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# Phosphatase orphan 1 inhibits myoblast proliferation and promotes myogenic differentiation

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## Abstract

Myogenesis includes sequential stages of progenitor cell proliferation, myogenic commitment and differentiation, myocyte fusion, and myotube maturation. Different stages of myogenesis are orchestrated and regulated by myogenic regulatory factors and various downstream cellular signaling. Here we identify phosphatase orphan 1 (Phospho1) as a new player in myogenesis. During activation, proliferation, and differentiation of quiescent satellite cells, the expression of Phospho1 gradually increases. Overexpression of *Phospho1* inhibits myoblast proliferation but promotes their differentiation and fusion. Conversely, knockdown of *Phospho1* accelerates myoblast proliferation but impairs myotube formation. Moreover, knockdown of *Phospho1* decreases the OXPHO protein levels and mitochondria density, whereas overexpression of *Phospho1* upregulates OXPHO protein levels and promotes mitochondrial oxygen consumption. Finally, we show that Phospho1 expression is controlled by myogenin, which binds to the promoter of *Phospho1* to regulate its transcription. These results indicate a key role of Phospho1 in regulating myogenic differentiation and mitochondrial function.

### Keywords

mitochondria; MyoG; myogenesis; Phospho1

## 1 | INTRODUCTION

The generation of skeletal muscle during embryonic development and regeneration processes relies on the myogenesis of muscle progenitor cells.[1] Myogenesis is a highly orchestrated process, comprised of several distinct cell stages, including the expansion of

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AUTHOR CONTRIBUTIONS

Y. Peng, F. Yue, G. Yang, and S. Kuang conceived the project and designed the experiments. Y. Peng, F. Yue, J. Chen, K. Huang, and W. Xia performed the experiments and analyzed the data. Y. Peng, F. Yue, and S. Kuang prepared the manuscript.

myogenic progenitors, commitment of progenitors to myoblasts, differentiation of myoblasts into myocytes, and fusion of myocytes into multinucleate myofibers.[2,3] These processes are tightly and sophisticatedly controlled by the sequential expression of a series of myogenic regulator factors (MRFs), including Myf5, MyoD, MyoG, and MRF4.[4,5] The expression of Myf5 and MyoD is responsible for the commitment of myogenic progenitors to myoblasts.[6–8] MyoD and MyoG together initiate the onset of myogenic differentiation, while MyoG alone drives the terminal differentiation of myoblasts and the fusion of myocytes to form myofibers.[9,10] MRF4 plays a complicated role during myogenesis as it determines skeletal muscle identity in Myf5:Myod double-mutant mice and also negatively regulates adult skeletal muscle growth by repressing MEF2 activity.[11–13] Beyond the MRFs, a number of cellular signaling has been demonstrated to play important roles in regulating myogenesis during development and muscle regeneration, which includes but is not limited to Notch, Wnt, LKB1/AMPK, PI3K/PTEN, as well as LncRNAs.[2,14–19]

Phosphatase, orphan 1 (Phospho1), a soluble cytoplasmic phosphatase, contains three peptide motifs that are conserved within the haloacid dehalogenase (HAD) superfamily of magnesium-dependent enzymes.[20] Phospho1 exhibits high specific activities toward phosphoethanolamine (PE) and phosphocholine (PC), thus is related to glycerophospholipid biosynthesis.[21] Phospho1 was first identified in chicken and has been implicated in the generation of inorganic phosphate (Pi) for matrix mineralization.[22] Inhibition of Phospho1 by Lansoprazole in developing chicken embryos diminished the mineralization of leg and wing bones.[23] Consistently, *Phospho1* knockout mouse has spontaneous fractures, bowed long bones, osteomalacia, and scoliosis in postnatal stages.[24,25] Besides the function in bone, Phospho1 is also expressed in a wide range of tissues such as brain, colon, heart, joints, muscle, mammary gland as well as breast cancer cells, suggesting a general role for Phospho1 in cellular phosphate metabolism.[20] Recent study showed that methylation of the *Phospho1* locus in blood DNA was associated with a decreased risk for type 2 diabetes, and the methylation level was decreased in skeletal muscles of diabetic patients compared to nondiabetic controls.[26] Therefore, the function of Phospho1 appears to be multifaceted.

In the present study, we found that Phospho1 is highly expressed in the skeletal muscle and its expression is markedly upregulated during satellite cell activation and myogenic differentiation. Using gene knockdown and overexpression tools, we show that the perturbation of Phospho1 expression affects mitochondrial function and myogenesis. We also identify MyoG as an upstream regulator of Phospho1.

## 2 | MATERIALS AND METHODS

### 2.1 | Primary myoblast isolation and culture

Primary myoblasts were isolated from hindlimb skeletal muscles of 1-month-old WT mice. In detail, hindlimb muscles were dissected, minced to approximately 1 $mm^3$, then digested in 5 mL of collagenase type II (Worthington) and dispase II mixture (Roche) for 24 minutes, with an addition of F10 medium (containing 20% FBS, HyClone) to stop the digestion. Cell mixtures were then filtered, centrifuged, and cultured in F-10 Ham's medium supplemented with 20% FBS, 4 ng/mL of basic FGF (Promega), and 1% penicillin-streptomycin (HyClone) on collagen-coated cell culture plates at 37°C, 5% $CO_2$. For differentiation,

primary myoblasts were seeded on Matrigel-coated cell culture plates and induced to differentiation by replacing with the Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 2% horse serum and 1% penicillin-streptomycin when the cell density was 80%.

### 2.2 | Single myofiber isolation and culture

Single myofibers were isolated from EDL muscles of 2-month-old mice. Briefly, EDL muscles were dissected carefully for digestion in a collagenase I medium (2 mg/mL, Sigma) made with DMEM for 1 hour at 37°C. EDL muscles were transferred to a horse serum-coated Petri dish (60-mm) with 5 mL of prewarmed DMEM and single myofibers were released by gently flushing muscles with a large bore glass pipette. Released single myofibers were then transferred and cultured with DMEM (Gibco) containing 20% FBS, 4 ng/mL of bFGF, and 1% penicillin-streptomycin in a horse serum-coated 60-mm dish at 37°C for indicated days.

### 2.3 | Muscle injury and regeneration

Tibialis anterior (TA) muscle regeneration was induced by cardiotoxin (CTX) injection. Mice were anesthetized using a ketamine-xylazine cocktail, and then CTX was injected (50 μL of 10 μM solution, Sigma) into the TA muscle. TA muscles were harvested at the indicated days postinjection to assess the completion of regeneration and repair.

### 2.4 | Adenovirus generation

The cDNA of Phospho1 (NM_153104.3) was cloned into the pAdTrack-CMV vector. After sequencing, the vector with the correct insert of Phospho1 was digested by PmeⅠ and then transformed into the pAdEasy competent cell. The positive recombinant plasmid was identified by PacⅠ digestion. Then, 4 μg of linearized recombinant plasmid was transfected into HEK293A cells seeded on a 10-cm dish using the Lipofectamine 2000 (Invitrogen). After 2 weeks, the cells were collected, and the recombinant adenovirus was extracted by three freeze-thaw-vortex cycles. Two more rounds of HEK293A cell infection were performed to amplify the adenovirus. The recombinant adenovirus with the empty pAdTrack-CMV plasmid was also generated as a control for further experiments.

### 2.5 | Lentivirus-mediated gene stable knockdown C2C12 cell lines

The shRNA of Phospho1 was designed using online software: https://rnaidesigner.invitrogen.com/rnaiexpress/, with a scrambled shRNA as control (Table 1). The annealed shRNA was inserted into the pLKO.1 vector and then was co-transfected with pCMV-VSV-G and pCMV delta R8.2 plasmids into HEK293T cells seeded on a 10-cm dish. After 48 hours transfection, the culture media were filtered through a 0.45 μm filter to remove the cellular debris and the lentivirus was collected. To generate the stable gene knockdown cell line, C2C12 cells (at 30% confluence) were infected by Phoshho1 or scramble shRNA lentivirus and subjected to puromycin (1 μg/mL) treatment after 48 hours infection to select shRNA-expressing cells. Puromycin was added in culture medium for two generations to get the pure lentivirus infected cells.

### 2.6 | Luciferase assay

HEK293T cells were seeded onto 48-well plates. The MyoG cDNA was inserted into pcDNA3.1 vector to overexpress MyoG. The pGL3-Phospho1 promoter-luciferase plasmid pGL3-P(616)(between −409 bp and +206 bp) and pGL3-P(320) (between −104 bp and +206 bp) were generated. For the transfection, each well was co-transfected with 80 ng Renilla plasmids, 250 ng pGL3-P/pGL3-basic, and 250 ng MyoG-pcDNA3.1 plasmids. Twenty-four hours after transfection, cells were lysed and analyzed following with the manual of Dual-Luciferase Reporter Assay System (Promega).

### 2.7 | Immunofluorescence staining and MitoTracker staining

For immunofluorescence staining, single myofibers or cultured cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes, quenched with 100 mM glycine for 10 minutes, and then incubated in blocking buffer (5% goat serum, 2% BSA, 0.1% Triton X-100, and 0.1% sodium azide in PBS) for 2–3 hours. Samples were then incubated with primary antibodies at 4°C overnight and then secondary antibodies and DAPI at room temperature for 1 hour (see Table 2 for antibody information).

For mitochondria staining, 0.5 μM MitoTracker Red CMXRos (Cell signaling) was added to the culture medium and cells were incubated with MitoTracker for 40 minutes at 37°C, 5% $CO_2$ incubator. Cells were then fixed by ice-cold methanol for 10 minutes at −20°C and processed to immunostaining with MF20 antibody.

### 2.8 | ChIP-qPCR

ChIP-qPCR was performed on primary myoblasts differentiated for 2 days. In brief, myotubes were fixed with 1% PFA for 10 minutes at room temperature with shaking, followed by the addition of 125 mM glycine for 5 minutes at room temperature. Cells were harvested and lysed with ChIP cell lysis buffer containing 20 mM Tris, 0.1% SDS, 1% Triton-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitor on ice for 10 minutes. The obtained nuclei were resuspended and sonicated to fragmentize the nuclear chromatin. The DNA fragments were then incubated with mouse IgG (as control) or MyoG antibody conjugated with agarose A/G beads (Santa Cruz Biotechnology) at 4°C overnight. The immunoprecipitates were eluted and incubated with Proteinase K overnight at 65°C to reverse the crosslinks. DNA fragments were purified using the Phenol-Chloroform method and subjected to the quantitative PCR.

### 2.9 | Total RNA extraction and real-time PCR

Total RNA was extracted from tissues/cells using TRIzol reagent according to the manufacturer's instructions. The purity and concentration of the total RNA were determined using a spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific). Three micrograms of total RNA were subjected to reverse transcription using random primers with M-MLV reverse transcriptase (Invitrogen). Real-time PCR was carried out in a LightCycler 96 PCR System (Roche) with SYBR Green Master Mix. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes (see Table 1 for primer information).

### 2.10 | Protein extraction and western blot analysis

Cells/tissues were lysed with radio-immunoprecipitation assay (RIPA) buffer with protease inhibitor (Sigma). Protein concentrations were determined using BCA Protein Assay Reagent (Pierce Biotechnology). Protein supernatant was denatured with Laemmli buffer and boiled for 10 minutes. For western blot, samples were loaded on PAGE gel and then transferred onto PVDF membrane (Millipore). The membrane was blocked in 5% fat-free milk for 2 hours, and blotted with primary antibodies at 4°C overnight, followed by the incubation with secondary antibodies at room temperature for 1 hour (see Table 2 for the antibody information).

### 2.11 | Statistical analysis

All analyses were conducted with Student's *t*-test, with a two-tail distribution. All experimental data were represented as mean ± sem. Comparison with $P$ value < .05 was considered significant.

## 3 | RESULTS

### 3.1 | Phospho1 is abundantly expressed during myogenic differentiation

We first compared the expression of Phospho1 in various mouse tissues and found that *Phospho1* mRNA was detectable in all examined tissues at different levels. The highest expression level was found in brown adipose tissues (BAT), skeletal muscles (extensor digitorum longus (EDL) and soleus (Sol)), and cardiac muscles (Figure 1A). Among different types of skeletal muscles, the protein of PHOSPHO1 was highly expressed in fast-twitch tibialis anterior (TA), gastrocnemius (Gas), and EDL muscles, compared to the lower level in Sol muscle (Figure 1B). During skeletal muscle regeneration, the mRNA level of Phospho1 sharply declined at 3 days postinjury (dpi) when muscle was degenerated, followed by gradual increases during muscle regeneration (Figure 1C). These results indicate that Phospho1 was enriched in differentiated myocytes and myofibers. In support of this, immunostaining of PHOSPHO1 in cultured primary myoblasts showed that PHOSPHO1 immunofluorescence was stronger in $MYOG^+$ differentiating myocytes at Day 1 of differentiation and markedly elevated in newly formed myotubes at Day 3 (Figure 1D). These observations were confirmed by qRT-PCR and Western blot analyses, both showing that the expression of Phospho1 was upregulated during myogenesis, following a similar trend of MyoG expression (Figure 1E,F). We further examined the PHOSPHO1 expression level in quiescent, activated, and proliferating satellite cells (SCs) on cultured single myofibers by immunofluorescence staining. Interestingly, 75% of quiescent SCs had no detectable levels of PHOSPHO1, whereas 25% of quiescent SCs were PHOSPHO1 positive (Figure 1G). In contrast, immunoreactivity of PHOSPHO1 was observed in all SCs after culture for 12 hours when SCs were activated and remain at high level in proliferating SCs (Figure 1G). Taken together, these data indicated that Phospho1 is expressed abundantly during myogenic differentiation.

### 3.2 | Phospho1 inhibits the proliferation of myoblasts

To investigate the role of Phospho1 in myogenesis, we performed the gain-of-function analysis using adenovirus-mediated overexpression of Phospho1 in cultured primary myoblast. The overexpression of Phospho1 was confirmed by qRT-PCR that showed ~450-folds increase of Phospho1 mRNA level in myoblasts after infected with Phospho1 adenovirus (Phospho1-OE) for 72 hours, compared to control cells infected with GFP-expressing adenovirus (Figure 2A). The immunofluorescence of GFP (the adenovirus-Phospho1 vector contains GFP cDNA) showed ~80% infection efficiency without influencing cell viability (Figure 2B,D). Notably, less $KI67^+$ myoblasts were observed in the Phospho1-OE group (Figure 2B), and the percentage of $KI67^+$ to $GFP^+$ cells in Phospho1-OE treated was about 50% of that in the GFP control group (Figure 2C). In consistency, we found a significant reduction of EDU-labeled myoblasts in the Phospho1-OE group (Figure 2D,E). These results suggest that overexpression of *Phospho1* inhibits myoblast proliferation.

In parallel, we performed the loss-of-function analysis in the C2C12 cell line with two independent lentiviral shRNAs. The transfection of shRNA1 achieved an 87% reduction of *Phospho1* mRNA in C2C12 cells, whereas shRNA2 did not induce the reduction of *Phospho1* (Figure 3F). Therefore, shRNA1 lentivirus was used to establish a stable *Phospho1* knockdown C2C12 cell line (Phospho1-KD). In contrast to the reduced number of EDU-labeled myoblasts after Phospho1-OE, EDU-labeled cells were increased by 44% in the Phospho1-KD group (Figure 2G,H). Moreover, qRT-PCR results showed that the expression of *CDK4* was significantly upregulated in Phospho1-KD C2C12 cells, compared to the control cells (Figure 2I). The protein level of CDK4 was also significantly increased in Phosphol-KD C2C12 cells (Figure 2J). Thus, these results indicate that *Phospho1* negatively regulates myoblast proliferation.

### 3.3 | Phospho1 promotes differentiation and fusion of myoblasts

Given that the expression of *Phospho1* was upregulated during myoblast differentiation, we next investigated the role of *Phospho1* in regulating myoblast differentiation and fusion. We infected the primary myoblasts with Phospho1-OE and control adenovirus, and then induced them to differentiate for 4 days. Immunostaining of MYOG indicated a notably increased differentiation in the Phospho1-OE group, represented by 87% in the Phospho1-OE group as compared to 74% $MYOG^+$ cells in the control group (Figure 3A,B). Moreover, longer and more nucleated myotubes were observed in cultures of Phospho1-OE compared to the GFP cultures by the immunostaining of MF20 (sarcomeric myosin heavy chain) (Figure 3C). Quantitative analysis showed that in comparison with the GFP control, Phospho1-OE cultures exhibited more myotubes with myonuclei number >5, concurrently fewer unfused myocytes with single myonuclei (Figure 3D). Accordingly, qRT-PCR analysis showed that overexpression of *Phospho1* upregulated the expression of MyoD and MyoG significantly, as well as the fast myofiber marker gene Myh1 (Type IIX myofiber) and Myh4 (Type IIB myofiber), and slow myofiber marker gene Myh7 (indicated Type I) (Figure 3E). Consistent with the changes in gene expression, Phospho1-OE up-regulated the protein levels of MYOG and MF20 (Figure 3F). These data suggest that overexpression of Phospho1 promotes myoblast differentiation and fusion.

We also evaluated differentiation efficiency in Phospho1-KD C2C12 cells. Immunofluorescence staining of MYOG and MF20 showed that after differentiation for 5 days, both the differentiation and fusion were dramatically decreased in Phospho1-KD cells (Figure 4A), representing 59% and 29% of those in the control cells, respectively (Figure 4B,C). As expected, the expression of myogenic genes *MyoD* and *MyoG* was significantly reduced after Phospho1-KD (Figure 4D). The expression of myofiber specific gene *Myh1* and *Myh4* were significantly decreased, while *Myh7* and *Myh2* were not changed (Figure 4D). Similarly, Western blot analysis showed a decrease in the expression of MYOG and MF20 upon knockdown of Phospho1, but not the expression of MYOD (Figure 4E). Altogether, knockdown of Phospho1 inhibited the C2C12 differentiation and myotube formation.

### 3.4 | Perturbation of Phospho1 affects mitochondrial protein abundance and function

A recent study reported that Phospho1 participates in UCP1-independent thermogenesis in brown adipocyte and knockdown of Phospho1 decreases the oxygen consumption rate of inguinal white adipocyte.[27] Given the high energy demand of skeletal muscle cells, we hypothesized that Phospho1 might also play an important role in the mitochondrial function of myoblasts. To test this, we first interrogated the changes in mitochondrial related genes in myotubes differentiated for 4 days in response to knockdown or overexpression of Phospho1. Phospho1-KD significantly downregulated the mitochondrial related genes *Cox1* and *Cox8b* as well as mitochondrial biogenetic genes *PGC1a* and *TFAM*, whereas Phospho1-OE upregulated the expression of *Cox8b*, *PGC1a*, and *TFAM* (Figure 5A,B). Consistently, the expression of representative proteins involved in oxidative phosphorylation (OXPHOS) NDUF8, SDHB, UQCRC2, and ATP5A were all decreased in Phospho1-KD C2C12 cells (Figure 5C). In support of this, MitoTracker staining in live myotubes after differentiation for 4 days showed much less MitoTracker signaling in Phospho1-KD C2C12 cells (Figure 5D,E). In contrast, more MitoTracker signaling was observed in Phospho1-OE myotubes (Figure 5D,E). Moreover, we examined the mitochondrial respiration rate in newly differentiated myotubes after infected with Phospho1-OE adenovirus by Seahorse Bioanalyzer. Phospho1-OE significantly increased the basal and maximal oxygen consumption rate (OCR) (Figure 5F,G). Collectively, these data reveal an important role of Phospho1 in the regulation of mitochondrial protein expression and respiratory function during myoblast differentiation.

### 3.5 | MYOG transcriptionally regulates Phospho1 expression in myoblasts

As Phospho1 critically regulates differentiation and fusion of myoblasts, we next sought to identify the upstream regulator of Phospho1 during the myogenesis. We first analyzed the transcription factor binding sites in the *Phospho1* promoter. This analysis led to the identification of two conserved MYOG binding sites spaced 133 bp apart and located within 500 bp upstream of Exon 1 (Figure 6A). We then analyzed the luciferase activity of Phospho1 promoters with MyoG binding sites (pGL3-P(616)) or without MyoG binding sites (pGL3-P(320)) in response to overexpression of MyoG (Figure 6B). The data showed that the luciferase activity of pGL3-P(616) was significantly higher than that of pGL3-P(320) and pGL3-basic plasmids, and the pGL3-P(616) activity was significantly higher when MyoG was overexpressed. This suggests that MyoG can bind to the Phospho1

promoter and regulate its transcription (Figure 6B). In addition, we designed a primer pair flanking these two binding sites and performed ChIP-qPCR on genomic DNA pulled down by IgG and MYOG antibody. The data showed increased Phospho1 promoter enrichment in the MYOG pulldown sample, suggesting that MYOG, indeed, bind the Phospho1 promoter (Figure 6C). To determine if MYOG binding functionally regulates Phospho1 transcription, we infected myoblasts with MyoG-OE adenovirus. Three days after infection, the expression of *Phospho1* is significantly upregulated in MyoG-OE myoblast compared to control myoblasts (Figure 6D). Hence, these results provided strong evidence that MyoG is an upstream transcriptional regulator of *Phospho1*.

## 4 | DISCUSSION

Understanding regulators that enhance myogenesis may lead to the identification of new targets for improving muscle regeneration and function in muscle diseases. Here, we report that the *Phospho1* is highly expressed in muscle tissues and induced during myogenic differentiation. Using stable Phospho1-KD C2C12 myoblasts, we find that knockdown of Phospho1 promotes proliferation and inhibits differentiation. Conversely, adenovirus-mediated overexpression of Phospho1 in primary myoblasts inhibited proliferation and promoted differentiation. Importantly, we found that Phopsho1 positively regulates the mitochondrial respiration of myoblasts. Moreover, the transcription of Phospho1 is regulated by the master myogenic transcriptional factor MyoG. Our study adds Phospho1 to the repertoire of regulators in controlling myogenesis.

Studies have shown that Phospho1 is essential for skeletal mineralization.[23–25] Phospho1 global knockout mice exhibit multiple defects such as growth retardation, smaller body weight, and severe bone defects.[24] The growth retardation and reduced body weight of the *Phospho1* KO mice are consistent with our observation of differentiation defects in Phospho1 null myoblasts. However, in mice, these defects may also be secondary effects of abnormal bone development. Future studies using myoblast-specific Phospho1 conditional KO mice will address the specific role of Phospho1 in myogenesis in vivo.

Apart from Phospho1 investigated in this study, previous studies have revealed a number of phosphatases that play a role in myogenesis. For example, loss of the phosphatase and tensin homolog (Pten) disrupts the quiescence of adult satellite cells, but Pten deletion in embryonic myoblasts promotes their proliferation and induce muscle hypertrophy.[28,29] SH2-containing protein tyrosine phosphatase positively regulates myogenesis via RhoA.[30] MAPK phosphatases 5 controls proliferation and differentiation of myoblasts through JNK and p38 MAPK pathways, respectively.[31] Skeletal muscle and kidney enriched inositol polyphosphate phosphatase negatively regulate myogenesis through inhibiting IGF2 production.[32] Inhibition of protein phosphatases 1 and 2A represses the differentiation of C2C12 cells.[33] Identifying the role of Phospho1 in myogenesis further points to the roles of its substrates. PC and PE, two substrates of PHOSPHO1, are dynamically regulated during chicken embryonic myogenesis.[34] The high expression of Phospho1 in skeletal muscles relative to other tissues in mice is consistent with its expression pattern in other animals annotated in the bgee.org database, suggesting that Phospho1 might play a conserved role in skeletal muscle function. This notion is supported by our series of functional assays showing

that Phospho1 promotes myogenic differentiation and mitochondrial function. Although Phospho1 is expressed in proliferating myoblasts, Phospho1 overexpression inhibits the proliferation of myoblasts, suggesting that its level must be fine-tuned during the proliferation and differentiation of myoblasts. As the initiation of differentiation requires cell cycle exit,[35] we speculate that Phospho1 might function as a cell cycle checkpoint controlling the transition from proliferation to differentiation states in myoblasts. In this regard, high levels of Phospho1 may promote premature differentiation at the expense of proliferation. Interestingly, in the resting muscles, Phospho1 is readily detected in a small portion of quiescent satellite cells. Given that quiescent satellite cells are heterogeneous in regards to myogenic commitment,[35,36] it would be interesting to determine if Phospho1$^{+}$ and Phospho1$^{-}$ satellite cells represent different cell fates. These observations together suggest that Phospho1 might regulate satellite cell function in a cell state-dependent manner.

Skeletal muscles are classified into two functionally different types: fast- and slow-twitch muscles based on their contractile protein expression and other biochemical and physiological characteristics. Fast-twitch muscles contain predominantly Type IIX and IIB myofibers with fewer mitochondria, relying on anaerobic respiration to produce energy for fast muscle contractions of short duration. In contrast, slow-twitch muscles contain more Type I and IIA myofibers with more mitochondria, which supports aerobic respiration to produce energy for endurance activities.[37,38] We observed that Phospho1 is more abundantly expressed in fast-twitch muscles (Gas, EDL, and TA) than slow-twitch muscles (Sol). We hypothesized that Phospho1 might be important for type II fiber formation or function. In support of this, overexpression of Phospho1 significantly upregulated the type II fiber marker genes Myh1(type IIX) and Myh4 (type IIB) in comparison with the changes in Myh2 (type IIA) and Myh7 (type I). Conversely, knockdown of Phospho1 significantly decreased the gene expression of Myh1 and Myh4. These results indicate that Phospho1 might regulate the differentiation and formation of type II fibers. In preclinical mouse models of muscle-wasting diseases, such as Duchenne Muscular Dystrophy (DMD) and cancer cachexia, type II fibers are more prone to wasting compared to type I fibers.[39,40] In this scenario, Phospho1 agonists may represent a potential treatment for cachexia and other muscle-wasting diseases.

As a phosphatase, Phospho1 has a high activity toward PE and PC to generate inorganic phosphate.[21] A previous study has shown that increase of cytosolic PE metabolism resulted in the inhibition of mitochondrial respiration.[41] This implies that Phospho1 may be involved in the cellular energy metabolism by regulating PE levels. Indeed, recent studies demonstrated that knockdown of Phospho1 significantly decreased cellular OCR in mouse inguinal adipocytes, which is dependent on creatine metabolism but independent of UCP1.[27] In accordance with these studies of Phospho1 in mitochondrial energy metabolism, our results show that Phospho1 promotes mitochondrial biogenesis and respiration in differentiated myoblasts. Creatine metabolism is essential for muscle energy metabolism and function,[42,43] particularly in fast-twitch fibers.[44] Given that Phospho1 is highly expressed in fast-twitch muscles and participated in creatine-driven energy expenditure in beige fat,[27] we speculate that the mitochondrial respiration regulated by Phospho1 in myoblasts may be associated with creatine metabolism. It will be interesting to determine the substrate of Phospho1 in muscle that mediates energy metabolism.

We observed that the Phospho1 expression pattern is similar to that of MyoG during myoblast differentiation, prompting us to examine if the expression of Phospho1 is regulated by MyoG. Through the analysis of the Phospho1 promoter, we found two E-box motifs that can be recognized by the MyoG transcription factor.[45] As expected, ChIP-qPCR data further verified that MyoG can bind to the Phospho1 promoter. Consistently, overexpression of MyoG significantly increased the Phospho1 expression in primary myoblast. Interestingly, Myog is not required for Phospho1 expression as the promoter with Myog binding domains exhibit higher than basal activity in the absence of Myog. These studies identify a transcriptional mechanism that upregulates Phospho1 expression during myogenic differentiation.

In summary, our study based on embryonic myoblast cell line (C2C12) and postnatal primary myoblasts reveals a key role of Phospho1 in myogenesis and mitochondrial function. Several limitations await to be resolved in future studies. First, the in vivo function of Phospho1 warrants investigation using conditional knockout models that are not currently available. Second, although the C2C12 and primary myoblasts were used to represent both embryonic and postnatal myoblasts, it is crucial to perform gain-of-function and loss-of-function studies in both cell types. This has not been accomplished in the current studies due to technical obstacles. Third, the observed changes in mitochondrial gene expression and respiratory function in response to the perturbation of Phospho1 expression may be partially due to alterations in myogenic differentiation, which is known to shift metabolism in myoblasts.[46,47] Nevertheless, our findings broaden the understanding of the function of lipid phosphatases in myogenesis.

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## Abbreviations:

| | |
|---|---|
| **BAT** | brown adipose tissue |
| **EDL** | extensor digitorum longus (muscle) |
| **FBS** | Fetal Bovine Serum |
| **FGF** | Fibroblast Growth Factor |
| **Gas** | gastrocnemius (muscle) |
| **MRFs** | myogenic regulator factors |
| **MyoG** | Myogenin |

| | |
|---|---|
| **OE** | overexpression |
| **OCR** | oxygen consumption rate |
| **PC** | phosphocholine |
| **PE** | phosphoethanolamine |
| **Phospho1** | Phosphatase, orphan 1 |
| **Sol** | soleus (muscle) |
| **TA** | tibialis anterior (muscle) |

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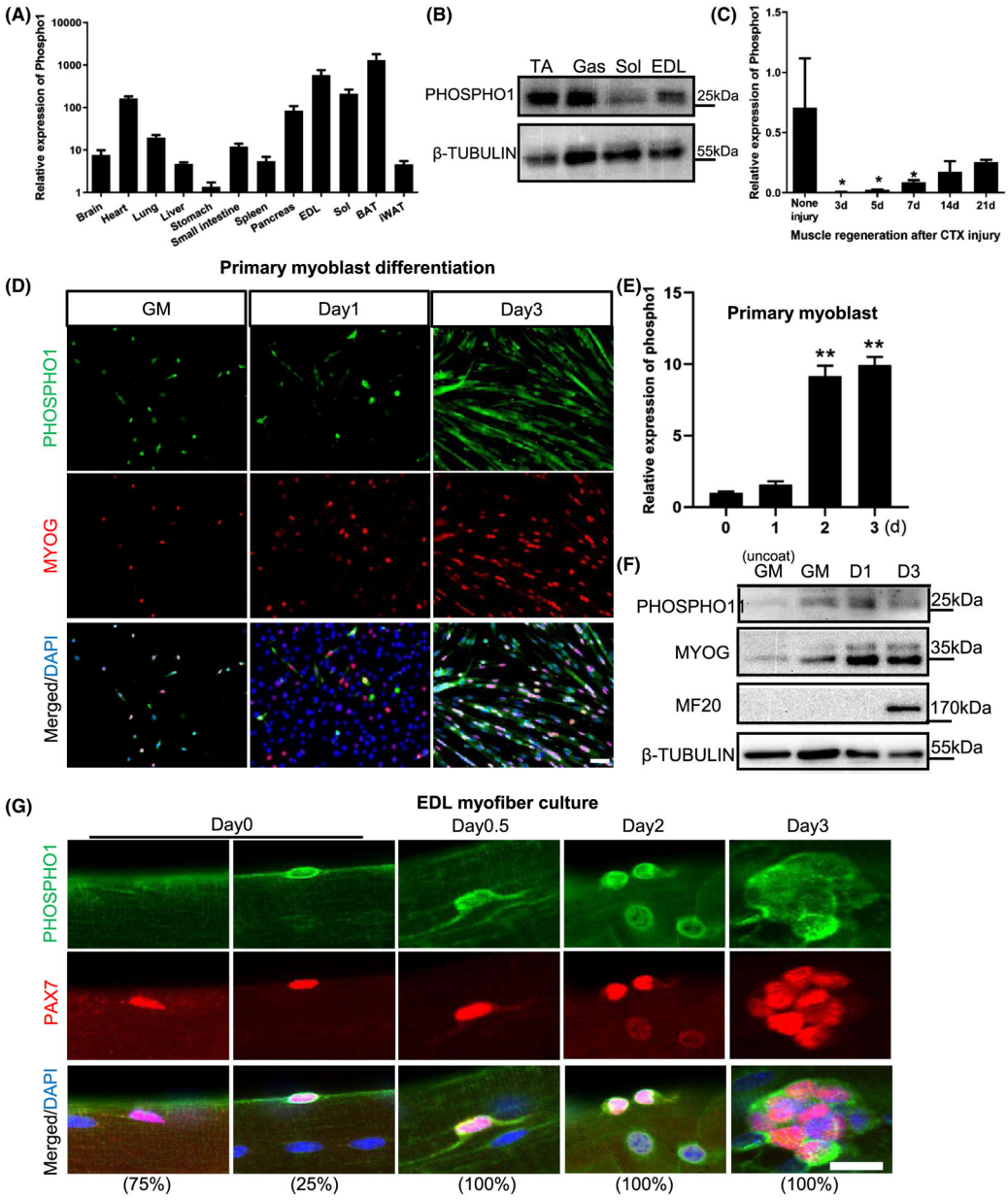


**FIGURE 1.**

Phospho1 expression pattern in muscle tissues and cells. A, qRT-PCR detection of Phospho1 expression in different mouse tissues (n = 4). B, Western blot showing the PHOSPHO1 protein level in TA, Gas, Sol, and EDL muscles. C, Relative levels of Phospho1 following CTX induced muscle injury and regeneration (n = 3). D, Immunofluorescent staining of PHOSPHO1 and MYOG during myoblast differentiation (scale bar: 50 μm). qRT-PCR (E) and Western blot (F) showing Phospho1 mRNA and protein level during the myogenic differentiation. G, PHOSPHO1 immunofluorescence in PAX7 positive SCs in freshly isolated EDL myofiber (0 hour) or myofibers cultured for 0.5–3 days (quantification based on n = 20 myofibers, Scale bar:20 μm). Data represented as mean ± s.e.m. (*t* test: $*P < .05$)

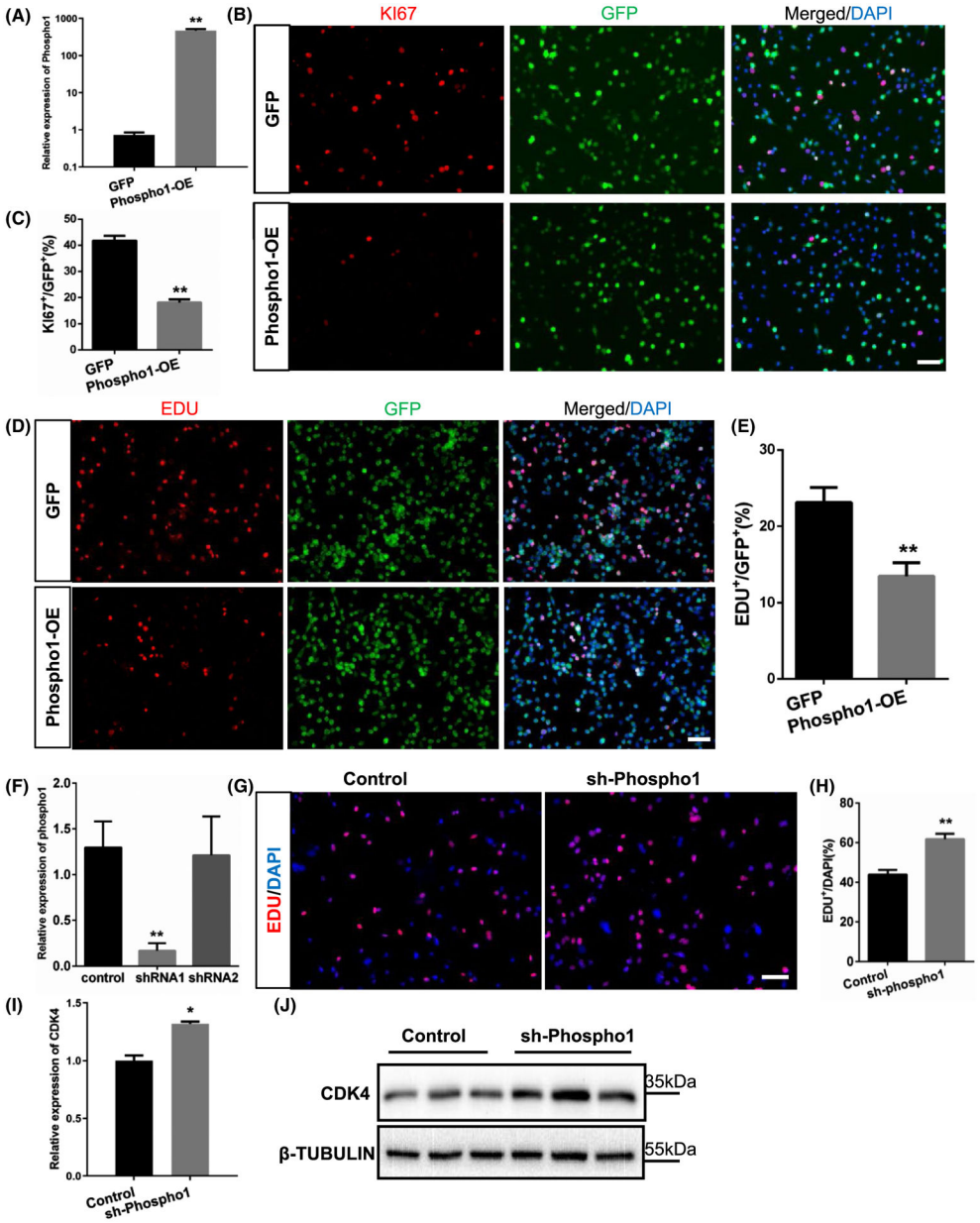


**FIGURE 2.**

Phospho1 inhibits the proliferation of myoblasts. Phospho1 overexpressing and control (GFP-expressing) myoblasts were cultured for 72 hours. A, Relative mRNA levels of Phospho1 in control and OE myoblasts (n = 3). B-C, Immunofluorescence staining (B) and quantification (C) of the proliferating KI67$^{+}$ cells (n = 11, scale bar: 50 μm). D-E, EDU staining (D) and quantification (E) of GFP control and Phospho1-OE cells (n = 15, scale bar: 50 μm). F, Control and Phospho1 knockdown C2C12 cells were cultured for 48 hours, and the relative mRNA level of Phospho1 was measured by qPCR. G-H, Immunofluorescence staining (G) and quantification(H) of EDU (scale bar: 50 μm) in control and Phospho1-KD C2C12 cells (n = 10). I, Relative mRNA level of the cell cycle gene CDK4 in control and sh-

Phospho1 C2C12 cells. J, Western blot showing the CDK4 expression in control and Phospho1 knockdown C2C12 cells. Data represented as mean ± s.e.m. (*t* test: ***P* < .01)

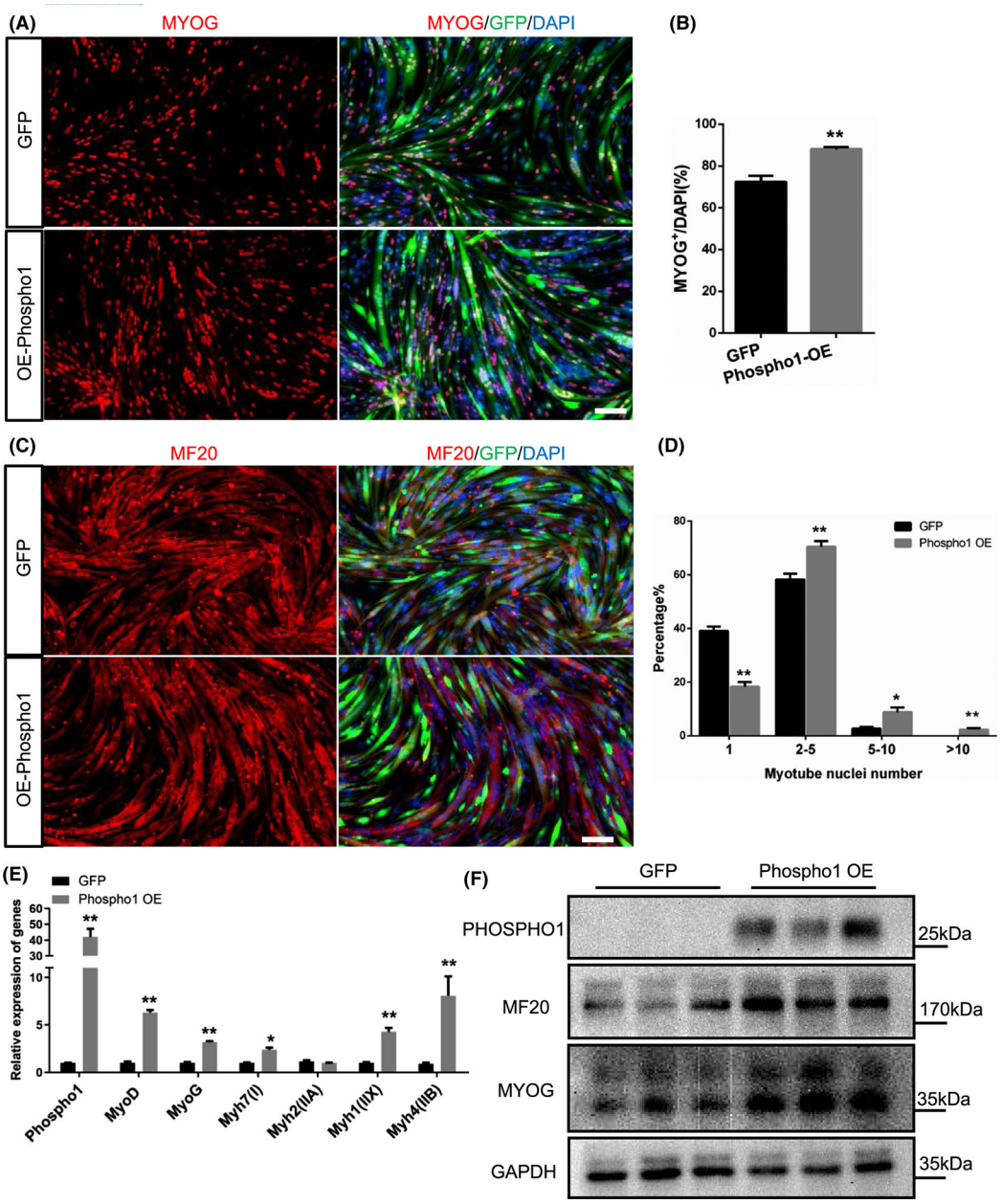


**FIGURE 3.**

Overexpression of Phospho1 promotes the differentiation and fusion of myoblast. Myoblasts were infected with GFP control/Phospho1 overexpressed adenovirus when cell density was 80% and then induce differentiation for 4 days. A-B, Immunofluorescence (A) and quantification (B) of $MYOG^+$ cells (n = 5, scale bar: 100 μm). C, Immunofluorescence of MF20 (scale bar: 100 μm). D, The of nuclei numbers in myotubes (n = 5). E, qRT-PCR detection of myogenic genes and fiber type genes expression. F, Western blot showing protein levels of myogenic proteins in myoblasts differentiated for 4 days. Data represented as mean ± s.e.m. (*t* test: $*P < .05$, $**P < .01$)

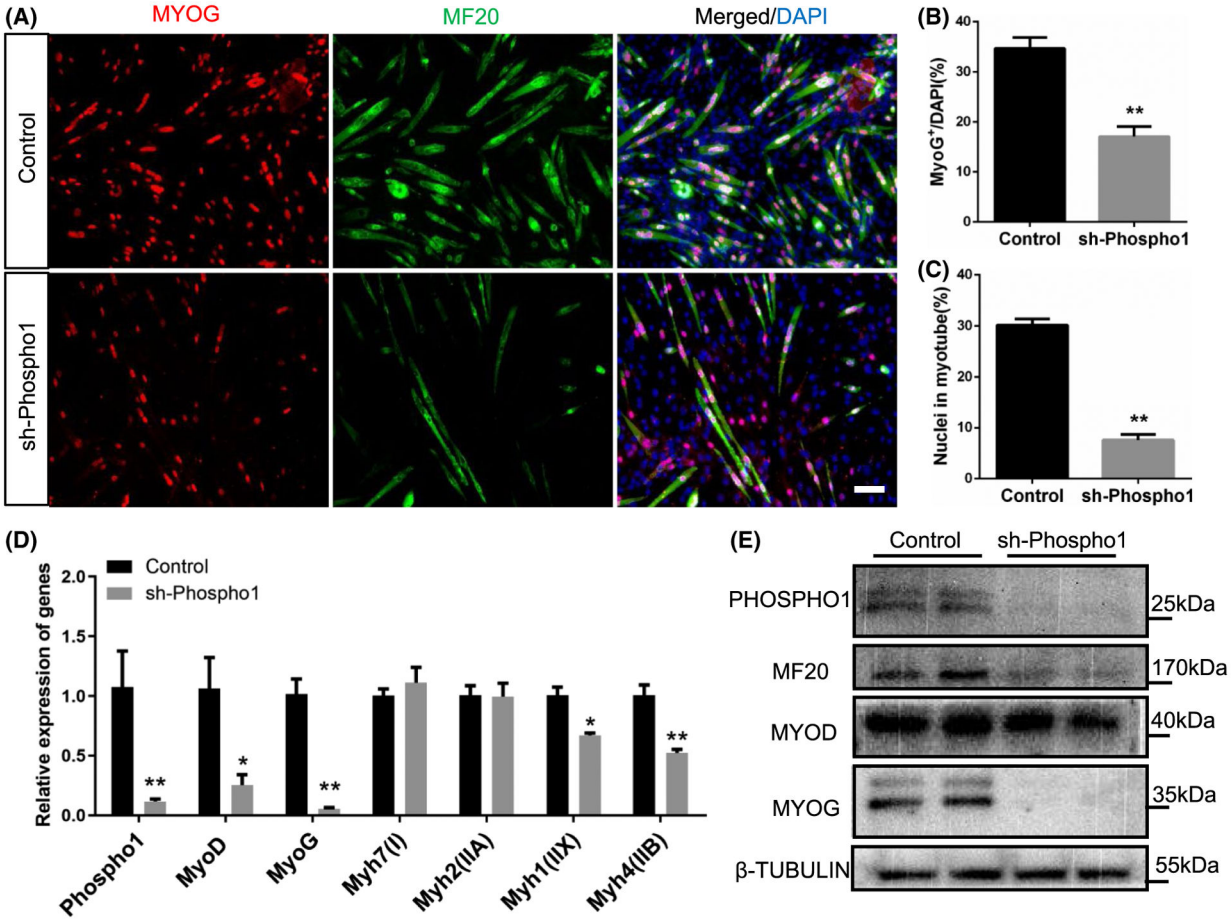


**FIGURE 4.**

Knockdown of Phospho1 inhibits differentiation and fusion of myoblasts. Control and sh-Phospho1 C2C12 cells were induced to differentiate at 80% confluence and differentiated for 5 days. A, MYOG and MF20 immunofluorescence staining (scale bar: 100 μm). B, Percentage of$MYOG^+$ cells (n = 5). C, Percentage of nuclei within myotubes (n = 5). D, qRT-PCR showing the relative mRNA levels of myogenic genes and myosin heavy chain genes in control and Phospho1 knockdown cells. E, Western blot showing the protein levels of myogenic factors in control and Phospho1 knockdown cells. Data represented as mean ± s.e.m. (*t* test: **P* < .05, ***P* < .01)

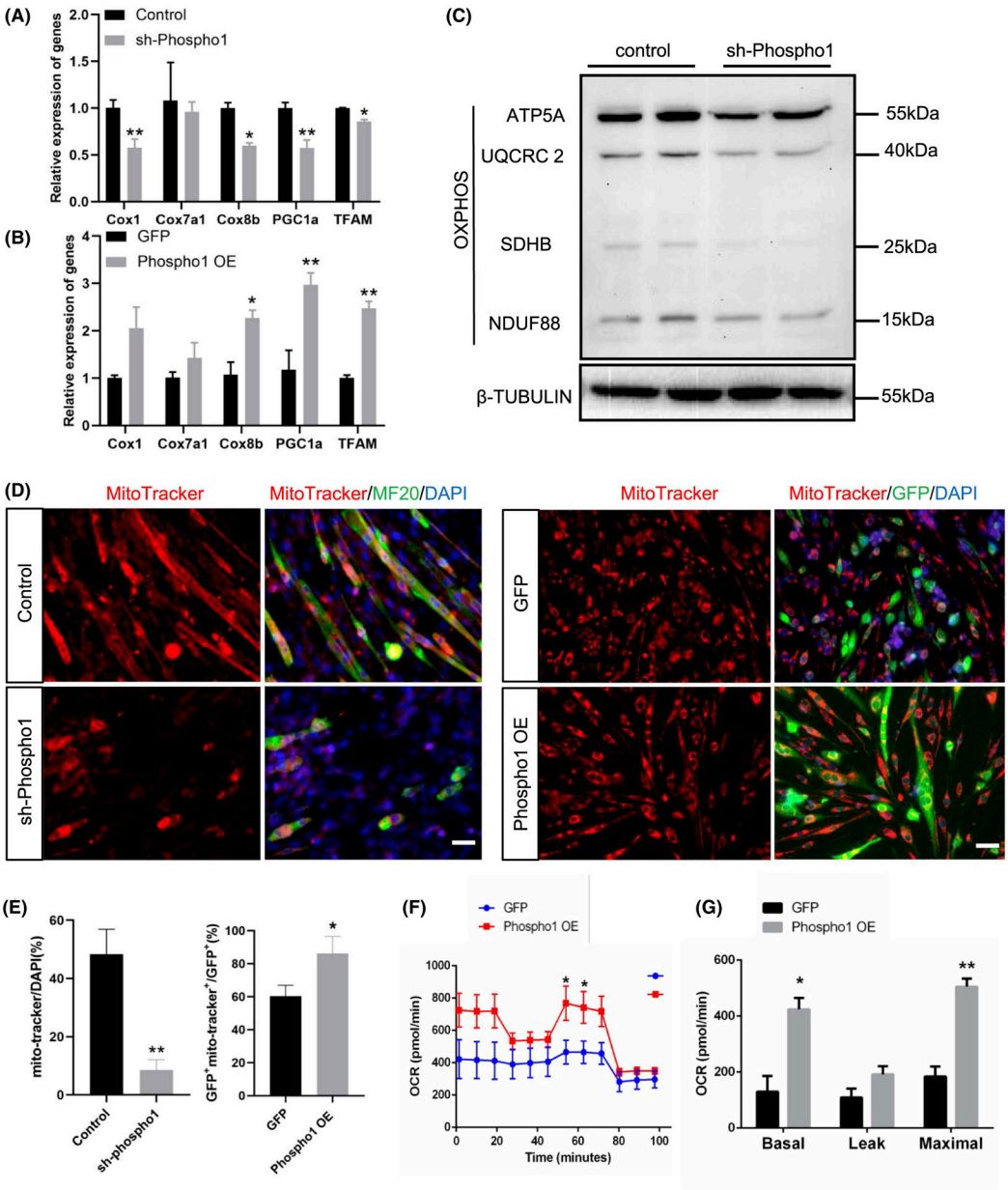


**FIGURE 5.**

Phospho1 regulates mitochondrial gene expression and respiration. qRT-PCR quantification of relative mRNA levels of mitochondrial related genes at 4 days after differentiation in Phospho1-OE (A) and knockdown myoblasts (B) (n = 3). C, Western blots showing protein levels of OXPHO complexes in control and knockdown C2C12 cells differentiated for 4 days. D-E, Immunofluorescence staining (D) and quantification (E) of MitoTracker in Phospho1 OE myoblast and knockdown C2C12 cells differentiated for 4 days (scale bar: 50 μm, n = 3). F, Seahorse analysis of oxygen consumption rate (OCR) in control and Phospho1 overexpressing myoblasts differentiated for 3 days. G, Quantification of the basal, leak and maximal OCR (n = 4). Data represented as mean ± s.e.m. (*t* test: **P* < .05, ***P* < .01)

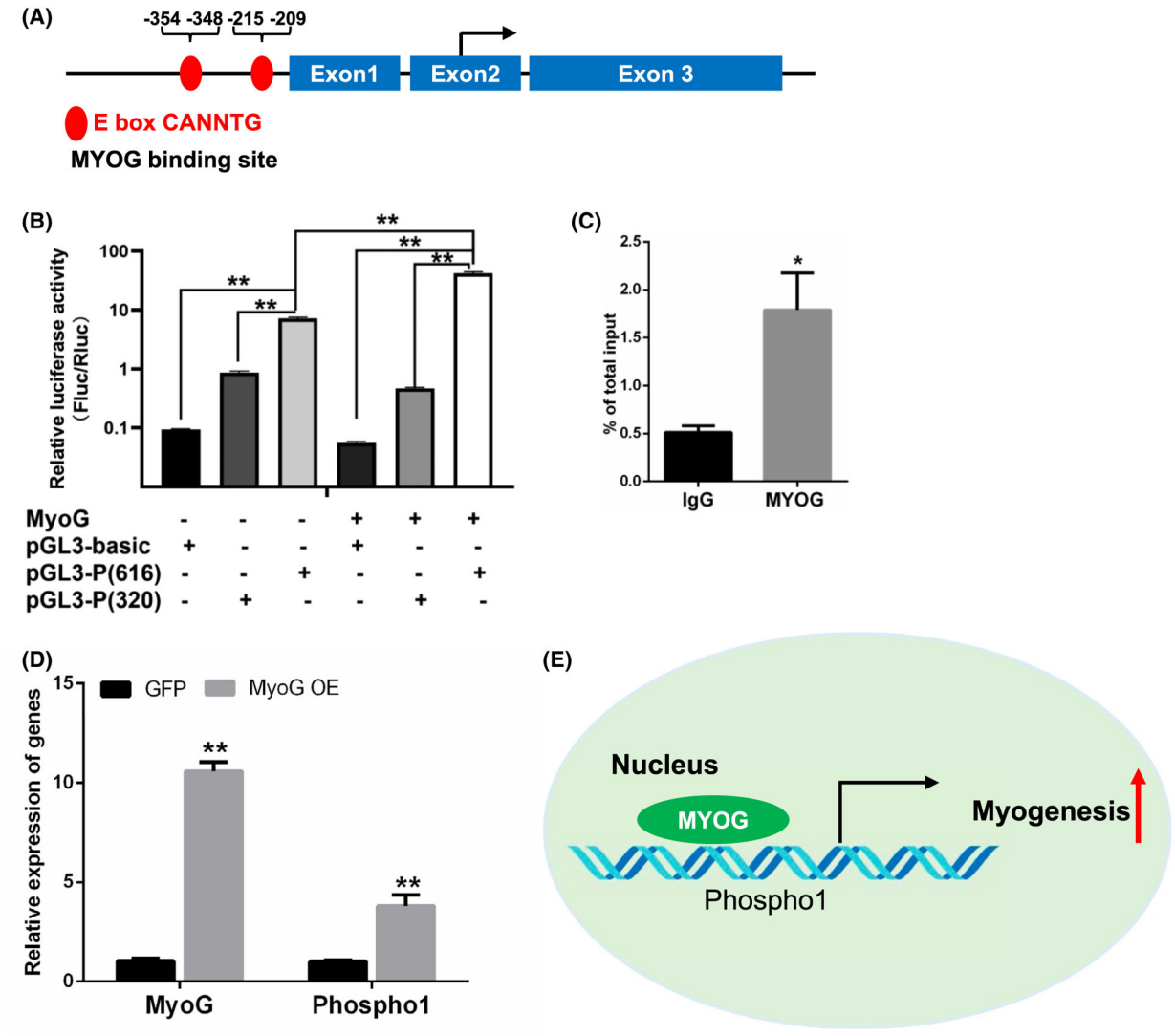


**FIGURE 6.**

MYOG is a transcriptional regulator of Phospho1 in myoblasts. A, Schematic of Phospho1 promoter showing two consensus MYOG binding sites. B, Luciferase activity analysis of Phospho1 promoter (pGL3-P(616): between −409 bp and 206 bp harboring the MyoG binding sites; and pGL3-P(320): between −104 bp and +206 bp without the MyoG binding sites (n = 6). C, ChIP-qPCR showing the enrichment of P(616) DNA in MyoG pull down group using myoblasts differentiated for 2 days (n = 4). D, qRT-PCR showing MyoG and Phospho1 expression levels in primary myoblasts 3 days after infection with MyoG and control adenovirus (following 1 day in growth medium and 2 days in differentiation medium). E, Schematic summary Phospho1 function in myogenesis. Data represented as mean ± s.e.m. (*t* test: ** $P < .01$)

**TABLE 1**

Sequences of primers used in this study

| **Genes** | **Primer sequence** | |
|---|---|---|
| 18S | F: AGTCCCTGCCCTTTGTACACA | R: CGATCCGAGGGCCTCACTA |
| Phospho1 | F: TTCTCATTTCGGATGCCA | R: TGAGGATGCGGCGGAAT |
| CDK2 | F: CCTGCTTATCAATGCAGAGGG | R: TGCGGGTCACCATTTCAGC |
| CyclinD2 | F: GAGTGGGAACTGGTAGTGTTG | R: CGCACAGAGCGATGAAGGT |
| MyoD | F: GGCTACGACACCGCCTACTA | R: CGACTCTGGTGGTGCATCTG |
| MyoG | F: TGCCCAGTGAATGCAACTCC | R: TTGGGCATGGTTTCGTCTGG |
| Myh7 | F: ACTGTCAACACTAAGAGGGTCA | R: TTGGATGATTTGATCTTCCAGGG |
| Myh2 | F: ACTTTGGCACTACGGGGAAAC | R: CAGCAGCATTTCGATCAGCTC |
| Myh1 | F: GCGAATCGAGGCTCAGAACAA | R: GTAGTTCCGCCTTCGGTCTTG |
| Myh4 | F: AGGACCAACTGAGTGAAGTGA | R: GGGAAAACTCGCCTGACTCTG |
| Cox1 | F: ATGAGTCGAAGGAGTCTCTCG | R: GCACGGATAGTAACAACAGGGA |
| Cox7a1 | F: GCTCTGGTCCGGTCTTTTAGC | R: GTACTGGGAGGTCATTGTCGG |
| Cox8b | F: GCGAAGTTCACAGTGGTTCC | R: GGAACCATGAAGCCAACGAC |
| **shRNAs** | **Sequence (5′–3′)** | |
| shRNA1 | CCGGTTGGACTACGATGCCTATCTACTCGAGTAGATAGGCATCGTAGTCCAATTTTTG | |
| shRNA1 | AATTCAAAAATTGGACTACGATGCCTATCTACTCGAGTAGATAGGCATCGTAGTCCAA | |
| shRNA2 | CCGGCTCCTGCTTCGAGGTTATTCTCTCGAGAGAATAACCTCGAAGCAGGAGTTTTTG | |
| shRNA2 | AATTCAAAAACTCCTGCTTCGAGGTTATTCTCTCGAGAGAATAACCTCGAAGCAGGAG | |
| Scrambled shRNA | CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG | |
| Scrambled shRNA | AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG | |

**TABLE 2**

Antibodies used in this study

| (Antibody) | Host | Dilution (WB) | Dilution (IF) | (Source) |
|---|---|---|---|---|
| PAX7 | Mouse | | 1:10 | Developmental Studies Hybridoma Bank |
| MF20 | Mouse | 1:200 | 1:100 | Developmental Studies Hybridoma Bank |
| MYOD | Mouse | 1:250 | 1:200 | Santa Cruz Biotechnology |
| MYOG | Mouse | 1:500 | 1:500 | Developmental Studies Hybridoma Bank |
| OXPHOS | Rabbit | 1:1000 | | Abcam |
| CDK4 | Mouse | 1:1000 | | Santa Cruz |
| PHOSPHO1 | Rabbit | | 1:500 | Novus |
| PHOSPHO1 | Mouse | 1:250 | | Santa Cruz |
| KI67 | Rabbit | | 1:1000 | Abcam |
| β-TUBULIN | Rabbit | 1:1000 | | Abcam |
| GAPDH | Mouse | 1:1000 | | Santa Cruz |
| 568 goat polyclonal anti-mouse IgG1 | | | 1:1000 | Thermo Fisher |
| 488 goat polyclonal anti-rabbit IgG | | | 1:1000 | Thermo Fisher |
| 488 goat polyclonal anti-mouse IgG2b | | | 1:1000 | Thermo Fisher |
| Goat Anti-Mouse lgG(H + L)-HRP Conjugate | | 1:1000 | | Jackson Immuno Research Labs |
| Goat Anti-Rabbit lgG(H + L)-HRP Conjugate | | 1:1000 | | Jackson Immuno Research Labs |