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

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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.[1,2] 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.[1–4] 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.[5]

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

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Nonstandard Abbreviations and Acronyms	
CD31	cluster of differentiation 31
CM	conditioned media
EB	Evans blue
HIF-1α	hypoxia inducible factor-1α
MCP-1	monocyte chemoattractant protein-1
PDGF	platelet-derived growth factor
PGC-1α	peroxisome proliferation activator receptor-γ coactivator-1α
Sflt1	soluble fms-like tyrosine kinase-1 (also known as sVEGFR1: soluble VEGF receptor 1)
SPP1	secreted phosphoprotein 1 (also known as osteopontin)
TRE	tetracycline response element
VEGF	vascular endothelial growth factor

improved endurance running capacity.[8,9] 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.[10] We also recently showed that PGC-1α is required for exercise-induced angiogenesis.[11] 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.[10] 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[12] were obtained from Dr Kelly. Soluble VEGF receptor 1 mice were kindly provided by Dr Keshet, Jerusalem, Israel.[13] SPP1$^{-/-}$ mice were purchased from Jackson Laboratories. TRE-VEGFA 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. Primary skeletal myocytes, pericytes, and smooth muscle cells were isolated, cultured, and differentiated from hindlimbs of as described previously.[14] Full details are provided in the Online Data Supplement on culture conditions, conditioned media (CM) preparation, transwell migration assays viral infections, and reagent procurement, including antibodies and ELISAs.

Real-Time PCR and Microarrays

Total RNA was isolated from mouse tissue and cultured cells using the TRIZOL (Invitrogen) and Turbocapture (Qiagen) method, respectively, and subjected to reverse transcription and relative expression levels determined. For microarrays, RNA was probed with Affymetrix mouse 1.0 gene arrays data obtained was analyzed using the Gene Set Enrichment Analysis (Broad Institute of MIT and Harvard; see Online Data Supplement for full details).

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.[15]

Animal Surgeries

Unless otherwise specified animals were anesthetized with ketamine-xylazine before all surgical procedures. Vascular leak was determined by measurement of Evans blue (EB) leak as previously described.[16] Hindlimb ischemia surgeries were performed, measured, and scored as previously described.[17] Refer to Online Data Supplement for specific details on all surgical procedures.

Histological Analysis

Quantification of capillaries was performed computationally. Refer to Online Data Supplement for detailed protocol.

Statistical Analysis

The data are presented as mean±SE. Statistical analysis was performed with Student *t* test for all in vitro and in vivo experiments. *P* values of <0.05 were considered statistically significant.

Results

PGC-1α Induces Robust Angiogenesis in Adult Skeletal Muscle

We have shown before that constitutive expression of PGC-1α, beginning in utero, increases vascular density in skeletal muscle.[10] To test whether PGC-1α could induce angiogenesis in adult skeletal muscle, we used a previously described double-transgenic mouse model.[12] TRE-PGC-1α transponder mice, in which PGC-1α expression can be induced by the tetracycline-sensitive transactivator, were crossed with inducer mice that express the tet-off tetracycline-sensitive transactivator under the control of the muscle-specific muscle creatine kinase promoter. The resulting double-transgenic animals express PGC-1α in a muscle-specific manner and only in the absence of doxycycline in the chow (Figure 1A). Transgenic expression of PGC1α in these mice begins ≈1 week after removal of doxycycline (doxy), shortly after which the mice display increased mitochondrial biogenesis and fatty acid oxidation in skeletal muscle.[12]

Doxy chow was replaced with normal chow at 10 to 12 weeks. At 1-week intervals thereafter, groups of mice were euthanized, quadriceps muscles were harvested, and transverse frozen sections were cut and evaluated by immunohistochemical staining for cluster of differentiation 31 (CD31; also known as platelet-endothelial cell adhesion molecule-1),

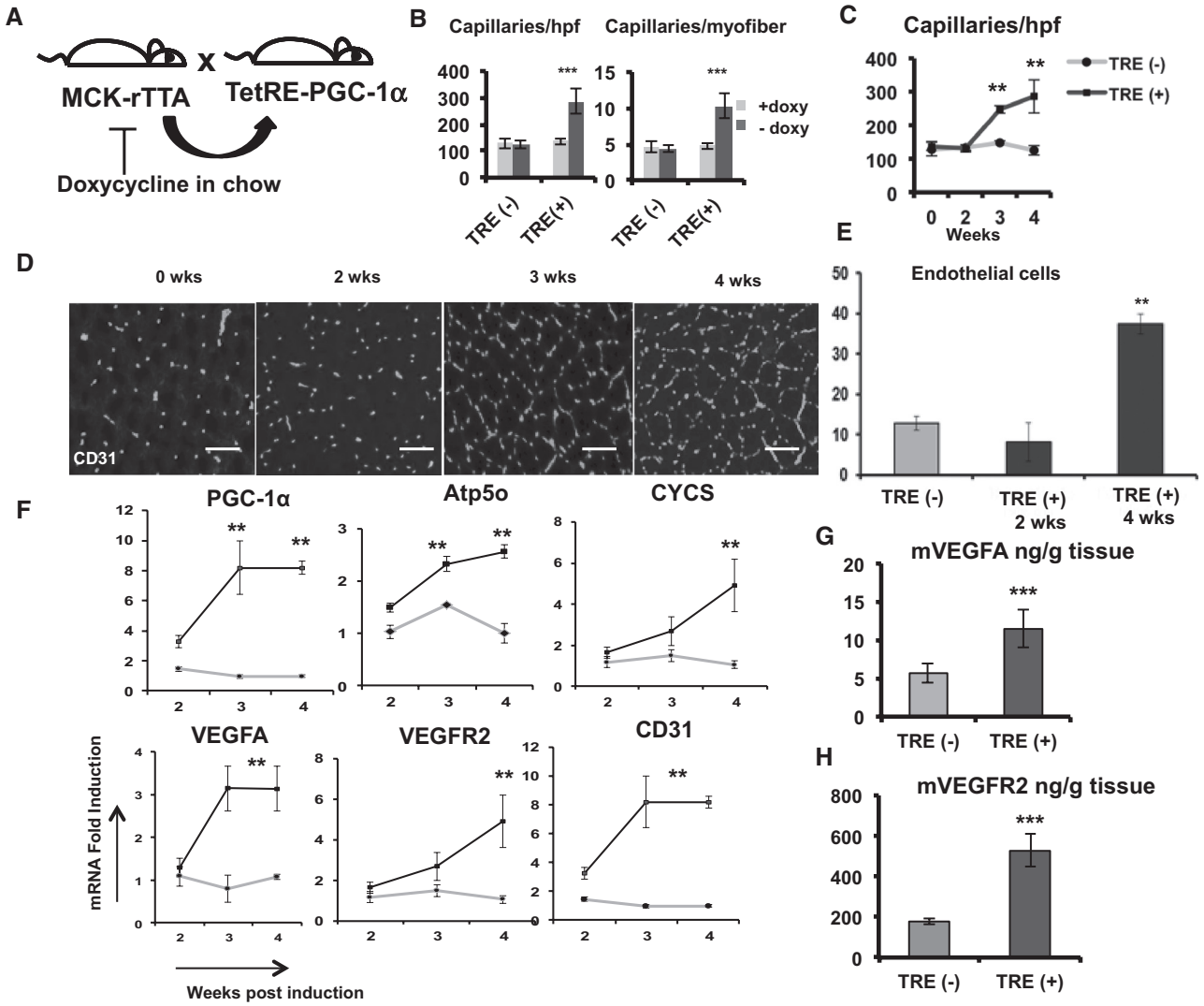

Figure 1. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) induces robust angiogenesis in adult skeletal muscle. A, Generation of the PGC-1α–expressing double-transgenic tetracycline response element (TRE) (+) mice: mice expressing the tetracycline transactivator (TTA) transactivator in muscle (muscle creatine kinase [MCK]-rTTA) were crossed with the TRE-PGC-1α transponder mice to generate double-transgenic TRE (+) mice. MCK-rTTA single transgenic mice (TRE (–) mice) were used as controls. **B**, Quantification of capillary density per high-power field (hpf) and per myofiber in transverse sections of the single transgenic TRE (–) and double-transgenic TRE (+) mice with (gray) or 4 weeks without doxy (black) chow. **C**, Capillary density in the quadriceps muscle of TRE (–) and TRE (+) mice at 1-week intervals after doxy removal. **D**, Representative CD31-stained transverse sections of TRE (+) mice after 0, 2, 3, and 4 weeks of doxy removal. **E**, Flow cytometry quantification of CD31-positive endothelial cells in the quadriceps muscle from TRE (–) and TRE (+) mice. **F**, Expression of mitochondrial and angiogenic genes in quadriceps muscle at the indicated times after induction of PGC-1α. **G** and **H**, vascular endothelial growth factor (VEGF; **G**) and VEGF receptor 2 (VEGFR2; **H**) ELISA of quadriceps muscle lysate after 3 weeks of PGC-1α induction. n>4 for each group, **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar, 100 μm. Error bars indicate SE. ATP5o indicates ATP synthase subunit O; and CYCS, cytochrome C oxidase.

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 doxy chow. Rebound from potential doxycycline-mediated inhibition of angiogenesis[18] 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.[12] 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.[12] 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,[19,20] and we have shown previously that PGC-1α regulates VEGFA expression.[10] 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,[13] 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

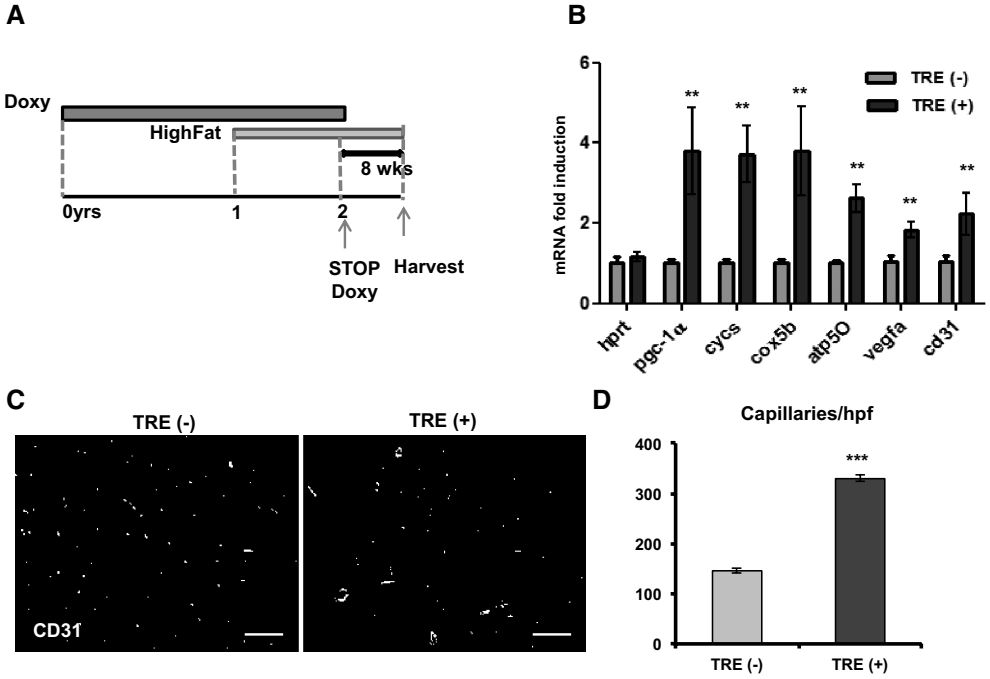

Figure 2. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) can induce angiogenesis in old diabetic animals. **A**, Experimental model to test PGC-1α–induced angiogenesis in old diabetic mice. **B**, Expression of mitochondrial and angiogenic genes in the quadriceps muscle after 8 weeks of doxy removal. **C**, Capillary density in the gastrocnemius muscle. **D**, Quantification of capillary number per high-power field. Error Bars are SE. n=4 per group, *significance with $P<0.05$, **$P<0.01$, ***$P<0.001$. Scale bar, 100 μm. TRE indicates tetracycline response element.

(Figure 2A). 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.[21] 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 ^{125}I-labeled albumin, which remains intravascular and can be quantified. Mice placed on a doxycycline-free diet for 4 weeks were injected with ^{125}I-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

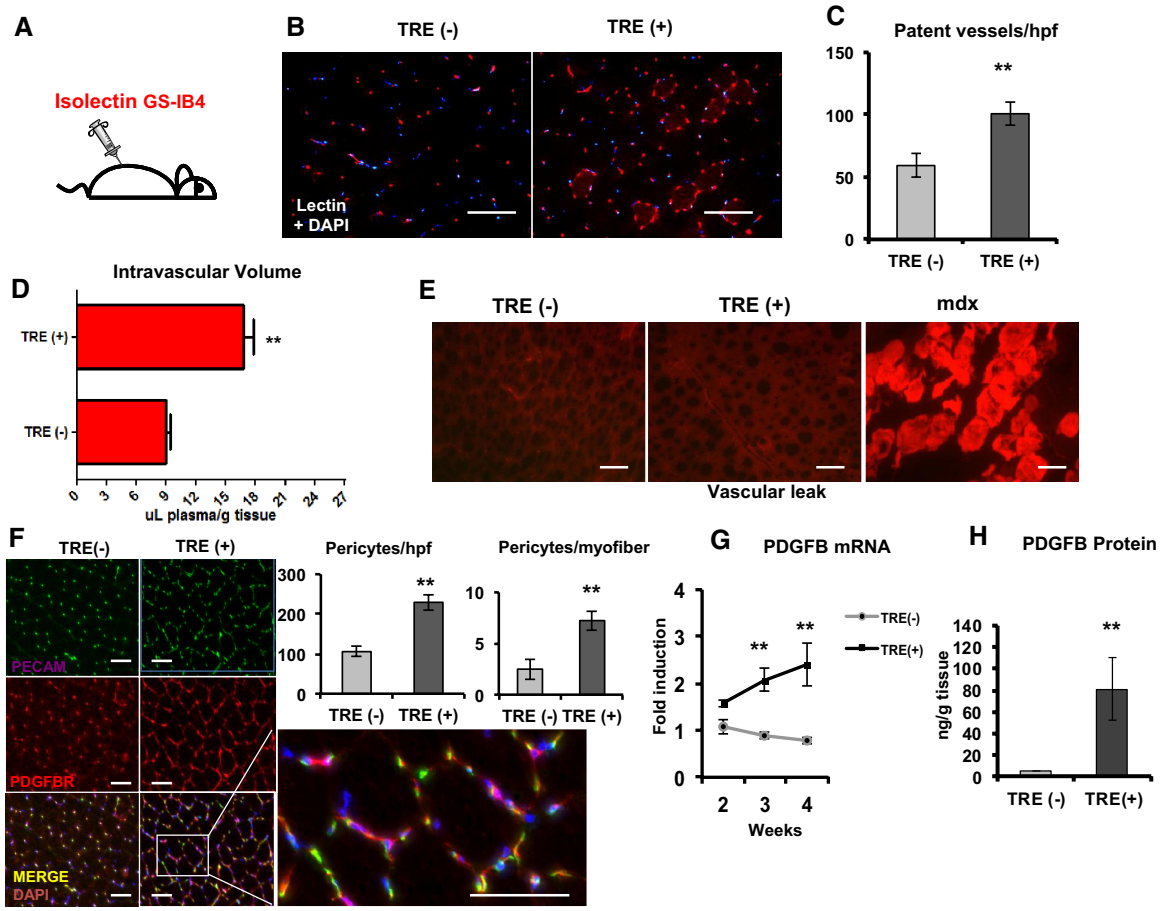

Figure 3. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α)–induced blood vessels are functional. A, Isolectin *Griffonia simplicifolia* (GS-IB4) was injected intravenously into wild-type and PGC-1α 12 to 16-week-old transgenic mice after 4 weeks of doxy removal. **B**, Visualization 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 ^{125}I-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 (Figure1B–1D; Online Figure ID) and isolectin 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.[22] A mouse model of muscle dystrophy (mdx mouse),[23] 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.[24–26]

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 PDGFBB.[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.[12] 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 monocyte-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 al[29] 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

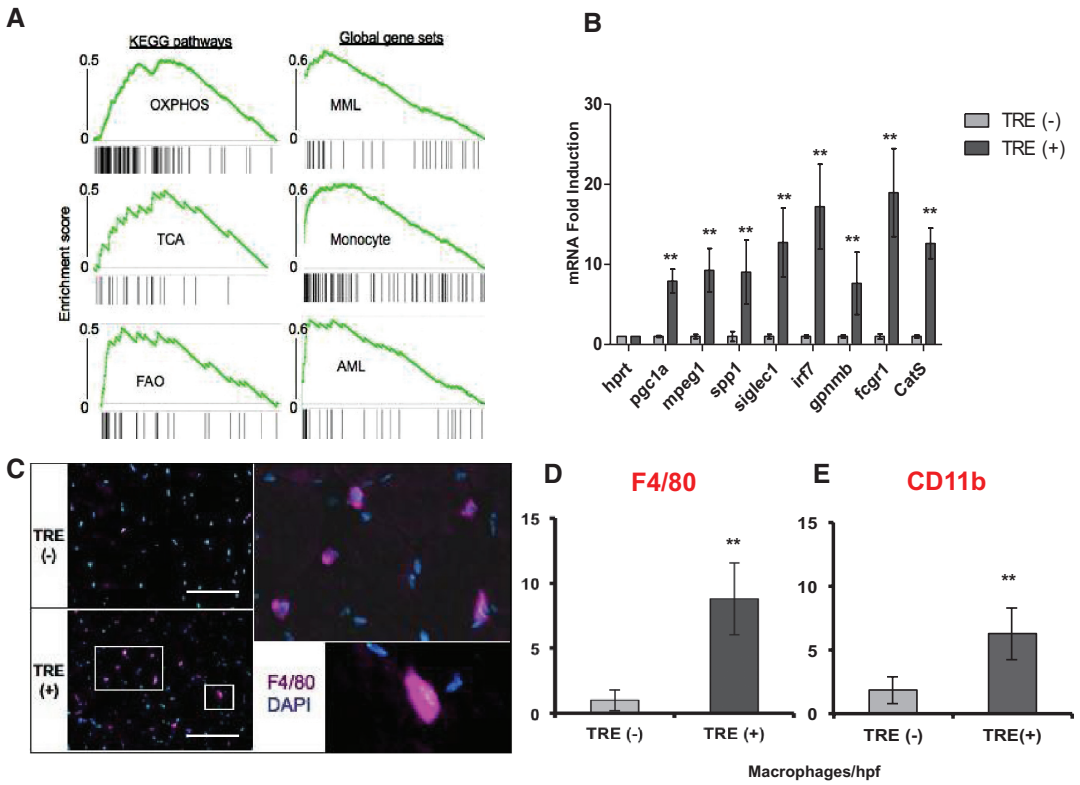

Figure 4. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) recruits macrophages to adult skeletal muscle. **A**, Gene set enrichment analysis of microarray data from gastrocnemius muscle of tetracycline response element (TRE (–)) and TRE (+) mice after 4 weeks of doxy removal. **B**, Real-time quantitative polymerase chain reaction validation of macrophage-specific genes induced in the microarray. **C**, F4/80-positive macrophage density in gastrocnemius muscle of TRE (–) and TRE (+) mice**. D**, Quantification of F4/80-positive macrophages. **E**, Quantification of CD11b-positive cells. Error bars are SE. n>4 per group, **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bar, 100 μm. AML indicates acute myeloid leukemia; DAPI, 4',6-diamidino-2-phenylindole; FAO, fatty acid oxidation; MML, monocytic myeloid leukemia; OXPHOS, oxidative phosphorylation; and TCA, tricarboxylic acid cycle.

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 myotubes, 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

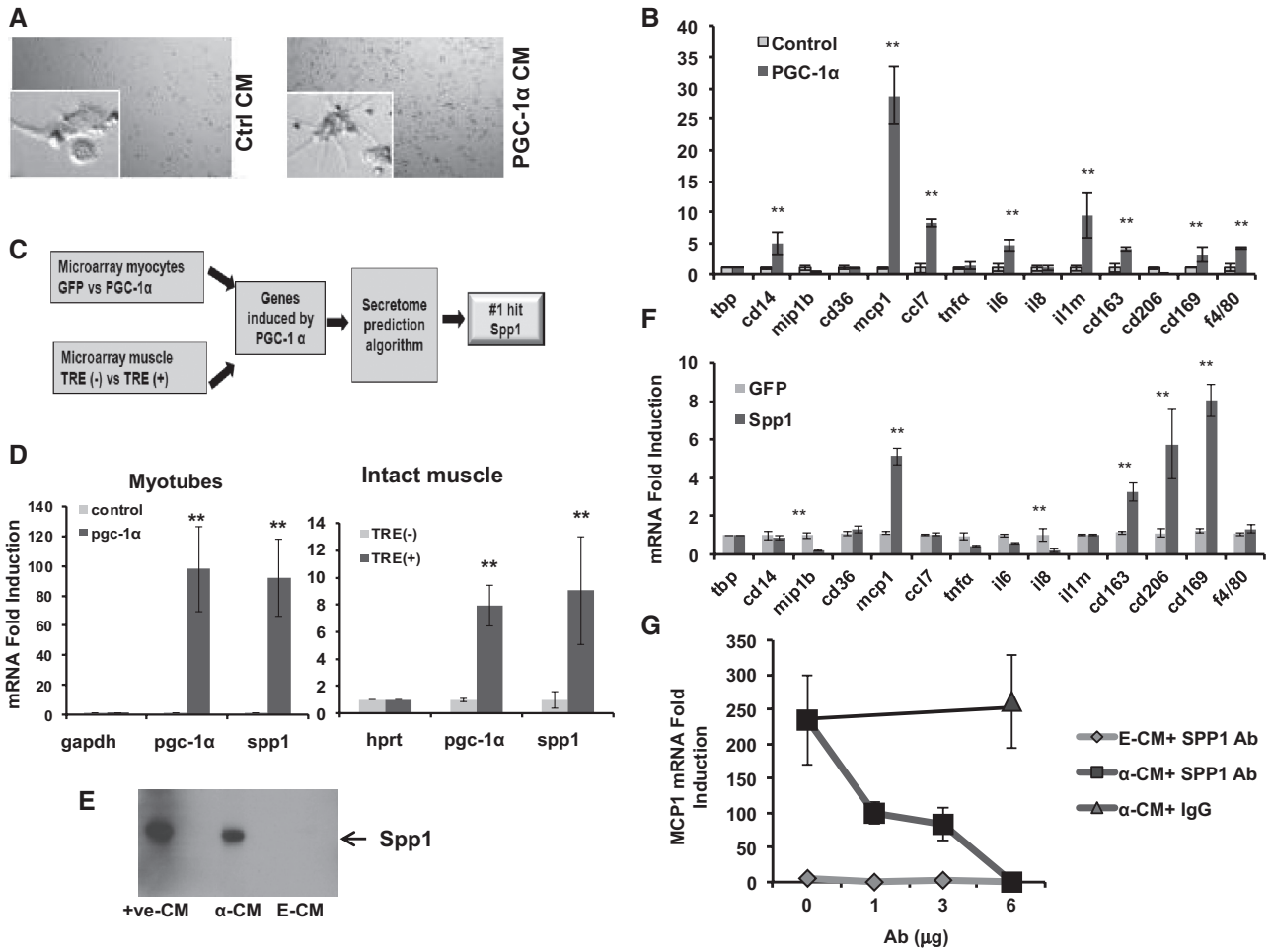

Figure 5. Peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) induces the secretion of secreted phosphoprotein 1 (SPP1) to regulate macrophage activation. **A**, Conditioned medium (CM) from myotubes overexpressing PGC-1α (PGC-1α CM) dramatically changes the morphology of macrophages. **B**, Expression of macrophage activation genes in THP1 cells treated with control and PGC-1α CM. **C**, Bioinformatic analyses of PGC-1α-expressing myotubes and muscle yield SPP1 as a lead candidate secreted factor that could impact macrophages. **D**, SPP1 expression in myotubes infected with control or PGC-1α retrovirus (**left**) and in gastrocnemius muscle from tetracycline response element (TRE (–)) and TRE (+) mice. **E**, Western blot of SPP1protein present in control and PGC-1α CM. Positive-CM: CM from 293 cells overexpressing SPP1. **F**, Expression of macrophage activation genes in THP1 cells treated with control and SPP1 CM. **G**, Monocyte chemoattractant protein-1 (MCP-1) gene expression after addition of SPP1-neutralizing antibody to control or PGC-1α CM. Error bars are SE. n=3 per group. **P*<0.05, ***P*<0.01, and ****P*<0.001. GFP indicates green fluorescent protein.

treated with CM from myotubes overexpressing PGC-1α versus control myotubes. Twenty four hours later, the medium was removed and replaced with fresh medium, thus removing the myotube CM and allowing the THP1 macrophages to condition the fresh medium (Figure 6A). Human umbilical cord endothelial cells were then seeded into the top chamber, and migrated cells were counted 12 hours later. As shown in Figure 6B and 6C, macrophages that had been educated by CM from PGC-α–expressing myotubes dramatically increased their ability to stimulate adjacent endothelial cells to migrate. Similar experiments with the differentiated 10 T1/2 model of pericytes[32,33] (Figure 6D and 6E) and smooth muscle cells (Figure 6F and 6G) yielded similarly strong 2- to 3-fold increases in cell migration. These results thus indicate that PGC-1α expression in myotubes has a strong, indirect, effect on numerous vascular cells, via activation of macrophages.

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.[34–37] 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.

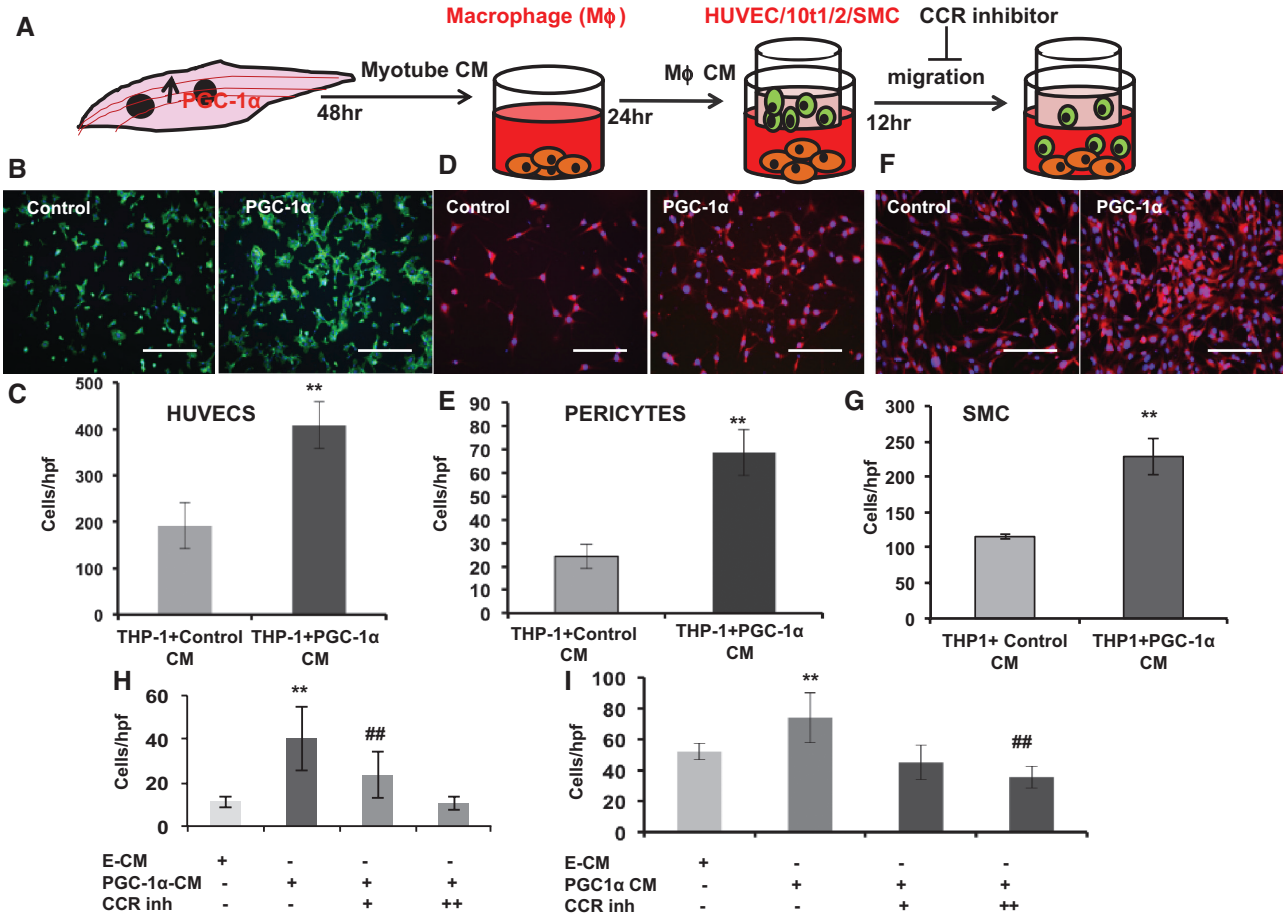

Figure 6. Conditioned macrophages secrete monocyte chemoattractant protein-1 (MCP-1) to recruit vascular cells. A, Experimental design for migration assay with human umbilical cord endothelial cells (HUVECs) and 10 t1/2 (pericytes). **B**, Migration of HUVECS. **C**, Quantification of migrated cells. **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.

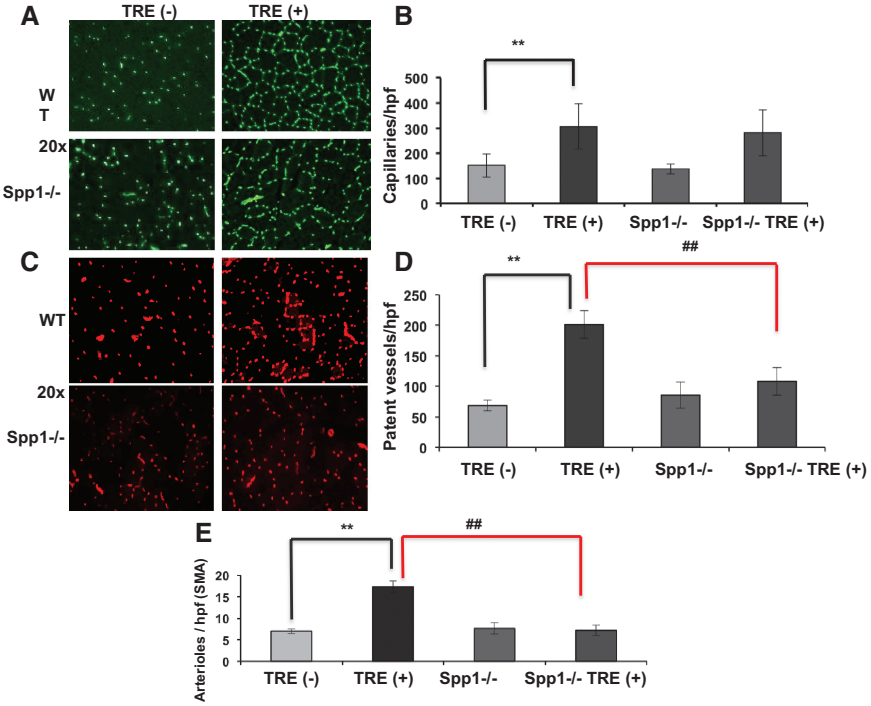

Figure 7. Secreted phosphoprotein 1 (SPP1) is required for vessel patency and arteriolar formation. A, Capillary density in the quadriceps after 4 weeks of doxy removal. **B**, Quantification of capillary endothelial cells/high power field (×20). **C**, Patent capillaries in quadriceps muscle after 4 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 tetracycle 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 isolectin 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.[19,38–40] 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.[17] We first used young 16-week-old C57/Bl6 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.[17] 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.[10,17,30] 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 ambulation 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.[41–43] To date, however, clinical trials have largely failed.[44–55] 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.[41,45] Angiogenesis is a complex process, not easily recapitulated by simple delivery of 1 or 2 angiogenic factors.[44,50] 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,[56] but a recent phase III trial failed to show benefit.[57] However, activation of HIF-1α typically inhibits metabolism, in particular oxidative metabolism,[5] which likely worsens oxidative tasks, such as walking.[57] 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 cells 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

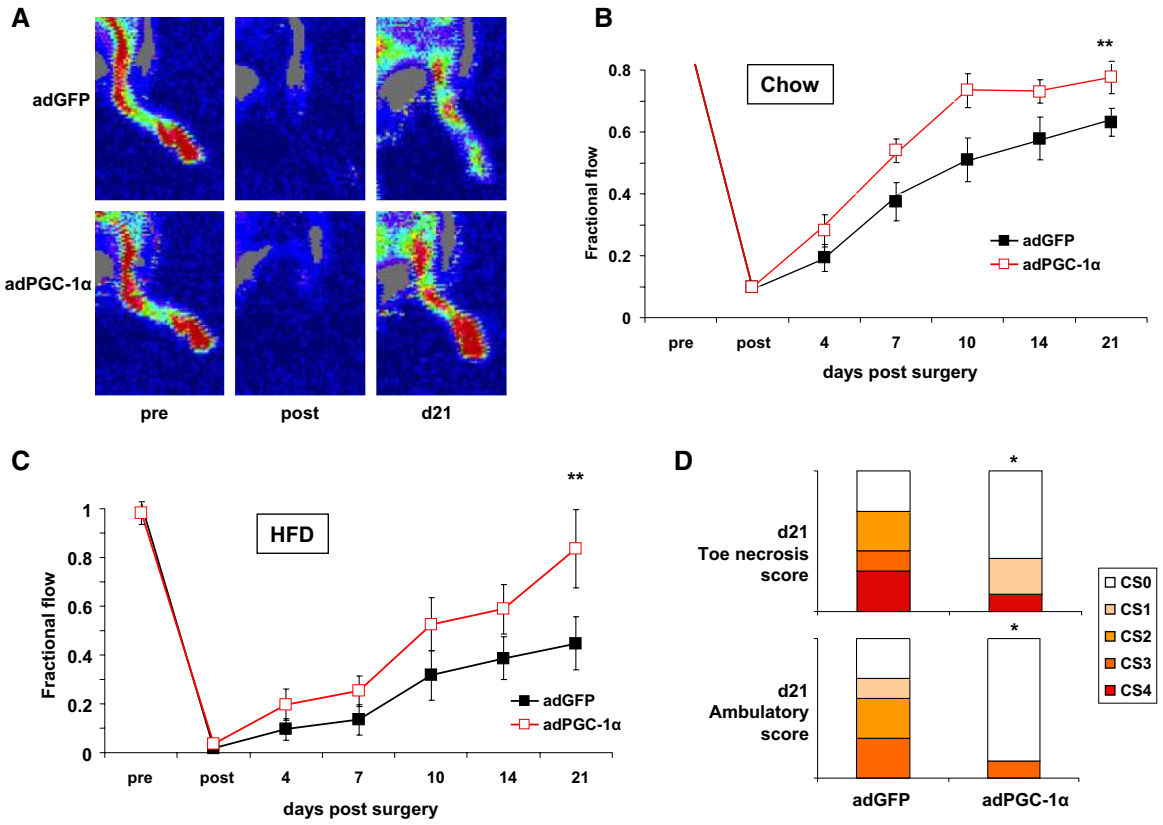

Figure 8. Adenoviral delivery of peroxisome proliferation activator receptor-γ coactivator-1α (PGC-1α) improves blood flow recovery after hindlimb ischemia. A, Sample laser Doppler images of blood flow recovery pre, immediately post, and 21 days after hindlimb ischemia surgery with concurrent injection 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 21days 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.

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

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.[30,75] 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,[76] an important maturation step in angiogenesis. Physiological angiogenesis in skeletal muscle likely does not occur by sprouting angiogenesis (ie, tip-cell formation), however, but rather via intussusception, a process that remains poorly understood.[2,77] 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.

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Disclosures

None.

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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 complication 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 mimetic 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.