates FoxO1 and FoxO3a mRNA, protein levels and phosphorylation status

FoxO mRNA and protein expression first were assessed in plantaris muscle in response to an acute exercise

Table 1. Morphometric measurements of *FoxO*$^{L/L}$ and *FoxO*$^{\Delta}$ mice before and after training

		Pre-train body mass (g)	Post-train body mass (g)	Soleus/body mass (mg g^{-1})	Plantaris/ body mass (mg g^{-1})	Gastrocnemius/ body mass (mg g^{-1})
Sedentary	*FoxO*$^{L/L}$	25.4 ± 1.0	26.1 ± 1.1	0.24 ± 0.02	0.47 ± 0.03	3.73 ± 0.06
	FoxO$^{\Delta}$	23.3 ± 1.3	24.3 ± 1.3	0.26 ± 0.02	0.47 ± 0.02	3.80 ± 0.11
1 day	*FoxO*$^{L/L}$	25.2 ± 1.1	25.2 ± 1.1	0.25 ± 0.01	0.52 ± 0.02	3.69 ± 0.13
	FoxO$^{\Delta}$	23.1 ± 2.1	23.1 ± 2.1	0.22 ± 0.01	0.52 ± 0.04	3.71 ± 0.10
7 day	*FoxO*$^{L/L}$	22.9 ± 1.0	21.2 ± 1.0	0.27 ± 0.02	0.50 ± 0.03	4.03 ± 0.20
	FoxO$^{\Delta}$	21.0 ± 1.2	20.8 ± 1.2	0.29 ± 0.02	0.49 ± 0.02	3.92 ± 0.07
10 day	*FoxO*$^{L/L}$	23.2 ± 1.8	23.0 ± 1.7	0.26 ± 0.01	0.54 ± 0.01	3.83 ± 0.13
	FoxO$^{\Delta}$	20.0 ± 1.3	25.5 ± 1.3	0.23 ± 0.01	0.50 ± 0.02	3.92 ± 0.07
14 day	*FoxO*$^{L/L}$	26.6 ± 1.4	25.7 ± 1.3	0.25 ± 0.01	0.50 ± 0.02	3.83 ± 0.13
	FoxO$^{\Delta}$	25.3 ± 1.4	25.3 ± 1.4	0.23 ± 0.01	0.47 ± 0.02	3.91 ± 0.10

All values are mean ± SEM; $n = 6$ per group. A significant training effect was observed only in the soleus/body mass ratio ($P < 0.01$).

bout. FoxO1 mRNA was elevated above sedentary levels immediately after exercise, with further significant increases detectable after 2 h of recovery (1.8 ± 0.33 and 5.5 ± 0.18, respectively, *vs.* 1.0 ± 0.09; $P < 0.05$; Fig. 1*A*). Increases in FoxO3a mRNA also were detected immediately following exercise and after 2 h of recovery (1.4 ± 0.1 and 1.8 ± 0.05, respectively, *vs.* 0.9 ± 0.06; $P < 0.05$; Fig. 1*B*). Protein levels of FoxO1 and FoxO3a were not altered immediately following the exercise bout. However, significant increases in both proteins were observed 2 h following the exercise bout when compared to levels in sedentary muscle (1.7 ± 0.3 *vs.* 1.0 ± 0.06 and 2.2 ± 0.2 *vs.* 1.1 ± 0.06, respectively; $P < 0.05$, Fig. 1*C* and *D*). Phosphorylation of FoxO1 and FoxO3a proteins by Akt and JNK has been shown to regulate both FoxO protein localization, stability and transcriptional activity (Burgering & Medema, 2003; Accili & Arden, 2004; Tzivion, 2011). p-[Thr183/Tyr185]-JNK showed minimal fluctuation in response to the acute exercise challenge ($P > 0.05$, Fig. 2*A*). In contrast, p-[ser473]-AKT relative to total AKT was elevated immediately subsequent to an acute bout of training, returning to basal levels following 2 h of rest (2.6 ± 0.04 and 1.2 ± 0.1 *vs.* 1.1 ± 0.06 for sedentary; $P < 0.01$, Fig. 2*B*). We assessed the phosphorylation of FoxO proteins at residues thr24 and thr32, which are consensus phosphorylation sites for AKT within FoxO1 and FoxO3a, respectively. p-(thr24)-FoxO1 levels relative to total FoxO1 were reduced significantly 2 h after an acute exercise bout compared to sedentary levels (0.6 ± 0.04 *vs.* 1.0 ± 0.03; $P < 0.01$, Fig. 2*C*). p-[thr32]-FoxO3a relative to total FoxO3a increased immediately after exercise (1.6 ± 0.08 *vs.* 1.0 ± 0.0; $P < 0.001$), and was reduced to basal levels after 2 h of recovery ($P < 0.01$, Fig. 2*D*).

Repeated exercise attenuates the acute exercise-induced increases in FoxO1 and FoxO3a proteins

To assess the consequences of repeated training on FoxO1 and FoxO3a mRNA and protein levels, we analysed plantaris muscle from mice that underwent treadmill running for 1–14 days. Based on the elevation in FoxO proteins observed after 2 h of recovery from an acute exercise challenge, all muscles were extracted 2 h after the final training bout in this protocol. FoxO1 mRNA increased significantly after a single bout of exercise (3.4 ± 0.6 *vs.* 1.1 ± 0.2 for sedentary). This response was attenuated significantly after 14 days of exercise training (1.7 ± 0.4 *vs.* 3.4 ± 0.6 for a single exercise bout, $P < 0.05$, Fig. 3*A*). FoxO3a mRNA was significantly lower after 7 and 14 days of training compared to a single exercise bout (0.8 ± 0.1 and 0.9 ± 0.04, respectively, *vs.* 1.8 ± 0.2; $P < 0.05$, Fig. 3*B*). Similar to the effect of training on mRNA levels, both FoxO1 and FoxO3a protein levels were significantly reduced after 10 and 14 days of training compared to a single exercise bout (1.0 ± 0.1 and 0.9 ± 0.09, respectively, *vs.* 1.5 ± 0.4 for FoxO1 and 0.8 ± 0.1 and 0.9 ± 0.04, respectively, *vs.* 1.8 ± 0.2 for FoxO3a; $P < 0.05$, Fig. 3*C* and *D*).

These changes in protein level suggest a reduced functional influence of FoxO proteins following extended training. The subcellular localization of FoxO proteins may provide an additional indication of the ability of FoxO to regulate target genes. Thus, we performed nuclear and cytoplasmic extractions on the gastrocnemius muscles from sedentary mice, as well as those that underwent an acute exercise bout or 10 days of repeated exercise, as significant changes were observed in FoxO1 and FoxO3a at these time points. Cytoplasmic levels of FoxO1 and

FoxO3a increased after 10 days of training, compared to sedentary or acute exercise levels (1.7 ± 0.2 *vs.* 1.0 ± 0 or 0.9 ± 0.05 for FoxO1 and 1.6 ± 0.06 *vs.* 1.0 ± 0 or 1.1 ± 0.07 for FoxO3a; $P < 0.01$, Fig. 4*A* and *C*). At the same time, nuclear levels of both FoxO1 and FoxO3a were significantly lower after 10 days of training compared to sedentary levels (0.6 ± 0.09 *vs.* 1.0 ± 0.0 and 0.4 ± 0.1 *vs.* 1.0 ± 0.0, respectively; $P < 0.05$, Fig. 4*B* and *D*).

We recently established that THBS1 is a direct transcriptional target of FoxO1 in endothelial cells (Roudier *et al.* 2013). Consistent with the observed changes in FoxO1 and FoxO3a protein levels, THBS1 protein levels were elevated 2 h after a single acute bout of training compared to sedentary (1.8 ± 0.2 *vs.* 1.0 ± 0.0; $P < 0.05$, Fig. 5*A* and *B*). However, this increase in THBS1 protein was not observed following 10 and 14 days of training (0.9 ± 0.01 and 0.8 ± 0.06, respectively, *vs.* 1.6 ± 0.01; $P < 0.01$ *vs.* day 1, Fig. 5*B*).

Endothelial cell-directed deletion of FoxO1/3a/4 inhibits the acute exercise-induced expression of THBS1

Our data thus far indicate that acute exercise increases FoxO1 and FoxO3a levels, while repeated exercise attenuates this response. Considering the angiostatic function of FoxO proteins within other contexts (Paik *et al.* 2007; Roudier *et al.* 2013), we next hypothesized that endothelial cell-directed deletion of FoxO proteins would enhance the angiogenic response to exercise training. We utilized a murine genetic model of conditional combined deletion of *FoxO1,3,4*, in which the inducible *Mx1* promoter drives Cre expression specifically within cells that exhibit interferon α/β responsiveness, which includes the endothelium, haematopoietic cells, as well as hepatocytes but, notably, not the skeletal myocytes (Hayashi *et al.* 2004; Paik *et al.* 2007). A primary benefit of this model is that it circumvents the embryonic lethality observed with constitutive endothelial

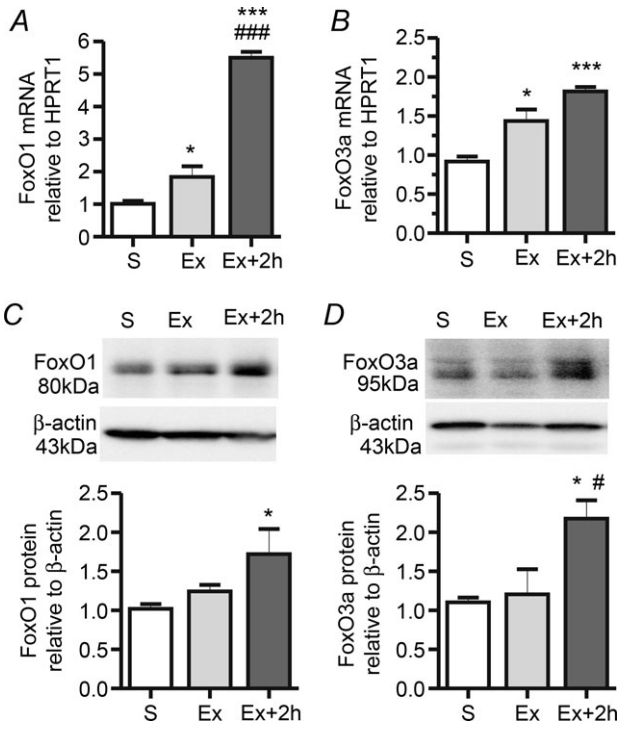

Figure 1. Acute aerobic exercise elicits changes in FoxO1 and FoxO3a mRNA and protein
FVB/n mice ($n = 4$) exercised for 1 day or remained sedentary (S). Muscles were extracted immediately (Ex), or 2 h after training (Ex+2 h). *A* and *B*, FoxO1 and FoxO3a mRNA levels were assessed by qPCR and expressed relative to HPRT1. *C* and *D*, FoxO1 and FoxO3a protein was assessed by Western blot, and expressed relative to β-actin. A significant training effect ($P < 0.05$, one-way ANOVA) was observed in all panels. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* sedentary; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs.* Ex (Tukey *post hoc* analysis).

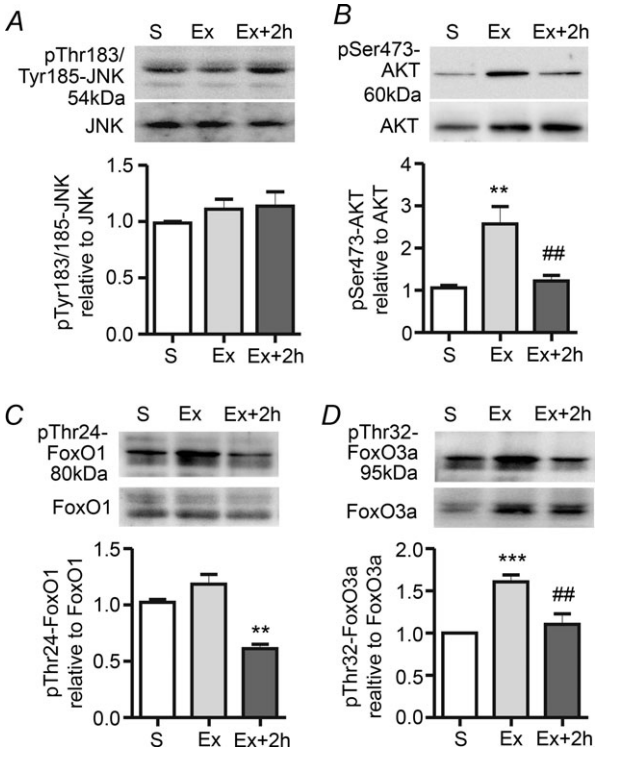

Figure 2. The phosphorylation status of Akt and FoxO proteins is altered by acute exercise
FVB/n mice ($n = 4$) exercised for 1 day or remained sedentary (S). Muscles were extracted immediately (Ex), or 2 h after training (Ex+2 h). *A*, p-[Thr183/Tyr185]-JNK was assessed relative to total JNK protein. *B*, p-[Ser473]-AKT was assessed relative to total AKT protein. *C*, p-[thr24]-FoxO1protein was assessed relative to FoxO1. *D*, p-[thr32]-FoxO3a protein was assessed relative to FoxO3a. A significant training effect ($P < 0.05$, one-way ANOVA) was observed in all panels except for p-JNK. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* sedentary; $^{\#\#}P < 0.01$ *vs.* Ex (Tukey *post hoc* analysis).

cell-specific deletion of *FoxO1* (Sengupta *et al.* 2012). Also, the simultaneous targeting of all three members of the FoxO family avoids possible functional compensation by FoxO3 or FoxO4 (Paik *et al.* 2007). We previously reported a strong endothelial cell deletion of FoxO1 and FoxO3a within the skeletal muscle microcirculation using this murine model (Roudier *et al.* 2013). In the current study, the efficacy of endothelial cell deletion was confirmed by analysis of FoxO protein levels within endothelial cells isolated from $FoxO^{\Delta}$ and $FoxO^{L/L}$ mice. There was an approximately 80% deletion of FoxO1 and FoxO3a proteins in the endothelial cells of $FoxO^{\Delta}$ mice compared to $FoxO^{L/L}$ mice (Fig. 6*A*), consistent with our previous findings. FoxO4 protein was not detectable in the endothelial cells from either genotype. We also assessed FoxO1 and FoxO3a levels in the whole muscle protein extracts of sedentary and 1 day exercised $FoxO^{L/L}$ and $FoxO^{\Delta}$ mice (Fig. 6*B*). The continued presence of FoxO proteins within whole muscle extracts was expected, as this model does not result in deletion of FoxO1 and FoxO3a within the skeletal myocytes. Notably, FoxO1 and FoxO3a levels were reduced significantly in the muscle extracts of 1 day exercised $FoxO^{\Delta}$ mice compared to their $FoxO^{L/L}$ counterparts (FoxO1: 1.5 ± 0.1 *vs.* 1.0 ± 0.1 in day 1 $FoxO^{L/L}$ and $FoxO^{\Delta}$, respectively; $P < 0.01$; FoxO3a: 1.4 ± 0.1 *vs.* 0.9 ± 0.2 in day 1 $FoxO^{L/L}$ and $FoxO^{\Delta}$, respectively; $P < 0.05$). A similar reduction in FoxO1 and FoxO3a protein levels were observed in the $FoxO^{\Delta}$ mice after 7 days of training (FoxO1: 1.5 *vs.* 0.7 in day 7 $FoxO^{L/L}$ and $FoxO^{\Delta}$, respectively; FoxO3a: 1.2 *vs.* 0.5 in day 7 $FoxO^{L/L}$ and $FoxO^{\Delta}$, respectively). These results collectively demonstrated that deletion of endothelial FoxOs abolishes the upregulation of FoxO proteins induced by exercise. There were no differences in body weight or in muscle mass/body weight ratios when comparing $FoxO^{L/L}$ and $FoxO^{\Delta}$ mice (Table 1).

THBS1 mRNA levels were measured to determine whether the endothelial deletion of FoxO proteins would modulate the response of THBS1 to exercise. In $FoxO^{L/L}$ mice, THBS1 mRNA levels increased robustly after an acute bout of exercise and this increase was no longer observed following 7 days of training (2.9 ± 0.6 *vs.* 0.7 ± 0.1; $P < 0.001$, Fig. 6*C*). In the mice with endothelial-directed deletion of FoxO gene products ($FoxO^{\Delta}$), basal levels of THBS1 mRNA were unaffected (1.0 ± 0.2 *vs.* 0.9 ± 0.1; $P > 0.05$), but the increase in THBS1 mRNA following an acute bout of exercise was abolished completely (0.9 ± 0.2 *vs.* 2.9 ± 0.6; $P < 0.001$, Fig. 6*C*). This effect also was observed for THBS1 protein, as the increase in THBS1 protein following one bout of exercise in $FoxO^{L/L}$ mice was not detected in the $FoxO^{\Delta}$ mice (1.9 ± 0.3 *vs.* 0.9 ± 0.2; $P < 0.05$, Fig. 6*D*).

Deletion of FoxO1/3a/4 results in an earlier angiogenic response to exercise training

Capillary to muscle fibre ratio (C/F) was assessed as an indicator of angiogenesis (Fig. 7*A*). C/F was affected

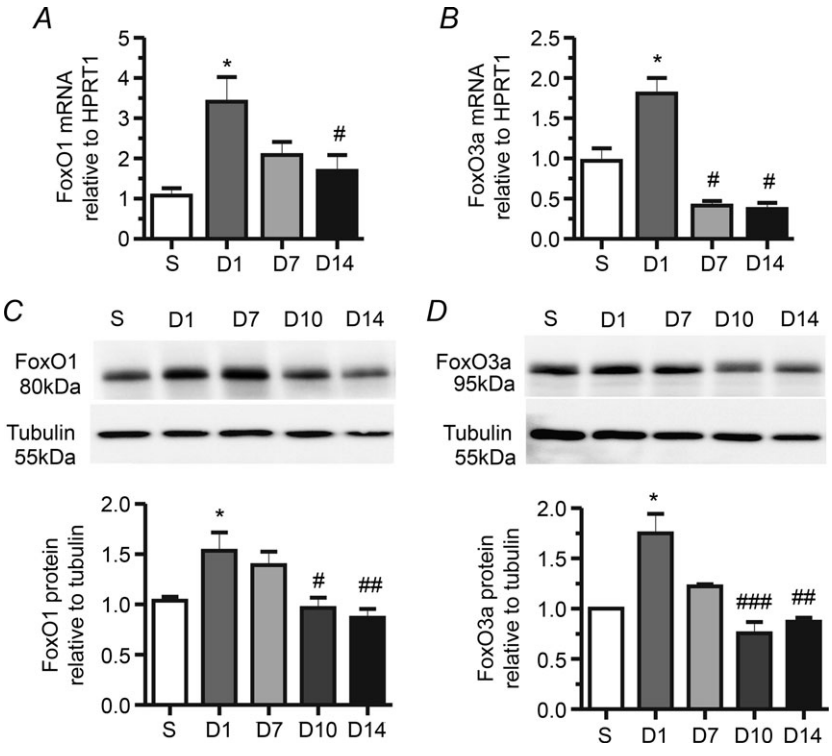

Figure 3. Repeated exercise training attenuates the mRNA and protein levels of FoxO1 and FoxO3a
FVB/n $FoxO^{L/L}$ mice ($n = 6$) trained for 1, 7, 10 or 14 days (D1, D7, D10, D14) or remained sedentary (S). Muscles were extracted 2 h after the final training session. *A* and *B*, FoxO1 and FoxO3a mRNA were assessed relative to HPRT1. *C* and *D*, FoxO1 and FoxO3a protein relative to tubulin. A significant training effect ($P < 0.05$, one-way ANOVA) was observed for both FoxO1 and FoxO3a mRNA and proteins. $^{*}P < 0.05$ *vs.* sedentary, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.01$ *vs.* D1 (Tukey *post hoc* analysis).

significantly by training ($P < 0.001$) and by FoxO deletion ($P < 0.01$, Fig. 7*B*). Importantly, the C/F of sedentary $FoxO^{\Delta}$ mice was not different from their $FoxO^{L/L}$ counterparts (1.63 ± 0.05 *vs.* 1.68 ± 0.04; $P > 0.05$). In response to training, a significant increase in C/F above resting levels was not evident in the $FoxO^{L/L}$ mice until 14 days of treadmill exercise (1.95 ± 0.11 *vs.* 1.68 ± 0.04; $P < 0.05$). In contrast, a substantially earlier angiogenic response was observed in the $FoxO^{\Delta}$ mice, with C/F significantly increased above sedentary following just 7 days of exercise training (1.94 ± 0.06 *vs.* 1.63 ± 0.05; $P < 0.05$). Notably, the C/F of 7 day trained $FoxO^{\Delta}$ mice was significantly greater than the C/F of $FoxO^{L/L}$ mice at the same time point (1.94 ± 0.06 *vs.* 1.70 ± 0.06; $P < 0.05$). No significant differences between the C/F of $FoxO^{\Delta}$ and $FoxO^{L/L}$ mice were observed at the 14 day time point (2.04 ± 0.05 *vs.* 1.95 ± 0.11; $P > 0.05$).

Discussion

In this study, we identify a physiological regulatory mechanism that modulates the onset of capillary growth in response to an exercise stimulus. We demonstrate that the transcription factors FoxO1 and FoxO3a are temporally regulated during exercise training, correlating with changes in expression of the angiostatic matrix protein THBS1. Furthermore, we provide evidence that endothelial-directed deletion of FoxO1/3a/4 abolishes the exercise-induced increase in THBS1 mRNA and results in an accelerated angiogenic response to exercise training. Together, these data indicate that FoxO proteins are critical regulators of exercise-induced angiogenesis.

The initial responsiveness of FoxO1 and FoxO3a to exercise is attenuated with repeated exercise. We showed a significant increase in the mRNA and protein levels of FoxO1 and FoxO3a 2 h after the completion of an acute bout of exercise. In contrast, by 10 and 14 days of training, the increase in FoxO mRNA and protein during the post-exercise recovery phase no longer occurred. This suggests a reduced functional role of these proteins with aerobic exercise training. The reduced nuclear levels of FoxO1 and FoxO3a levels observed after 10 days of training support this hypothesis.

Modulation of FoxO1 and FoxO3a protein levels may occur through both transcriptional and post-transcriptional regulatory mechanisms. Previous studies have

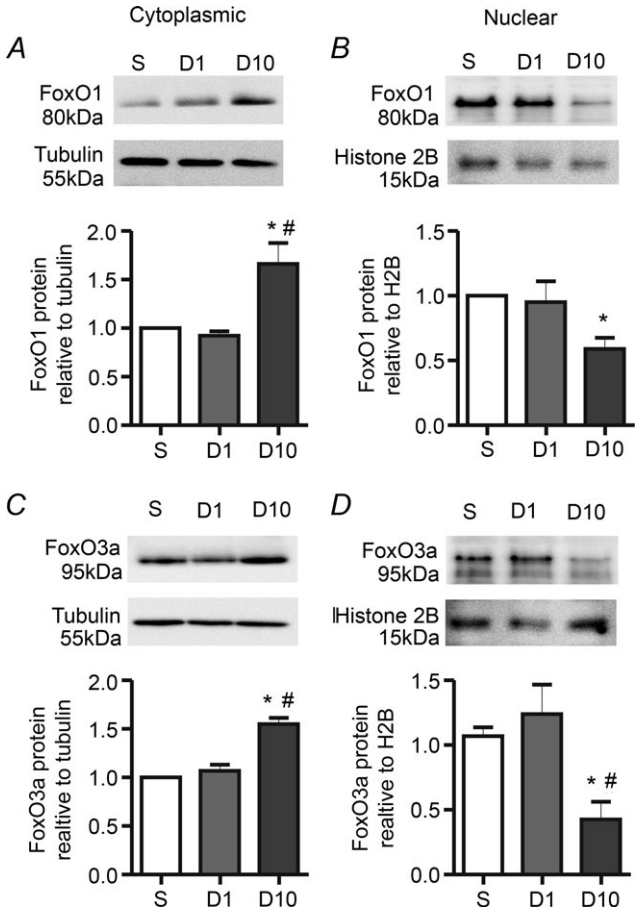

Figure 4. FoxO1 and FoxO3a nuclear exclusion is evident after 10 days of repeated exercise
Nuclear and cytoplasmic extracts were obtained from sedentary, 1 day and 10 day trained $FoxO^{L/L}$ mice (S, D1, D10) 2 h after their final bouts of exercise ($n = 4$). *A* and *C*, cytoplasmic levels of FoxO1 and FoxO3a proteins, respectively, relative to tubulin. *B* and *D*, nuclear levels of FoxO1 and FoxO3a proteins, respectively, relative to Histone 2B. Significant training effects were observed in both cytoplasmic and nuclear extracts for FoxO1 and FoxO3a ($P < 0.05$, one-way ANOVA). $^{*}P < 0.05$ *vs.* sedentary, $^{\#}P < 0.05$ *vs.* D1 (Tukey *post hoc* analysis).

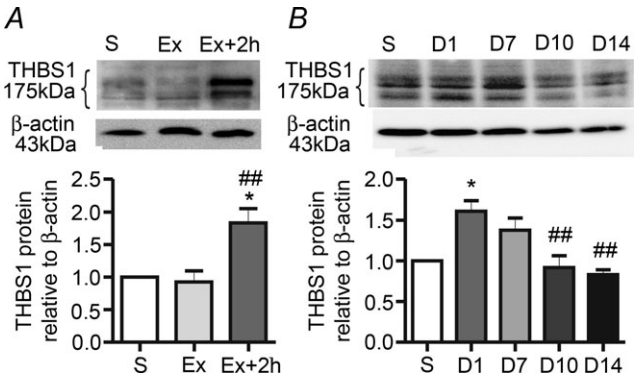

Figure 5. Exercise-induced modulation of the angiostatic protein THBS1
A, FVB/n mice ($n = 4$) exercised for 1 day or remained sedentary (S). Muscles were extracted immediately (Ex), or 2 h after training (Ex+2 h). *B*, FVB/n $FoxO^{L/L}$ mice ($n = 6$) trained for 1, 7, 10 or 14 days (D1, D7, D10, D14) or remained sedentary (S), and all muscles were extracted 2 h after the final training session. In both, THBS1 protein was assessed relative to β-actin. For densitometric analysis of THBS1, intensities of the three bands evident at ~ 175 kDa were summed together. $^{*}P < 0.05$ *vs.* sedentary; $^{\#\#}P < 0.01$ *vs.* Ex (Tukey *post hoc* analysis).

reported increases in FoxO1 mRNA in rodent (Russel *et al.* 2005; Pasiakos *et al.* 2010) and human (Mahoney *et al.* 2005) skeletal muscle following an acute exercise bout, consistent with our observed changes in mRNA and total protein level. Independent of changes in mRNA level, FoxO protein localization, degradation and transcriptional activity are subject to regulation by kinases. In particular, AKT and JNK are recognized to modulate FoxO subcellular localization. AKT phosphorylation targets FoxO for nuclear export, poly-ubiquitination and proteosomal degradation (Burgering & Medema, 2003; Accili & Arden, 2004; Tzivion *et al.* 2011) while JNK phosphorylation facilitates FoxO translocation back to the nucleus (Sunayama *et al.* 2005). We found that AKT is phosphorylated transiently immediately following acute exercise, as has been observed by other researchers (Nader & Esser, 2001; Sakamoto *et al.* 2003). We also observed significantly lower levels of FoxO1 phosphorylation at the consensus Akt site Thr-24 2 h post-exercise, at which point AKT phosphorylation was no longer elevated. JNK-induced phosphorylation can oppose Akt-driven phosphorylation on FoxO proteins. However, we did not observe any change in phosphorylation of JNK after a single exercise bout. Nonetheless, it is possible that

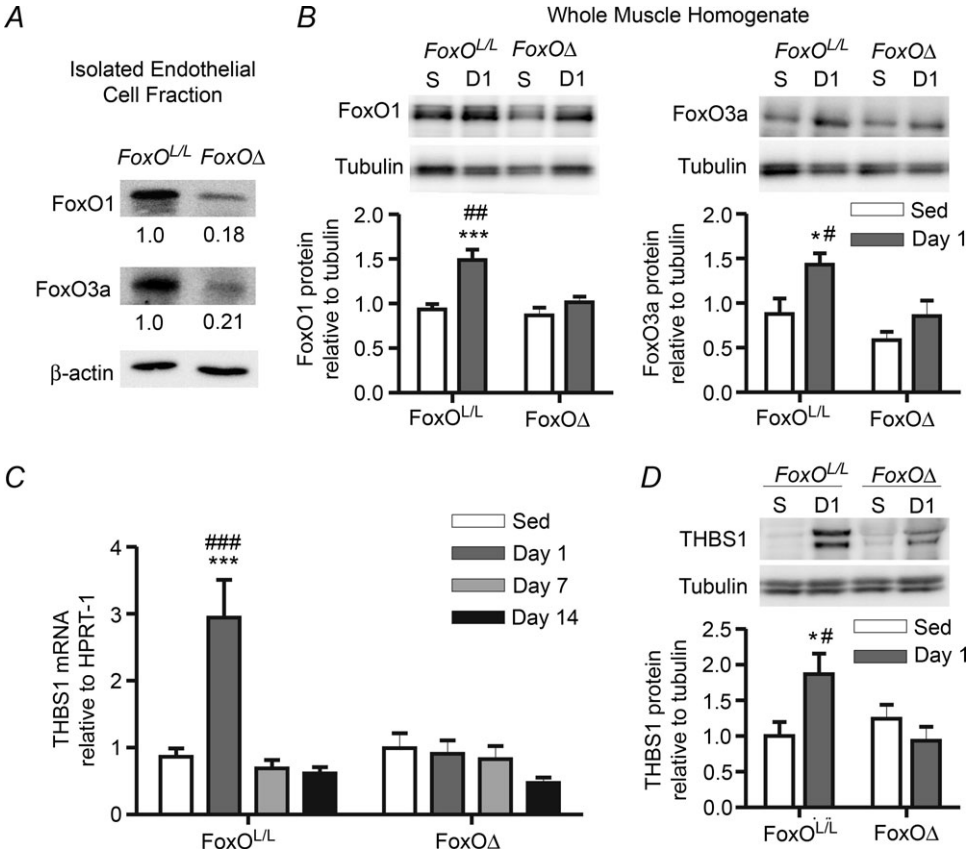

Figure 6. *FoxO*$^{\Delta}$ mice express reduced levels of endothelial FoxO1 and FoxO3a, and have an abrogated THBS1 response to exercise

A, FoxO1 and FoxO3a protein levels were analysed in endothelial cells isolated from epididymal fat of *FoxO*$^{L/L}$ and *FoxO*$^{\Delta}$ mice ($n = 3$). Representative blots are shown, with the respective densitometric values (relative to β-actin) listed under each blot. *B*, muscles were extracted from *FoxO*$^{L/L}$ and *FoxO*$^{\Delta}$ sedentary (S) mice, or 2 h after an acute exercise bout (D1). FoxO1 and FoxO3a protein levels were assessed by Western blot to determine the extent of deletion from whole gastrocnemius extracts, and quantified relative to tubulin. Significant exercise and genotype effects were observed (two-way ANOVA) for both FoxO1 ($P = 0.0008$ and $P = 0.006$, respectively) and FoxO3a ($P = 0.01$ and $P = 0.009$, respectively). *C*, *FoxO*$^{L/L}$ and *FoxO*$^{\Delta}$ mice ($n = 6$) trained for 1, 7 or 14 days, or remained sedentary (S) and muscles were extracted 2 h after training. THBS1 mRNA levels were measured by qPCR, normalizing to the housekeeping gene HPRT. Significant training ($P < 0.0001$) and genotype ($P < 0.01$) effects were observed (two-way ANOVA). *D*, THBS1 protein was assessed by Western blot of whole gastrocnemius extracts and quantified relative to tubulin. Significant exercise ($P < 0.05$) and genotype ($P < 0.05$) effects were observed (two-way ANOVA). $^{*}P < 0.05$; $^{***}P < 0.001$ *vs.* sedentary *FoxO*$^{L/L}$; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ *vs.* *FoxO*$^{\Delta}$ day 1 (Bonferroni *post hoc* analysis).

this level of phosphorylated JNK contributes to restrain the extent of Akt-dependent phosphorylation of FoxO proteins.

After 10–14 days of exercise training, a bout of exercise failed to induce increases in FoxO1 or FoxO3a protein. This training adaptation may reflect a reduced production of FoxO proteins (consistent with the measurements of mRNA) and/or an increased rate of ubiquitination and degradation of the FoxO proteins. For example, our laboratory previously described that the expression of E3 ubiquitin ligase Mdm2 is increased by aerobic exercise training (Roudier *et al.* 2012), and that Mdm2 and FoxO1 binding is negatively correlated to the level of pAkt (Milkiewicz *et al.* 2011). Interestingly, we observed that mice with reduced expression of Mdm2 have a higher level of FoxO1 protein as well as elevated levels of FoxO targets THBS1 and a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS-1) (Roudier *et al.* 2012). We have observed continued elevation of pAkt levels with repeated exercise (data not shown), although the direct influence of Mdm2 on FoxO1 or FoxO3a degradation remains to be investigated in the context of aerobic exercise.

The matricellular protein THBS1 exerts significant anti-proliferative and pro-apoptotic effects on endothelial cells (Volpert, 2000; Bornstein, 2001; Lawler & Lawler, 2012). While several angiostatic proteins have been identified within skeletal muscle (as reviewed by Olfert & Birot, 2011), to date THBS1 is the only one that has been established as a significant regulator of skeletal muscle capillarization (Malek & Olfert, 2009; Audet *et al.* 2013). The modulation of THBS1 levels with exercise training matched closely with that of FoxO1 and FoxO3a protein levels, with THBS1 mRNA and protein increasing substantially after a single bout of exercise, together with a loss of this response after 7 or 10 days of training, in the case of mRNA and protein, respectively. Our results are congruent with published reports that THBS1 mRNA increased after 1 day of training, returning to resting levels after 5 days of training (Olfert *et al.* 2006). We previously reported that FoxO1 is a direct transcriptional regulator of the angiostatic protein THBS1 in endothelial cells (Roudier *et al.* 2013). Thus, the increase in THBS1 mRNA suggests an increased transcriptional activity of FoxO proteins. It was somewhat surprising that neither FoxO1 nor FoxO3a levels were elevated in nuclear extracts of 1 day

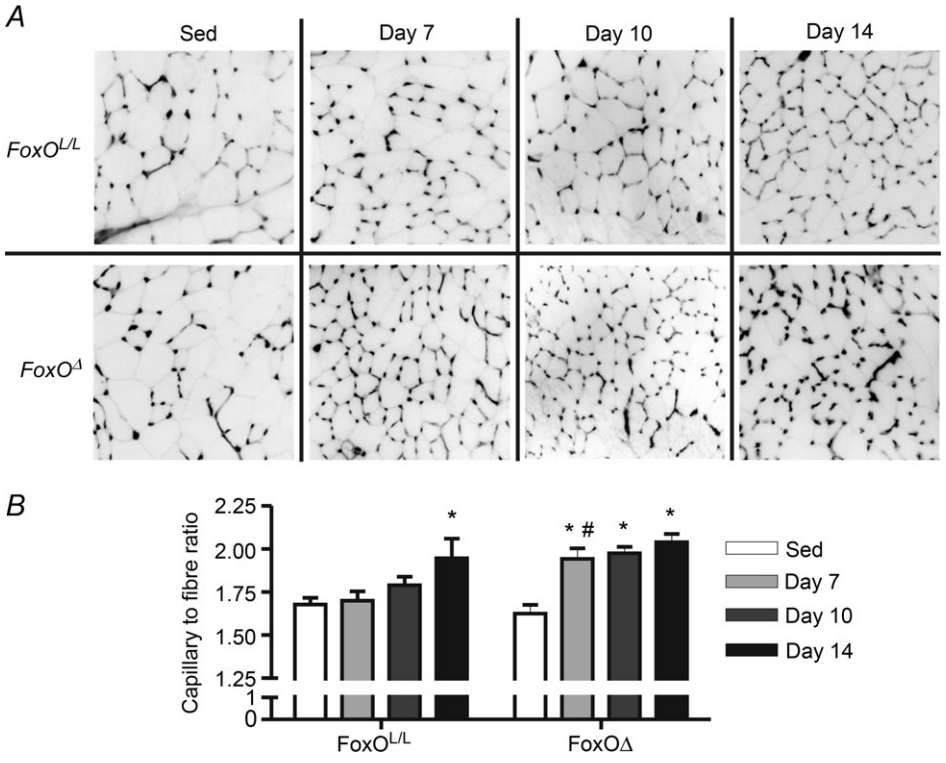

Figure 7. Deletion of FoxO1/3a/4 accelerates the angiogenic response to exercise training
FoxO$^{L/L}$ and *FoxO*$^{\Delta}$ mice ($n = 6$) trained for 7, 10 or 14 days, or remained sedentary. *A*, plantaris muscle cross-sections were stained with isolectin to indicate the presence of capillaries. Greyscale fluorescence images have been inverted to enhance visualization of muscle fibres. *B*, capillary to muscle fibre ratio was calculated as an indicator of angiogenesis. Significant genotype ($P < 0.01$) and training ($P < 0.0001$) effects were observed (two-way ANOVA). $^{*}P < 0.05$ *vs*. sedentary of the same genotype, $^{\#}P < 0.05$ *vs*. day 7 *FoxO*$^{L/L}$ (Bonferroni *post hoc* analysis).

trained mice compared to their sedentary counterparts. Nonetheless, it is possible to have an increase in FoxO transcriptional activity independently of shifts in nuclear localization. For example, deacetylation of FoxO proteins by SIRT1 and SIRT2 has been shown to occur in response to oxidative stress and this can increase FoxO-dependent transcription of target genes (Brunet *et al.* 2004; Motta *et al.* 2004). AMPK also is activated during exercise (Richter & Ruderman, 2009) and can phosphorylate and activate the FoxO members, preferentially FoxO3a (Greer *et al.* 2007), as well as enhance SIRT deacetylation activity (Canto *et al.* 2009). These studies indicate that FoxO location and activity is established by a complex network of kinases. Further work is required to elucidate the regulatory mechanisms underlying the exercise-induced shifts in FoxO protein and activity.

We recently demonstrated that endothelial cell FoxO expression is associated with repression of angiogenesis within ischaemic skeletal muscle (Roudier *et al.* 2013). Through use of a mouse model of endothelial-directed conditional combined deletion of FoxO1/3a/4, we bring evidence now for a physiological role of endothelial cell FoxO proteins in regulating the initiation of exercise-induced angiogenesis. First, we establish that the acute increase in THBS1 mRNA following an exercise bout is endothelial FoxO-dependent, as this response was completely absent in those mice with endothelial cell-directed FoxO deficiency. In contrast, it is notable that endothelial FoxO deficiency did not alter basal levels of THBS1 mRNA in sedentary animals, indicating that the appropriate stimulus must be present to trigger the FoxO-dependent transcription of THBS1. While our data provide strong support for the key role of FoxO proteins in the upregulation of THBS1 following an acute bout of exercise, we cannot exclude the involvement of additional transcriptional or post-transcriptional regulatory mechanisms contributing to the regulation of THBS1 protein at later time points of exercise training.

In rodents, endurance training has been reported to elicit an angiogenic response after 12–15 days, depending on the intensity and type of training (Adolfsson *et al.* 1981; Lloyd *et al.* 2003). Consistent with those reports, we observed a significant increase in C/F ratio in $FoxO^{L/L}$ mice following 14 days of training in the current study. $FoxO^{\Delta}$ mice, which express reduced endothelial cell levels of FoxO1/3a/4, exhibited an accelerated angiogenic response to repeated exercise compared to $FoxO^{L/L}$ littermates, with significant increases in C/F observed after just 7 days of training in the $FoxO^{\Delta}$ mice. The reduced levels of FoxO in the $FoxO^{\Delta}$ mice throughout the training period effectively served to relieve the repressive functions of FoxO proteins associated with acute exercise, permitting an earlier angiogenic response. This finding highlights the significant physiological role of endothelial cell FoxO proteins in establishing the balance between angiogenic and angiostatic proteins within skeletal muscle. For example, an acute exercise bout promotes the production of angiogenic VEGFA as well as the angiostatic THBS1. THBS1 is reported to interfere with VEGF signalling through reducing the phosphorylation of VEGFR2 (Zhang *et al.* 2009; Chu *et al.* 2013). Our data are consistent with the concept that FoxO-dependent upregulation of THBS1 promotes a state of endothelial cell quiescence (Iruela-Arispe *et al.* 1991; Tolsma *et al.* 1997) and that the absence of this influence in the endothelial FoxO-deleted mice facilitates endothelial cell activation and proliferation in response to the presence of VEGFA. However, considering the complexity of the angiogenic sprouting process, it is probable that the actions of endothelial cell FoxO proteins involve the orchestration of additional angiostatic factors, and these additional targets represent an area for future investigation.

In the current study, animals ran on the treadmill daily at a set duration and intensity. It is well known that both exercise intensity and duration exert an influence on the activation of kinases (e.g. Akt, AMPK, MAPK) (as reviewed by Sakamoto and Goodyear, 2002), and thus could also impact the extent of FoxO expression and activity. Considering the intermittent stimulus associated with repeated bouts of exercise, the temporal dynamics of the attenuation of FoxO proteins with training also may be expected to vary dependent on the training frequency. Assessment of the roles of these exercise parameters to establish the time window required to suppress FoxO proteins may be of benefit in optimizing the training parameters that can provoke the most rapid increase in muscle capillarization.

An initial burst in both pro-angiogenic and angiostatic factors has been reported in response to an acute exercise bout. Furthermore, multiple studies have indicated that training-induced increases in basal VEGFA, as well as exercise-induced increases in VEGFA, are observed after 5–7 days of repeated exercise (reviewed by Haas *et al.* 2012). While these changes are not as profound as the initial response of VEGFA to an acute exercise bout, an elevated level of VEGFA is maintained within the muscle. In contrast, we demonstrate that increased FoxO and THBS1 levels are not maintained after 7, 10 or 14 days of exercise. This is consistent with a permissive scenario in which the balance between pro- and anti-angiogenic factors is shifted in favour of angiogenesis (Olfert & Birot, 2011). Our data illustrate that the endothelial cell FoxO proteins play a role in repressing this shift, as their down-regulation accelerates the angiogenic process. Following the formation of nascent capillaries, maturation of these vessels requires re-establishment of endothelial cell quiescence, strengthening of endothelial cell junctions, basement membrane deposition

and pericyte recruitment. These events involve the Ang1-Tie2, PDGFB, TGFβ and dll4-Notch signalling pathways (Herbert & Stainier, 2011; Ehling *et al.* 2013). It is tempting to speculate that angiostatic regulators such as the FoxO proteins may promote these events. There is some evidence in support of this hypothesis: for example, FoxO was shown to co-operate with Notch to promote transcription of Notch target genes; THBS1 promotes the activation of latent TGFβ; FoxO proteins directly interact with Smad transcription factors to enhance Bmp/TGFβ signalling (Murphy-Ullrich & Poczatek, 2000; Gomis *et al.* 2006; Kitamura *et al.* 2007). However, these interactions, and the specific role of FoxO proteins in the maturation of newly formed capillaries, remain to be established within the context of exercise-induced angiogenesis.

Exercise training is an established model of physiological angiogenesis, but it is also recognized as an effective therapeutic approach to restore the microvascular deficiencies that accompany chronic diseases such as peripheral artery disease, obesity and chronic heart failure. To date, research has centred on the identification and stimulation of pro-angiogenic pathways while there has been little advancement in knowledge of the regulation of angiostatic factors in response to exercise training. Our study indicates that the acute exercise-induced increase in FoxO1 and FoxO3a proteins is down-regulated by repeated exercise, and provides novel evidence that a further reduction in endothelial FoxO levels results in an earlier angiogenic response. We also demonstrate that the angiostatic protein THBS1 is regulated with a similar expression pattern to FoxO proteins during exercise training. Taken together, we demonstrate a significant role for endothelial cell FoxO proteins in regulating the onset of the angiogenic response to exercise training and suggest that this occurs due to their transcription of downstream angiostatic targets such as THBS1. Beyond the value of elucidating this critical physiological pathway, our finding provides insight into a fundamental angiostatic mechanism wherein an individual's capacity to modulate endothelial cell FoxO protein levels may impact the efficacy with which capillary growth is induced in response to exercise.

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Additional Information

Competing interests

None to declare.

Author contributions

D.S., E.R., O.B. and T.L.H. contributed to the conception and design of experiments. D.S., E.R., S.T.K.L., E.N., O.B. and T.L.H. were involved in the collection, analysis and interpretation of data. Experiments were conducted in the labs of O.B. and T.L.H. D.S., E.R. and T.L.H. drafted the manuscript and all authors contributed to the critical revision of the manuscript and approved the final submission.

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