Rationale: Mechanisms of angiogenesis in skeletal muscle remain poorly understood. Efforts to induce physiological angiogenesis hold promise for the treatment of diabetic microvascular disease and peripheral artery disease but are hindered by the complexity of physiological angiogenesis and by the poor angiogenic response of aged and patients with diabetes mellitus. To date, the best therapy for diabetic vascular disease remains exercise, often a challenging option for patients with leg pain. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α), a powerful regulator of metabolism, mediates exercise-induced angiogenesis in skeletal muscle.

Objective: To test whether, and how, PGC-1α can induce functional angiogenesis in adult skeletal muscle.

Methods and Results: Here, we show that muscle PGC-1α robustly induces functional angiogenesis in adult, aged, and diabetic mice. The process involves the orchestration of numerous cell types and leads to patent, nonleaky, properly organized, and functional nascent vessels. These findings contrast sharply with the disorganized vasculature elicited by induction of vascular endothelial growth factor alone. Bioinformatic analyses revealed that PGC-1α induces the secretion of secreted phosphoprotein 1 and the recruitment of macrophages. Secreted phosphoprotein 1 stimulates macrophages to secrete monocyte chemoattractant protein-1, which then activates adjacent endothelial cells, pericytes, and smooth muscle cells. In contrast, induction of PGC-1α in secreted phosphoprotein 1−/− mice leads to immature capillarization and blunted arteriolarization. Finally, adenoviral delivery of PGC-1α into skeletal muscle of either young or old and diabetic mice improved the recovery of blood flow in the murine hindlimb ischemia model of peripheral artery disease.

Conclusions: PGC-1α drives functional angiogenesis in skeletal muscle and likely recapitulates the complex physiological angiogenesis elicited by exercise. (Circ Res. 2014;115:504-517.)

Key Words: angiogenesis effect ■ cytokines ■ gene action regulation ■ genetic therapy ■ intercellular signaling peptides and proteins ■ macrophages ■ muscle, skeletal ■ osteopontin ■ peripheral arterial disease ■ PGC-1alpha protein, mouse

Exercise is a potent stimulus for angiogenesis in adult skeletal muscle, and is one of the few instances of non-pathological angiogenesis that occurs in mammals after development. However, how exercise orchestrates the complex process of physiological angiogenesis in skeletal muscle remains poorly understood. One prevailing notion has been that the metabolic needs of exercising muscle lead to local hypoxia, activation of the transcription factor hypoxia inducible factor-1α (HIF-1α), and induction of vascular endothelial growth factor (VEGF) and angiogenesis. However, hypoxia has been difficult to show in muscles undergoing endurance exercise, a potent stimulus for angiogenesis. Moreover, deletion of HIF-1α increases, rather than decreases, capillary density in skeletal muscle.

The transcriptional coactivator peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) is a powerful regulator of mitochondria and metabolism in multiple tissues.6,7 In skeletal muscle, PGC-1α orchestrates the induction of hundreds of genes involved in mitochondrial biology, including components of the electron transport chain and β oxidation. Transgenic animals overexpressing PGC-1α in skeletal myocytes have markedly more mitochondria and as a result have...
We recently showed that PGC-1α also regulates VEGF and other angiogenic factors. PGC-1α induces VEGF in a HIF-independent fashion by coactivating estrogen-related receptor-α on a novel enhancer in the first intron of the VEGF gene. We also recently showed that PGC-1α is required for exercise-induced angiogenesis. Together, these observations suggest that PGC-1α likely orchestrates physiological angiogenesis in skeletal muscle. Indeed, transgenic induction of PGC-1α in myocytes beginning prenatally (via the muscle creatine kinase promoter) leads to dramatic induction of capillary density. It is unclear, however, if this can be recapitulated outside the plastic environment of in utero development, and especially in older and diabetic contexts where endothelial dysfunction is prominent. The cellular and molecular mechanisms by which PGC-1α orchestrates angiogenesis are also not known.

We show here, using an inducible transgenic model, that PGC-1α robustly induces angiogenesis in adult, aged, and diabetic mice. The vessels are abundant and functional, likely recapitulating physiological angiogenesis. Mechanistically, we uncover a novel role for macrophages, the secreted factors (secreted phosphoprotein 1 ([SPP1]; also known as osteopontin), and monocyte chemoattractant protein-1 (MCP-1), not previously known to be involved in physiological angiogenesis. Finally, we show that adenoviral delivery of PGC-1α to skeletal muscle accelerates recovery from limb ischemia in mice.

**Methods**

**Animals**

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Muscle creatine kinase -tetracycline transactivator and tetracycline response element (TRE)-PGC-1α-inducible mice were obtained from Dr Kelly. Soluble VEGF receptor 1 mice were kindly provided by Dr Keshet, Jerusalem, Israel. SPP1 mice were purchased from Jackson Laboratories. TRE-VEGF mice were generated by homologous recombination at the HPRT locus. All transgenic animals were maintained hemizygous on a mixed C57Bl/6 and 129 strain unless otherwise stated. Full details are provided in the Online Data Supplement.

**Cells and Reagents**

Human umbilical cord endothelial cells, 10T1/2, THP1, and C2C12 cells were maintained using standard growth media conditions.
an endothelial cell–enriched marker. As shown in Figure 1B, marked increases in angiogenesis were noted in the double-transgenic mice (TRE (+)) but not in single transgenic mice (TRE (−)) or in double-transgenic mice maintained on doxycycline. Rebound from potential doxycycline-mediated inhibition of angiogenesis is not likely because the single transgenic mice, which were switched to normal chow, did not induce angiogenesis. Capillary density in the induced animals more than doubled from 100 capillaries per high-power field (caps/hpf) to 250 caps/hpf (Figure 1B). The number of capillaries surrounding each fiber increased from 5 to 10 (Figure 1B), consistent with the absence of significant change in fiber size after transgene induction. Similar increases in capillary density were seen in tibialis anterior and gastrocnemius, and the increase in capillary density was also detectable by staining with CD144 (VE-Cadherin; Online Figure IA–ID).

We next tested the time course of angiogenesis induction at 0, 2, 3, and 4 weeks after doxy removal. As shown in Figure 1C and 1D, capillary density was induced as early as 3 weeks after doxy removal. Flow cytometry analysis of cells enzymatically removed from quadriceps revealed a near quadrupling of CD31+ endothelial cells at 4 weeks (Figure 1E), indicating that the marked increase in CD31 staining seen in stained transverse sections reflects increases...
in endothelial cell number, rather than size or projections. Real-time quantitative polymerase chain reaction revealed 2- to 3-fold increases in the expression of mitochondrial genes (ATP synthase subunit O and cytochrome C oxidase) after 3 weeks of induction, as shown previously. Quantitative polymerase chain reaction analysis of angiogenic genes revealed 3-fold increase in VEGFA and VEGF receptor 2 expression at 3 and 4 weeks, and a 8-fold increase in CD31 mRNA expression (Figure 1F). Consistent with these findings, levels of VEGFA and VEGF receptor 2 protein were induced 2.5-fold at 3 weeks, as measured by ELISAs with protein extracts from whole muscle (quadriceps) (Figure 1G and 1H). Together, these results demonstrate that PGC-1α can robustly induce angiogenesis in adult skeletal muscle, even after the increased plasticity present during the developmental period.

**PGC-1α-Mediated Angiogenesis in Skeletal Muscle Is VEGFA Dependent**

Endothelial migration is a hallmark of angiogenesis. Therefore, we tested whether PGC-1α expression in myocytes could stimulate the migration of adjacent endothelial cells. C2C12 cells were made to differentiate into myotubes in the bottom wells of modified Boyden chambers (Transwell system) and human umbilical cord endothelial cells were seeded into the top chambers. As shown in Online Figure IIA, infection of C2C12s with adeno-PGC-1α markedly stimulated the migration of the endothelial cells. VEGFA is crucial for many, but not all, forms of angiogenesis, and we have shown previously that PGC-1α regulates VEGFA expression. To test whether the migration of endothelial cells was dependent on VEGF secretion, the CM in the above coculture experiment was supplemented with soluble VEGF receptor 1 (soluble fms-like tyrosine kinase 1 [sFlt1]), which binds to and inhibits VEGF family members. As shown in Online Figure IIB, blocking of VEGF led to complete inhibition of human umbilical cord endothelial cell migration.

To test whether VEGF is required for PGC-1α-induced angiogenesis in intact animals, we next used transgenic mice that express human sFlt1 fused to an IgG1 Fc fragment, under control of a tetracycline-sensitive transactivator-responsive promoter (a kind gift of Eli Keshet). These mice were crossed with the PGC-1α-inducible transgenic mice, yielding triple transgenic mice in which removal of doxy leads to the simultaneous induction of PGC-1α and sFlt1 in skeletal muscle (Online Figure IIC). Groups of 12-week-old triple transgenic mice, and controls, were switched from doxy chow to normal chow, and the capillary density was evaluated in quadriceps 4 weeks after induction. As shown in Online Figure IID to IIF, sFlt1 expression completely inhibited the increase in capillary density mediated by PGC-1α. The induction of angiogenesis by PGC-1α in skeletal muscle thus requires VEGFA in intact animals.

**PGC-1α Can Induce Angiogenesis in Aged and Diabetic Animals**

To test whether PGC-1α can induce angiogenesis in old and diabetic mice, we maintained PGC-1α double-transgenic mice on a 45% high-fat diet (containing doxycycline to maintain the transgene inhibited) starting at the age of 12 months. Twelve months later, at 2 years of age, doxycycline was removed from the diet for 8 weeks to allow induction of PGC-1α.
Glucose tolerance tests confirmed that the mice had become diabetic (Online Figure IIIA and IIIB). There was no difference in the body weights of the wild-type and transgenic mice. After 8 weeks of PGC-1α induction, there was a 4-fold increase in PGC-1α expression (Figure 2B) and a 2-fold increase in VEGFA and CD31 in muscle (Figure 2B). Transverse sections of the gastrocnemius stained for CD31 revealed a 2-fold increase in capillary density in the PGC-1α–induced old diabetic animals, from 120 caps/hpf in the TRE (−) animals to 325 caps/hpf in the TRE (+) double transgenics (Figure 2C and 2D). Similar results were obtained from 2-year-old transgenic nondiabetic animals maintained on a regular chow diet (Online Figure IIIC–IIIE). These data thus demonstrate that aged and diabetic muscle beds remain exquisitely responsive to angiogenic stimulation by PGC-1α.

PGC-1α Induces the Formation of Patent, Functional, Nonleaky Blood Vessels

We next sought to test the functional integrity of the vessels induced by PGC-1α. A critical parameter of functional vessels is patency. To gauge the blood vessel patency, intravascular injections of isolectin *Griffonia simplicifolia* were used. *Griffonia simplicifolia* lectin avidly binds N-glucosamine oligomers on the luminal side of the endothelium, but does not escape the vessel lumen, thus staining only patent vessels. Mice were injected intravenously and euthanized 15 minutes later and the muscles were harvested and sectioned. Fluorescent imaging showed a 2-fold increase in the number of patent vessels per high-power field in the induced TRE (+) mice when compared with the TRE (−) mice (Figure 3A–3C).

Another measure of vessel patency is the amount of intravascular volume contained in a muscle bed. To measure this, we used intravenous injections of 125I-labeled albumin, which remains intravascular and can be quantified. Mice placed on a doxycycline-free diet for 4 weeks were injected with 125I-labeled albumin, and 5 minutes later quadriceps and tibialis anterior muscles were removed, and the radioactive content was measured by gamma counting. As shown in Figure 3D, the PGC-1α–expressing double-transgenic mice had a 2-fold increase in the intravascular volume of the quadriceps muscle, compared with the TRE (−) mice.

Vascular leak in the quadriceps muscle of tetracycline response element (TRE (−)), TRE (+), and mdx mice. **Figure 3. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α)–induced blood vessels are functional.** A, Isolectin *Griffonia simplicifolia* (GS-IB4) was injected intravenously into wild-type and PGC-1α 12 to 16-week-old transgenic mice after 4 weeks of doxy removal. B, Visualization of intravascular isolectin the gastrocnemius muscle after 4 weeks of doxy removal. C, Quantification of lectin-stained patent capillaries per high-power field (hpf). D, Measurement of intravascular volume in the quadriceps muscle of wild-type and PGC-1α transgenics 5 minutes after injection of 125I-BSA. E, Vascular leak in the quadriceps muscle of tetracycline response element (TRE (−)), TRE (+), and mdx mice. F, Capillary and pericyte density in the gastrocnemius muscle after 3 weeks of PGC-1α expression. Graphs show quantification of pericytes/hpf and pericytes/myofiber. G, Platelet-derived growth factor (PDGF) B gene expression after 0, 2, 3, and 4 weeks of doxy removal. H, PDGFB protein expression after 3 weeks of PGC-1α expression. Error bars are SE. n=3 to 4 per group, *significance with *P<0.05, **P<0.01, ***P<0.001. Scale bar, 100 μm. DAPI indicates 4’,6-diamidino-2-phenylindole; and PECAM, platelet endothelial cell adhesion molecule.
mice (TRE (+)) revealed a 2-fold increase in intravascular volume in muscle beds when compared with the single transgenic TRE (−) mice. This strong increase in intravascular volume is consistent with the increase in capillary content seen by immunostaining (Figure 1B–1D; Online Figure ID) and isletin staining (Figure 3B and 3C). The blood vessels induced by PGC-1α are thus patent and capable of sustaining blood flow.

One critical parameter of vessel integrity is the impermeability to macromolecules. To test this, we used injections of EB dye, a macromolecule that normally remains intravascular but easily extravasates in the presence of aberrantly permeable vessels. A mouse model of muscle dystrophy (mdx mouse), in which EB is well known to extravasate, was used as a positive control. Double-transgenic mice that had been induced for 4 weeks, and controls, were injected with EB intraperitoneally, and muscles were harvested after 16 hours. Fluorescent microscopy showed that blood vessels induced by PGC-1α did not leak EB, despite the dramatic increase in vessel density. In contrast, as a positive control, mdx mice had strong EB staining (Figure 3E). PGC-1α–induced blood vessels are thus not leaky, and thus differ from those in most tumor vessels, or vessels induced by VEGFA injection alone into muscle.23–26

In sharp contrast to the above findings, induction of VEGFA alone, achieved by similar skeletal muscle–specific double-transgenic expression, led to induction of dramatically disorganized neovascularization, with the appearance of large sinusoid formations (Online Figure IVA), and a dramatic increase in capillary leak (Online Figure IVB and IVC). Induction of VEGFA is thus necessary (Online Figure II) but not sufficient for complete PGC-1α–mediated angiogenesis.

Vessel maturation, including the establishment of patency and impermeability, also involves the recruitment of perivascular cells, such as pericytes, and the formation of new arterioles via recruitment of smooth muscle cells. Immunostaining of transverse sections of quadriceps muscle with antibodies against platelet-derived growth factor (PDGF) receptor β platelet-derived growth factor receptor-β, a pericyte-enriched marker, showed a >2-fold increase in the number of pericytes per high-power field in the TRE+ mice when compared with the TRE (−) mice after 3 weeks of induction of PGC-1α (Figure 3F). Higher order vessels (arterioles) were also increased in number in TRE (+) mice, as measured by smooth muscle actin staining (Online Figure VA and VB). The formation of larger arteries, however, did not seem to be induced, as evaluated by micro-CT in either the inducible model (Online Figure VC and VD) or the constitutive model of PGC-1α muscle expression (Online Figure VE and VF). Pericytes are known to migrate to angiogenic areas in response to the ligand PDGFB.27–28

Real-time quantitative polymerase chain reaction analysis of the RNA from quadriceps muscle at 0, 2, 3, and 4 weeks of PGC-1α expression showed that the TRE (+) mice had up to a 2-fold increase in PDGFB gene expression when compared with the TRE (−) mice (Figure 3G). Adenoviral delivery of PGC-1α to primary myotubes also induced PDGFB dramatically, and this required the presence of the transcription factor estrogen-related receptor-α (Online Figure VG). PDGFBB protein levels were elevated 30-fold in TRE (+) mice when compared with the TRE (−) mice (Figure 3H). Together, these data demonstrate that PGC-1α orchestrates the formation of patent, nonleaky, and functional, pericyte-covered blood vessels in adult skeletal muscle. Interestingly, the large increase in capillaries and arterioles had no effect on systemic blood pressure, systemic arterial resistance, cardiac output, or Frank Starling relationships, as evaluated by invasive hemodynamic measurements (Online Figure VIA and VIB).

**Induction of PGC-1α Recruits Macrophages to Skeletal Muscle**

To gain understanding of the genes and pathways activated by PGC-1α during the angiogenesis process, we conducted an expression microarray analysis using RNA from muscles of the PGC-1α transgenic and control mice after 4 weeks of induction. Gene set enrichment analysis revealed a strong activation of oxidative phosphorylation, tricarboxylic acid cycle and fatty acid oxidation programs in the PGC-1α–expressing TRE (+) mice (Figure 4A), as expected.2 Surprisingly, the gene sets most strongly induced by PGC-1α were those of myeloid cells (Figure 4A). Indeed, of the 30 most-induced genes on the microarray, 14 were genes known to be expressed strongly in macrophages (Online Table I). Real-time quantitative polymerase chain reaction analysis of RNA from the quadriceps muscle confirmed these findings (Figure 4B). These observations suggested that induction of PGC-1α in skeletal muscle leads to infiltration of macrophages. To test this, sections from quadriceps of transgenic animals were immunostained with antibodies to the monocye-specific marker CD11b and the macrophage-specific marker F4/80. This revealed a dramatic 4-fold increase in the number of monocytes/macrophages, from 2 to 8 per high-power field, in the double-transgenic TRE (+) mice when compared with the TRE (−) single transgenics (Figure 4C–4E). Thus, surprisingly, the induction of PGC-1α in skeletal muscle cells leads to a strong recruitment of macrophages, despite the absence of any overt damage, or of any biomechanical or ischemic stress.

Grunewald et al29 have reported that VEGF-activated endothelial cells can recruit monocytes and macrophages during the process of angiogenesis. To test whether endothelial activation and angiogenesis were required for the PGC-1α–induced recruitment of macrophages, we used the transgenic mice that express human sFlt1 fused to an IgG1 Fc fragment described above, crossed with the PGC-1α–inducible transgenic mice. As shown in Online Figure VIIA and VIIB, sFlt1 expression had no effect on the recruitment of macrophages, even though it completely inhibited the increase in capillary density seen in the inducible PGC-1α transgenics (Online Figure II). The recruitment of macrophages by PGC-1α thus occurs independently of angiogenesis or signaling by VEGF family members.

**Myocytes Expressing PGC-1α Secrete SPP1 to Regulate Macrophage Activation**

The above findings suggested that PGC-1α causes the secretion from myocytes themselves of factors that can affect...
macrophage function. To test this idea, CM was harvested from differentiated C2C12 myotubes overexpressing PGC-1α and then placed on peripheral blood monocytes/macrophages. Within 24 hours, striking changes were seen in the morphology of the macrophages treated with PGC-1α CM when compared with control CM (Figure 5A). The morphological changes, including the appearance of pseudopodia, suggested that macrophages were activated by the PGC-1α CM. Consistent with this notion, macrophages treated with PGC-1α CM showed a marked elevation in the expression of macrophage activation genes (Figure 5B).

To begin to identify key factors secreted by muscle cells expressing PGC-1α, we used bioinformatic approaches to generate a list of candidate proteins induced and secreted by PGC-1α. RNA from myotubes and from intact muscles overexpressing PGC-1α were probed with Affymetrix arrays, and genes induced by PGC-1α in both conditions were subjected to publicly available algorithms to predict the subset of genes that are likely secreted (SignalP 3.0; Figure 5C). The most dramatically induced gene on this list was SPP1, a protein known to modulate monocyte/macrophage biology.30,31 Increased expression of SPP1 gene was noted in both myotubes (80-fold) and intact muscle (9-fold) overexpressing PGC-1α (Figure 5D). SPP1 protein was abundantly detectable in PGC-1α CM when compared with control CM (Figure 5E). PGC-1α thus strongly induces the secretion of SPP1 from myocytes.

Treatment of macrophages with SPP1 led to the induction of a subset of the same genes that were induced by CM from myotubes expressing PGC-1α (Figure 5F compared with Figure 5B), including MCP-1 (6-fold), CD163 (2-fold), and CD169 (4-fold). These data suggested that SPP1 mediates some of the effects of the PGC-1α-CM on macrophages. To test this directly, we treated the PGC-1α CM with SPP1-neutralizing antibody before adding the CM to the THP1 cells, revealing a dose-dependent decrease in MCP-1 gene expression, with a complete block on the addition of 6 μg of SPP1 antibody but not control IgG (Figure 5G). Together, these results show that PGC-1α induces SPP1 secretion from myocytes, which in turn activates macrophages to express MCP-1 and other markers of activation.

**Conditioned Macrophages Secrete MCP-1 to Recruit Vascular Cells**

The pronounced activation of macrophages by PGC-1α–expressing myocytes suggested that the macrophages may assist the orchestration of PGC-1α–induced angiogenesis. We first investigated this notion by testing whether macrophages that had been educated by PGC-1α–expressing myocytes would, in turn, affect the function of vascular cells. THP1 monocytes were first plated into bottom chambers of transwells and treated with phorbol myristate acetate to induce differentiation into macrophages. The cells were then
We showed above that CM from PGC-α—expressing myotubes strongly induced the expression of MCP-1, in a manner dependent on the secretion of SPP1 (Figure 5G). MCP-1 secreted from macrophages can recruit endothelial cells, pericytes, and smooth muscle cells. This suggested that the activity present in THP1-CM in the above experiments maybe be, at least in part, MCP-1. To test this, we used inhibitors to the MCP-1 receptors. Endothelial cells express 2 receptors for MCP-1, C-C chemokine receptor 1 and 2. Treatment of THP1 CM with a cocktail of C-C chemokine receptor 1 and 2 antagonists completely abrogated the ability of the CM to induce endothelial cell migration (Figure 6H). Induction of pericyte migration was similarly abrogated by the addition of the inhibitor cocktail (Figure 6I). Together, these results indicate that PGC-1α in myotubes induces the secretion of SPP1, which in turn activates macrophages to secrete MCP-1, ultimately contributing to the stimulation of endothelial cell and pericyte migration.
Figure 6. Conditioned macrophages secrete monocyte chemoattractant protein-1 (MCP-1) to recruit vascular cells. A, Experimental design for migration assay with human umbilical cord endothelial cells (HUVECs) and 10 T1/2 (pericytes). B, Migration of HUVECs. C, Quantification of migrated cells. D, Migration of differentiated 10 T1/2 cells. E, Quantification of migrated cells. F, Migration of A0184 cells. G, Quantification of migrated cells. H, Quantification of migrated HUVECs with or without C-C chemokine receptor (CCR) inhibitors. I, Quantification of migrated 10 T1/2 cells with or without CCR inhibitors. Error bars are SE. n=4 per group. *P<0.05, **P<0.01, and ***P<0.001. Scale bar, 100 μm. CM indicates conditioned medium; and SMC, smooth muscle cells.

Figure 7. Secreted phosphoprotein 1 (SPP1) is required for vessel patency and arteriolar formation. A, Capillary density in the quadriceps after 4 weeks of doxy removal. B, Quantification of capillary endothelial cells/high power field (×20). C, Patent capillaries in quadriceps muscle after 4 weeks of doxy removal. D, Quantification of patent vessels per high-power field (×20). E, Quantification of arterioles (smooth muscle actin)/lpf (×5). Error bars, SE. n=4 per group. *, # Significance with P<0.05, **P<0.01, and ***P<0.001. Scale bar, 100 μm. TRE indicates tetracycline response element.
PGC-1α Induces Aberrant Vessel Formation In Vivo in the Absence of SPP1

To test in vivo directly, the role of SPP1 in vessel formation in response to PGC-1α, SPP1−/− mice were crossed with the double-transgenic TRE (+) mice to produce SPP1−/− TRE (+) mice. Expression of PGC-1α was induced for 4 weeks, after which the mice were injected with lectin (as described above), and muscles were harvested and stained for CD31 and smooth muscle actin, as described above. As shown in Figure 7A and 7B, the absence of SPP1 had no effect on the marked increase in vascular density (CD31 staining) seen after induction of PGC-1α. In sharp contrast, the absence of SPP1 almost completely abrogated the 3-fold increase in isoelectin staining seen after induction of PGC-1α (Figure 7C and 7D). These data indicate that SPP1, although dispensable for capillary formation per se, is essential for the development of vessel patency in vivo. Vessel patency allows for blood flow, and blood flow is thought to be crucial for the formation of new arterioles. As shown in Figure 7E, the absence of SPP1 also completely abrogated the increase in arterioles (smooth muscle actin staining) seen after the induction of PGC-1α. Together, these data demonstrate that SPP1 is essential for proper maturation of vessels induced by PGC-1α in intact animals.

Adenoviral Injection of PGC-1α Improves Blood Flow Recovery in Young and Old Diabetic Mice

To begin to address the translational potential of PGC-1α induction in skeletal muscle, we used adenovirus-expressing PGC-1α, versus control virus-expressing green fluorescent protein, in the murine hindlimb ischemia model. We first used young 16-week-old C57/B16 mice on normal chow. Adenovirus encoding either PGC-1α or green fluorescent protein was injected into the hindlimb at the time of hindlimb ischemia surgery, as described. Blood flow recovery was then monitored for the ensuing 21 days, using noninvasive laser Doppler imaging. As shown in Figure 8A and 8B, 60% recovery of blood flow was seen in control-injected mice after 21 days, as observed by others. In contrast, animals injected with PGC-1α virus had significantly accelerated recovery of blood flow, reaching 60% recovery within 10 days (Figure 8A and 8B). PGC-1α thus improves recovery from hindlimb ischemia in young animals. We next tested the PGC-1α virus in 1 year-old, high-fat fed, diabetic animals. As shown in Figure 8C, blood flow recovery in the control-injected animals was markedly reduced when compared with the young control animals (Figure 8B), reaching only 40% recovery after 21 days. Consistent with this, most animals developed severe amputation defects and limb necrosis (Figure 8D), outcomes that are rarely seen in younger mice (not shown). In contrast, animals injected with PGC-1α virus had significantly accelerated recovery of blood flow, reaching >70% recovery after 21 days (Figure 8C), and the clinical outcomes were markedly improved in PGC-1α–injected animals (Figure 8D).

Discussion

Therapeutic angiogenesis has been pursued extensively as a potential treatment for ischemic disorders, such as peripheral artery disease. To date, however, clinical trials have largely failed. Several clinical factors likely underlie these failures, including trial designs, difficulties with patient recruitment, and heterogeneous patient populations. In addition, important biological hurdles have also become evident. Angiogenesis is a complex process, not easily recapitulated by simple delivery of 1 or 2 angiogenic factors. Agents capable of coordinating this complex process have remained elusive. One approach has been to target the so-called master regulators. This has been tried primarily with HIF-1α, with promising early results, but a recent phase III trial failed to show benefit. However, activation of HIF-1α typically inhibits metabolism, in particular oxidative metabolism, which likely worsens oxidative tasks, such as walking. A second, equally important biological hurdle to therapeutic angiogenesis is that most patients receiving such therapy have significant endothelial dysfunction that renders them less responsive to many angiogenic stimuli, but most preclinical trials have involved young, healthy animals. Here, we show that PGC-1α can produce sustainable functional angiogenesis in adult skeletal muscle. PGC-1α orchestrates the complex recruitment of multiple cell types (Online Figure VIII), resulting in new blood vessels that are patent, nonleaky, and functional. PGC-1α adenoviral injections improved blood flow in a murine model of peripheral arterial disease. Moreover, PGC-1α robustly induced angiogenesis, and improved blood flow after hindlimb ischemia, in old and diabetic animals. These data highlight PGC-1α as a potential therapeutic agent in the treatment of peripheral artery disease.

Recovery of blood flow after hindlimb ischemia in the mouse, and improvements in blood flow and function in humans with peripheral artery disease, is a complex process that likely includes the formation of new collaterals, capillaries, and arterioles. The induction of PGC-1α in skeletal muscle does not seem to induce the formation of new higher order arteries beyond arterioles. We propose here that PGC-1α likely predominantly affects local effects at the level of capillaries and arterioles. These effects likely allow optimal redistribution of local blood flow to match metabolic demand. In addition, increased cutaneous capillary flow could contribute to reduced tissue necrosis, via improved wound healing, independently of flow to the muscle component of the hindlimb. Tissue flow is known to be autoregulated to match oxygen supply to demand, but because the transgenic mice are not more active than their control littermates, and demand is not altered, supply is therefore also not altered. Thus, we do not see evidence that resistance at baseline is decreased, or that flow is increased. Interestingly, this suggests that, in these mice, the formation of new arterioles is not occurring in response to increase in mass baseline flow although local fluctuations cannot be excluded. However, exercise not only robustly induces PGC-1α expression in skeletal muscle in rodents and humans but also has numerous other effects, including increases in blood flow, cardiac output, etc. The genetic model used here thus separates the effects of PGC-1α from other exercise-induced effects. The data thus allow us to conclude that PGC-1α...
is sufficient to induce functional angiogenesis, but it is likely that other factors, such as hemodynamic effects of exercise (or ischemia), contribute to subsequent formation of higher order vessels.

The induction of PGC-1α likely recapitulates normal angiogenesis in muscle. Postnatal physiological (ie, nonpathological) angiogenesis is relatively rare, limited primarily to uterine changes during the estrous cycle, and to exercise-induced angiogenesis in skeletal muscle. Mechanisms of physiological angiogenesis are poorly understood, but likely differ significantly from those of pathological angiogenesis (eg, in cancer or retinopathy). HIF-1α, for example, plays a prominent role in pathological angiogenesis,58–61 whereas deletion of HIF-1α in skeletal muscle leads to more blood vessels, rather than fewer.5 By contrast, PGC-1α expression is strongly induced by exercise, and deletion of PGC-1α in skeletal muscle does block exercise-induced angiogenesis, indicating PGC-1α likely mediates this process.11 PGC-1α–induced angiogenesis thus likely closely recapitulates exercise-induced angiogenesis. In addition, PGC-1α likely contributes to the increase in capillary:fiber ratio consistently seen in oxidative fibers, where PGC-1α expression is high. This coordination allows the delivery of oxygen/fuel via the vasculature to match the consumption of oxygen/fuel in mitochondria-rich oxidative fibers.

The mechanisms of physiological angiogenesis in muscle remain poorly understood. Several studies have revealed a robust infiltration of macrophages in pathological angiogenesis, such as found in ischemia and cancer.62–64 In these conditions, the macrophage infiltrate is felt to be part of the underlying inflammatory response. Exposure of macrophages to hypoxia results in their secretion of angiogenic molecules, such as VEGF, placental-induced growth factor, fibroblast growth factor 2, and PDGF.65–67 However, these processes are unlikely to be at play in our inducible PGC-1α model because (1) there is no hypoxia, (2) there is no trauma, and (3) there is no generalized inflammation, but instead a specific recruitment of macrophages. The data thus reveal an alternative mechanism for macrophage recruitment during angiogenesis: via PGC-1α–induced secretion of SPP1. SPP1 is a secreted noncollagenous sialic acid–rich protein that plays an important role in modulating numerous cell behaviors.68–70 The protein is a powerful regulator of leukocyte migration and an inducer of angiogenic cytokines.71–74

Our data indicate that the recruited macrophages act, at least in part, to help orchestrate multicellular angiogenesis. SPP1 stimulates the macrophages to induce MCP-1, which in turn activates endothelial cells, pericytes, and smooth muscle cells. Deletion of SPP1 in intact animals leads to

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**Figure 8. Adenoviral delivery of peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) improves blood flow recovery after hindlimb ischemia.**

A, Sample laser Doppler images of blood flow recovery pre, immediately post, and 21 days after hindlimb ischemia surgery with concurrent injection of green fluorescent protein (GFP) vs PGC-1α adenovirus at the time of ligation. B, Quantification of blood flow of animals in A. Data presented as fractional flow compared with nonischemic leg. n=7 per group. C, Quantification of blood flow in the ischemic limbs of high fat-fed (HFD) diabetic mice after injection of GFP or PGC-1α adenovirus. n=7 per group. D, Measurement of toe necrosis and ambulatory scores 21 days after hindlimb ischemia surgery and adenoviral injection. *P<0.1 by Mann–Whitney U test, **P<0.01 by 2-way ANOVA with Bonferroni correction. CS0–S4 indicates clinical scores 0 to 4.
aberrant PGC-1α-induced vasculature, most notably the diminution of patent blood vessels, and blunted arteriolarization. An SPP1-macrophage-MCP-1 axis thus seems to be important for vessel maturation during physiological angiogenesis. Interestingly, previous work has shown that SPP1 expression is induced by hindlimb ischemia, and that SPP1 knockout mice have decreased recruitment of macrophages and significantly impaired blood flow recovery after hindlimb ischemia. In these contexts, it is also interesting to note that macrophages have recently been implicated in the process of tip-cell to tip-cell anastomosing in neuronal angiogenesis, an important maturation step in angiogenesis. Physiological angiogenesis in skeletal muscle likely does not occur by sprouting angiogenesis (i.e., tip-cell formation), however, but rather via intussusception, a process that remains poorly understood. Our data thus suggest that macrophages may be involved in both forms of vessel maturation.

In summary, the current study demonstrates that PGC-1α is a powerful orchestrator of functional angiogenesis in skeletal muscle. Angiogenesis can be robustly activated in either young or old and diabetic mice. And adenoviral delivery of PGC-1α improves the response to hindlimb ischemia in both of those contexts. The study thus provides novel mechanistic insights into physiological angiogenesis in skeletal muscle.

Sources of Funding

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Disclosures

None.

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34. Arellíva TI, Kukhina NB, Antonova OA, Krasnikova TL. MCP-1-stimulated chemotaxis of monocytes and endothelial cells is dependent on activation of different signaling cascades. Cytokine. 2005;31:439–446.


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**Novelty and Significance**

**What Is Known?**

- Peripheral artery disease (PAD) and microvascular disease are major complications of diabetes mellitus.
- Exercise is currently the best-known therapeutic intervention in the treatment of PAD.
- Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) is induced by exercise in skeletal muscle and is required for exercise-induced angiogenesis.

**What New Information Does This Article Contribute?**

- Induction of PGC-1α in the adult murine muscle induces the formation of new functional blood vessels.
- PGC-1α activates a cascade of events, including the production of secreted phosphoprotein 1, leading to the recruitment of macrophages required for vessel maturation.
- Viral delivery of PGC-1α in significantly improves blood flow recovery in the murine hindlimb ischemia model of PAD.

PAD and microvascular disease are major complications of diabetes mellitus and are major contributors to limb amputations. Therapeutic attempts at increasing the formation of new blood vessels have thus far been unsuccessful, likely in part because of poorly formed or leaky blood vessels. Exercise is currently the best-known treatment for PAD although patient participation is often difficult. We use here a known mimic of the exercise response, the transcriptional regulator PGC-1α, to induce new capillary blood vessels in the adult mouse. The blood vessels formed are patent, not leaky, and fully functional. PGC-1α activates a complex cascade of events, including the secretion of secreted phosphoprotein 1, which recruits macrophages involved in vessel maturation. Adenoviral delivery of PGC-1α to a limb ischemic model improved blood flow recovery, highlighting the therapeutic potential of targeting the PGC-1α pathway. Taken together, the results show that PGC-1α coordinates the formation of new blood vessels analogous to those achieved with exercise, and potentially offers a new therapeutic target for the treatment of PAD.
PGC-1α Induces SPP1 to Activate Macrophages and Orchestrate Functional Angiogenesis in Skeletal Muscle

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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-1α-inducible mice 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. 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 PDGFRβ 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. 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-1α 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 cDNA 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, $5\times10^4$ 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 1 cm distally, and the section of the artery between the two ligatures was removed. Cautery was used to control the bleeding, and three injections of $1 \times 10^8$ 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. 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

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 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 = 100 µm.
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.
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.
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

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