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Real-time imaging of peroxisome proliferator-activated receptor-γ coactivator-1α promoter activity in skeletal muscles of living mice

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ADULT MAMMALIAN SKELETAL MUSCLES are heterogeneously specialized as a result of embryonic and postnatal development, varying in contractile properties, metabolic capacities, ultrastructures, and susceptibility to fatigue (20, 24). However, they remain plastic such that alterations in contractile load, hormonal shifts, or systemic diseases induce stable, long-term adaptation (4, 29), as has been demonstrated by endurance exercise. Orchestrated signal transduction from neuromuscular activity to gene regulation machinery ensures efficient and timely phenotypic changes to meet the altered functional demand.

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a transcriptional coactivator cloned from a differentiated brown fat cell line (22), is considered to be a key regulator in mediating the genetic reprogramming during skeletal muscle adaptation. PGC-1α mRNA and protein are highly expressed in slow, oxidative fibers compared with fast, glycolytic fibers (11, 32). By interacting with nuclear regulatory proteins, PGC-1α stimulates target gene expression during various biological processes (23). The most striking property of PGC-1α in skeletal muscle is that its overexpression in cultured myoblasts induces mitochondrial biogenesis and stimulates oxidative respiration (34), and its overexpression in skeletal muscle in transgenic mice results in enhanced mitochondrial biogenesis and remarkable fast-to-slow fiber type switching (11). Consistent with the notion that increased expression of PGC-1α plays a key role in skeletal muscle adaptation, it has been reported that endurance exercise induces PGC-1α mRNA and protein expression in the recruited skeletal muscles (1, 8, 21, 26), which prompted us to search for a novel innovative experimental approach to study PGC-1α gene regulation in vivo.

The profound effects of increased PGC-1α protein expression are probably the result of its ability to enhance activities and expression of transcription factors, such as nuclear respiratory factor 1 (NRF-1), myocyte enhancer factor 2 (MEF2), and host cell factor (HCF) (15). More importantly, PGC-1α positively regulates its own expression, playing a positive-feedback function (9). Therefore, it is critical to elucidate the signal transduction pathway and the molecular mechanism for contractile activity-induced PGC-1α transcription in skeletal muscle. Previous studies in cultured myocytes have linked multiple signals, such as Ca2+ and nitric oxide, to PGC-1α gene regulation (17, 18) and have confirmed that both the MEF2 and cAMP response element (CRE) consensus sequences play an important regulatory role in PGC-1α gene transcription (9). However, the sequence elements required for PGC-1α gene regulation in skeletal muscle in vivo have not been investigated because of the lack of a suitable experimental model.

Mammalian striated muscles possess unique architectural features such that direct injection of naked plasmid DNA (direct plasmid injection) can result in a sustained transgene expression (30). It was this finding more than a decade ago that triggered substantial interest in studying promoter regulation in skeletal muscle in vivo (2, 5, 25, 31, 35, 38). However, because of the variability in transgene expression and the limitation of single-sample collection from each injection, the use of this technique to study skeletal muscle gene regulation has been primarily limited to comparing promoter activities of contractile proteins and metabolic enzymes between slow- and fast-
twitch muscle fibers. We began to establish a unique combinatorial approach to study promoter regulation in vivo owing to two recent technological developments. The first development was electrical pulse-mediated gene transfer in skeletal muscle, which has been shown to increase the efficiency of gene transfer by roughly 100-fold over the aforementioned direct plasmid injection (16). The second development was optical bioluminescence imaging, allowing for the detection of in vivo luciferase activity from the same animal repeatedly (13, 33). In this study, we have combined these two techniques and used the combinatorial approach with site-directed mutagenesis to determine the role of the MEF2 and CRE sequence elements in the PGC-1α promoter in vivo. Our findings address the essential role of these sites in the PGC-1α promoter in mediating increased transcription following nerve stimulation and prove the feasibility of using this relatively simple approach in studying transcriptional regulation in vivo.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (8 wk old) were obtained commercially (Jackson Laboratory) and housed in temperature-controlled quarters (21°C) with a 12:12-h light-dark cycle. Mice were provided with water and chow (Purina) ad libitum. Experimental protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Plasmid DNA constructs. RSVL was constructed by subcloning the firefly luciferase reporter coding sequence downstream of the Rous sarcoma virus (RSV) promoter enhancer. pG3L-Basic is a construct carrying the firefly luciferase coding sequence without a promoter and was used as a negative control (Promega). PGC-1αL, a construct containing a 3.1-kb 5′-flanking region of the mouse PGC-1α gene upstream of the firefly luciferase coding sequence, and PGC-1αL(ΔMEF2), a mutant construct with mutations of two consensus MEF2 binding sites (−2901 and −1539) in the 3.1-kb PGC-1α promoter, were kindly provided by E. N. Olson (7). PGC-1αLΔCRE was generated by site-directed mutagenesis from PGC-1αL as described previously (9). Briefly, a forward primer, 5′-AGGGCTTCCTGTGAGTGGTTGAGTGCAAGCCACGAG-3′, and a reverse primer, 5′-TGCTGCACAAATCTGCTTAGCTACTCAAGCCAGCGCCT-3′, (mutated nucleotides are underlined) were used in a PCR-based site-directed mutagenesis using the QuickExchange sited-directed mutagenesis kit (Stratagene). All plasmid DNAs were transformed and amplified in DH-5α bacteria, purified using the plasmid maxi kit (Qiagen), and dissolved at a concentration of 0.5 mg/ml in 0.9% NaCl solution.

Electric pulse-mediated gene transfer. Electric pulse-mediated gene transfer was performed as described previously (16). Briefly, under anesthesia (50 mg/kg ip pentobarbital sodium), the hindlimbs were shaved with an electric hair clipper and treated by application of iodine twice followed by 70% ethanol twice. An incision of ~1 cm was made on the left lateral side of the thigh to expose the deep peroneal nerve. Two stainless steel electrodes were secured under the deep peroneal nerve with two sutures for each electrode. To accommodate the change in the size of the animal, the two electrodes were kept at a distance of ~3 mm apart. The contralateral TA was sham operated. Motor nerve stimulation was initiated within 30 min after the surgery and lasted for 2 h, during which the mouse was kept under anesthesia. The stimulation parameters were 0.25 ms in duration and 10 Hz in frequency, as previously described (37), but the amplitude was adjusted between 1 and 3 V to maintain maximal contractions and minimize possible damage.

Optical bioluminescence imaging. In vivo bioluminescence images were acquired under anesthesia (50 mg/kg ip pentobarbital sodium) with the IVIS system (Xenogen). Repeated measurements were made for every mouse immediately before (control) and after the 2-h motor nerve stimulation at different time points (0, 1, 2, 3, and 4) h while the mice were kept under anesthesia by additional intraperitoneal injection of pentobarbital sodium at 25 mg/kg if needed. All mice were allowed to recover from anesthesia between 4 and 24 h after nerve stimulation. Mice were anesthetized again at 24 h after motor nerve stimulation to obtain data. The imaging system consists of a cooled (−105°C) charge-coupled device camera mounted on a light-tight imaging box and a computer for image acquisition and analysis. After injection of luciferin (~140 mg/kg ip), mice were placed on an imaging platform in a supine position. Images were acquired with a 15-cm field of view, a binning factor of 10, and an exposure time of 2 min. The Xenogen LivingImage (version 2.11) software captured and displayed the bioluminescence signals as a pseudocolor overlay on a photographic image of the animals for subsequent analysis.

The images were analyzed similarly to the manner described previously (3). A region of interest (ROI) was manually placed over the region to be analyzed, with the size of the ROI held constant across all images of a given hindlimb. The total flux in photons·s−1·cm−2·sr within the ROI was measured (where sr is steradian). To determine the effects of nerve stimulation, the ratio of the total flux from the stimulated muscle to that from the contralateral control muscle was determined for each time point.

Semiquantitative RT-PCR. Semiquantitative RT-PCR analysis was performed as described previously (36) to measure endogenous PGC-1α mRNA expression in TA muscle in response to increased contractile activity. PGC-1α mRNA data were normalized by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for each sample and expressed as relative change compared with the contralateral control muscle. The PCR primers used are as follows: GAPDH forward primer, 5′-GGGACGAGGTGCAAGGAGAT-3′; GAPDH reverse primer, 5′-GTTGTCAGGGTGTATAAGAAA-3′; PGC-1α forward primer, 5′-AAACCTTGACTAGCTGCT-3′; and PGC-1α reverse primer, 5′-TCTTGTGGTTGTGCTGGCA-3′. Semiquantitative RT-PCR analysis was performed as described previously (36) to measure endogenous PGC-1α mRNA expression in TA muscle in response to increased contractile activity. PGC-1α mRNA data were normalized by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for each sample and expressed as relative change compared with the contralateral control muscle. The PCR primers used are as follows: GAPDH forward primer, 5′-GGGACGAGGTGCAAGGAGAT-3′; GAPDH reverse primer, 5′-GTTGTCAGGGTGTATAAGAAA-3′; PGC-1α forward primer, 5′-AAACCTTGACTAGCTGCT-3′; and PGC-1α reverse primer, 5′-TCTTGTGGTTGTGCTGGCA-3′. Semiquantitative RT-PCR analysis was performed as described previously (36) to measure endogenous PGC-1α mRNA expression in TA muscle in response to increased contractile activity. PGC-1α mRNA data were normalized by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for each sample and expressed as relative change compared with the contralateral control muscle. The PCR primers used are as follows: GAPDH forward primer, 5′-GGGACGAGGTGCAAGGAGAT-3′; GAPDH reverse primer, 5′-GTTGTCAGGGTGTATAAGAAA-3′; PGC-1α forward primer, 5′-AAACCTTGACTAGCTGCT-3′; and PGC-1α reverse primer, 5′-TCTTGTGGTTGTGCTGGCA-3′. Semiquantitative RT-PCR analysis was performed as described previously (36) to measure endogenous PGC-1α mRNA expression in TA muscle in response to increased contractile activity. PGC-1α mRNA data were normalized by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for each sample and expressed as relative change compared with the contralateral control muscle. The PCR primers used are as follows: GAPDH forward primer, 5′-GGGACGAGGTGCAAGGAGAT-3′; GAPDH reverse primer, 5′-GTTGTCAGGGTGTATAAGAAA-3′; PGC-1α forward primer, 5′-AAACCTTGACTAGCTGCT-3′; and PGC-1α reverse primer, 5′-TCTTGTGGTTGTGCTGGCA-3′. To determine the effects of nerve stimulation, the ratio of the total flux from the stimulated muscle to that from the contralateral control muscle was determined for each time point.

Electrical pulse-mediated gene transfer. Electric pulse-mediated gene transfer was performed as described previously (16). Briefly, under anesthesia (50 mg/kg ip pentobarbital sodium), both tibialis anterior (TA) muscles were injected with 15 μg of plasmid DNA (30 μl) by using a 0.5-ml insulin syringe with a 28-gauge needle at a rate <0.015 ml/min. Within 1 min after injection, electrical field was delivered to the injected TA muscle by a S88K square-pulse stimulator (Grass Telefactor) through a model 533 two-needle array (BTX). Eight pulses at 100 ms in duration, 1 Hz in frequency, and 100 V in amplitude (200 V/cm) were applied to the muscle by placing the needle arrays on the medial and lateral sides of the TA muscle so that the electrical field was perpendicular to the long axis of the myofibers. The mice were allowed to recover for 4 days before electrode implantation, nerve stimulation, and in vivo luciferase assay. The recovery period of 4 days was previously determined to be sufficient for transgene expression (16). To minimize the impact of muscle regeneration as reported recently (2), the contralateral TA muscle was treated identically and used as a control.

Motor nerve stimulation. Electrod implantation was performed as described previously in rats (37). Briefly, under anesthesia (50 mg/kg ip pentobarbital sodium), the hindlimbs were shaved with an electric hair clipper and treated by application of iodine twice followed by 70% ethanol twice. An incision of ~1 cm was made on the left lateral side of the thigh to expose the deep peroneal nerve. Two stainless steel electrodes were secured under the deep peroneal nerve with two sutures for each electrode. To accommodate the change in the size of the animal, the two electrodes were kept at a distance of ~3 mm apart. The contralateral TA was sham operated. Motor nerve stimulation was initiated within 30 min after the surgery and lasted for 2 h, during which the mouse was kept under anesthesia. The stimulation parameters were 0.25 ms in duration and 10 Hz in frequency, as previously described (37), but the amplitude was adjusted between 1 and 3 V to maintain maximal contractions and minimize possible damage.

Statistics. Differences between relative PGC-1α mRNA level in the stimulated and the contralateral control muscles were analyzed by paired t-test. Luciferase activity in the stimulated TA muscle was normalized by that in the contralateral control, and the differences among different time points were analyzed by ANOVA followed by Dunnett's test to locate the differences. P < 0.05 was accepted as statistically significant.
RESULTS

Contractile activity induces PGC-1α mRNA expression in mouse TA muscles. To determine whether increased contractile activity results in an upregulation of PGC-1α mRNA in mouse skeletal muscle, we stimulated the peroneal nerve at 10 Hz continuously. A 2-h stimulation followed by a 1-h recovery resulted in a 1.6-fold increase \((P < 0.05)\) in PGC-1α mRNA in the stimulated TA muscle compared with the contralateral control (Fig. 1, A and B). Preliminary results showed that increased PGC-1α mRNA expression was transient (not shown), as was observed in mouse plantaris muscle in response to a single bout of voluntary running (Pohnert SC, Akimoto T, Zhang M, Gumbs C, Rosenberg PB, Williams RS, and Yan Z, unpublished observations).

To determine whether short-term (2 h) nerve stimulation induces increased expression of PGC-1α protein in TA muscle, we performed Western immunoblot analysis for whole muscle homogenates. No significant increase in PGC-1α protein expression was detected between 0 and 6 h after the nerve stimulation (Fig. 1, C and D). Preliminary data showed no detectable changes in PGC-1α protein abundance up to 24 h after stimulation (not shown).

PGC-1α-luciferase reporter gene expression can be detected in vivo after gene transfer. To determine whether electric pulse-mediated gene transfer results in PGC-1α reporter gene expression at a level above the background in skeletal muscle, we injected plasmid DNA constructs (Fig. 2A) into mouse TA muscles, followed by electrotransfer. Luciferase activities in vivo were measured 4 days after the gene transfer. As shown in Fig. 2, B and C, TA muscles injected with the constitutively active RSVL and the promoterless pGL3-Basic constructs had luciferase activities at 9.9 ± 4.5 \(\times\) 10\(^6\) and 6.5 ± 0.3 \(\times\) 10\(^3\) photons \(-\text{s}^{-1}\) \(-\text{cm}^{-2}\) \(-\text{sr}\), respectively. PGC-1α-L and its mutant forms, PGC-1α-L(ΔCRE) and PGC-1α-L(ΔMEF2), had luciferase activities at 7.2 ± 1.9 \(\times\) 10\(^7\), 2.0 ± 1.0 \(\times\) 10\(^5\), and 8.6 ± 5.1 \(\times\) 10\(^5\) photons \(-\text{s}^{-1}\) \(-\text{cm}^{-2}\) \(-\text{sr}\), respectively.

Motor nerve stimulation results in increased PGC-1α reporter gene expression. To determine whether the proximal 3.1-kb mouse PGC-1α promoter (7) contains sufficient sequence elements for contractile activity-dependent responsiveness, we transferred PGC-1α-L into TA muscles and stimulated the muscle via the peroneal nerve at 10 Hz. Continuous nerve stimulation for 2 h resulted in a threefold increase in luciferase activity that lasted for at least 3 h, followed by a decline to the basal level at 24 h after stimulation (Fig. 3, A and B). RSVL, as a negative control, was not altered by motor nerve stimulation.

Mutation of the CRE site or both MEF2 sites abolishes contractile activity-induced PGC-1α promoter activation. To determine the importance of MEF2 and CRE sites in the PGC-1α promoter for the responsiveness to muscle contraction, we generated PGC-1α-L(ΔCRE) and PGC-1α-L(ΔMEF2), in which the CRE site (−222) or both MEF2 sites (−2901 and −1539) were mutated, respectively. As shown in Fig. 3, A and B, mutations of the CRE site or both MEF2 sites completely abolished contractile activity-induced activation of PGC-1α promoter activity.
DISCUSSION

In this study, we combined electric pulse-mediated gene transfer and optical bioluminescence imaging to study promoter function in vivo in mouse skeletal muscle under the...
influence of increased contractile activity. The main findings of
the present study are that contractile activity stimulates the
PGC-1α promoter and that the increased promoter activity is
dependent on the presence of the CRE (−222) and the MEF2
sites (−2901 and −1539) in the PGC-1α promoter. These
findings provide direct in vivo evidence that the CRE and the
MEF2 binding sites are required for contractile activity-in-
duced PGC-1α mRNA expression in skeletal muscle.

Previous studies have established that contractile activity
enhances PGC-1α mRNA expression in rats and humans (1, 8,
21, 26). It was not known whether a similar induction of
PGC-1α mRNA occurs in mice. Our results show a transiently
increased PGC-1α mRNA expression in the stimulated TA
muscle in mice (Fig. 1, A and B). Similar transient induction of
PGC-1α mRNA in plantaris muscle in response to voluntary
running also has been observed in mice (Pohnert et al., unpub-
lished observations). Thus a single bout of contractile activity
is sufficient to induce a transient increase in PGC-1α mRNA
expression in mouse skeletal muscle.

It is worth noting that the observed increase of PGC-1α
mRNA (1.6 fold) was less than that of the reporter gene
activity (3–4 fold), although both increases have a transient
pattern. The differences could attributable to different regulat-
ory mechanisms between the endogenous PGC-1α promoter
on the genome and the reporter gene in the episomal plasmid
DNA. It is also possible that endogenous PGC-1α mRNA is
subject to a posttranscriptional regulation, such as mRNA
degradation, whereas the luciferase mRNA is not. Finally, the
two readouts were measured with different assays, which may
result in the final differences in quantitative results. In our
experimental setting, luciferase activity in total muscle lysate
presumably resembles more closely the transcriptional activity
of the PGC-1α luciferase reporter gene.

A recent study (27) reported that PGC-1α protein in skeletal
muscle nuclear fraction increases transiently following two
separate sessions of endurance exercise in rats. Short-term
nerve stimulation (2 h) in mouse TA muscle in the present
study did not result in increased PGC-1α protein expression
(Fig. 1, C and D), despite the increase in PGC-1α mRNA,
whereas long-term voluntary running (4 wk) resulted in a
twofold increase in PGC-1α protein in plantaris muscles.
Likewise, we did not observe a significant increase in PGC-1α
protein expression in plantaris muscles following a single bout
of voluntary running (12 h) (not shown). Long-term nerve
stimulation and voluntary running both have been shown
previously to induce significant increases in mitochondrial
biogenesis and measurable fiber type switching in mice.

There are at least two possible reasons for the apparent
discrepancy between the findings in this study that there was no
detectable increase in PGC-1α protein following a single bout
of motor nerve stimulation or voluntary running and the pre-
vious findings in rats that endurance exercise induced a tran-
sient increase in PGC-1α protein in the skeletal muscle nuclear
extacts. First, the previously reported increase in PGC-1α
protein was induced by two separate sessions of exercise;
additional sessions of exercise may elicit other regulatory
events leading to increased PGC-1α protein expression. Re-
peted bouts of contractile activities are likely needed to induce
detectable increases in PGC-1α protein in skeletal muscle.
Second, technical differences are also worth considering. We
measured PGC-1α protein in whole muscle homogenate,
whereas investigators in the previous study employed nuclear
isolation. However, the negative finding in PGC-1α protein
expression in the muscle homogenate does not negate the
possible functional role of PGC-1α in exercise-induced skele-
tal muscle adaptation. A subtle increase in PGC-1α protein in
the nucleus, which may not be detectable, may still play an
important functional role in mediating genetic reprogramming
during skeletal muscle adaptation.

Our finding that expression of PGC-1α luciferase reporter
gene in skeletal muscle after gene transfer could be reproduc-
ibly detected by optical bioluminescence imaging is technically
important because it made possible the study of PGC-1α
promoter regulation in vivo. It set the stage for further analysis
for the PGC-1α promoter activity and possibly other genes of
interest in response to increased contractile activity in vivo.
The transient induction pattern of the reporter gene activity is
similar to that of PGC-1α gene transcription in human subjects
after a 3-h knee extensor exercise (21), suggesting that the
luciferase activity is reflecting the endogenous PGC-1α tran-
scription. RSVL, under the control of the constitutively active
RSV promoter, had luciferase activity ~10-fold higher than
that of the PGC-1α luciferase reporter gene but did not respond
to the motor nerve stimulation. This finding indicates that
muscle contractile activity does not have general effects on the
luciferase reporter gene. Collectively, these findings suggest
that the sequence elements responsible for exercise-induced
increase of PGC-1α gene transcription reside in the 3.1-kb
mouse PGC-1α promoter.

The transient induction of the PGC-1α reporter gene in this
study is consistent with the findings in a recent study (21), in
which the human PGC-1α gene expression peaked at 2 h after
one-leg extensor exercise in sedentary human subjects. The
increase in PGC-1α transcription in those human subjects is
more profound (~10 fold) than the increase in PGC-1α lucif-
erase reporter gene activity reported in this study (~3 fold).
The difference could be attributable to the differences between
mice and humans, between nerve stimulation and one-leg
extensor exercise, and/or between luciferase reporter gene and
nuclear run-on analyses. However, the overall responsiveness
of the PGC-1α gene to increased contractile activity appears to
be preserved among different species.

An intriguing and important question is how increased
contractile activity transduces the signals to activate PGC-1α
transcription. Extensive research in cardiovascular develop-
ment has led to the current understanding of the PGC-1α gene
regulation in cardiac myocytes (12, 14). Briefly, phosphoryla-
tion of HDAC5 promotes HDAC5 nuclear export, leading to
derepression of MEF2 activity (14) and elevated PGC-1α gene
expression. This mechanism plays an essential role in mainte-
nance of mitochondrial biogenesis in the heart (7). However,
it is not known whether the same regulatory mechanism accounts
for the exercise-induced adaptation in skeletal muscle. A recent
study in cultured skeletal muscle cells (9) has suggested the
importance of the proximal MEF2 binding site (~1539) for
PGC-1α positive autoregulation. Consistently, mutation anal-
ysis in this study indicates that the MEF2 binding sites are
indispensable for contractile activity-induced PGC-1α gene
expression in vivo. Future research could focus on the role of
HDAC-MEF2 interaction and the upstream signals leading to
the upregulation of the PGC-1α gene in skeletal muscle.
Mouse PGC-1α promoter contains a CRE between the proximal MEF2 binding site and the transcription start site. It has been shown that this sequence element is essential in mediating PGC-1α gene upregulation induced by calcineurin and CaMK IV activities (9). We have recently obtained evidence that activation of the p38 MAPK pathway participates in contractile activity-induced PGC-1α gene expression in skeletal muscle by using downstream effectors that include ATF2 (Pohnert et al. unpublished observations). It is possible that increased abundance and/or activity of CRE-binding transcription factors, such as ATF2, c-Jun, and CREB (CRE binding protein), stimulate PGC-1α transcription through the CRE site. Mutation of the CRE site abolished contractile activity-induced PGC-1α reporter gene expression, supporting the role of CRE in the regulation of the PGC-1α promoter. Further analysis in vivo should test the roles of the cognate transcription factors recruited to the CRE site in PGC-1α gene regulation.

Although this study proves the feasibility of using this experimental approach to study gene regulation in vivo in skeletal muscle, it does not negate the importance of conventional transgenic techniques for reporter gene study. For example, the transgene is expressed from an episomal plasmid DNA in this technique, which may not completely recapitulate the expression of the endogenous gene. Nevertheless, the new approach is a useful strategy for in vivo analysis of gene regulation with certain advantages over conventional transgenesis.

The transgenic approach used in this study helped us to better understand the molecular mechanisms for exercise training-induced skeletal adaptation. Recent findings suggest that certain effects of exercise can be mimicked in cultured myocytes (18, 19). However, exercise-induced skeletal muscle adaptation may not be faithfully replicated in tissue culture because of a lack of the unique architectural arrangement and integrated systemic influences in intact skeletal muscles. Our model can be used to study gene regulation in skeletal muscle in living animals under physiological conditions, providing a relatively simple alternative approach to the conventional transgenic technology. There are at least two technical advantages associated with this approach. First, this method allows for repeated measurements before and after a treatment, which minimizes the variability between individual animals and permits the use of paired statistical tests. Enhanced statistical power could minimize the number of animals needed for a given study. Second, many time points can be studied at essentially no additional cost. This option increases the likelihood that a true time course can be defined, which makes it less likely that an important regulatory event would be missed.

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