SUPPLEMENTAL MATERIAL

PGC-1α Induces SPP1 to Activate Macrophages and Orchestrate Functional Angiogenesis in Skeletal Muscle

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SUPPLEMENTAL ONLINE DETAILED METHODS

Animals

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. MCK-TTA and TRE-PGC-1**α**-inducible mice[1] were obtained from Dr. Daniel Kelly at Washington University St Louis (now at Sanford Burnham Institute, Florida) and are in a mixed C57Bl/6 and 129 strain. 10-12 week old mice were used for all experiments unless specified. Littermate controls were used in all experiments. No differences were observed between genders, and thus genders were mixed. Purina chow containing doxycycline (200 mg/kg), 45% fat were all purchased from Research Diet Inc., New Brunswick, NJ). For the old, diabetic mice experiments; 2 year old mice were used. sVEGFR1 mice were kindly provided by Dr. Eli Keshet, Jerusalem, Israel[2]. SPP1 -/- mice were purchased from Jackson Labs (Stock No. 004936). TRE-VEGFA mice were generated by subcloning the murine VEGFA120 pro-peptide into the HPRT-targeting vector pMP8-II containing the tet-responsive promoter. ES cells were then generated by homologous recombination, and chimeras were bred for germline transmission. Transgenic mice were then bred with MCK-TTA mice to drive expression in skeletal muscle. All transgenic animals were maintained hemizygous.

Cells and Reagents

Immunostaining was performed using anti-CD31 antibody (BD Pharmingen) and anti PDGFR**β** antibody (R&D Systems). Human umbilical cord endothelial cells (HUVECs) and C2C12 cells were maintained in endothelial basal medium-2 (EBM-2) and Dulbecco's modified Eagle's medium (DMEM; supplemented with 10% fetal bovine serum(FBS)), respectively. C2C12 cells were differentiated in DMEM (2% horse serum).

Primary skeletal myocytes were isolated from ERRαKO and wildtype animals were performed on entire hindlimb muscle after collagenase/dispase digestion, as described previously[3]. Cell were maintained in Ham's F10; supplemented with 20% FBS and 2.5ng/ml basic fibroblast growth factor (bFGF)) and switched into differentiation media (DMEM (5% horse serum)). Cells were infected with adenovirus expressing GFP or PGC-1α at a multiplicity of infection (MOI) of 10–30, for 48 hrs prior to harvesting and processing of mRNA.

THP-1 cells (ATCC) were seeded in DMEM and 20% FBS at $1x10^5$ cells/well in 24 well plates, and differentiated with 10ng/mL PMA for 48 hours. After differentiation, THP-1 cells were incubated with control, PGC-1**α** or SPP1 conditioned medium for 24 hrs and cells harvested for qPCR and western blots.

10T1/2 (ATCC) were cultured and maintained in DMEM with 10% FBS. Prior to migration assays 10T1/2 cells were differentiated with 1ng/mL TGF-**β** in DMEM.

CCR-1 (J-113863) and CCR-2 (sc-202525) antagonists were purchased from Tocris Bioscience and Santa Cruz respectively. SPP1 antibodies AF808 and SC-10593 purchased from R&D Systems and Santa Cruz for the neutralization and western blot respectively.

Real-time PCR

Total RNA was isolated from mouse tissue and cultured cells using the TRIZOL (Invitrogen) and Turbocapture (Qiagen) method, respectively. Samples for real-time PCR analyses were reverse transcribed (Applied Biosystems), and quantitative real-time PCRs were performed on the cDNAs in the presence of fluorescent dye (SYBR green; Bio-Rad). Relative expression levels were determined using the comparative cycle threshold method. The qPCR data was normalized against control primers for mu hprt, mu tbp and mu 36B4 (see Supplement Table II)

ELISA

Angiogenic gene panel ELISA was performed by Aushon Biosystems, Bellirica, MA using their Searchlight Assay services.

Measurement of Intravascular Volume

Intravascular volume was measured by injecting ^{125}I-BSA intravenously into wild type and PGC-1α transgenic mice after 4 weeks of transgene induction. The tracer was allowed to circulate for 5 minutes and then the amount of radioactivity in the muscle was measured in a gamma counter [4].

Lectin and Evans Blue injections

Animals were anesthetized with ketamine-xylazine and 50uL of Alexafluor 594 Isolectin GS-IB4 (Molecular Probes, Invitrogen) was administered intravenously, muscle harvested and embedded in OCT after 10 min. Evans blue dye (1% solution) was i.p.-injected at a concentration of 1% volume per gram of body weight. After 16 h, mice were sacrificed and skeletal-muscle frozen histological sections were prepared. Evans blue incorporation was analyzed by fluorescence microscopy[5].

Endothelial cell migration assay

Differentiated C2C12 myotubes in 24-well plates were infected with adenovirus expressing GFP or PGC-1α for 34 h. BSA or soluble Flt1 (100 ng/ml; R & D Systems) was added to the medium for 12 h. Then, $5x10^4$ cells of HUVECs were plated on the upper compartment of transwells (8.0 μm pore size) prewarmed with EBM-2 medium for 16 h at 37°C. HUVEC migration to the lower compartment of transwells was measured after 12 h. Migrated HUVECs were fixed with 4% paraformaldehyde in PBS for 20 min at RT, and cells that remained in the upper compartment were removed with cotton swabs. Cells were blocked with 5% BSA in PBS-Tween 20 (PBST; 0.2% Tween 20) and stained with phalloidin-FITC in PBST for 4 h to visualize filamentous actin. Transwell inserts were washed three times in PBST and mounted onto slides with DAPI mounting medium.

Conditioned Medium Preparation

C2C12 myotubes were infected with PGC-1α retrovirus selected for puromycin to obtain a stable cell line. These cells were incubated in serum free DMEM and the PGC-1α-conditioned medium was collected after 48 hrs. For preparation of SPP1 conditioned medium, HEK293T cells were transiently transfected with SPP1 expression plasmid and the transfection medium was changed to DMEM plus 0.5% FBS after 24hrs. This conditioned medium was collected after 48hrs. For THP-1 conditioned medium experiments, differentiated THP-1 cells were incubated with control or PGC-1α CM and then cocultured with HUVECs, pericytes or smooth muscle cells in transwell inserts and migration was measured after 12h as described in the previous section.

Western Blotting

Conditioned media from C2C12s overexpressing either PGC-1α or control virus were processed for western blotting (conditioned media from HEK 293 cells overexpressing SPP1 was used as a positive control). Media was collected after 48hrs and subjected to electrophoresis on 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (BioRad) for Western blot analysis. SPP1 antibody (SC-10593 Santa Cruz) was used and bands were detected by chemiluminescence following the manufacturer's instructions.

Hind limb ischemia

Animals were anesthetized with ketamine-xylazine and shaven anteriorly distal to the midriff, including both hind limbs. A skin incision was made over the left femoral artery, just distal to the inguinal ligament. The femoral artery was then visualized and ligated proximally at the inguinal ligament and again approximately 1cm distally, and the section of the artery between the two ligatures was removed. Cautery was used to control the bleeding, and three injections of $1x10^8$ pfu adeno-PGC-1α versus adeno-GFP control were done, two in the inner thigh and one in the gastrocnemius. The incision was then closed with sutures and the mice were monitored throughout the recovery process. The recovery of blood flow was tracked non-invasively by infrared Doppler scanning of the lower limb, excluding the foot [6]. Ambulatory score was as follows: 0=no impairment, 1=no toe flexion, 2=no plantar flexion, 3=no use of foot. Tissue necrosis score was: 0=no necrosis, 1=discoloration, 2=loss of 1-2 toes, 3=loss of 3-5 toes, 4=necrosis of the foot.

Microarray analysis

RNA was extracted from the WT and PGC-1α transgenics after 4 weeks of PGC-1α expression using the TRIZOL (Qiagen) method and used to probe Affymetrix mouse 1.0 gene arrays by the Dana Farber Cancer Institute, Boston, MA. The data obtained was analyzed using the Gene Set Enrichment Analysis (Broad Institute of MIT and Harvard).

Histological analysis

Quantification of capillaries was performed computationally, using Volocity (Improvision; PerkinElmer) and ImageJ software, on three random fields chosen from the mid portions of transverse sections from the indicated muscles. All choices of random fields and quantifications were performed blind.

Hemodynamic measurements

Invasive hemodynamic measurements were performed by the BIDMC Mouse Cardiac Physiology Core. Animals were anesthetized with isoflurane via precise vaporizer. The right carotid artery was dissected and catherized with a 1.0-Fr high-fidelity pressure catheter (Scisense). The catheters were advanced to the left ventricular (LV) chamber to obtain pressure volume (PV) loop data. Pressure signals were recorded at 2 kHz for 60 min, and analyzed using PowerLab software (Chart 4.1.2, ADInstruments). For blood pressure (BP) measurement a 1.2 Fr pressure catheter from Millar was used, measurements were recorded in carotid artery (not advanced to LV).

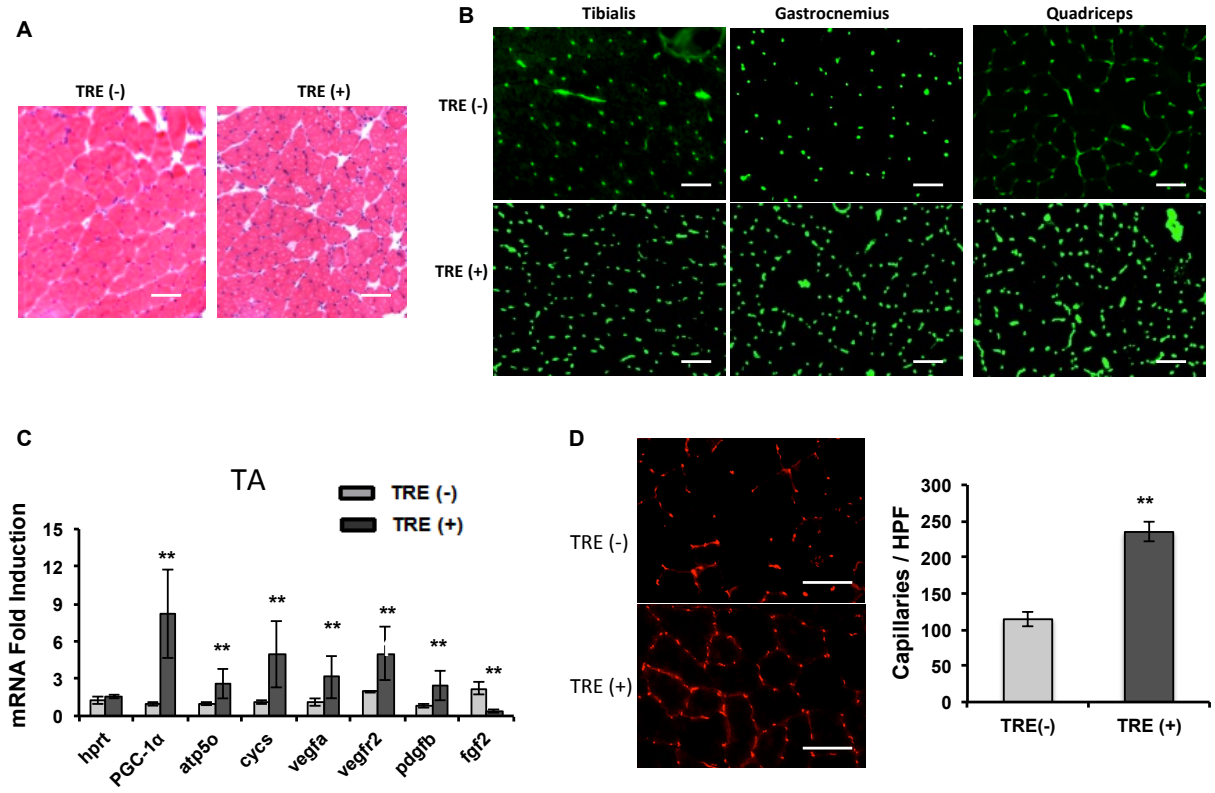
Vascular Cast and Micro-CT

Animals were anesthetized with ketamine-xylazine and perfused through the left ventricle with 20mls of 37°C heparinized saline (10U/ml heparin) followed by 20mls of formalin. After formalin perfusion, mice were perfused with 20mls of MICROFIL Yellow (Flow Tech Inc), MICROFIL was allowed to cure overnight at 4°C. Once the cast was set, hindlimbs were removed and scanned in a uCT35 scanner (Scanco Medical USA, Wayne PA) with the following conditions a 6 μm voxel size, 55 kVp, and 144 μA.

Statistical analysis

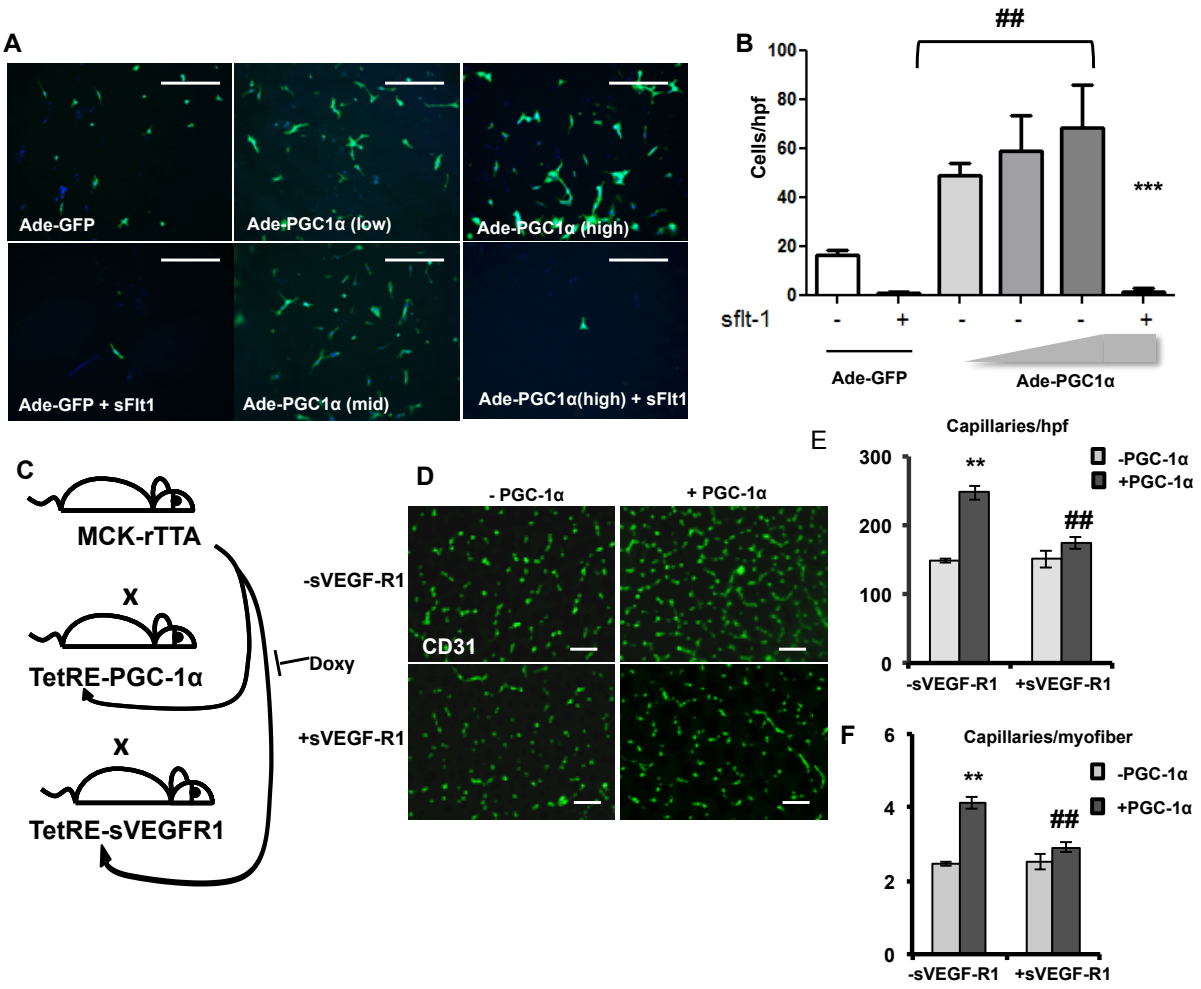
The data are presented as means ± SE. Statistical analysis was performed with Student's **t**-test for all in vitro and in vivo experiments. P-values of <0.05 were considered statistically significant.

SUPPLEMENTAL FIGURES



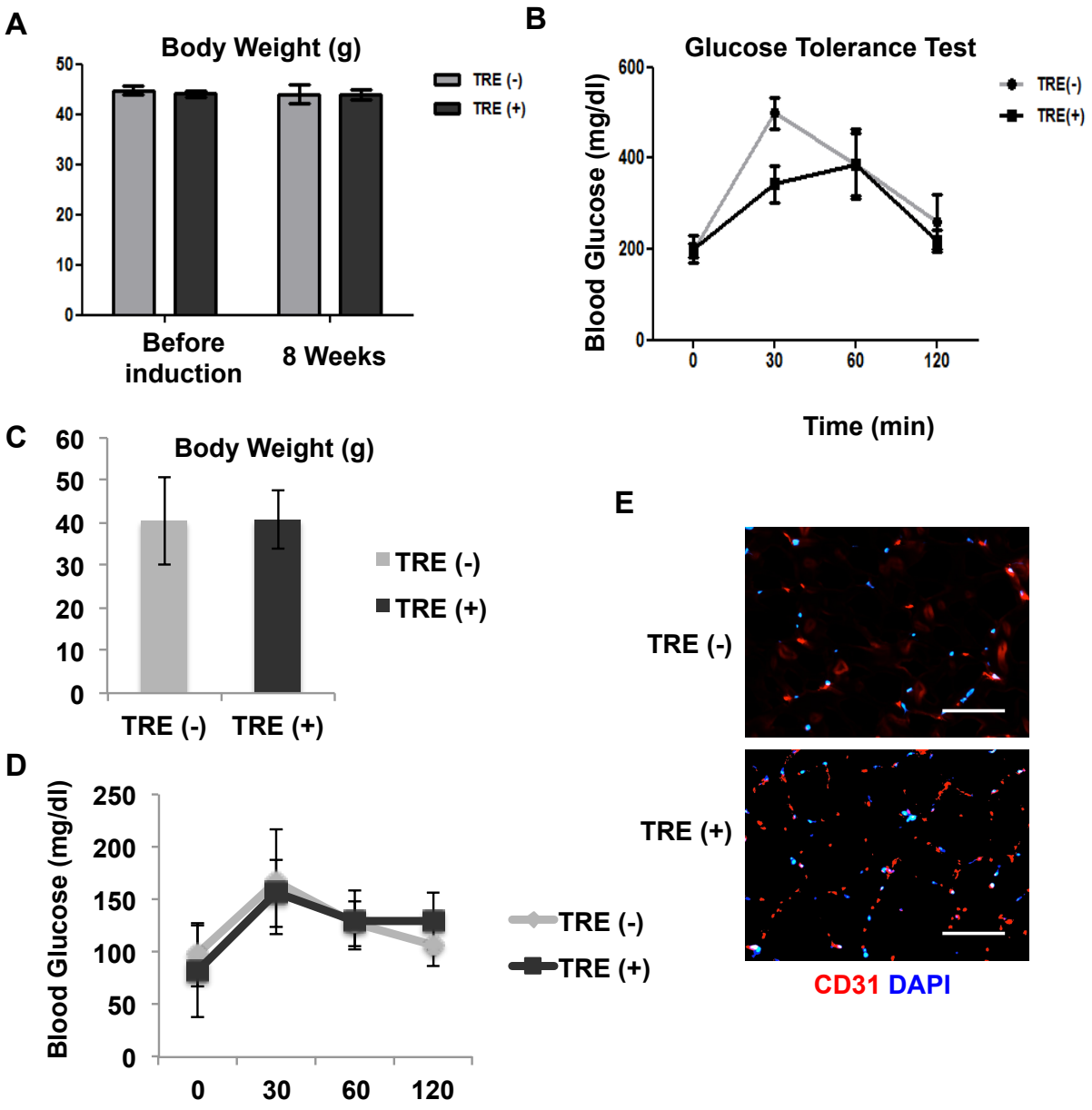
Supplemental Figure I

A, Hemotoxylin and eosin (H&E) staining of quadriceps muscle of PGC-1α expressing TRE (-) and TRE (+) mice. **B,** Capillary density in the tibialis, gastrocnemius and quadriceps muscle of TRE (-) and TRE (+) mice. **C,** Expression of mitochondrial and angiogenic genes in the tibialis muscles after 4 weeks of doxy removal. **D,** Capillary density measured by CD144 staining; sample images on left, and quantification on right. scale bar = 100μm. N=4/group, ** significance with $P<0.01$.



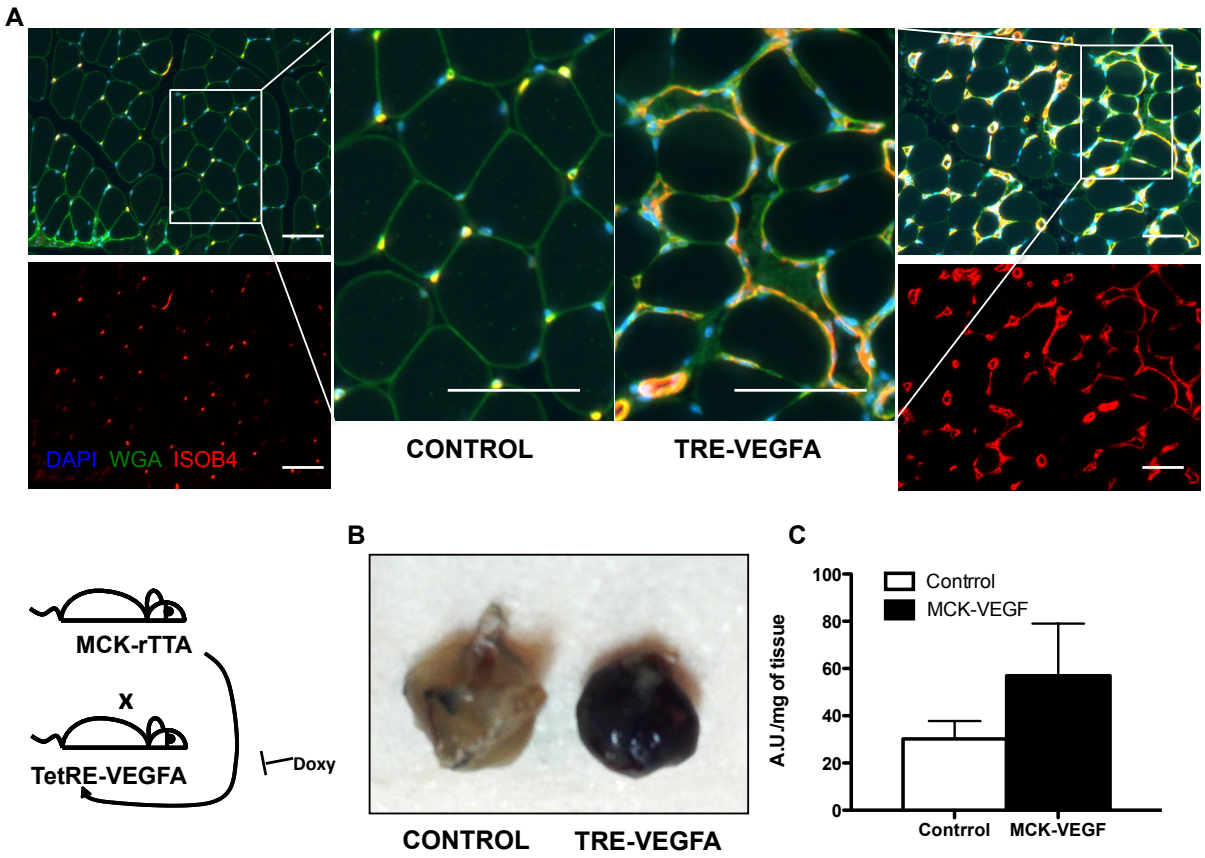
Supplemental Figure II: PGC-1α-mediated angiogenesis in skeletal muscle is VEGF-dependent

A, Endothelial cells (HUVECs) stained with phalloidin-FITC and DAPI (4, 6-diamidino-2-phenylindole), after migration in response to medium conditioned by C2C12 myotubes overexpressing PGC-1α versus GFP control. **B,** Quantification of migrated cells in A (hpf: high power field). **C,** Generation of mice expressing muscle specific sVEGFR-1 and PGC-1α under the control of the 'tet-off' promoter. Removal of doxycycline from chow results in the induction of PGC-1α and sVEGFR1 transgenes. **D,** Capillary density in the quadriceps muscle after 4 weeks of doxy chow removal. **E,** Quantification of capillary numbers per hpf and per myofiber. Error bars indicate SE. N=8/group, # * Significance with $P<0.05$, **$P<0.01$, ***$P<0.001$. scale bar = 100μm.



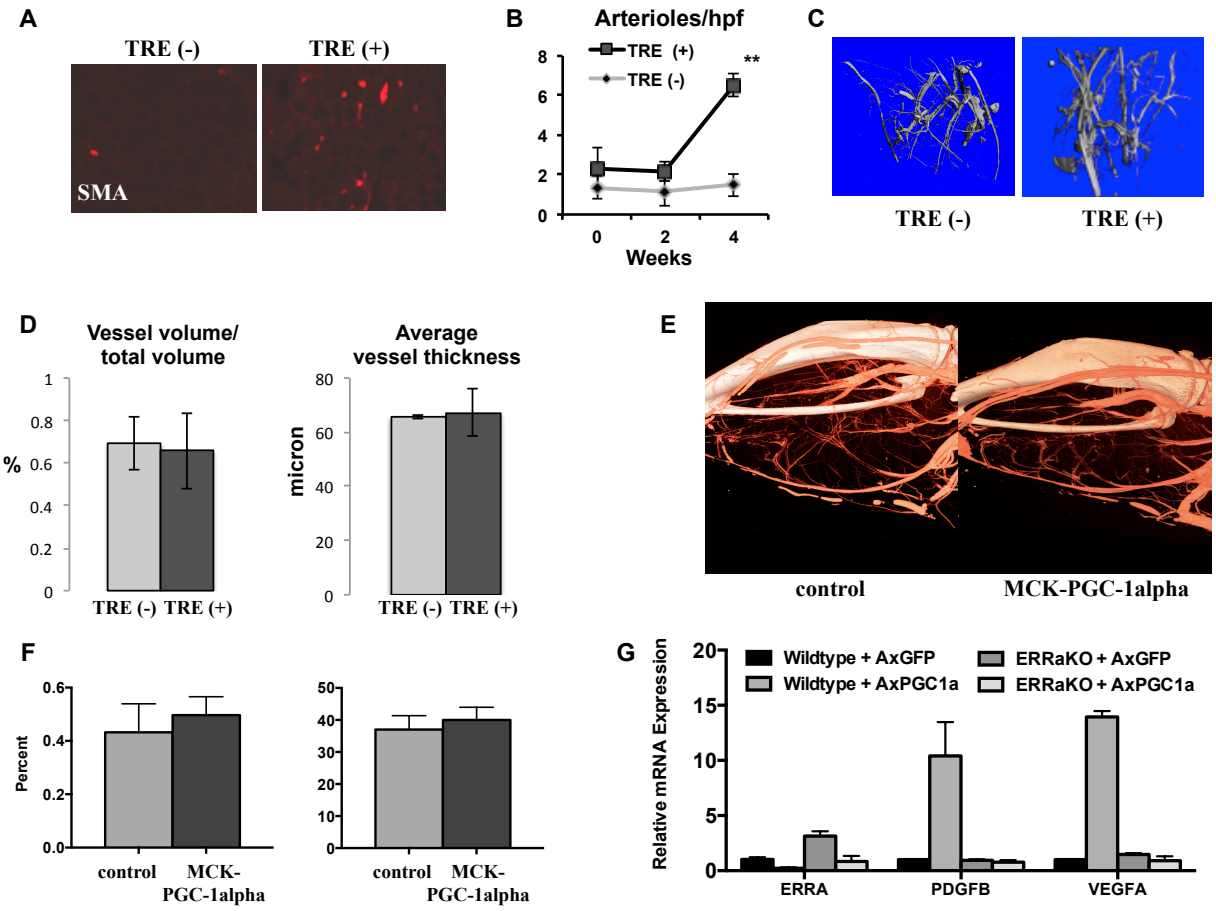
Supplemental Figure III

A, Body weights of TRE (-) and TRE (+) 2 yr-old high fat-fed diabetic mice. **B,** Glucose tolerance test of TRE (-) and TRE (+) old high fat-fed diabetic mice. **C,** Body weights of TRE (-) and TRE (+) 2 yr-old chow-fed mice. **D,** Glucose tolerance test of TRE (-) and TRE (+) 2-yr-old chow-fed mice. **E,** capillary density in TRE (-) and TRE (+) 2-yr-old chow-fed mice. scale bar = 100μm.



Supplemental Figure IV

A, Neovascularization in gastrocnemius of TRE (+) VEGFA mice. **B,** Evans Blue Dye leak into gastrocnemius of TRE (+) VEGFA mice. **C,** Quantification of B.

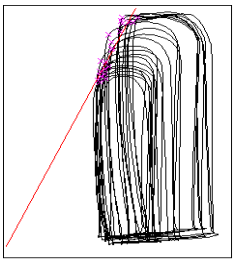


Supplemental Figure V

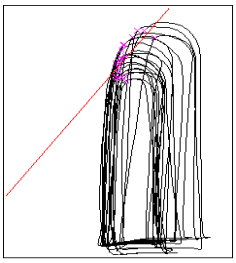
A, Arteriolar density of TRE (-) and TRE (+) quadriceps muscle after 4 weeks of PGC-1α induction. **B,** Quantification of arterioles at 0, 2 and 4 weeks of PGC-1α transgene expression. **C,** MicroCT visualization of arteries in TRE (-) and TRE (+) animals. **D,** Quantification of C. **E,** MicroCT visualization of arteries in control and MCK-PGC-1α animals. **F,** Quantification of E. G, gene expression of the indicated genes in primary skeletal muscle cells either ERRα -/- (ERRαKO) vs wildtype control, 48hrs after infection with adeno-PGC-1α (AxPGC1α) versus GFP-encoding control. Error Bars are SE. N=4/group, ** significance with P<0.01. scale bar = 100μm.

A

TRE (-)



TRE (+)

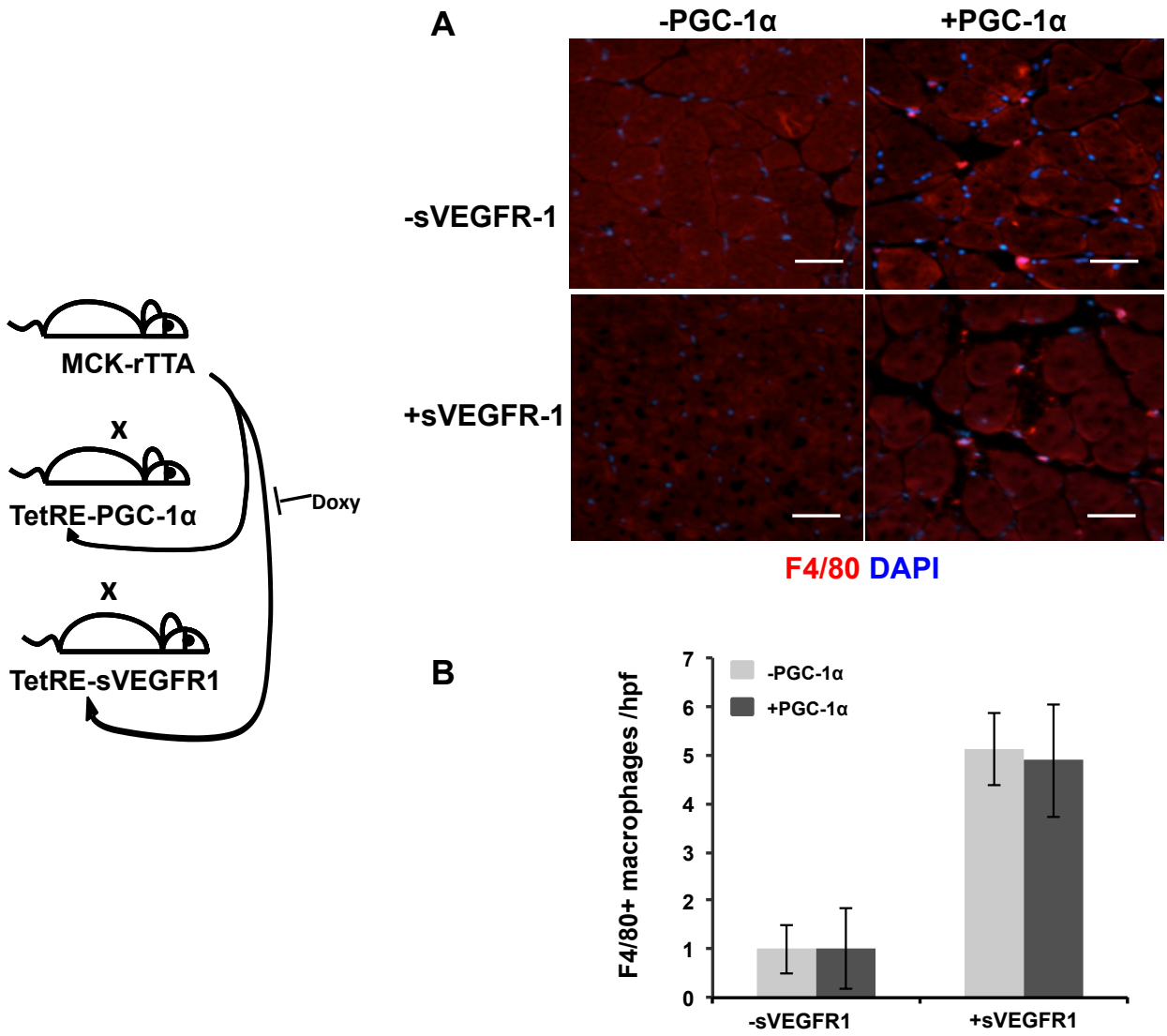


B

	HR	MAX LVP	MIN-LVP	EDM-LVP	MAX-LV_V	MIN-LV_V	SV (ul)	CO ml/min	EF (%)	SW	DP/DT(mmHg/ Sec) MAX	DP/DT(mmHg/ Sec) MIN	AP(mmHg) MAX	AP(mmHg) MIN	SVR mHRU
AVE TRE (-)	490.5	110.6	-0.44	6.14	52.7	30.0	15.7	7.7	32.6	1986.0	9894.7	-6066.5	110.0	74.4	12.2
AVE TRE (+)	499.9	114.5	1.03	9.58	55.8	34.0	16.6	8.3	31.7	1920.1	8254.0	-7159.0	115.0	76.9	10.9
SEM	19.8	3.7	1.0	1.7	4.9	5.1	2.1	1.1	4.2	125.4	433.0	3028.8	2.9	1.8	1.7
SEM	22.4	13.6	3.0	4.5	6.6	6.1	1.2	0.7	2.1	228.3	416.5	378.8	11.1	8.3	0.9
p-value:	0.752	0.743	0.593	0.422	0.699	0.613	0.765	0.676	0.872	0.782	0.025	0.761	0.613	0.722	0.559

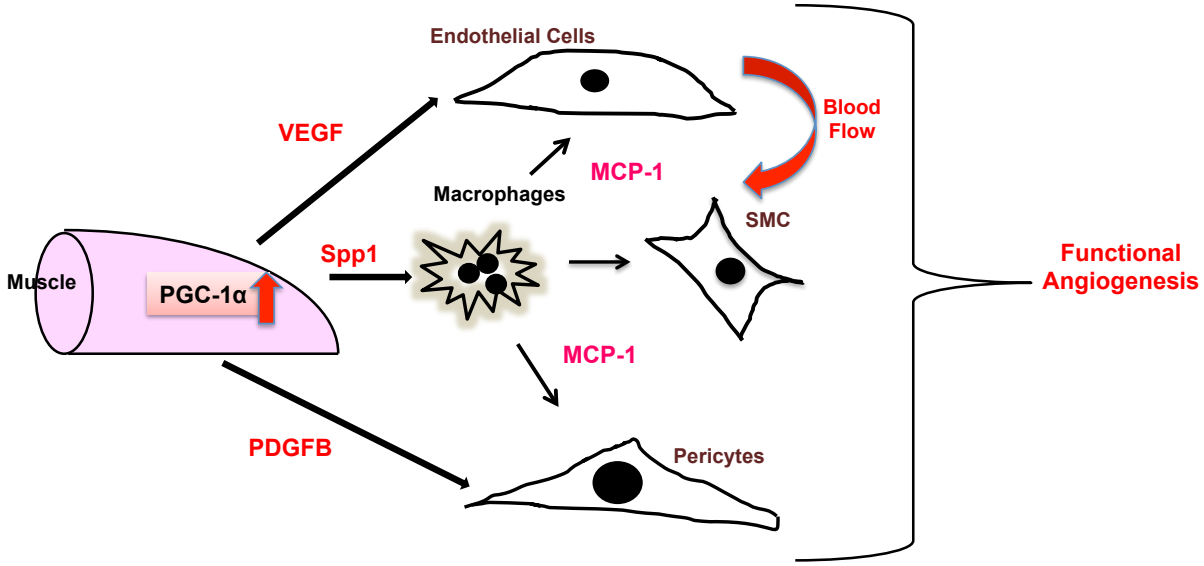
Supplemental Figure VI

A, Representative Frank Starling curves from TRE (-) and TRE (+) hearts. **B,** Invasive hemodynamic measurements in TRE (-) and TRE (+) hearts. N=5 per group.



Supplemental Figure VII

A, Representative images of F4/80 staining in gastrocnemius mice expressing muscle specific sVEGFR-1 and PGC-1α under the control of the 'tet-off' promoter (see Supplemental Figure II). **B,** Quantification of A. scale bar = 100μm.



Supplemental Figure VIII: Model for PGC-1α-mediated orchestration of different cell types, myokines, and cytokines to mediate functional angiogenesis in skeletal muscle.

probe set	gene	fold change
10403911	Gpx6: glutathione peroxidase 6	11.49
10569102	**Irf7: interferon regulatory factor 7**	10.98
10408557	**Serpinb1a: serine peptidase inhibitor b1a**	10.04
10538187	**Gpnmb: glycoprotein nmb**	9.07
10461594	Ms4a4c: membrane-spanning 4-domains a4c	8.71
10461721	**Mpeg1: macrophage expressed gene 1**	8.4
10342666		8.34
10494271	**Ctss: cathepsin S**	7.78
10500335	**Fcgr1: Fc receptor, IgG, high affinity I**	7.58
10338973		7.27
10523717	**Spp1: secreted phosphoprotein 1**	7.13
10387536	**Cd68: CD68 antigen**	7.12
10515378	Hpdl: 4-hydroxyphenylpyruvate dioxygenase-like	6.91
10509985	Gm693: gene model 693, (NCBI)	6.79
10348166	Chrng: cholinergic receptor, nicotinic, gamma	6.78
10598976	Timp1: tissue inhibitor of metalloproteinase 1	6.51
10524621	Oasl2: 2'-5' oligoadenylate synthetase-like 2	6.49
10481175	1110002H13Rik: RIKEN cDNA 1110002H13 gene	6.3
10339373		6.24
10342868		6.16
10461636		6.13
10547657	**C3ar1: complement component 3a receptor 1**	6.11
10603551	**Cybb: cytochrome b-245, beta polypeptide**	5.99
10375515	**Ifi47: interferon gamma inducible protein 47**	5.87
10565132	BC048679: cDNA sequence BC048679	5.86
10509410	**Rap1gap: Rap1 GTPase-activating protein**	5.64
10487823	**Siglec1: sialic acid binding Ig-like lectin 1**	5.38
10551883	**Tyrobp: TYRO tyrosine kinase binding protein**	5.33
10508734	Ptafr: platelet-activating factor receptor	5.27

Supplemental Table I. Top genes induced by PGC-1alpha in transgenic animals. In blue: macrophage/monocyte-associated genes.

Supplemental Table II. Primers for qPCR Analysis

Primer Name	Sequence
mHprt F	GTTAAGCAGTACAGCCCCAAA
mHprt R	AGGGCATATCCAACAACAAACTT
MTbp F	CCCTATCACTCCTGCCACACCAGC
mTbp R	GTGCAATGGTCTTTAGGTCAAGTTTACAGCC
m36b4 F	GGAGCCAGCGAGGCCACACTGCTG
m36b4 R	CTGGCCACGTTGCGGACACCCTCC
mPGC-1 Ex3-5 F	AGCCGTGACCACTGACAACGAG
mPGC-1 Ex3-5 R	GCTGCATGGTTCTGAGTGCTAAG
mAtp5o F	AGGCCCTTTGCCAAGCTT
mAtp5o R	TTCTCCTTAGATGCAGCAGAGTACA
mu Vegfa F	CTGTAACGATGAAGCCCTGGAG
mu Vegfa R	TGGTGAGGTTTGATCCGCAT
mu Pdgfb F	GGCAGCAGCAGAGCCTGCTG
mu Pdgfb R	GTTGGCGTTGGTGCGATCGATGAGG
mu CD31 F	CATTGAGGCGCAGGACCACGTG
mu CD31 R	GAAGGACTCCTGCACGGTGACG
mu Fgf2(bfgf) F	GCGAGAAGAGCGACCCACACG
mu Fgf2(bfgf) R	AAGCCAGCAGCCGTCCATCTTCC
mu Vegfr2 F	GGTACAGAAATGGAAGGCCC
mu Vegfr2 R	TGGTGAGGATGACCGTGTAG
mu Mpeg1 F	CTGGATGATAATAGCGTGTGC T
mu Mpeg1 R	CAAGACAGGTAGTTTCAGGGC
mu CD169 F	GCTGGTGGACAAGCGTTTC
mu CD169 R	TTCAAGTCTTTGAGCAACAGGT
mu Irf7 F	GAGACTGGCTATTGGGGGAG
mu Irf7 R	GACCGAAATGCTTCCAGGG
mu FcgR1 F	CAATGGTTTATCAACGGAACAGC
mu FcgR1 R	ATGCTATAACTAGGCGTGGAGA
mu SPP1 F	AGCAAGAAACTCTTCCAAGCAA
mu SPP1 R	GTGAGATTCGTCAGATTCATCCG
mu Cathepsin S F	AGA AGG GCT GCG TCA CTG AG
mu Cathepsin S R	GAA TGT ACC TTG AAC ACG TAG
mu gbnmb F	CAG GAA TGA TTT GGG ACT GAC C
mu gbnmbR	CCG GGA ACC TGA GAT GCT G
mu GAPDH f	AGGTCGGTGTGAACGGATTTG
mu GAPDH r	TGTAGACCATGTAGTTGAGGTCA
hu cd14 f	ACGCCAGAACCTTGTGAGC
hu cd14 r	GCATGGATCTCCACCTCTACTG
hu mip1b(ccl4) f	GATTACTATGAGACCAGCAGCC

hu mip1b(ccl4) r	TTCAGTTCCAGGTCATACACG
hu MCP-1 (ccl2) f	CAGAAGTGGGTTCAGGATTCC
hu MCP-1 (ccl2) r	ATTCTTGGGTTGTGGAGTGAG
hu ccl7 f	AGACCAAACCAGAAACCTCC
hu ccl7 r	TGAGCAGCAGACACAGAAG
huTNFa f	AGGTCTACTTTGGGATCATTGC
huTNFa r	GAAGAGGTTGAGGGTGTCTG
hu IL6 f	AAATTCGGTACATCCTCGACGG
hu IL6 r	GGAAGGTTCAGGTTGTTTTCTGC
hu il8 f	ACTGAGAGTGATTGAGAGTGGAC
hu il8 r	AACCCTCTGCACCCAGTTTTC
hu il1m f	CCTCATGCTCTGTTCTTGGG
hu il1m r	TGTCCTGCTTTCTGTTCTCG
hu CD163 f	ACTTGAAGACTCTGGATCTGCT
hu CD163 r	CTGGTGACAAAACAGGCACTG
hu CD206 f	GCTCCTGGTTTTTGCCTCTGT
hu CD206 r	CACTGGGACTCACTGCATCC
hu CD169 f	GCTGGTGGACAAGCGTTTC
hu CD169 r	TTCAAGTCTTTGAGCAACAGGT

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