SUPPLEMENTAL MATERIAL

1. DETAILED METHODS

Animal husbandry

Generation of the muscle-specific ERRγ over-expressing mice using human α-skeletal actin promoter, and backcrossing to C57Bl/6J background has been previously described [1]. These mice were bred and maintained in the vivarium at the Institute of Molecular Medicine at the University of Texas Health Science Center, Houston. Mice were housed under standard environmental conditions (20-22°C, 12 h-12 h light-dark cycle) and provided tap water *ad libitum*. Twelve to sixteen week old mice were used in all the experiments. The animals were maintained and treated according to the NIH Guide for Care and Use of Laboratory animals and were approved by the Animal Welfare Committee at the University of Texas Health Science Center, Houston.

Hindlimb ischemia and tissue collection

Hindlimb ischemia was achieved by complete femoral occlusion, adapting previously described protocols [2, 3]. Mice were anesthetized by intra-peritoneal injections of ketamine (100 mg/kg)/xylazine (10 mg/kg) in saline. Left hindlimbs were shaved and the femoral artery exposed at the level of the bifurcation to the muscle branch. Ischemia was induced by ligation and segmental resection of the femoral vessels proximal to the bifurcation with silk sutures (#7.0). Hindlimb muscles such as tibialis anterior (TA), EDL, gastrocnemius, soleus and plantaris were collected at various time points after the induction of ischemia, weighed, immersed in liquid nitrogen-chilled melting isopentane and stored at -80°C.

Laser Doppler blood flow measurement

Blood flow was measured in both the contralateral non-ischemic and ischemic TA with a deep tissue laser Doppler probe (Vasamedics Laserflo BPM2). Multiple measurements were made along the length of the TA and averaged for each muscle. Blood flow in the ischemic TA is reported as the % of the blood flow to contralateral non-ischemic hindlimb (ischemic/contralateral x 100).

Fluorescence microangiography

Fluorescence microangiography was performed, as previously described [1]. Briefly, anesthetized mice were subjected to intra-cardiac perfusion with 10 ml of PBS followed by fluorescent microsphere (0.1 μM) suspension. Next, the mice were euthanized, tissues collected and appropriately frozen. Transverse cryosections of the TA were processed and subjected to fluorescent microscopy to image skeletal muscle vasculature.

Ischemic muscle damage

Ischemic muscle damage was detected using Evans blue dye (EBD) exclusion test. EBD solution (1% W/V in saline) was prepared and sterilized by passage through a 0.22 um filter. It was intraperitoneally administered 24 hours prior to specimen

collection. Because EBD is impenetrable across intact membrane (or viable cells), it is excluded from intact skeletal muscle myofibers, but it selectively stains damaged myofibers. The EBD staining was visualized in muscle cryosections as florescence using excitation wavelength between 470 and 540 nm and an emission wavelength at 680 nm. Skeletal muscle damage was evaluated as the function of EBD staining in the cryosections.

Microfil perfusion and imaging

Whole-mount vascular mapping of the TA vasculature was performed by a modified protocol previously described by Limbourg et al (2009). In brief, a 12% (w/v) microfil pigment solution was prepared by using Gouache in 4% PFA. The microfil solution was administered by intra-cardiac route in anesthetized mice, and the TA muscles were dissected, followed by serial dehydration in alcohol. Next, the tissue was incubated in fresh transparency solution consisted of (1:1) benzylbezoate and benzylalcohol until tissue became transparent. After the muscles were processed, whole mount tissue images were taken on an inverted microscope.

Immunohistochemistry

Serial transverse cryosections (9 μm thick at intervals of 90 μm) were obtained from the mid-belly of the TA isolated from contralateral and ischemic hindlimbs of wild type and transgenic mice. Frozen muscle sections were processed for CD31 and isolectin immunohistochemical staining by using a rat anti-mouse monoclonal antibody (AbD serotec; MCA2388) and biotinylated isolectin B4 (Vector Laboratories), respectively. Fiber typing was performed by immunohistochemical staining of MHC type I, IIA, IIX and IIB using the mouse monoclonal antibodies A4.840, A4.74, 6H1 and BF-F3, respectively (Developmental Studies Hybridoma Bank), as described previously [4]. All primary antibodies were visualized using suitable Alexa Fluor® secondary antibodies from Molecular Probes. Isolectin was visualized by a DyLight® 488 streptavidin conjugate (Vector Laboratories) according to the supplier instructions. Negative control staining by omitting either the primary or the secondary antibody was included in all sets of experiments.

Digital image analysis and morphometrics

Immunostained sections were examined using a Zeiss Axioimager fluorescence microscope and images were captured using an Axiocam digital camera, as described previously [5]. In brief, quantification of both myofiber type as well as capillary density was performed using Zeiss Axiovision software version 4.8, while blinded for treatment. Digital image evaluation of muscle transverse sections was performed with the public domain NIH Image J program.

Cell culture

Muscle C2C12 cells stably expressing empty vector or dominant negative ERRγ (ERRGDN) were grown in 20% FBS-DMEM. For the experiments, confluent cells grown in 10 cm culture dishes were differentiated in 2% horse serum-DMEM for 24 h. Next, the

medium was changed and cells were subjected to normoxic or hypoxic conditions for additional 24 h. Hypoxia constituted placing the cells in a hypoxia chamber, equilibrating the chamber with 95% N2 and 5% CO2 and returning the chamber with the cells to the cell culture incubator. Cells under normoxic conditions were directly returned to the cell culture incubator. After 24 h, these cells were used for extracting RNA for gene expression. Additionally, secreted Vegfa concentration in the conditioned media was measured using Elisa, as described above.

Gene Expression

Gastrocnemius muscles from both the contralateral and ischemic limbs or C2C12 cells were used for studying gene expression, as described previously [1]. In brief, total RNA was prepared from skeletal muscles or C2C12 cell lysates using the RNeasy Mini Kit (Qiagen). Total RNA (5 µg) was reverse-transcribed to cDNA with SuperScript II Reverse Transcriptase (Invitrogen) and analyzed by quantitative real-time PCR on an ABI7900 cycler, using the Applied Biosystems SYBR Green PCR Master Mix. Primers were designed using the software Primer Express 3.0 (Applied Biosystems, TX) and are listed in the table below. All data were normalized to cyclophilin or gapdh.

Gene	Forward primer	Reverse Primer
cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT
EphrinA	GCACGTGCAGCTGAATGACT	GGCTGCATCTGCCACAGAGT
Fgf-1	GAAGCATGCGGAGAAGAACTG	CGAGGACCGCGCTTACAG
Gapdh	CACCATCTTCCAGGAGCGAG	CCTTCTCCATGGTGGTGAAGAC
Nrp1	CTGGTGAGCCCTGTGGTCTATT	CACATGAGAGCCGGACATGT
Vegfa-121	TGCAGGCTGCTGTAACGATG	CCTCGGCTTGTCACATTTTTCT
Vegfa-165	TGCAGGCTGCTGTAACGATG	GAACAAGGCTCACAGTGATTTTCT
Vegfa-189	TGCAGGCTGCTGTAACGATG	CTCCAGGATTTAAACCGGGATT

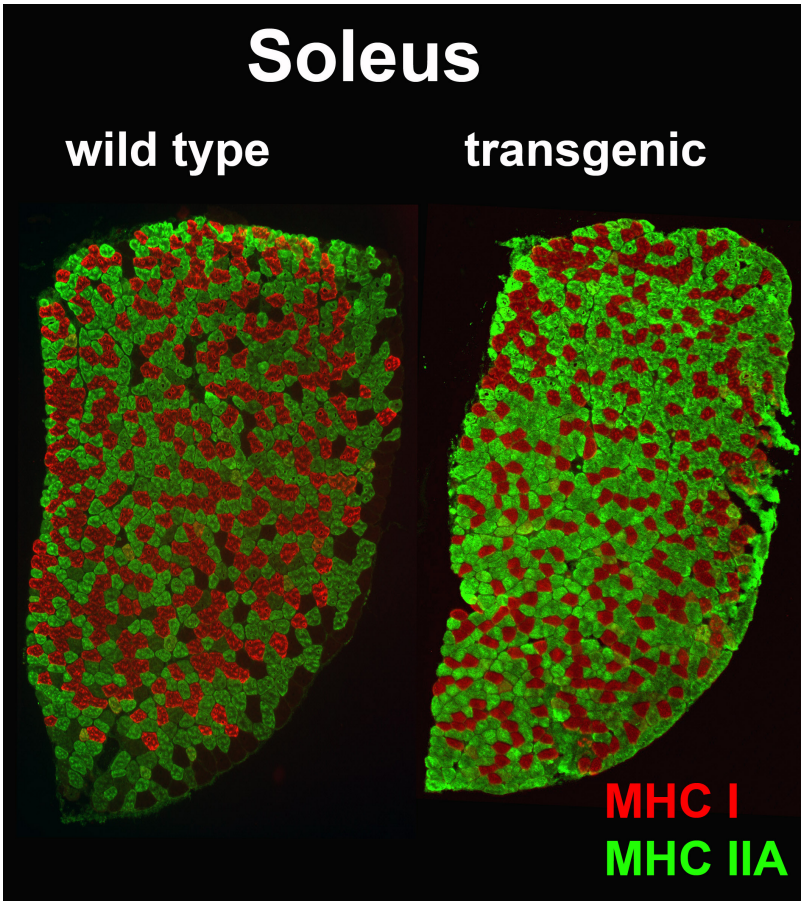
ERRγ luciferase assay

HEK 293T cells were plated in 96-well plates and cultured in DMEM supplemented with 10% FBS and 0.2% Normocine to reach 90% confluence. Cells were transfected with the Vegfa promoter-driven firefly luciferase (100 ng) along with a control plasmid (pCMX; 80 ng) or ERRγ plasmid (pCMX-ERRγ; 80 ng) using lipofectamine, according to the manufacturer's instructions (Invitrogen). Control renilla luciferase plasmid was included in all transfections for normalization. After transfection (24 h) a subset of cells was subjected to either normoxia or hypoxia for additional 24 h, followed by chemiluminescence measurement using the Dual-Glo Luciferase Assay System (Promega).

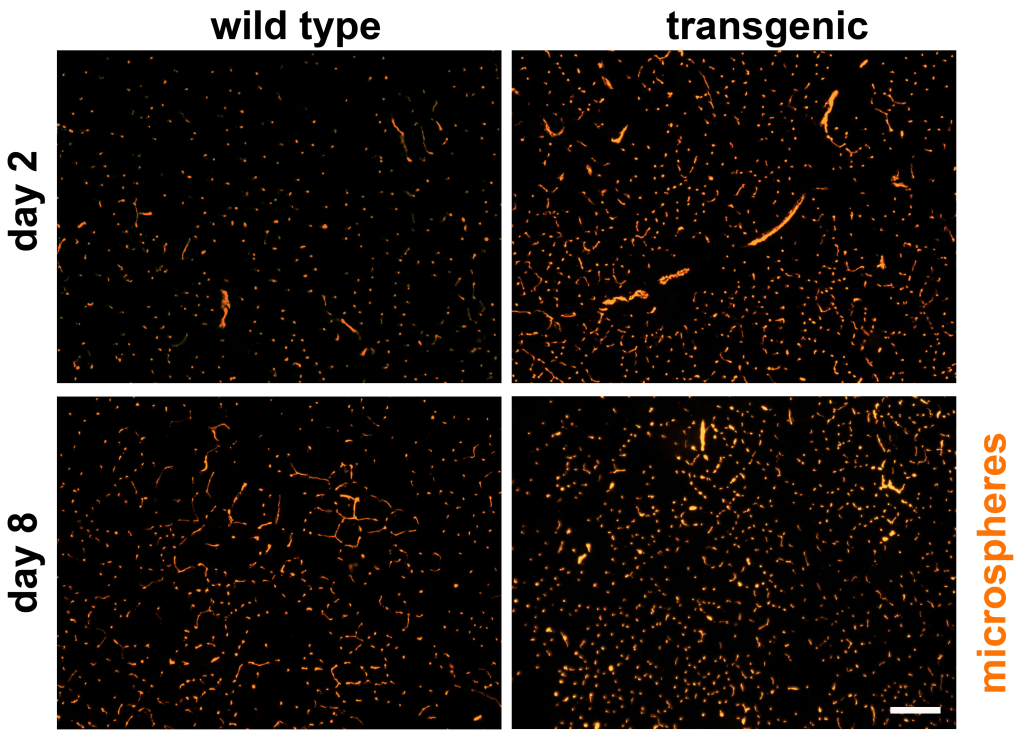
Chromatin Immunoprecipitation

ChIP assay was performed on C2C12 cells stably expressing ERRγ. Cells were grown in 10 cm tissue culture plates using DMEM supplemented with 20% FBS and Normocine. The cells were differentiated by culturing in differentiation medium (DMEM supplemented with 2% horse serum and Normocine) for 24 h, after reaching 70-80 % confluence. Next, the cells were subjected to hypoxia for 4hrs. ChIP was performed by using ChIP-IT Express Enzymatic Immunoprecipitation kit (Active Motif). DNA-protein crosslinking was performed using DMEM (without FBS) containing formaldehyde (1% final) at room temperature for 10 min with gentle rotation. Cells were scraped-off from the plates using police scrapper and pelleted at 2400 g for 10 min at 4°C. The cell pellets were re-suspended in cell lysis buffer containing protease inhibitor cocktail and incubated at 4°C for 30 min followed by efficient lysis using Dounce homogenizer. Samples were then centrifuged at 5000 rpm for 10 min at 4°C and the nuclear pellets were lysed with digestion buffer supplemented with protease inhibitors. DNA was sheared by using enzymatic shearing cocktail at 37°C for 20min. For antibody-enriched and IgG-antibody control samples, sheared chromatin was diluted in ChIP dilution buffer and pre-cleared using 20 µl of protein-G magnetic beads (Fisher Scientific) for 2 h at 4°C. For ChIP, 10% input samples were prepared and purified using the QIAquick Spin Kit (Qiagen). Pre-cleared chromatin was immunoprecipitated overnight with either a purified specific rabbit anti-ERRγ polyclonal antibody or rabbit IgG (Santa Cruz Biotechnology) and 25µl of protein-G magnetic beads. The beads were washed sequentially for 15 min at room temperature with three different buffers. First, beads were washed with buffer-I [0.1% SDS, 150 mM NaCl, 2 mM EDTA (pH 8), 20 mM Tris-HCl (pH 8)], then buffer-II [1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA (pH 8), 20 mM Tris-HCl (pH 8.1)] and finally with buffer-III (1% NP-40, 0.25 mM LiCl, 1% Na-deoxycholate, 1 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8)]. The beads were next washed briefly with TE buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8)] and de-crosslinked (1% SDS, 0.1 M NaHCO3) at 65°C overnight. De-crosslinked samples were purified using the QIAquick Spin Kit (Qiagen). PCR were performed using Phusion Taq-polymerase (Fisher Scientific) and primer pair for Vegfa promoter region (forward: CGGATTGTGGAAATCAGCAGACGA; reverse: AGAAGAGCCCAGAAGTTGGACGAA). PCR was performed with following condition; initial denaturation at 98°C for 45 s followed by 98°C for 20 s, 60°C for 20 s, 72°C for 20 s for 35 cycles.

2. SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

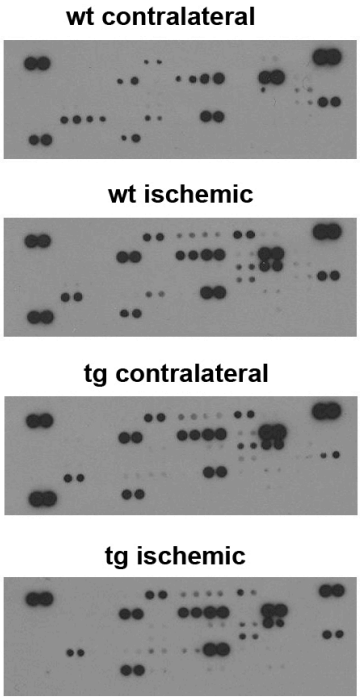


Online Figure I. Representative immunohistological images of the soleus depicting the total transverse muscle girth stained for MHC I (red) and IIA (green). IIX/IIB fibers are unstained (black). Note that the proportion of type I myofibers (red) is similar between the wild type and the transgenic soleus.

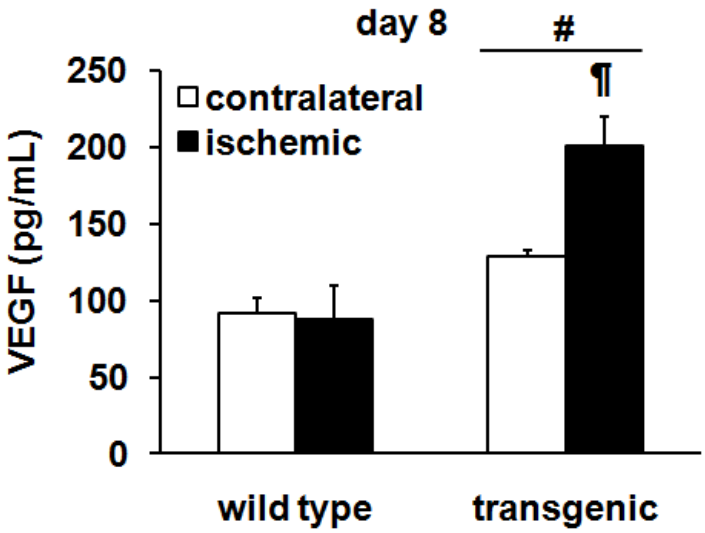


Online Figure II. Fluorescence microangiography in the contralateral non-ischemic muscle. Representative images of microsphere perfusion on day 2 and on day 8 in contralateral TA cryosections from wild type and transgenic mice. Scale 100 μm.

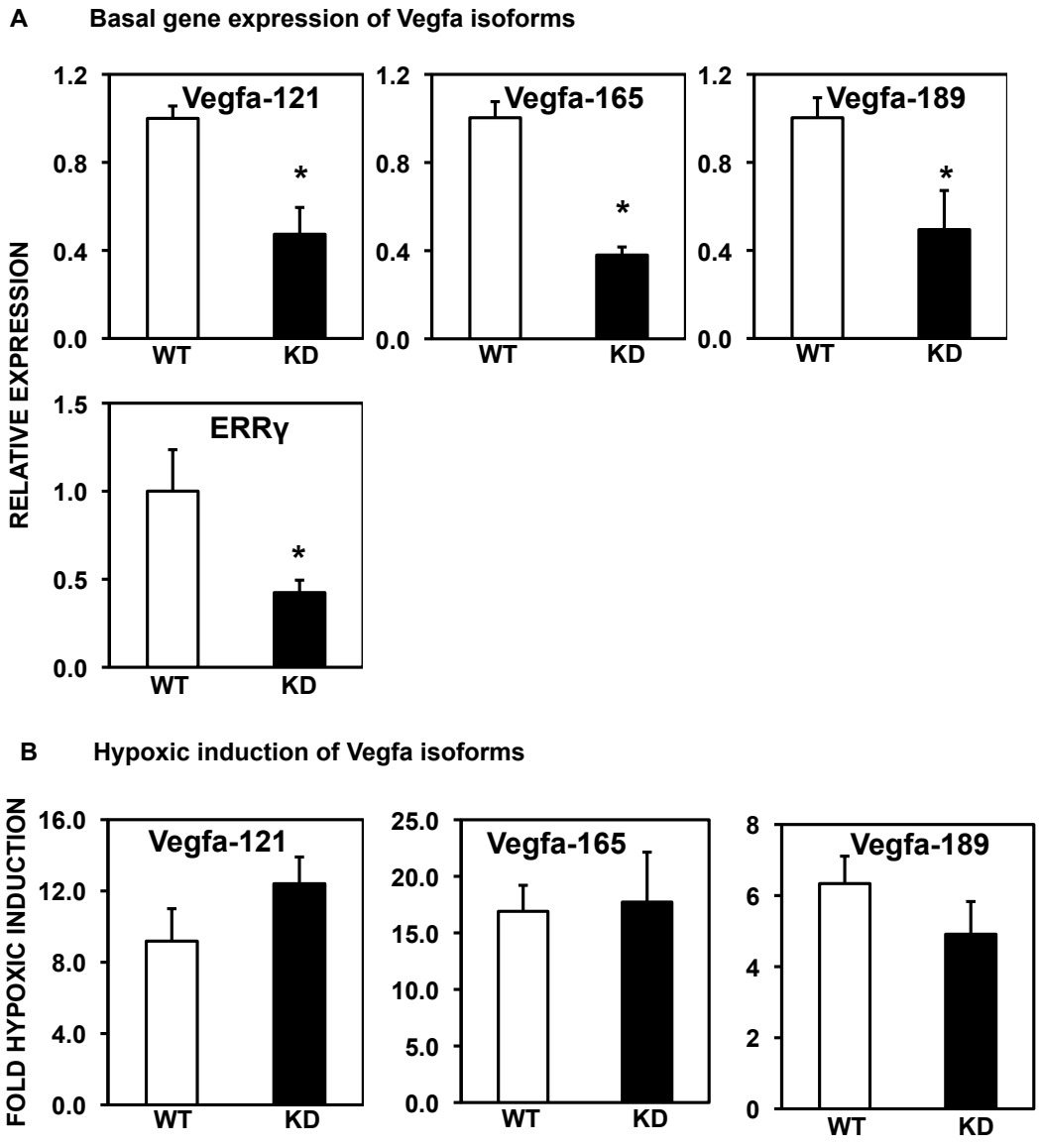
Factor	Fold-changes		
	Ischemia	Transgene	Ischemia + Transgene
Angiogenin	**7.4**	**8.8**	**16.3**
Angiopoietin-1	**2.5**	**4.7**	**1.4**
Angiopoietin-3	0.7	2.3	2.9
Coagulation Factor III	**16.3**	**13.2**	**0.7**
DPPIV (CD26)	**3.7**	**3.6**	**1.1**
Endoglin (CD105)	**2.4**	**3.2**	**1.2**
Endostatin/Collagen XVIII	1.2	1.4	1.1
Endothelin-1	**6.4**	**15.1**	**ND**
FGF-1	0.9	1.2	0.8
IGFBP-1	ND	ND	14.6
IGFBP-2	**10.0**	**17.0**	**0.6**
IGFBP-3	**8.0**	**8.6**	**6.5**
MMP-3 (pro and mature form)	**5.9**	**3.5**	**3.2**
NOV (IGFBP-9)	1.0	0.4	1.8
Osteopontin	1.3	1.0	1.2
PDGF-AA	**ND**	**ND**	**2.6**
PDGF-AB/PDGF-BB	**0.9**	**0.2**	**22.7**
Pentraxin-3	ND	ND	6.0
Platelet Factor 4	1.3	1.0	1.5
Prolactin	ND	1.3	ND
Serpin F1	**1.6**	**2.1**	**ND**
Thrombospondin-2	ND	ND	5.1
TIMP-4	**ND**	**ND**	**3.7**
VEGF-B	**ND**	**ND**	**2.0**



Online Figure III. Protein expression levels of various angiogenic regulators were determined in the indicated groups using a commercial protein array panel (R & D). The densiometric analysis was presented as fold change. Data is representative of N=5 animals/group. Fold change calculated as: Ischemia=wt ischemic/ wt contralateral; Transgene=tg contralateral/wt contralateral; Ischemia+Transgene=tg ischemic/tg contralateral. Factors induced by Ischemia, Transgene and/or Ischemia+Transgene are highlighted in bold.



Online Figure IV. VEGFA Elisa. VEGFA protein concentration in tissue lysates from the plantaris muscle on day 8 post-ischemia [two-way ANOVA, (#) $p<0.001$ significantly different from wild type; (¶) $p<0.001$ significantly different from transgenic contralateral, N=8 per genotype].



Online Figure V. Hypoxic response in mouse primary muscle cells. Following measurements were made in wild type (open bars) and ERRγ knockdown (KD; black bars) primary muscle cells. Isolation of primary cells from mouse muscles and stable KD of ERRγ using lentiviral siRNA was conducted, as we previously described [1]. **(A)** Basal expression of Vegfa121, 165 and 189 as well as ERRγ genes in differentiated (48 h) primary muscle cells. **(B)** Fold induction of Vegfa121, 165 and 189 gene expression in differentiated (48 h) primary muscle cells subjected to 24 h of hypoxia (N=6 per group). Values are represented as mean ± SD. Where indicated (*) represents statistically significant difference between WT and KD cells ($p<0.05$, Unpaired Student's t-test).

3. REFERENCES

1. Narkar VA, Fan W, Downes M, Yu RT, Jonker JW, Alaynick WA, Banayo E, Karunasiri MS, Lorca S, Evans RM. Exercise and pgc-1alpha-independent synchronization of type i muscle metabolism and vasculature by errgamma. *Cell Metab*. 2011;13:283-293
2. Limbourg A, Korff T, Napp LC, Schaper W, Drexler H, Limbourg FP. Evaluation of postnatal arteriogenesis and angiogenesis in a mouse model of hind-limb ischemia. *Nat Protoc*. 2009;4:1737-1746
3. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S. Microrna-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science*. 2009;324:1710-1713
4. Matsakas A, Foster K, Otto A, Macharia R, Elashry MI, Feist S, Graham I, Foster H, Yaworsky P, Walsh F, Dickson G, Patel K. Molecular, cellular and physiological investigation of myostatin propeptide-mediated muscle growth in adult mice. *Neuromuscul Disord*. 2009;19:489-499
5. Matsakas A, Mouisel E, Amthor H, Patel K. Myostatin knockout mice increase oxidative muscle phenotype as an adaptive response to exercise. *J Muscle Res Cell Motil*. 2010;31:111-125