Exercise and PGC-1α-Independent Synchronization of Type I Muscle Metabolism and Vasculature by ERRγ

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SUMMARY

How type I skeletal muscle inherently maintains high oxidative and vascular capacity in the absence of exercise is unclear. We show that nuclear receptor ERRγ is highly expressed in type I muscle and, when transgenically expressed in anaerobic type II muscles (ERRGO mice), dually induces metabolic and vascular transformation in the absence of exercise. ERRGO mice show increased expression of genes promoting fat metabolism, mitochondrial respiration, and type I fiber specification. Muscles in ERRGO mice also display an activated angiogenic program marked by myofibrillar induction and secretion of proangiogenic factors, neovascularization, and a 100% increase in running endurance. Surprisingly, the induction of type I muscle properties by ERRγ does not involve PGC-1α. Instead, ERRγ genetically activates the energy sensor AMPK in mediating the metabovascular changes in ERRGO mice. Therefore, ERRγ represents a previously unrecognized determinant that specifies intrinsic vascular and oxidative metabolic features that distinguish type I from type II muscle.

INTRODUCTION

Tissue vascular supply is tightly coupled to its oxidative capacity. This is especially evident in skeletal muscle beds enriched in either oxidative slow-twitch or glycolytic fast-twitch myofibers (Flück and Hoppeler, 2003; Pette and Staron, 2000). Slow-twitch muscles are characterized by high mitochondrial content, fatigue-resistant (type I) fibers, and dense vascularity to ensure a steady and prolonged supply of oxygen and nutrients (Annex et al., 1998; Cherwek et al., 2000; Ripoll et al., 1979). Fast-twitch (type II) muscles generally have lower oxidative capacity and a reduced blood supply and are fatigue sensitive. How the type I versus the type II muscle vasculature is specified to match oxidative capacity is unclear.

Previous studies have established that nuclear receptors such as PPARα, PPARδ, and ERRα, along with coregulators PGC-1α, PGC-1β, and Rip140 control diverse aspects of aerobic respiration, including fatty acid oxidation, oxidative phosphorylation, and mitochondrial biogenesis, in skeletal muscle (Arany et al., 2007; Huss et al., 2004; Lin et al., 2002; Minnich et al., 2001; Muoio et al., 2002; Seth et al., 2007; Wang et al., 2004). While signaling factors such as TGF-β1, platelet-derived growth factor, fibroblast growth factors (FGF) 1 and 2, and vascular endothelial growth factor (VEGF) are known to stimulate angiogenesis (Carmeliet, 2000; Ferrara and Kerbel, 2005; Gustafsson and Kraus, 2001), whether and how these factors orchestrate dense vascularization of aerobic muscles is unclear. One possibility is vascular arborization by coactivator PGC-1α that is induced by hypoxia and exercise (Arany et al., 2008). However, PGC-1α knockout mice are viable, still retain oxidative muscle, and have normal vasculature (Arany et al., 2008; Lin et al., 2004). Since the intrinsic enrichment of blood flow to aerobic muscles in the absence of exercise is unlikely to depend on PGC-1α induction, we speculate the existence of an alternative regulatory angiogenic pathway.

Estrogen receptor-related receptor γ (ERRγ), like other members of the ERR subfamily, is a constitutively active orphan nuclear receptor, though unlike ERRα and β, it is more selectively expressed in metabolically active and highly vascularized tissues such as heart, kidney, brain, and skeletal muscles (Giguère, 2008; Heard et al., 2000; Hong et al., 1999). In vitro studies suggest that ERRγ activates genes such as *Pdk4* and *Acadm* that play a regulatory role in oxidative fat metabolism (Huss et al., 2002; Zhang et al., 2006). Furthermore, a comprehensive gene expression analysis identified ERRγ as a key regulator of multiple genes linked to both fatty acid oxidation and mitochondrial biogenesis in cardiac muscles (Alaynick et al., 2007; Dufour et al., 2007). Expression of ERRγ is also induced in variety of tumors with hypermetabolic demands and abundant vasculature (Ariazi et al., 2002; Cheung et al., 2005; Gao et al., 2006). Therefore, we explored the potential of ERRγ in controlling the intrinsic angiogenic pathway in oxidative slow-twitch muscles. We found ERRγ to be exclusively and abundantly expressed in oxidative (type I) slow-twitch muscles. Transgenic expression of ERRγ in fast-twitch type II muscle triggers aerobic transformation, mitochondrial biogenesis, VEGF induction, and robust myofibrillar vascularization, all in the absence of exercise. These intrinsic effects of ERRγ do not depend on PGC-1α induction, but rather are linked to activation of the metabolic sensor AMPK. These findings reveal an exercise-independent ERRγ pathway that promotes and coordinates vascular supply and metabolic demand in oxidative slow-twitch muscles.

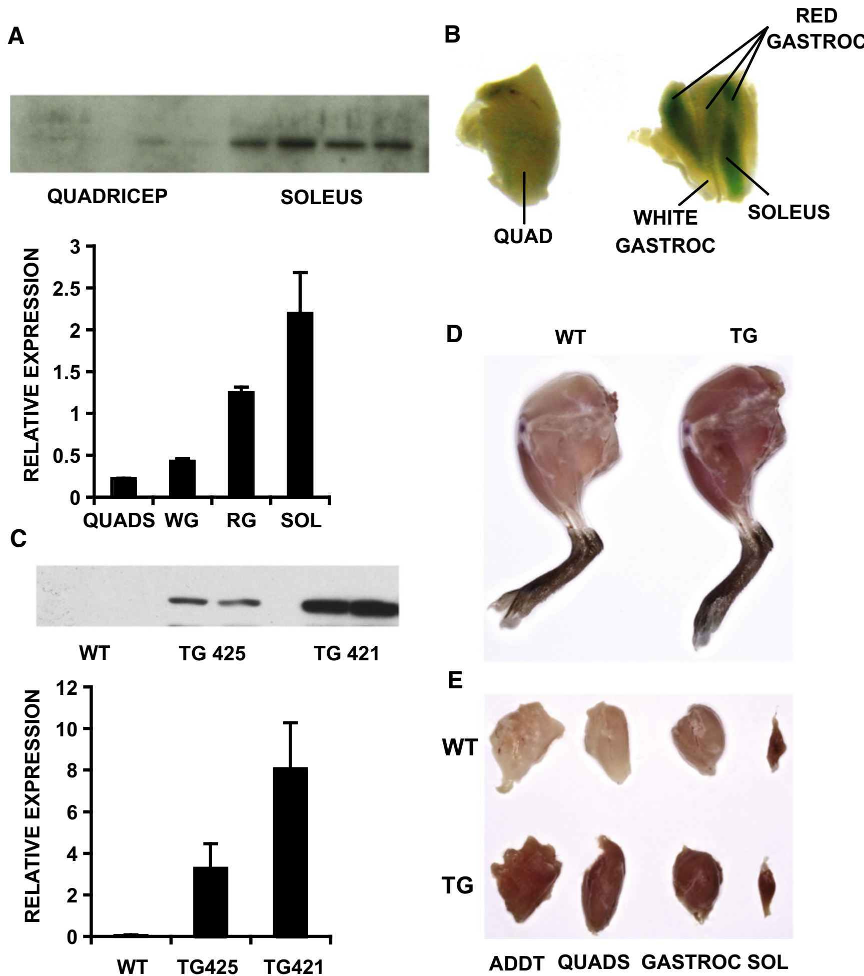

Figure 1. Skeletal Muscle ERRγ Expression
(A) ERRγ gene (lower panel) and/or protein (upper panel) expression in quadriceps (QUADS), white gastrocnemius (WG), red gastrocnemius (RG), and soleus (SOL) isolated from C57BL/6J mice (n = 4).
(B) Representative images of β-galactosidase-stained muscles.
(C) Expression of transgene transcript (lower panel) and protein (upper panel) in quadriceps of WT and TG founders 425 and 421.
(D) Representative hindlimbs from WT and transgenic mice.
(E) Dissected hindlimb muscle beds (adductor [ADDT], quadriceps, gastrocnemius [GASTROC], and soleus). In (A) and (C), data are presented as mean ± SD (n = 4). See Figure S1.

RESULTS

Skeletal Muscle ERRγ Expression

Because skeletal muscle is a functionally heterogeneous tissue consisting of both aerobic slow-twitch and glycolytic fast-twitch muscles, we re-evaluated ERRγ expression in the context of different myofibrillar beds. We found that ERRγ transcript is highly expressed in oxidative muscles such as soleus and red gastrocnemius, with minimal expression in glycolytic quadriceps and white gastrocnemius (Figure 1A, lower panel). ERRγ protein is undetectable in quadriceps but highly expressed in soleus (Figure 1A, upper panel).

Previously, we described viable ERRγ +/− mice in which a β-galactosidase protein-coding region without the promoter was introduced in-frame with the initiation site of the *Esrrg* gene (Alaynick et al., 2007) such that the enzyme mimics the expression of endogenous ERRγ. β-galactosidase staining of different muscle beds from ERRγ +/− adult mice further confirmed that the receptor is highly expressed in oxidative (e.g., soleus and red gastrocnemius) compared to the minimal levels in glycolytic muscles (e.g., quadriceps and white gastrocnemius) (Figure 1B).

Transgenic Muscle-Specific ERRγ Overexpression

The above expression pattern of ERRγ supports its presumptive role in oxidative and slow-twitch muscle biology. To test this idea, we generated transgenic mice selectively expressing ERRγ in skeletal muscles under the control of the human α-skeletal actin promoter (Muscat and Kedes, 1987; Wang et al., 2004). Two ERRγ-overexpressing (ERRGO) transgenic lines were obtained (TG 421 and 425) showing both transcript (lower panel) and protein (upper panel) in fast-twitch quadriceps (Figure 1C). Gross anatomical analysis of hindlimb muscles (Figure 1D) and dissection of individual muscle beds (Figure 1E) revealed enhanced red coloration (characteristic of oxidative fibers) in transgenic compared to WT muscle. Importantly, slow-twitch (soleus) muscle, already high in ERRγ expression, was not affected (Figure 1E), presumably because it is already fully oxidative. In addition, oxidative biomarkers myoglobin and cytochrome *c* were induced in the quadriceps of both the transgenic lines compared to WT mice (Figure S1). For subsequent studies we focused on TG 421, due to slightly higher biomarker expression in this progeny.

Fast- to Slow-Twitch Transformation of Skeletal Muscle by ERRγ

To study the transcriptional effect of ERRγ, muscle gene expression was measured in quadriceps from WT and ERRGO mice. In gene array analysis, we found that ERRγ regulated a total of 1123 genes in skeletal muscles, of which 623 genes were induced. Gene ontology-based classification of these genes is presented in Figure 2A. The majority of the upregulated genes belong to either mitochondrial biology (90) or oxidative metabolism (43), encoding various components of the fatty acid oxidation pathway as well as the oxidative respiratory chain, reflective of aerobic adaptation (described in Table S1). Furthermore, contractile genes, especially ones associated with slow myofibers,

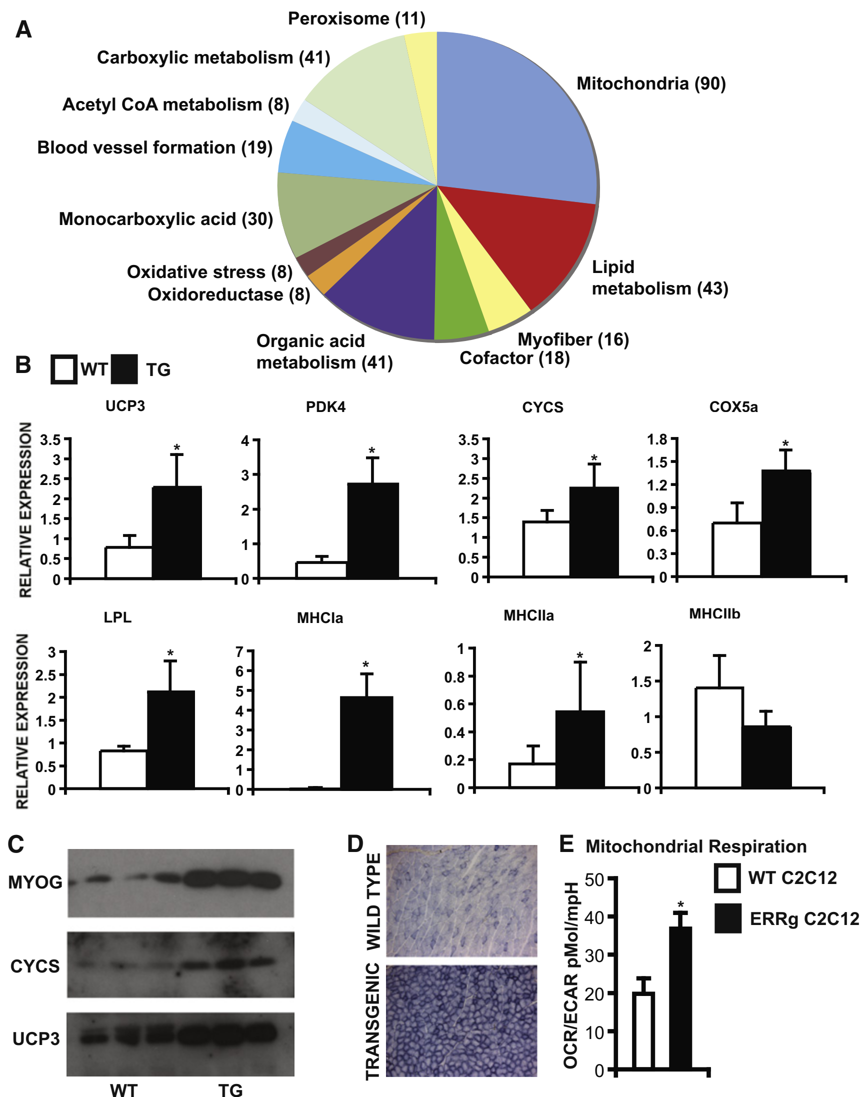

Figure 2. ERRγ Promotes Oxidative Muscle Transformation

(A) Gene ontology classification of positively regulated genes. Gene selection was based on $p < 0.05$ on Bonferroni's multiple comparison test for fold change (n = 3).

(B) ERRγ increases expression of oxidative metabolism (*Ucp3*, *Pdk4*, *Cycs*, *Cox5a*, *Lpl*) and oxidative muscle (*Mhc1a*, *Mhc2a*) but not glycolytic muscle (*Mhc2b*) biomarker genes. Data are presented as mean ± SD from n = 6 samples.

(C) ERRγ increases protein expression of myoglobin, cytochrome *c*, and uncoupling protein 3 (n = 3).

(D) Representative images of SDH-stained WT and transgenic gastrocnemius cryosections. Similar results were obtained from n = 4 mice.

(E) OCAR/ECAR ratio representing a shift in cellular energy production to oxidative phosphorylation. Data is presented as mean ± SD. * represents statistically significant difference between WT and transgenic mice or between WT and ERRγ-overexpressing C2C12 cells ($p < 0.05$, unpaired Student's t test). See Figure S2 and Tables S1, S2, and S4.

were also activated, raising the possibility of fast-to-slow transformation linked to the metabolic switch (Table S2).

We confirmed that key biomarker genes associated with oxidative metabolism (*Ucp3*, *Pdk4*, *Cycs*, *Cox5a*, *Lpl*) and oxidative myofibers (*Mhc Ia*, *Mhc IIa*) but not glycolytic myofibers (*Mhc IIb*) were induced by ERRγ in quadriceps of transgenic mice (Figure 2B). Conversely, many of the biomarker genes tested (*Ucp3*, *Cycs*, *Acscl1*, *Cox6a2*, *Ppara*) were found to be downregulated by siRNA-mediated ERRγ knockdown in primary cultured myotubes (Figure S2A) isolated from oxidative muscles (soleus and red gastrocnemius). Moreover, the oxidative changes were confirmed at the protein level, as exemplified by increased expression of myoglobin, cytochrome *c*, and UCP3 in transgenic relative to WT muscle (Figure 2C). Furthermore, staining of gastrocnemius cryosections for defining oxidative mitochondrial enzyme SDH activity revealed an increase in oxidative myofibers in ERRGO compared to WT mice (Figure 2D), which was confirmed by electron microscopy (data not shown).

To access the metabolic effects of ERRγ at the cellular level, we measured the mitochondrial bioenergetics in WT and ERRγ-overexpressing C2C12 cells using an extracellular flux analyzer. Specifically, we determined the oxygen consumption rate (OCR) (an indicator of mitochondrial respiration) along with the extracellular acidification rate (ECAR) (a measure of glycolysis) in these cells (Figures S2B and S2C). ERRγ expression significantly induced mitochondrial respiration (OCR) and reduced cellular glycolysis (ECAR), resulting in an 85% shift in the cellular energy production ratio toward oxidative phosphorylation (Figure 2E).

The above observations show that ERRγ promotes an overt conversion of glycolytic fast-twitch muscles such as quadriceps to an oxidative slow-twitch phenotype.

ERRγ Promotes Skeletal Muscle Vascularization

Intrinsic vascularization of slow-twitch myofibers enables a baseline of exercise-independent fatigue resistance. We speculated that ERRγ, by virtue of its restricted expression to type I fibers, could, in addition to promoting oxidative metabolism, simultaneously induce vessel formation to match the increased oxidative demand. To test this, we first stained muscle cryosections for PECAM 1 (CD31), an endothelial cell marker that is routinely used to detect angiogenesis and changes in tissue vasculature. We found that transgenic muscles showed increased PECAM 1 (Figure 3A) staining compared to WT. Similarly, transgenic muscle cryosections showed an increase in alkaline phosphatase staining, an alternative marker for tissue endothelium (Figure 3B). These findings point toward a possible induction of angiogenesis and muscle vascularization by ERRγ. To test

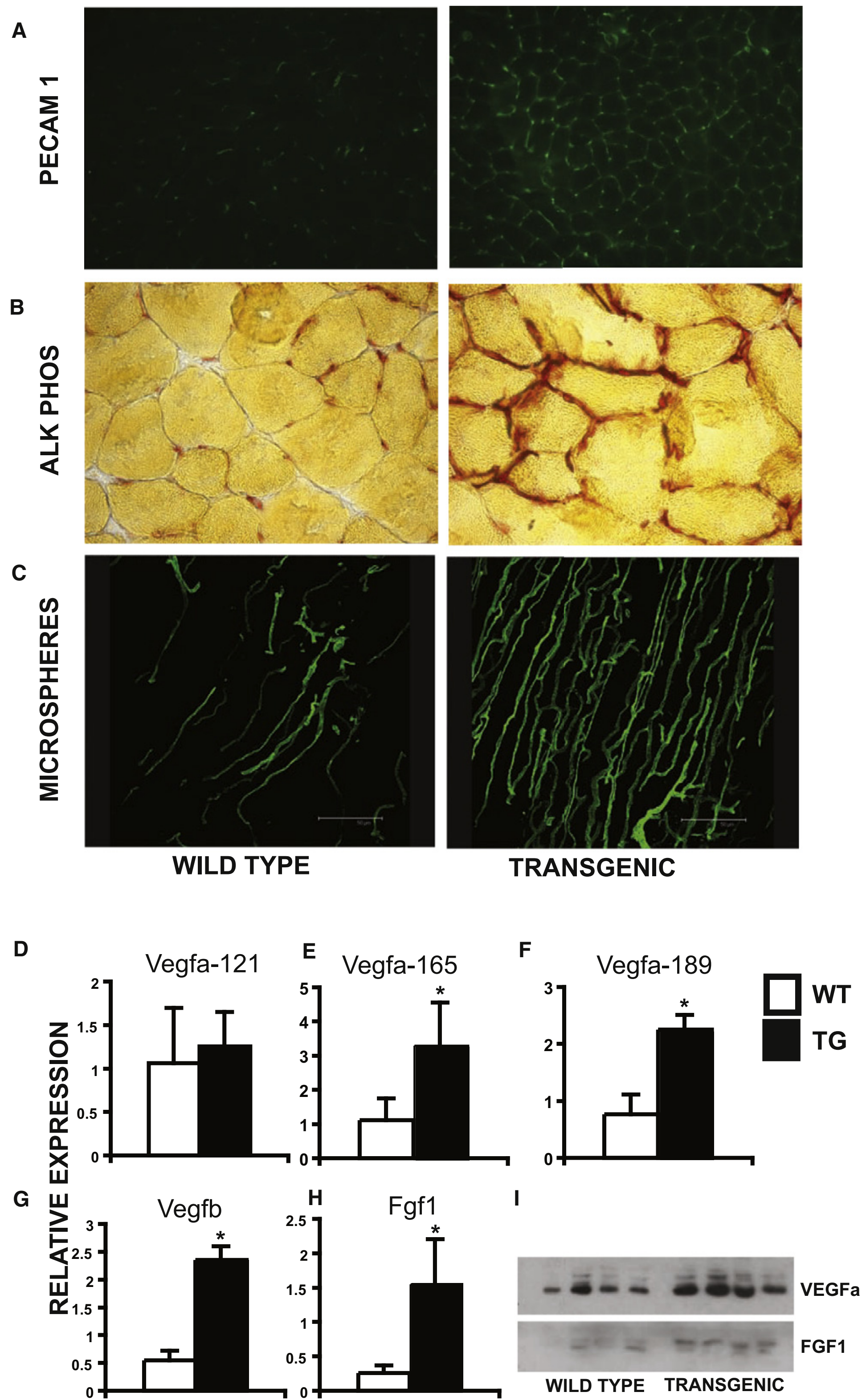

Figure 3. ERRγ Increases Muscle Vascularization

(A) Increased PECAM 1 staining in transgenic compared to WT gastrocnemius.

(B) Increased alkaline phosphatase staining in transgenic compared to WT tibialis muscles.

(C) Confocal images of microsphere perfused WT and transgenic gastrocnemius. Similar results were obtained from n = 4 experiments in (A)–(C).

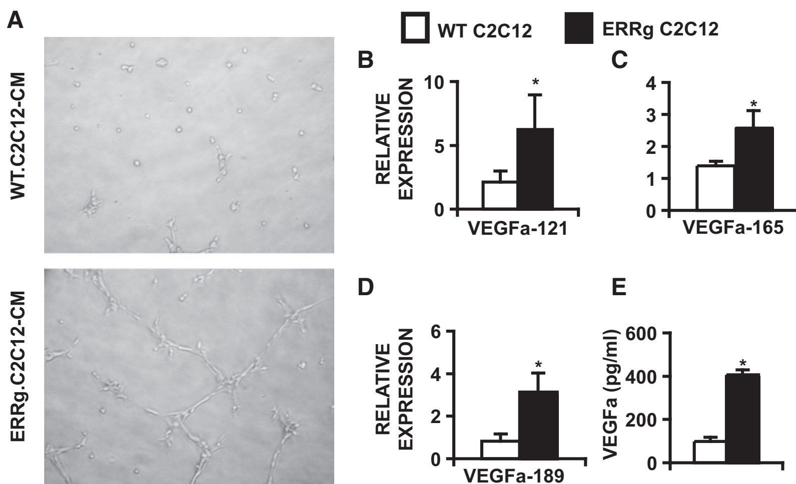

Figure 4. Paracrine Stimulation of Angiogenesis by ERRγ

(A) Tube formation in SVEC4-10 cells treated for 7–8 hr with conditioned media from WT and ERRγ-overexpressing C2C12 myotubes. Similar results were obtained from 4–6 experiments.

(B–D) Expression of *Vegfa* isoforms in WT and ERRγ-overexpressing C2C12 myotubes (n = 6).

(E) Vegfa concentrations (pg/ml) in conditioned media from 2 day differentiated WT and ERRγ-overexpressing C2C12 myotubes (n = 3). Data in (B)–(E) are presented as mean ± SD. * represents significant difference between WT and ERRγ-overexpressing C2C12 cells ($p < 0.05$, unpaired Student's t test). See Figure S3 and Table S3.

whether ERRγ supports formation of functional nonleaky blood vessels, we used microangiography following intraventricular perfusion of a fluorescent microspheres (0.1 μM). The impermeability of the microspheres allows their vascular retention, enabling confocal angiographic "vascular mapping" of intact and mature blood vessels. Examination of perfused microspheres in WT and transgenic gastrocnemius revealed an increase in muscle vascularity by ERRγ (Figure 3C), showing that ERRγ dually promotes oxidative fiber specification and neovascularization.

Paracrine Regulation of Muscle Vascularization of ERRγ

How might ERRγ expressed in myofibers regulate proximal vascular development? Gene expression studies (Figure 2A and Table S3) revealed increased expression of 25 angiogenic genes, including vascular endothelial growth factor A (*Vegfa*), in ERRGO quadriceps. Real-time PCR confirmed induction of two *Vegfa* isoforms (165 and 189) along with *Vegfb* and *Fgf1* in transgenic muscles (Figures 3D–3H). Moreover, ERRγ as well as ERRα and ERRβ increased the transcription of a *Vegfa* promoter-driven luciferase reporter in AD 293 cells (Figure S3). In addition, we confirmed that the protein levels of Vegfa and Fgf1 were increased in the quadriceps of the transgenic mice (Figure 3H), raising the specter that muscle ERRγ activates paracrine networks that are released into the microenvironment to promote neovascularization.

To directly test whether ERRγ triggers paracrine angiogenesis, we employed an SVEC4-10 (murine endothelial cells) tube formation assay. We reasoned that conditioned media from ERRγ-overexpressing muscle cells would contain the appropriate signals to induce tube formation in endothelial cells. Indeed, treatment of SVEC4-10 cells with conditioned media from ERRγ-overexpressing C2C12 myotubes stimulated tube formation in 7–8 hr (Figure 4A). To confirm that the conditioned media contains angiogenic signals, we examined the gene expression in cells and protein levels in the media (by ELISA) of a representative angiokine, Vegfa. We found that overexpression of ERRγ in C2C12 myotubes increases expression of *Vegfa*-121, -165, and -189 genes (Figures 4B–4D) and increases total Vegfa secretion (by 4-fold) in the media (Figure 4E). These results demonstrate that ERRγ can induce angiogenic factors such as myocellular Vegfa to increase angiogenesis in a paracrine fashion.

Physiological Effects of ERRγ-Remodeled Muscle

Aerobic exercise-induced remodeling of skeletal muscles depends on both an increase in oxidative capacity and new blood vessel formation, changes that are a critical part of the physiologic adaptation to training (Bloor, 2005; Egginton, 2009; Gavin et al., 2007; Gustafsson and Kraus, 2001; Jensen et al., 2004; Waters et al., 2004). Therefore, we investigated the potential of ERRγ to promote physiological remodeling. First, in metabolic cage oxymetric studies, we found that the transgenic mice exhibited an increase in oxygen consumption (during both the light and dark cycles) in concert with the observed increased oxidative metabolism and blood supply to skeletal muscles (Figure 5A). Second, the ERGGO mice have a lower respiratory exchange ratio (RER) compared to the WT mice, indicative of a tendency to preferentially oxidize fat over carbohydrate in the transgenic skeletal muscles (Figure 5B). The ambulatory activities of WT and transgenic mice were comparable and therefore unlikely to contribute to changes in oxymetric parameters (Figure S4A). These combined changes led us to explore whether ERRGO mice acquired enhanced running endurance. ERRγ transgenic mice were able to run longer and further compared to the WT littermates (Figure 5C). Finally, the ERRGO mice were subjected to a high-fat/high-carbohydrate diet to establish whether the induction of endurance muscle and oxidative RER affected global metabolic balance. As expected, ERRGO mice gained 35% less weight than WT controls on a high-fat diet (Figure S4B). These findings demonstrate that targeting of ERRγ increases oxidative metabolism and blood

(D–H) Expression of *Vegfa-121*, *Vegfa-165*, *Vegfa-189*, *Vegfb*, and *Fgf1* transcript levels in WT and transgenic quadriceps. Data are presented as mean ± SD from n = 6 samples.

(I) ERRγ increases VEGFa and FGF1 protein expression (n = 4). * represents significant difference between WT and transgenic mice ($p < 0.05$, unpaired Student's t test).

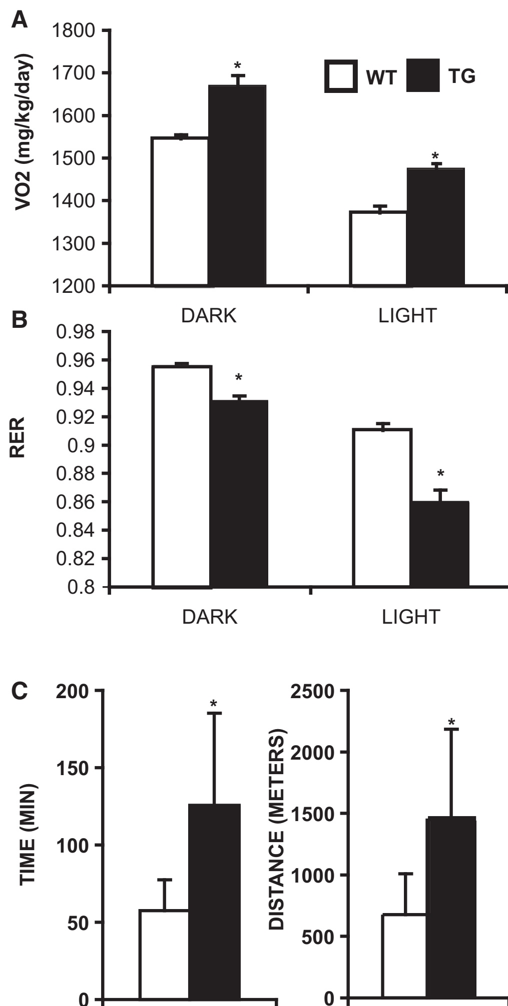

Figure 5. Physiological Effect of ERRγ Overexpression

(A and B) Average oxygen consumption (n = 6–7) (A) and average RER (n = 6–7) (B) during the light and the dark cycle over a period of 24 hr in WT and transgenic mice.

(C) Running endurance as a function of time and distance (n = 6). Data are presented as mean ± SEM in (A) and (B) and as mean ± SD in (C). * indicates statistically significant difference between the two groups ($p < 0.05$, unpaired Student's t test). See Figure S4.

supply to skeletal muscle, leading to increased oxygen consumption, better endurance, and resistance to weight gain.

PGC-1α-Independent Regulation of Aerobic Muscle by ERRγ

PGC-1α is induced by hypoxia and exercise to promote HIF1α-independent vascularization of type II muscle (Arany et al., 2008) and further activated by posttranslational modifications such as deacetylation (Jäger et al., 2007; Puigserver et al., 2001; Rodgers et al., 2005). Therefore, we asked whether the ERRγ-induced changes in the muscle were due to the induction and/or activation of PGC-1α. The levels of PGC-1α mRNA, protein, and acetylation remained unchanged in the ERRγ-transformed skeletal muscle (Figures 6A and S5A). Interestingly, of the two additional ERR isoforms that can mediate PGC-1α signaling, ERRβ, but not ERRα, was also significantly induced in transgenic muscle (Figure 6A) (Mootha et al., 2004; Schreiber et al., 2003; Huss et al., 2002).

How might ERRγ control metabolism, VEGF induction, and vasculature remodeling in ERRGO mice in absence of enhanced PGC-1α signaling? We focused on the alternative aerobic master-regulator—AMPK—because of its known role in metabolic (Fujii et al., 2007, 2008) and vascular adaptation (Zwetsloot et al., 2008). While AMPK is normally induced by exercise or hypoxia, surprisingly, we found it to be constitutively activated in ERRGO muscle (Figures 6B and 6C). The AMPK activation was further validated by measuring phospho-ACC levels (an AMPK target and a biomarker of AMPK activity), which we found to be higher in the transgenic compared to the WT muscles (Figure S5B). ATP consumption is critical to AMPK activation, as AMP stimulates and ATP inhibits the enzyme (Xiao et al., 2007). Indeed, we found that ATP levels were lower in ERRγ-overexpressing compared to control C2C12 muscle cells, providing a biochemical basis for the observed AMPK activation (Figure S5C). (Note that we use cultured muscle cells for measuring ATP levels because ERRγ overexpression promotes both angiogenic gene expression as well as oxidative respiration in a fashion similar to transgenic muscle.) Interestingly, in WT mice, we found that AMPK is more active in predominantly oxidative slow-twitch compared to predominantly glycolytic fast-twitch muscle in resting state (Figures 6B and 6C). Indeed, a synthetic activator, AICAR, at a dose (500 mg/kg/day) previously shown to stimulate AMPK in anaerobic muscle and improve aerobic performance (Narkar et al., 2008), was able to direct aspects of skeletal muscle transformation in a fashion similar to ERRγ (Figure 6D). These observations suggest a convergence between ERRγ and AMPK pathways that comprise an exercise-independent mechanism to direct intrinsic vascularization and oxidative metabolism in type I muscle, as depicted in Figure 6E.

DISCUSSION

Oxidative slow-twitch muscle beds are highly vascularized, pointing to an underlying regulatory network that integrates blood flow to myocellular metabolism. A transcriptional pathway specifying intrinsic differences between type I and II muscles has not previously been identified. Discovery of the components of this network has implications in treating cardiovascular diseases commonly linked to peripheral vascular degeneration due to ischemia. Here, we show that in the skeletal muscle, ERRγ is exclusively expressed in highly vascularized aerobic muscles. Transgenic overexpression of ERRγ is sufficient to enable anaerobic muscles to acquire enhanced oxidative capacity and dense vasculature. The observed morphological remodeling is linked to induction by ERRγ of genes controlling oxidative phosphorylation, fatty acid oxidation, and oxidative slow-twitch myofibers as well as a parallel induction of proangiogenic genes involved in paracrine regulation of vasculature. At a functional level, these genetic changes impart high oxygen-consuming and exercising capacity as well as resistance to diet-induced obesity to the ERRGO mice. Surprisingly, these effects are independent of PGC-1α and instead are associated with ERRγ-directed AMPK activation in the muscle. Therefore, ERRγ regulates blood supply

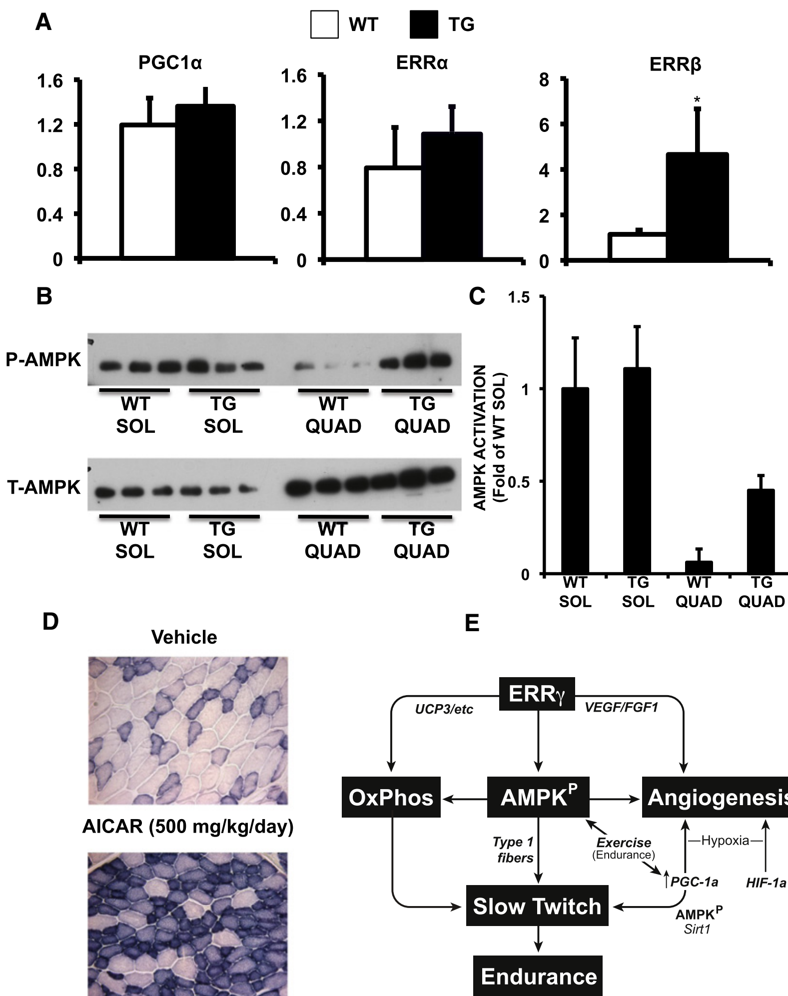

Figure 6. PGC-1α-Independent Regulation by ERRγ

(A) Relative expression of *PGC-1a*, *Erra*, and *Errb* genes in WT and transgenic muscle (n = 6). Data are presented as mean ± SD. * represents significant difference between WT and transgenic mice ($p < 0.05$, unpaired Student's t test).

(B) Phospho-AMPK (upper panel) and total-AMPK (lower panel) in soleus (SOL) and quadriceps (QUAD) of WT and transgenic mice (n = 3).

(C) Quantification of AMPK activation (phospho- to total-AMPK ratio) by densitometric analysis, presented as fold of WT soleus (n = 3). Data is presented as mean ± SD.

(D) Representative images of SDH staining of muscle cryosections from vehicle and AICAR (500 mg/kg/day for 4 weeks) treated mice. Similar results were obtained from n = 3 mice.

(E) Synchronization of metabolism and vasculature by ERRγ in aerobic muscle. See Figure S5.

to aerobic muscles and, perhaps, is a transcriptional gauge of myocellular supply and demand.

Although skeletal muscle adapts to exercise by increasing oxidative metabolism and vascular supply via induction of transcriptional regulators such as PGC-1α (Arany et al., 2008; Baar et al., 2002; Huss et al., 2002; Pilegaard et al., 2003; Russell et al., 2003, 2005), how type I fibers achieve intrinsic vascularization even in the absence of exercise is poorly understood. We show here that one such molecular pathway involves nuclear receptor ERRγ—highly expressed in oxidative slow-twitch muscles. Targeted expression of ERRγ to quadriceps and white gastrocnemius, where the receptor is typically not expressed, morphologically endows these muscles with dense vascular supply and numerous slow-twitch characteristics. Recently, it was reported that muscle-specific overexpression of a constitutively active ERRγ (VP16-ERRγ) imparts an oxidative metabolic phenotype to the skeletal muscle (Rangwala et al., 2010). However, the effect of VP16-ERRγ on muscle vascularization was not evaluated in these mice.

Genome-wide expression analysis revealed that ERRγ acts by coordinately inducing gene networks promoting mitochondrial biogenesis, oxidative transformation, and angiogenesis. The ERRγ program includes mobilization and oxidation of fat (e.g., *Acadl*, *Acadm*, *Cpt1b*, *Cpt2*, *Lpl*), electron transport (e.g., *Atp5h*, *Cox6a2*, *Ndufab1*, *Ndufb2m*, *Ndufv1*, *Sdhb*), mitochondrial biogenesis (e.g., *Mfn1*), and formation of energy-efficient, slow-contractile muscle (e.g., *Tnnc1*, *Tnni1*, *Tnnt1*). The observed changes constituting transformation of the contractile apparatus to a slow phenotype and increase in oxidative metabolic genes reflected in profound increase in mitochondrial (SDH) staining represents a fiber type switch. Notably, ERRγ also induces key transcriptional inducers of oxidative metabolism including *Esrrb*, *Ppara*, *Ppard*, and *Ppargc1b* (Table S4) (Lin et al., 2002; Minnich et al., 2001; Muoio et al., 2002; Wang et al., 2004). Therefore, it is likely that ERRγ is a critical upstream genetic switch that may determine metabolic fate by presiding over the expression of multiple aerobic regulators.

We hypothesize that the vascular program triggered by myocellular ERRγ activates a transcriptional program that directs secretion of paracrine signals into skeletal muscle microenvironment to induce angiogenesis. This model is strongly supported by our observation that conditioned media from ERRγ overexpressing C2C12 myotubules is able to induce endothelial cell tube formation in culture. Indeed, ERRγ transcriptionally induced all isoforms of angiokine *Vegfa* in C2C12 myotubes, resulting in increased Vegfa secretion into the media. Vegfa is a key regulator of angiogenesis critical for guiding endothelial cells to their targets (Grunewald et al., 2006; Springer et al., 1998). Furthermore, ERRγ stimulates the *Vegfa* promoter containing putative ERR binding sites that is known to transcribe all *Vegfa* isoforms (Arany et al., 2008). Vegfa mRNA and protein expression are also induced in ERRGO muscle. These findings collectively raise the possibility of direct transcriptional activation of angiogenic genes by ERRγ. However, it is important to note that the angiogenic effects of ERRγ cannot be solely attributed to Vegfa induction and secretion. For example, ERRγ additionally activates the expression of *Fgf1* and *Cxcl12*, known to regulate endothelial cell proliferation and migration (Forough et al., 2006; Gupta

et al., 1998; Partridge et al., 2000; Shao et al., 2008; Zheng et al., 2007), along with *Efnb2*, proposed to recruit mural cells that are required for vessel maturation (Foo et al., 2006). Additionally, upregulated factors such as *Notch4* as well as *Sox17* are transcriptional regulators of vasculogenesis (Hainaud et al., 2006; Leong et al., 2002; Matsui et al., 2006). In this aspect, ERRγ seems to serve a function similar to HIF1α, a known master regulator of angiogenesis during hypoxia (Pajusola et al., 2005). Interestingly, it was recently demonstrated that ERRs might physically interact with HIF1α in regulating its transcriptional activity (Ao et al., 2008). Whether such a mechanism is relevant to our model remains to be determined. Along these lines, HIF1α mRNA levels—a marker for chronic hypoxia—did not change in ERRGO compared to WT muscles (data not shown), indicating an absence of hypoxia or its involvement in the vascular effects of ERRγ (Hoppeler and Vogt, 2001a, 2001b). Furthermore, HIF1α is known to negatively regulate oxidative metabolism (Mason et al., 2004, 2007) and is therefore unlikely to contribute to ERRγ-mediated remodeling of skeletal muscles.

ERRGO mice exhibited increased oxygen consumption, decreased RER, high running endurance, and resistance to diet-induced weight gain. These changes are physiological hallmarks of increased aerobic capacity in mice and are a direct consequence of engineering highly oxidative and vascularized muscle by ERRγ. While similar remodeling of skeletal muscle and aerobic physiology are triggered by exercise, our data prove that generation of a fully functional "endurance vasculature" is not exercise dependent (Bloor, 2005; Egginton, 2009; Gavin et al., 2007; Gustafsson and Kraus, 2001; Jensen et al., 2004; Waters et al., 2004). Reciprocally, the extent to which ERRγ signaling in skeletal muscle contributes to exercise adaptation remains to be determined.

A surprising finding of our study was lack of change in the expression of PGC-1α, a known and inducible regulator of aerobic muscles, in the ERRγ-transformed muscle. One alternative possibility is posttranslational activation of PGC-1α without change in its expression (Jäger et al., 2007; Puigserver et al., 2001; Rodgers et al., 2005). Deacetylation of PGC-1α is critical for its activation in the skeletal muscle (Cantó et al., 2010; Gerhart-Hines et al., 2007; Lagouge et al., 2006). However, ERRγ overexpression did not lead to deacetylation of PGC-1α, which remained comparably acetylated in both the WT and ERRGO muscles. The lack of posttranslational activation of the cofactor in ERRGO mice is further underscored by a previous report that nongenomic activation of PGC-1α typically leads to its transcriptional induction, which we did not observe in these studies (Jäger et al., 2007). Along the same lines, it was recently shown that both PGC-1α and β are dispensable for fiber type specification in the skeletal muscle (Zechner et al., 2010). In contrast, we find that an alternative aerobic master regulator, AMPK, is activated by ERRγ in the skeletal muscles. AMPK is typically activated by exercise (Fujii et al., 2000; Winder and Hardie, 1996; Wojtaszewski et al., 2000) and is essential for exercise-mediated switch to aerobic myofibers in the skeletal muscle (Röckl et al., 2007). Indeed, transgenic activation of AMPK in the skeletal muscle increases the proportions of oxidative myofibers in absence of any exercise (Röckl et al., 2007). Similarly, we found that chemical activation of AMPK by AICAR triggers aerobic transformation of type II muscle. However, AMPK alone is unlikely to mediate all the ERRγ effects, and contribution by additional metabolic regulators (e.g., calcineurin, SIRT1, etc.) in ERRGO mice cannot be ruled out. This is possible because, unlike ERRγ, AMPK activation apparently does not lead to a complete transformation to a type I phenotype, but to more intermediate type IIa and IIx oxidative myofibers (Röckl et al., 2007). In this context, it is peculiar that we found AMPK to be naturally and selectively active in soleus (predominantly type I myofibers) compared to quadriceps (predominantly type II myofibers). Previous studies have suggested AMPK activity to be similar between soleus and EDL (also predominantly made up of type II myofibers) (Dzamko et al., 2008; Jensen et al., 2007; Jørgensen et al., 2004). Speculatively, this discrepancy may have technical attributes or may even be linked to possible differences in recruitment of EDL and quadriceps for postural activity that might affect basal AMPK activation. Nevertheless, our results demonstrate that in the context of overexpression, ERRγ is sufficient to initiate both metabolic and vascular pathways to drive aerobic remodeling of sedentary muscle independently of PGC-1α by recruiting alternative regulators such as AMPK (see Figure 6E).

Multiple diseases, including obesity and diabetes, are commonly linked to deregulation of both oxidative metabolism and vascularity. A shared therapeutic approach to these conditions includes exercise that activates a plethora of transcriptional pathways to increase aerobic metabolism and vascularization to ultimately enhance performance (Bloor, 2005; Egginton, 2009; Gavin et al., 2007; Gustafsson and Kraus, 2001; Jensen et al., 2004; Waters et al., 2004). Our findings present a possibility of therapeutically exploiting ERRγ to simultaneously regulate oxidative capacity and vascularity. High expression levels of this receptor in tissues most prone to metabolic and vascular diseases (e.g., heart, skeletal muscle, brain, and kidney) further potentiates its value as a potential pharmacologic target (Ariazi et al., 2002; Cheung et al., 2005; Gao et al., 2006; Giguère, 2008; Heard et al., 2000; Hong et al., 1999). In summary, our studies show that ERRγ controls mitochondrial function and metabolism together with angiogenesis that anatomically synchronizes vascular arborization to oxidative metabolism.

EXPERIMENTAL PROCEDURES

Animals

Mouse ERRγ cDNA was placed downstream of the human α-skeletal actin promoter and upstream of the SV40 intron/poly (A) sequence. The purified transgene was injected into C57BL/6J × CBA F1 zygotes. Two transgenic founders (TG 425 and 421) were obtained that were backcrossed for five generations with C57BL/6J. All experiments used age-matched (2–3 months) and sex-matched (male) transgenic and WT littermates. Mice were maintained on a normal chow diet. ERRγ +/− mice and tissue β-galactosidase staining have been described previously (Alaynick et al., 2007).

Drug Treatment

Male C57BL/6J mice (8 weeks old) were intraperitoneally injected with vehicle or AICAR (500 mg/kg/day), as previously described (Narkar et al., 2008).

Gene and Protein Expression Analysis

RNA was extracted using TRIzol extraction method from quadriceps or soleus isolated from WT and transgenic mice. Additionally, protein lysates were prepared from quadriceps and analyzed by western blotting with myoglobin (Dako), CYCS (Santa Cruz), UCP3 (Affinity Bioreagents), phospho-AMPKα

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(Cell Signaling, cat. no. #2535), and total-AMPKα (Cell Signaling, cat. no. #2532) antibodies. Note that the AMPK antibodies detect both the α1 and 2 catalytic subunits of AMPK (Narkar et al., 2008).

Microarray Analysis

Global gene expression analysis was performed in quadriceps from WT and transgenic mice, as previously described (Narkar et al., 2008).

Muscle Staining and Immunohistochemistry

SDH, PECAM/CD31, and alkaline phosphates staining are described in the Supplemental Experimental Procedures.

Fluorescence Microangiography

Blood vessel mapping was performed as previously described (Johnson et al., 2004; Springer et al., 2000). Briefly, a red fluorescent microsphere (0.1 μM) suspension was intraventricularly perfused (10 ml, 1 ml/min), followed by euthanasia and tissue collection. Longitudinal cryosections (10 μM) of frozen gastrocnemius were processed and subjected to confocal microscopy to image skeletal muscle vasculature.

Cell Culture, In Vitro Angiogenesis, and Vegfa ELISA

See Supplemental Experimental Procedures.

Oxymetery and Treadmill Assays

Oxygen consumption, RER, and ambulatory activity were measured in 3-month-old WT and transgenic male mice (n = 6–7/group) of comparable weight using Comprehensive Lab Animal Monitoring System to obtain oxymetric measurements (Columbus Instruments). These mice were first acclimated in the monitoring system for 1 day, followed by data collection for 24 hr to include a 12 hr light and dark cycle. For each animal, the average of all the data points within the light or dark phase was used as a representative value of the respective cycle. Diurnal differences between the light and dark cycles were detectable in all animals, validating the method of data collection.

Endurance was determined in WT and transgenic mice (n = 6/group), as previously described (Narkar et al., 2008). Treadmill protocol is described in Supplemental Experimental Procedures.

Data Analysis

Data was analyzed using either one way ANOVA with an appropriate post hoc test or unpaired Student's t test, as indicated.

ACCESSION NUMBERS

The global gene expression data has been deposited in the NCBI Gene Expression Omnibus under the GEO series accession number GSE22086.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at doi:10.1016/j.cmet.2011.01.019.

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