

LETTERS

HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1 α

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Ischaemia of the heart, brain and limbs is a leading cause of morbidity and mortality worldwide. Hypoxia stimulates the secretion of vascular endothelial growth factor (VEGF) and other angiogenic factors, leading to neovascularization and protection against ischaemic injury¹. Here we show that the transcriptional coactivator PGC-1 α (peroxisome-proliferator-activated receptor- γ coactivator-1 α), a potent metabolic sensor and regulator², is induced by a lack of nutrients and oxygen, and PGC-1 α powerfully regulates VEGF expression and angiogenesis in cultured muscle cells and skeletal muscle *in vivo*. PGC-1 α ^{-/-} mice show a striking failure to reconstitute blood flow in a normal manner to the limb after an ischaemic insult, whereas transgenic expression of PGC-1 α in skeletal muscle is protective. Surprisingly, the induction of VEGF by PGC-1 α does not involve the canonical hypoxia response pathway and hypoxia inducible factor (HIF). Instead, PGC-1 α coactivates the orphan nuclear receptor ERR- α (oestrogen-related receptor- α) on conserved binding sites found in the promoter and in a cluster within the first intron of the *VEGF* gene. Thus, PGC-1 α and ERR- α , major regulators of mitochondrial function in response to exercise and other stimuli, also control a novel angiogenic pathway that delivers needed oxygen and substrates. PGC-1 α may provide a novel therapeutic target for treating ischaemic diseases.

Ischaemia is a profound metabolic challenge with potentially catastrophic consequences. PGC-1 α is a potent modulator of oxidative metabolism in numerous settings². In particular, PGC-1 α powerfully regulates oxidative phosphorylation, mitochondrial biogenesis, and respiration^{3,4}. To investigate a possible role for PGC-1 α in ischaemia, cultured C2C12 myotubes were deprived of nutrients and maintained in 0.2% oxygen. This led within 3 h to an induction of more than tenfold in PGC-1 α mRNA, as measured by quantitative PCR (Fig. 1a), and an increase in PGC-1 α protein, as detected by western blotting (Supplementary Fig. 1). The expression of PGC-1 α remained elevated when treatment was continued for 48 h (Supplementary Fig. 2) and returned to baseline within 3 h after restoration of complete medium and 21% oxygen (Fig. 1a). This induction of PGC-1 α was not unique to C2C12 myotubes and was observed in nearly all cell lines tested, including fibroblasts, striatal neurons, hepatocytes and primary skeletal muscle cells (Supplementary Fig. 3). Either removal of nutrients or placement in 0.2% oxygen alone led to a threefold induction of PGC-1 α expression, whereas the combined treatment led to a synergistic 12-fold induction (Fig. 1b). Hypoxia is well known to induce the expression of a broad genetic programme, at least in part through stabilization of the transcription factors HIF-1 α and HIF-2 α (ref. 5). This programme includes angiogenic genes such as VEGF and glycolytic genes such as *GLUT1*. To examine whether

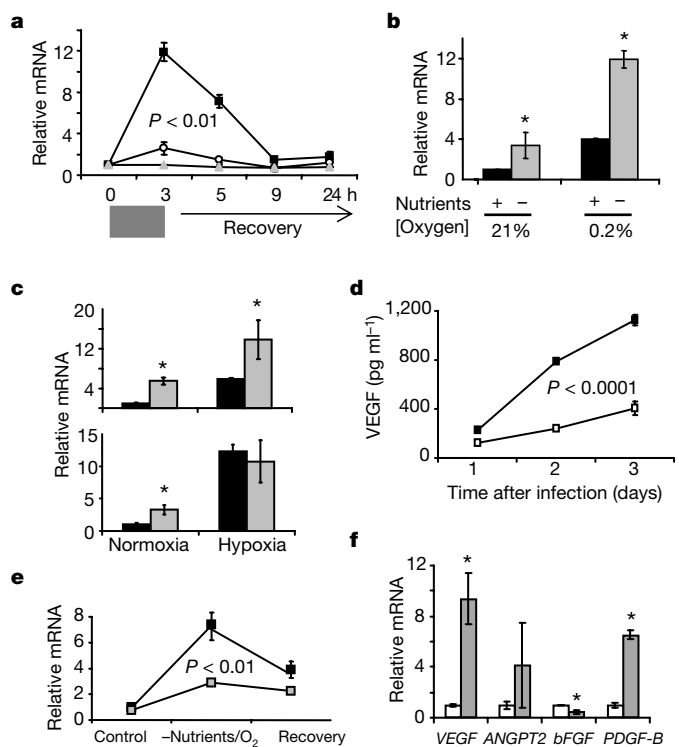


Figure 1 | PGC-1 α regulates VEGF in response to deprivation of nutrients and oxygen. **a**, C2C12 myotubes were deprived of oxygen (0.2%) and nutrients for 3 h and then restored to complete medium. Gene expression was measured by quantitative PCR. Squares, PGC-1 α ; circles, PGC-1 β ; triangles, PRC (PGC-1-related gene). **b**, Myotubes were deprived of oxygen or nutrients, or both, for 3 h, and expression of PGC-1 α mRNA was measured. **c**, Myotubes were infected with adenovirus expressing GFP (black bars) or PGC-1 α (grey bars) for 24 h and then maintained in either 21% or 0.2% oxygen for 16 h. Top, VEGF mRNA; bottom, GLUT1 mRNA. **d**, Myotubes were infected as in **c**, and VEGF in the culture medium was measured. Open squares, GFP; filled squares, PGC-1 α . **e**, Primary muscle cells derived from PGC-1 α wild-type (black squares) and knockout (grey squares) animals were treated as in **a**; expression of VEGF mRNA was measured. **f**, Primary myotubes were infected with adenovirus expressing GFP (white bars) or PGC-1 α (grey bars) and expression of the indicated mRNAs was measured. ANGPT2, angiopoietin-2; bFGF, basic fibroblast growth factor. Error bars indicate s.e.m.; $n > 3$ per group in all panels. Asterisk, $P < 0.05$ compared with control.

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PGC-1 α contributes to the regulation of this programme, C2C12 myotubes were infected with adenovirus expressing PGC-1 α or green fluorescent protein (GFP) as control. PGC-1 α expression led to a fourfold induction of VEGF mRNA, which was further increased to 14-fold in the presence of 0.2% oxygen (Fig. 1c and Supplementary Fig. 4). The induced VEGF transcript is functional, as demonstrated by an increased secretion of VEGF protein into the cell culture medium (Fig. 1d). All alternatively spliced forms of VEGF were induced by PGC-1 α (Supplementary Fig. 5). To determine whether PGC-1 α is required for the induction of VEGF, primary skeletal muscle cells were acquired from PGC-1 α ^{-/-} and wild-type animals. Treating these cells for 3 h with 0.2% oxygen and nutrient deprivation led to an 8-fold induction of VEGF in wild-type cells, and this was reduced to threefold in PGC-1 α ^{-/-} cells (Fig. 1e). The induction of GLUT1 was similarly blunted in PGC-1 α ^{-/-} cells (Supplementary Fig. 6). Hence, under these conditions, PGC-1 α contributes greatly to the VEGF gene response to nutrient and oxygen deprivation. Angiogenesis in response to hypoxia or other insults is a complex programme, requiring coordination by multiple signals⁶. VEGF has a dominant function in recruiting the endothelium, whereas platelet-derived growth factor (PDGF)-BB recruits mural cells to support and encase the endothelium. Angiopoietin 2, in the presence of VEGF, facilitates the sprouting of new vessels from existing vessels. All three of the genes encoding these proteins are inducible by PGC-1 α , as shown by the adenoviral delivery of PGC-1 α to primary skeletal muscle cells (Fig. 1f). Together, these data show that PGC-1 α is markedly induced by nutrient and oxygen deprivation and, in turn, regulates a wide programme of genes involved in the coordination of neovascularization.

We have previously described transgenic animals that express PGC-1 α in some skeletal muscle beds at levels that are about tenfold

those in wild-type controls⁷. To test whether PGC-1 α can elicit neovascularization *in vivo*, we examined the skeletal muscle of these animals, compared with wild-type littermate controls. Expression of VEGF was induced in all muscle beds examined in the transgenic animals (Fig. 2a, top panel), except in the fibre type I-rich soleus muscle; the transgene is less well expressed in soleus and there is little induction of PGC-1 α above wild-type levels (data not shown). Expression of other angiogenic factors, including PDGF-B and angiopoietin 2, was also induced in transgenic skeletal muscle (Fig. 2a, bottom panel). The density of capillaries was induced markedly in multiple muscle beds of transgenic animals in comparison with wild-type littermate controls, as determined by staining for the endothelial marker CD31 (Fig. 2b and Supplementary Fig. 7). The number of capillaries per high-power field increased from 100 to 300, and the number of capillaries per fibre similarly increased from five to ten. Hence, PGC-1 α powerfully induces angiogenesis *in vivo*.

We next tested whether PGC-1 α was required for the normal vascular response of skeletal muscle to ischaemia. PGC-1 α ^{-/-} animals and wild-type controls were subjected to ligation and ablation of the femoral artery. In wild-type animals this procedure causes nearly complete cessation of blood flow to the hind limb. This is then followed by progressive neovascularization and return of blood flow to the limb over the ensuing weeks (ref. 8 and Fig. 2c–e). By contrast, the return of blood flow is severely blunted in PGC-1 α ^{-/-} animals (Fig. 2c, d); only about 30% of blood flow is recaptured in PGC-1 α ^{-/-} animals 14 days after surgery, in contrast with more than 50% in wild-type littermate controls. Moreover, in transgenic animals expressing PGC-1 α in skeletal muscle, the return of blood flow after ligation of the femoral artery is markedly accelerated (Fig. 2e and Supplementary Fig. 8): 75% of blood flow is recaptured in these animals within six days after surgery, in

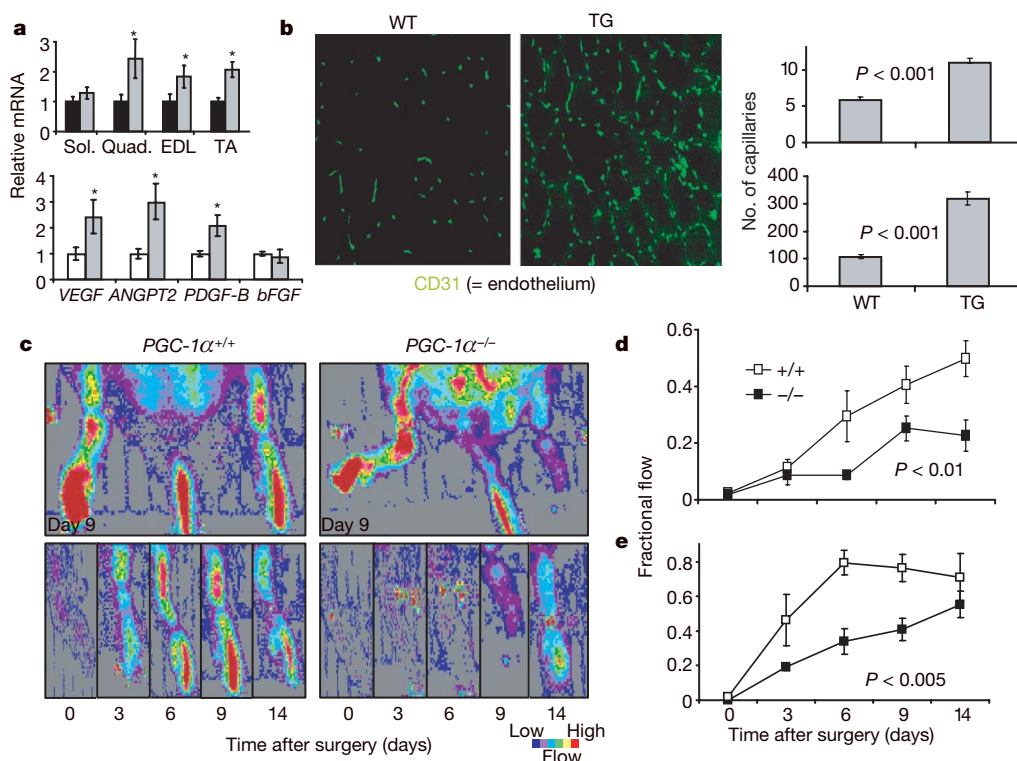


Figure 2 | PGC-1 α regulates angiogenesis and blood flow recovery after ischaemia *in vivo*. **a**, Top: expression of VEGF mRNA in wild-type (black bars) and muscle creatine kinase (MCK)-PGC-1 α transgenic (grey bars) animals. Bottom: expression of the indicated mRNAs in quadriceps from wild-type (white bars) and MCK-PGC-1 α transgenic (grey bars) animals. Sol., soleus; quad., quadriceps; EDL, extensor digitorum longus; TA, tibialis anterior. $n = 5$ per group. **b**, Left: transverse sections of TA muscle from wild-type (WT) and transgenic (TG) animals stained for CD31. Right:

quantification of capillaries per fibre (top) and per high-power field (bottom). $n = 6$ high-power fields from three mice per group. **c**, **d**, PGC-1 α ^{+/+} and PGC-1 α ^{-/-} mice subjected to femoral artery ligation were subsequently monitored by infrared imaging. Sample images are shown in **c**, and quantification of blood-flow recovery is shown in **d**. $n = 8$ per group. **e**, Blood flow recovery after treating WT (filled squares) and MCK-PGC-1 α TG (open squares) mice as in **c**. $n = 6$ per group. Error bars indicate s.e.m. Asterisk, $P < 0.05$ compared with control.

contrast with less than 40% in wild-type controls. Hence, under these conditions, PGC-1 α contributes significantly to the physiological response to hindlimb ischaemia. PGC-1 α does not seem to affect functional VEGF signalling in endothelial cells (Supplementary Figs 9 and 10), which is consistent with a primary role for PGC-1 α in skeletal muscle cells.

The regulation of *VEGF* in response to hypoxia is thought to be mediated primarily through the transcription factors HIF-1 α and HIF-2 α (ref. 9). However, the regulation of *VEGF* by PGC-1 α does not seem to involve this pathway. The hypoxic response element (HRE) that is targeted by HIF is well characterized and, when placed upstream of a luciferase reporter gene, can be activated by the addition of HIF-1 α or HIF-2 α , or by hypoxia itself. However, the addition of PGC-1 α has no effect on this activity, even if tested in the presence of exogenous HIF-1 α or HIF-2 α , or at reduced oxygen tension (Supplementary Fig. 11). The same is true if the HRE is tested in the context of a larger fragment of the *VEGF* promoter (Supplementary Fig. 12). Delivery of PGC-1 α to cells by means of adenovirus has no effect on *HIF-1\alpha* gene expression or protein stability, even though *VEGF* is robustly induced (Fig. 3a). HIF-1 α is also not altered in *PGC-1\alpha*^{-/-} cells (Supplementary Fig. 13). HIF-1 α and HIF-2 α form obligate heterodimers with the aryl hydrocarbon receptor nuclear translocator (ARNT) subunit of HIF, and in cells lacking ARNT there is no HIF activity¹⁰. However, when PGC-1 α is delivered by means of adenovirus to cells lacking ARNT, a fourfold induction of *VEGF* is still seen, equivalent to that seen in wild-type control cells (Fig. 3b). Hence, the induction of *VEGF* by PGC-1 α does not require the HIF pathway.

Similarly, the induction of *PGC-1\alpha* by the deprivation of nutrients and oxygen is apparently independent of HIF. The promoter region of *PGC-1\alpha*, placed upstream of a luciferase reporter gene, is not affected by the addition of a constitutively active form of HIF-1 α , whereas the promoter region of *VEGF* is strongly activated (Supplementary Fig. 14). A number of small molecules, including CoCl₂,

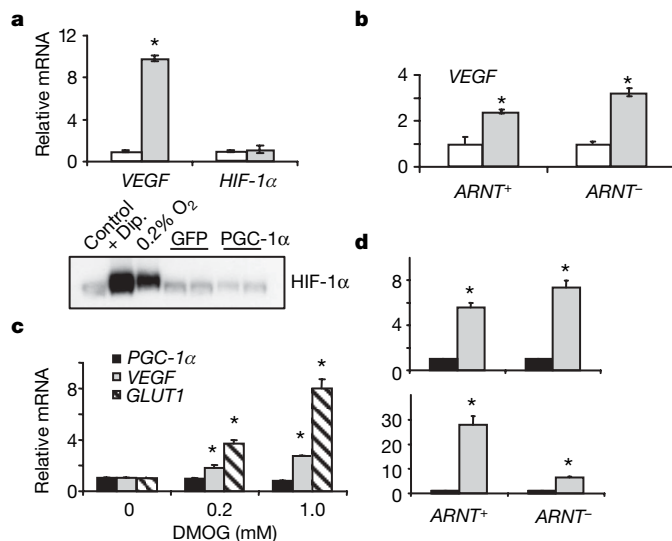


Figure 3 | PGC-1 α is induced by nutrient/oxygen deprivation and regulates VEGF independently of the HIF pathway. **a**, Primary skeletal muscle cells were infected with adenovirus expressing GFP (white bars) or PGC-1 α (grey bars). After 48 h, *HIF-1\alpha* mRNA (top), and protein (bottom) was measured. HIF-1 α protein levels after treatment for 6 h with dipyridamole (dip.) or hypoxia (0.2% O₂) are shown as controls. ct, control. **b**, ARNT⁻ and ARNT⁺ cells were infected with adenovirus expressing GFP (white bars) or PGC-1 α (grey bars), and *VEGF* mRNA was measured. **c**, C2C12 myotubes were treated with dimethylallyl glycine (DMOG) for 24 h, and expression of the indicated mRNAs was measured. **d**, ARNT⁻ and ARNT⁺ cells were deprived of oxygen and nutrients for 3 h (grey bars), and expression of *PGC-1\alpha* mRNA (top) and *VEGF* mRNA (bottom) was measured. Black bars, controls. Error bars indicate s.e.m.; $n > 3$ per group in all panels. Asterisk, $P < 0.05$ compared with control.

deferrioxamine and dimethylallyl glycine, can activate the HIF pathway and induce *VEGF*, *GLUT1* and other target genes; these molecules, however, have no effect on *PGC-1\alpha* expression (Fig. 3c and Supplementary Fig. 15). Finally, the induction of *PGC-1\alpha* by the deprivation of nutrients and oxygen is intact in cells lacking ARNT (Fig. 3d, top panel, and Supplementary Fig. 16), whereas the induction of canonical HIF targets is blunted (Fig. 3d, bottom panel, and Supplementary Fig. 17). Together, these data clearly show that PGC-1 α has a critical function in an angiogenic pathway that is independent of the canonical HIF pathway.

PGC-1 α is known to co-activate several transcription factors, including many members of the MEF2 (myocyte enhancer factor-2), FOXO (forkhead transcription factor O) and nuclear receptor families². Plasmids expressing several of these transcription factors, with or without PGC-1 α , were co-transfected into 10T1/2 cells along with a reporter plasmid containing 2 kilobases of the *VEGF* promoter driving the luciferase gene. The addition of PGC-1 α to nuclear respiratory factor (NRF)-1 or NRF-2 (Fig. 4a), MEF2s or FOXO1 (data not shown) had no effect on luciferase activity. The addition of PGC-1 α to the orphan nuclear receptor ERR- α , in contrast, led to an eightfold induction of luciferase activity (Fig. 4a). ERR- α is known to interact physically and functionally with PGC-1 α and is involved in the activation of programmes of fatty acid oxidation and oxidative phosphorylation¹¹⁻¹⁶. The induction of luciferase activity by PGC-1 α plus ERR- α did not require the integrity of the HIF-responsive element in the *VEGF* promoter (Supplementary Fig. 18). Instead, it required the integrity of two putative ERR- α -binding sequences, both of which are perfectly conserved across vertebrate species (Supplementary Fig. 19). To examine the role of ERR- α in regulating endogenous VEGF, primary skeletal muscle cells were infected with adenovirus expressing ERR- α . This led to a threefold induction of *VEGF* mRNA, compared with infection with a control virus expressing GFP alone (Fig. 4b). As a critical test for whether ERR- α is required for the PGC-1 α -mediated induction of *VEGF*, primary mouse embryonic fibroblasts were prepared from ERR- α ^{-/-} and wild-type animals, and the expression of VEGF was evaluated after infection with adenovirus expressing PGC- α . In wild-type mouse embryonic fibroblasts, PGC-1 α induced *VEGF* expression sevenfold, compared with virus expressing GFP alone (Fig. 4c). In sharp contrast, the induction of *VEGF* by PGC-1 α was completely abrogated in ERR- α ^{-/-} mouse embryonic fibroblasts. The induction of *PDGF-B* by PGC-1 α was also abrogated in these cells (Supplementary Fig. 20). Consistent with these findings is our observation that the expression of *VEGF* in ERR- α ^{-/-} primary skeletal muscle cells was reduced by about 50% (Supplementary Fig. 21). Thus, PGC-1 α seems to stimulate VEGF expression at least in part by the coactivation of ERR- α .

ERR- α recognizes the consensus DNA sequence AAGGTCA¹⁷. A search through the 25 kilobases surrounding the murine *VEGF* gene revealed the existence of 11 such sites, whereas only three or four would have been predicted by chance alone (Fig. 4d). Of these 11 sites, 6 are perfectly conserved between human, mouse and rat (red arrows in Fig. 4d); strikingly, 5 of them are clustered within regions of high homology in the first intron of the *VEGF* gene. A 1,200-base-pair region encompassing these five sites was amplified by polymerase chain reaction and cloned upstream of the SV40 promoter and a luciferase reporter gene. Co-transfection of *PGC-1\alpha* and ERR- α with this reporter plasmid led to a synergistic sevenfold induction of luciferase activity (Fig. 4e, left half). By contrast, PGC-1 α and ERR- α had no effect on a control plasmid containing the SV40 promoter alone (Fig. 4e, right half). The induction of luciferase activity was dependent on the integrity of the conserved ERR- α sites (Supplementary Fig. 22). Chromatin immunoprecipitation assays revealed that PGC-1 α can occupy these ERR- α sites as well as the ERR- α sites found on the promoter (Fig. 4f). Thus, the first intron of the *VEGF* gene contains a putative enhancer region in which several conserved

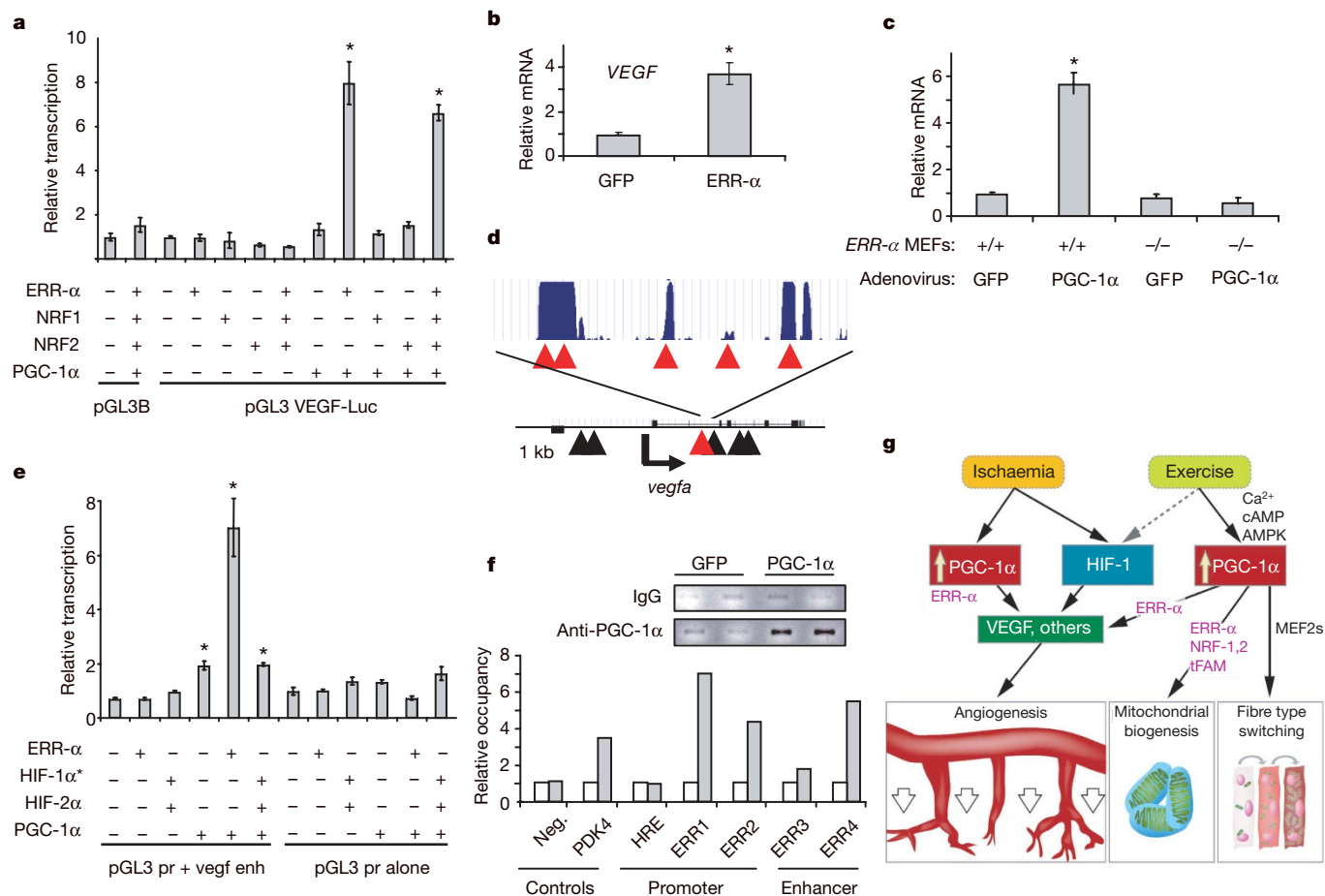


Figure 4 | PGC-1 α regulates expression of VEGF through coactivation of ERR- α . **a**, Luciferase (Luc) activity in cells transfected with VEGF or control promoter constructs, plus vectors expressing the indicated proteins. **b**, VEGF mRNA in primary skeletal myotubes infected with adenovirus expressing GFP or PGC-1 α . **c**, ERR- α ^{+/+} and ERR- α ^{-/-} mouse embryonic fibroblasts evaluated as in **b**. **d**, Diagram of the murine *vegfa* gene region. Arrowheads indicate consensus ERR- α binding sites; red arrowheads indicate sites perfectly conserved between mammals. kb, kilobase. The histogram shows interspecies homology. See Supplementary Fig. 15 for details. **e**, Luciferase activity in cells transfected with the indicated plasmids, plus a reporter

construct containing the region depicted in **d**. pr, SV40 promoter; vegf enh, *vegfa* enhancer. **f**, Chromatin immunoprecipitations in C2C12 myotubes infected with the indicated adenovirus. White bars, IgG; grey bars, PGC-1 α . ERR1–ERR4, putative ERR- α -binding sites in the VEGF promoter and enhancer (see Supplementary Figs 13 and 15); PDK4, known site in the *PDK4* promoter; HRE, hypoxic responsive element; neg., unrelated site. **g**, Speculative model for the role of PGC-1 α in the regulation of angiogenesis during exercise and in response to ischaemia. AMPK, AMP-activated protein kinase; tFAM, mitochondrial transcription factor A. Error bars indicate s.e.m. Asterisk, $P < 0.05$ compared with control.

ERR- α -binding sites are recognized by ERR- α and coactivated by PGC-1 α to elicit the robust induction of VEGF transcription.

The data here show that PGC-1 α is a mediator of signalling in response to deprivation of nutrients and oxygen, and that it powerfully regulates VEGF and other angiogenic factors to elicit neovascularization *in vivo*. The regulation of VEGF in response to hypoxia is thought to be mediated primarily through the well-known HIF factors⁹. Surprisingly, the novel PGC-1 α /ERR- α pathway described here is apparently independent of the HIF pathway. PGC-1 α ^{-/-} mice are viable, suggesting that PGC-1 α is not essential in embryonic vascularization. Angiogenesis in the adult occurs in both physiological and pathological contexts¹. The robust induction of vascularization by PGC-1 α , and its critical function in the response to limb ischaemia, strongly implicate PGC-1 α in the angiogenic response to ischaemia, providing protection against further ischaemic insults (Fig. 4g). PGC-1 α is also robustly induced by exercise and mediates known responses to exercise such as fibre-type switching and mitochondrial biogenesis^{3,7}. On the basis of these observations and the data presented here, we speculate that the PGC-1 α /ERR- α pathway also mediates exercise-induced neovascularization (Fig. 4g). This elegantly links the regulation of consumption of oxygen by mitochondria to the delivery of oxygen and nutrients by the vasculature. Angiogenesis is also crucial to tumour progression and

metastasis. The interface of metabolism with cancer progression has been the subject of renewed scrutiny in recent years¹⁸. It will be of great interest to elucidate the role of PGC-1 α and ERR- α in this interface, given the important function of these molecules in metabolic control.

Human clinical trials that examine the efficacy of VEGF delivery as therapy in various settings, including chronic limb ischaemia, have yielded disappointing results^{6,19,20}. In large part this may be because the use of VEGF alone seems to lead to immature, leaky vessels¹⁹. The generation of fully functional vessels requires the coordinated action of numerous signals, such as PDGF-BB and the angiopoietins⁶. One therapeutic approach to this problem may be to modulate a transcriptional regulator that coordinates these signals appropriately^{6,21}. The PGC-1 α /ERR- α pathway provides such an opportunity.

METHODS SUMMARY

Nutrient and oxygen deprivation was induced by placing cells in Hanks balanced salt solution and 0.2% oxygen. Hindlimb ischaemia was induced by ligation of the femoral artery, as described⁸. Infections with adenovirus and transfections were performed as described²². One-way analysis of variance with repeated values was used to analyse data from femoral ligations. Two-tailed independent Student's *t*-tests were used to determine all other *P* values.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Animals. All animal experiments were performed in accordance with procedures approved by the Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent chow diet with 12-h/12-h light/dark cycles. Hindlimb ischaemia was induced by ligation of the femoral artery, as described⁸. Blood flow was measured with a MoorLDI2 infrared scanner.

Cell culture, adenovirus infections, and transient transfections. Cells were maintained in DMEM medium in the presence of 10% FBS. 10T1/2 fibroblasts, C2C12 myoblasts, *ARNT*⁺ and *ARNT*⁻ hepatoma cells (2712 and 2717) were from ATCC. Primary skeletal muscle cells were prepared as described²³. Mouse embryo fibroblasts were prepared by homogenizing day-13.5 embryos from *ERR-α* heterozygous matings. Experiments were performed on cells that were passaged two to four times. Nutrient and oxygen deprivation was induced by placing cells in Hanks balanced salt solution and 0.2% oxygen. Transfections of 10T1/2 cells were performed with Superfect (Qiagen) with 0.2 μg of reporter plasmid plus 0.1 μg of the indicated expression plasmids and 0.01 μg of a control plasmid encoding *Renilla* luciferase. After 24 h, cells were treated as indicated; after a further 24 h, luciferase assays were performed (Promega Dual-Luciferase). Cells were infected with adenovirus at a multiplicity of infection of 10–30, and mRNA expression was measured 48 h later. The adenovirus expressing *ERR-α* was generated by Welgen. The adenovirus expressing PGC-1α has been described.

Reagents and gene expression. All reagents were from Sigma unless otherwise indicated. VEGF enzyme-linked immunosorbent assay kit was from Calbiochem. Total RNA was prepared with the Trizol method (Invitrogen). mRNAs were quantified with quantitative real-time PCR by using SYBR green dye and an ABI 7300 machine. Genome analyses were performed with the University of California Santa Cruz genome browser (<http://genome.ucsc.edu/>). Chromatin immunoprecipitations were performed with the EZChip kit (Upstate) in accordance with the manufacturer's protocol. One-way analysis of variance with repeated values was used to analyse data from femoral ligations. Two-tailed independent Student's *t*-tests were used to determine all other *P* values.

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