

FoxO1 regulates muscle fiber-type specification and inhibits calcineurin signaling during C2C12 myoblast differentiation

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Abstract Adult skeletal muscle fibers can be categorized into slow-oxidative and fast-glycolytic subtypes based on specialized metabolic and contractile properties. The Forkhead box O1 (FoxO1) transcription factor governs muscle growth, metabolism, and cell differentiation, and has been shown to be involved in regulating muscle fiber type specification. However, to date, the mechanism behind FoxO1-mediated fiber type diversity is still unclear. In this article, FoxO1 being expressed preferentially in fast twitch fiber enriched muscles is reported. Moreover, the authors also detected that FoxO1 expression decreased in both fast and slow muscles from mice undergoing endurance exercise which induced a fast-to-slow fiber type transition. Using C2C12 myoblast, constitutively active FoxO1 mutant altered the proportion of muscle fiber type composition toward a fast-glycolytic phenotype and attenuated calcineurin phosphatase activity. In addition, a transcriptionally inactive FoxO1 by resveratrol triggered the expression of genes related to slow-oxidative muscle but not sufficient to induce a complete slow fiber transformation. Taken together, these results suggest that FoxO1 up-regulates fast fiber-type formation and down-regulates muscle oxidative capacity at least in part through inhibition of the calcineurin pathway.

Keywords FoxO1 · Fiber-type · Myosin heavy chains · Calcineurin · C2C12 myoblasts

Introduction

Skeletal muscle is composed of heterogeneous specialized muscle fibers that differ in their biochemical, physiological, and metabolic properties [1]. On the basis of specific myosin heavy chain (MyHC) isoform expression, adult skeletal muscle fibers are generally categorized as MyHC I (slow oxidative), MyHC IIa (fast oxidative), MyHC IIx/d (fast glycolytic), and MyHC IIb (fast glycolytic) [2, 3]. Fiber composition in adult skeletal muscle is regulated in response to changes in physical activity, environment, or pathological conditions [1, 4]. For example, endurance exercise training increases the percentage of slow fiber transforming the myofibers to an increased oxidative metabolism [5–7].

The O subfamily of Forkhead/winged helix transcription factors (FoxO) has been shown to regulate various cell functions including metabolism [8, 9], cell cycle [10, 11], and muscle atrophy [12, 13]. FoxO1 transcriptional activity is regulated by a variety of signaling mechanisms including phosphatidylinositol-3 kinase (PI3K)-mediated activation of AKT, which directly phosphorylates FoxO, leading to nuclear export and inactivation [14, 15]. In addition, FoxO1 can modulate cell differentiation and stimulate myotube fusion of primary mouse myoblasts [16, 17]. However, the physiological role of FoxO1 in skeletal muscle is still unclear. Meanwhile, FoxO1 skeletal muscle transgenic mice showed a marked decrease in skeletal muscle mass, and the expression of slow fiber genes, but not that of fast fiber genes decreased [18]. Reciprocally, conditional ablation of FoxO1 expression in the soleus muscle leads to reduced slow fiber and increased fast fiber formation [19]. It seems to be controversial how FoxO1 plays a critical role in muscle fiber-type composition postnatally. Therefore, the effects of FoxO1 on the muscle fiber-type specification still need to be clarified.

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At present, the intracellular pathways that transduce physiological stimuli into molecular signals that alter fiber-type gene expression are largely unknown. Several reports suggest that calcineurin is responsible for the formation of slow-twitch fibers [20, 21]. Furthermore, it has been reported that FoxO1 protein decreased calcineurin phosphatase activity in the cardiomyocytes [22, 23]. Moreover, using gain- and loss-of-function in C2C12 myoblasts demonstrated that calcineurin inhibited FoxO factors and prevented myotube atrophy [24]. Given that calcineurin is important for the formation of slow fibers and the interaction between FoxO1 and calcineurin, FoxO1 might be involved in calcineurin pathway regulating muscle fiber transition.

To gain insight into the potential role of FoxO1 in control of differentiation of muscle fiber type, the authors subjected mice to endurance swimming training which could promote the transformation of fast- to slow-twitch fiber. It was found that in controlled conditions, FoxO1 expression was much higher in fast than in slow muscle fibers, and it was significantly depressed in both slow-oxidative and fast-glycolytic muscles by long-lasting training. In this study, the authors demonstrated that in C2C12, myoblast overexpression of FoxO1 induced the transformation of slow to fast fiber type and markedly decreased calcineurin phosphatase activity. Accordingly, inhibition of FoxO1 by resveratrol (RSV) increased the expression of genes related to slow-oxidative muscles, but without a significant transition in muscle fiber types. Together, these results suggest that FoxO1 protein regulates slow to fast myofiber switching and attenuates muscle oxidative capacity at least in part through inhibition of the calcineurin pathway.

Materials and methods

Animals and endurance exercise program

Twelve male C57BL/6J mice, at 4 week old, were obtained from the Fourth Military Medical University (FMMU, China). They were reared at 25°C under artificial lighting for 12 h from 7 AM to 7 PM daily and had free access to water and food. After 2 week acclimation to the new environment, they were randomly assigned to a swimming training group (trained, $n = 6$) and a sedentary group (untrained, $n = 6$). They were then exercised by a modification of the procedure described previously [25]. In brief, the mice in the trained group were accustomed to swimming training for a period of 4 week, 6 days/week, during which time exercise was for 10 min initially and then was extended by 10 min daily until the animals swam continuously for 1 h. Water temperature was controlled

every 10 min to be maintained at 35–36°C. Mice swam as a group in a bath measuring $51 \times 71 \text{ cm}^2$ (diameter, height). Group swimming was used because it promotes more vigorous exercise than when mice are allowed to swim alone [26].

Muscle dissection

About 24 h after the previous session, mice were euthanized by decapitation and were exsanguinated. Samples of slow-oxidative and fast-glycolytic muscles were obtained by dissection of soleus and gastrocnemius muscles, which were quickly excised from both hindlimbs of the animals. The specimens were immediately cooled in liquid nitrogen and stored at -80°C for subsequent analyses.

Plasmids, antibodies and reagents

Plasmids for pcDNA3-FLAG-tagged wild-type FoxO1 (FoxO1-WT) and Akt phosphorylation-resistant mutant of FoxO1 (FoxO1-A3, where the three Akt phosphorylation sites Thr24, Ser256, and Ser319 were converted to alanine) were gifted by Huang et al. [27]. The following antibodies were purchased: anti-FoxO1, anti-phospho-FoxO1 (Ser256), anti-MyoD, anti- β -actin, and anti-Flag (Santa Cruz Biotechnology, USA); anti-sarcomeric MyHC (clone MF-20, DSHB); anti-Myogenin (Millipore, USA); anti-MyHC slow (clone NOQ7.5.4D, Sigma); and anti-MyHC fast (clone MY-32, Sigma). Inhibitor reagent RSV was purchased from Sigma.

Cell culture and induction of differentiation

Mouse C2C12 myoblasts were grown on gelatin-coated dishes in Dulbecco's modified Eagle's medium (Gibco) supplemented with 4.5 g/l D-glucose, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10% fetal bovine serum (Hyclone Laboratories), and antibiotics (penicillin: 100 IU ml^{-1} , streptomycin: $100 \mu\text{g ml}^{-1}$) in 5% CO_2 at 37°C. When the cells achieved 70% confluence, they were differentiated in 2% horse serum (Gibco) for 3 days. Under these conditions, cells were found to be healthy, viable, and not undergoing necrosis or apoptosis, as observed using phase contrast microscope (Fig. 3a–c).

Transfection

C2C12 skeletal muscle cells were transfected at 70–80% confluence with 8 μg of DNA and 20 μl of Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. In brief, transfection reagent was incubated with

plasmid DNA constructs in Opti-MEM (Invitrogen) at room temperature for 20 min. Then, this transfection mixture was applied to the proliferating cells and incubated for 5 h at 37°C. For stable transfections, following incubation, the transfection medium was removed and replaced with growth medium containing 15% FBS and 400 µg/ml G418 (Gibco) over several days until 7 days after all the cells in the control untransfected flask had been eliminated by G418. All subsequent experiments on stably transfected cells were conducted in the absence of G418. At this stage, cells were subjected to differentiation medium consisting of DMEM plus 2% horse serum for 3 days to induce differentiation into myotubes. After differentiation, multiple clones were isolated and subjected to preliminary analysis, and a single clone that was representative of the pool was chosen on the basis of FoxO1 expression.

RNA extraction and quantitative real time RT-PCR

Muscle tissue or C2C12 myotube lysates were homogenized and extracted for total RNA using TRIzol reagent (TaKaRa) by standard techniques. The integrity of RNA was checked on 2% agarose gels, and total RNA concentration was estimated by a spectrophotometer (Thermo). Two micrograms of total RNA was reverse-transcribed to synthesize cDNA by using the PrimeScript RT-reagent Kit for RT-PCR (TaKaRa) after treatment with DNase I (TaKaRa) to remove contaminating genomic DNA. Real time PCR amplification reactions were carried out by Bio-Rad iQ5 by SYBR Premix Ex TaqTM II (TaKaRa) chemistry detection under amplification conditions. Quantification of the mRNA data was done by the comparative threshold cycle ($\Delta\Delta CT$) method, with the modification that the relative efficiency of each primer was included in the calculation. The specificity of the PCR amplification was always verified with melting curve analysis. Table 1 provides details of primers of the genes studied.

Western analysis

Protein was collected from mice skeletal muscles and C2C12 myotubes. Protein lysate from each sample was denatured at 95°C for 5 min. Total protein for each sample was diluted in a loading dye consisting of Tris, 10% SDS, 2-mercaptoethanol and bromophenol blue and then separated on 7.5% SDS-PAGE gel at 75 V for 6 h. The gels were then transferred onto PVDF membranes (Millipore) at 30 V for 16 h at 4°C. Blots were incubated in blocking solution consisting of 4% non-fat dry milk and 0.05% Tween-20 in PBS for 2 h. The primary monoclonal antibodies were diluted in blocking solution and incubated with the blots for 2 h at room temperature. Blots were washed three times in PBS/Tween for 5 min each. Blots

Table 1 Primers (S sense, A antisense) for gene

Gene	Primer	Sequence 5' → 3'
<i>MyHCI</i>	S	GCCTGGGCTTACCTCTCTATCAC
	A	CTTCTCAGACTTCCGCAGGAA
<i>MyHCIIa</i>	S	CAGCTGCACCTTCTCGTTTG
	A	CCCGAAAACGGCCATCT
<i>MyHCIIx</i>	S	GGACCCACGGTCGAAGTTG
	A	CCCGAAAACGGCCATCT
<i>MyHCIIb</i>	S	CAATCAGGAACCTTCGGAACAC
	A	GTCCTGGCCTCTGAGAGCAT
<i>FoxO1</i>	S	TCAAGGATAAGGGCGACAGC
	A	TGTCCATGGACGCAGCTCTT
<i>Gadd45α</i>	S	AGACCGAAAGGATGGACACG
	A	TGACTCCGAGCCTTGCTGA
<i>MCIP1.4</i>	S	AGTCCCTGATTGCCTGTGT
	A	TTTGGCCCTG GTCTCACTTT
<i>TnI slow</i>	S	AGTTGAGAGGAAATCCAAGAT
	A	CTTCAGCTTCAGGTCCTTGAT
<i>Myoglobin</i>	S	CACCATGGGGCTCAGTGATG
	A	CTCAGCCCTGGAAGCCTAGC
<i>β-actin</i>	S	CGTGAAGATGACCCAGATCA
	A	CACAGCCTGGATGGCTACGT

were then incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were washed as before and detection with Bio-Rad GS-800 densitometer and analyzed using Quantity One software. All Western analyses were repeated as two independent experiments.

Immunofluorescence

The immunostaining of muscle fibers in culture was performed by using mAbs against total MyHC. On the third day of culture, myotubes were fixed and processed for immunohistochemistry. Cells were rinsed with Dulbecco's PBS (DPBS), fixed in 4% paraformaldehyde for 60–90 min, washed in DPBS, incubated in DPBS supplemented with 1% BSA and 0.05% permeabilization solution, and blocked for 30 min with 10% goat serum and 1% BSA in DPBS. Cultures were then incubated overnight with primary antibodies against sarcomeric MyHC (clone MF-20, DSHB), followed by the secondary antibody Cy3 anti-mouse IgG1. The secondary antibody was diluted 1:100 in DPBS containing 10% goat serum, and incubation was performed for 1 h at room temperature.

Calcineurin phosphatase activity assay

Calcineurin activity was measured spectrophotometrically using the Calcineurin Cellular Assay Kit Plus (BIOMOL).

In brief, cells were lysed in lysis buffer provided by the kit. Phosphatase activity was measured by detection of free phosphate released from the calcineurin-specific RII phosphopeptide, and normalized to protein content. Assays were performed in quadruplicate in three independent experiments.

Statistical analysis

All transfection experiments were carried out 3–5 times in triplicate. Main and interactive effects were analyzed by One-way ANOVA using SPSS13.0 software. When justified by One-way ANOVA, differences between individual group means were analyzed by Fisher's PLSD test. Differences were considered statistically significant at $P < 0.05$.

Results

Endogenous *FoxO1* and *MyHCs* genes expression in skeletal muscle

In mammalian skeletal muscle, myofibers are mainly classified into fast-glycolytic and slow-oxidative fibers based on specific MyHC isoform expression. As a first step to explore the potential relationship between FoxO1 and muscle fiber expression in muscle, the authors examined the expression pattern of *FoxO1* and *MyHCs* in fast- and slow-twitch skeletal muscles by quantitative real time RT-PCR. As shown in Fig. 1, mouse slow-oxidative soleus consists of about 60% *MyHC I* slow fibers and 40% *MyHC IIa* fast oxidative fibers, while fast-glycolytic gastrocnemius is mainly composed of *MyHC IIb* and *MyHC IIx* fast fibers (>90%, together).

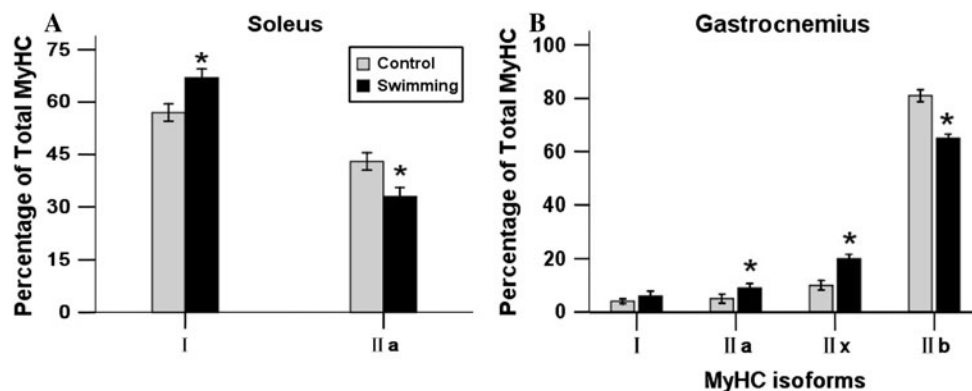


Fig. 1 Myosin heavy chain (MyHC) isoforms mRNA distribution in soleus and gastrocnemius of untrained (gray bars) and mice swimming trained (black bars) for 4 weeks normalized against β -actin. **a** Quantitative real-time RT-PCR (qRT-PCR) showed soleus consists of *MyHC I* and *MyHC IIa* fibers. After chronic training, the

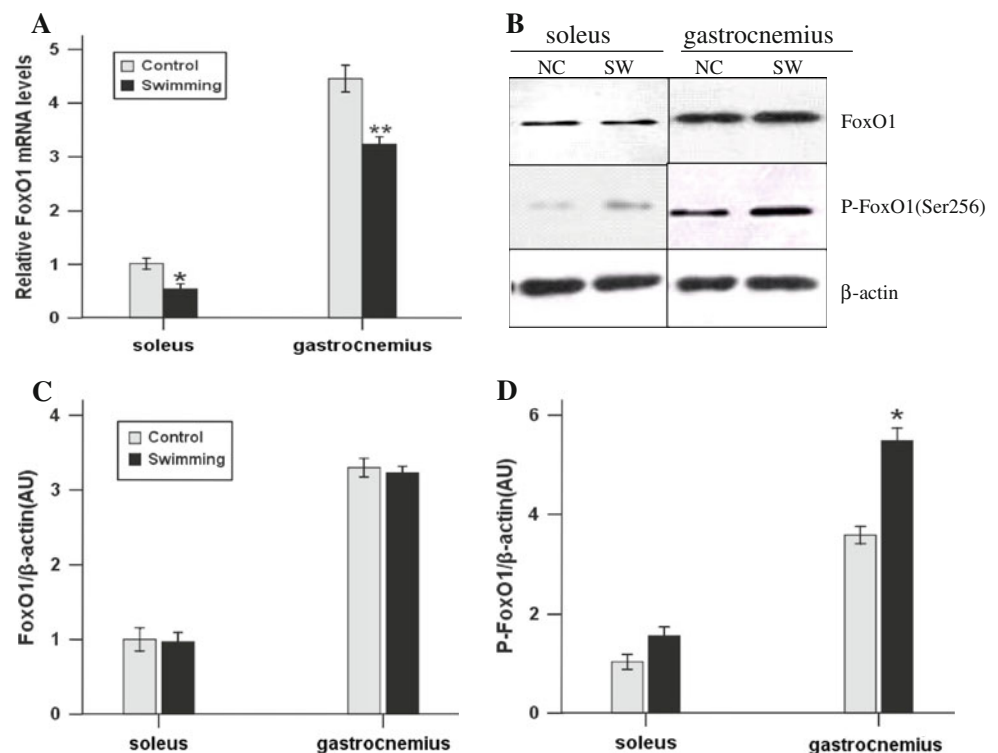
percentage of *MyHC I* was increased but *MyHC IIa* was decreased in soleus. **b** Gastrocnemius is mainly composed of *MyHC IIb* and *MyHC IIx* fibers. Compared to untrained mice, *MyHC IIa* and *IIx* were up-regulated but *MyHC IIb* was down-regulated in gastrocnemius. Values are means \pm SEM; $n = 6$ in each group. * $P < 0.05$

Inactivation of FoxO1 after long-term swimming training

The real-time PCR also showed that *FoxO1* expression was significantly related to muscle fiber type distribution and expressed preferentially in muscle enriched in fast fibers ($P < 0.05$) (Fig. 2a). Accordingly, the comparison between gastrocnemius and soleus in control mice showed that FoxO1 protein expression was 3–4 times greater in the fast than in the slow muscles ($P < 0.05$) (Fig. 2b, c). To further understand the relationship between FoxO1 and the specification of muscle fiber type, the authors subjected mice to swimming training [25, 28]. After an chronic training based on 4 weeks with daily swimming training, the authors found an increase in the percentage of *MyHC I* and a concomitant decrease in *MyHC IIa* in soleus while an up-regulation of *MyHC IIa* and *IIx*, but a down-regulation of *MyHC IIb* in gastrocnemius of trained mice compared to untrained mice ($P < 0.05$) (Fig. 1a, b). These data suggest that the endurance swimming training program was enough to switch the fiber from fast to slow-twitch type.

FoxO1 expression at the level of mRNA was markedly affected by training, since a significant reduction of *FoxO1* mRNA was detected in both slow-oxidative and fast-glycolytic muscles of trained mice when compared to untrained mice (Fig. 2a). Surprisingly, Western blot did not detect any change in the amount of total FoxO1 protein in both soleus and gastrocnemius muscles of the trained and the untrained mice (Fig. 2b, c). However, an increase in FoxO1 phosphorylation at Ser256 was observed in both slow and fast muscles after long-term endurance exercise (Fig. 2b, d). These results may reflect that the loss of FoxO1 function could contribute to the transformation of fast to slow twitch fibers in vivo.

Fig. 2 FoxO1 loses activity after swimming training. **a** qRT-PCR showed *FoxO1* mRNA expression pattern in soleus and gastrocnemius of untrained and swimming trained mice. *FoxO1* is expressed preferentially in gastrocnemius and a significant reduction of *FoxO1* mRNA contents both in two muscles compared to untrained mice. **b–d** Western blot analysis of FoxO1 protein expression in soleus and gastrocnemius of untrained (NC) and trained (SW). Western blot results showed FoxO1 protein expression was higher in gastrocnemius than in soleus muscles. An increase in FoxO1 phosphorylation at Ser256 compared to untrained mice. Values are means \pm SEM; $n = 6$ in each group. * $P < 0.05$, ** $P < 0.01$



FoxO1 induces slow to fast-twitch fiber transition

The authors have demonstrated that FoxO1 is selectively highly expressed in fast muscle, and changes after fiber-type transition in vivo. Next, the authors used C2C12 mouse cell line to investigate the role of FoxO1 in muscle fiber determination in vitro. First, the authors stably transfected C2C12 mouse cell line with plasmids expressing FoxO1-WT and FoxO1-A3 (the constitutively active FoxO1) followed by the exposure of the cells to differentiation medium (Fig. 3e). Then the authors detected the time-course expression of early (MyoD), intermediate (myogenin), and late (MyHC) myogenic transcription factors during the differentiation of skeletal muscle C2C12 myoblasts (Fig. 3g). Because MyoD is the predominant myogenic factor in fast fibers while myogenin is the predominant factor in slow fibers [29], the authors found that the overexpression of FoxO1 mutant had a little reduction in MyoD expression but an approximate 40% decrease in myogenin protein on day 3 of differentiation (Fig. 3f, h).

The real-time PCR results obtained so far had described the relative abundance of each endogenous MyHC isoform and the changes of each *MyHC* gene isoform in FoxO1-A3 cells on day 3 of differentiation (D3). In control-infected C2C12 myotubes, *MyHC IIx* mRNA was the most highly expressed adult isoform, followed by *MyHC IIb*, *MyHC I*, and *MyHC IIa* (Fig. 4a). The authors demonstrated that C2C12 myotubes induced by FoxO1 was attributable to combine effects of the up-regulation of *MyHC2x* and the

down-regulation of *MyHC I*, *MyHC IIa* and *MyHC IIb* (Fig. 4a).

To evaluate whether the transcriptional effects of FoxO1 were translated into protein changes, Western blot analysis was performed. The results indicated that FoxO1 enhanced fast MyHC protein but suppressed MyHC slow protein (Fig. 4b,c), which was consistent with the MyHCs transcript expression (Fig. 4a). Notably, the relative expression of the total sarcomeric MyHC protein was also decreased in FoxO1-expressing cells. These results suggest that FoxO1 plays a critical role in the myofiber specification toward fast-twitch type.

FoxO1 decreases oxidative capacity in C2C12 myotube

To determine whether a decrease in FoxO1 function induces the differentiation of slow fiber type, cells were treated with resveratrol (RSV, 100 μ M), an activator of Sirt1, which inhibited the endogenous FoxO1 activity [30–32]. As expected, *Gadd45 α* , which is known to be regulated by FoxO transcription factors [33, 34], was significantly decreased (Fig. 5a). The results showed that treatment of cells with RSV increased the oxidative fibers related gene *TnI slow* and *myoglobin* (Fig. 5b) whereas it was not enough to change the ratio of fast glycolytic to slow oxidative-type muscle fibers (Fig. 5c).

To test whether RSV's effects on oxidative fibers function are mediated by FoxO1, the overexpression of FoxO1 largely blocked the RSV-induced increase in *TnI*

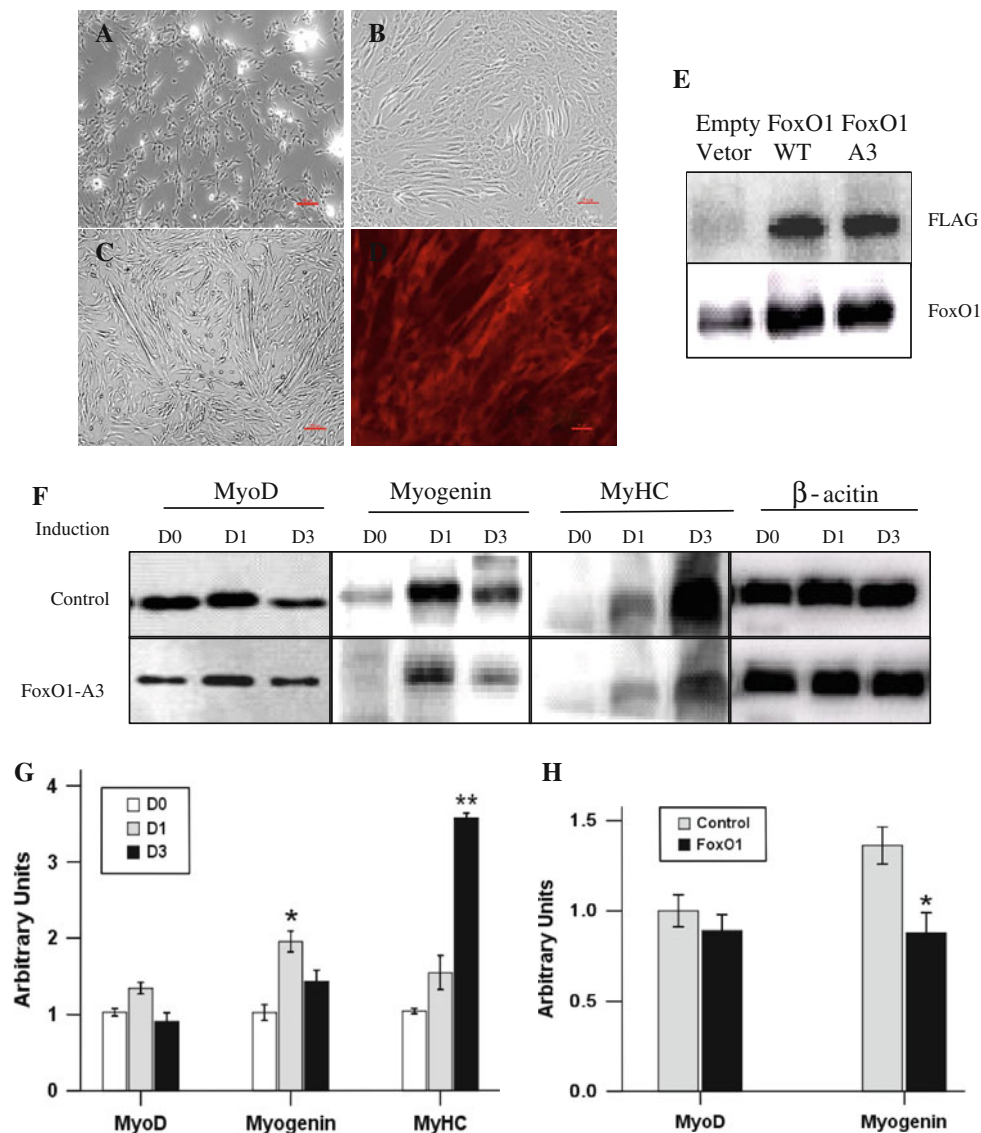


Fig. 3 FoxO1 regulates C2C12 myoblast differentiation. Proliferating C2C12 cells were infected for 2 days in proliferation medium (PM) and followed by 3 day in differentiation medium (DM). **a–d** Differentiation and Morphometric analysis of C2C12 myoblast differentiation at 0 day (D0), 1 day (D1) and 3 day (D3). C2C12 cells were immunostained with anti-MyHC antibody. Bar 100 μ m. **e** Cell lysates derived from control empty vector pcDNA3.1, FoxO1-WT, and FoxO1-A3 infected cells were subjected to Western analysis for FLAG and FoxO1 expression. **f** Western blot detected the time-course expression of myogenic markers (MyoD, myogenin, and MyHC)

slow and myoglobin expression (Fig. 5b). Importantly, in FoxO1-infected C2C12 cells addition of RSV reversed FoxO1-induced muscle fiber switch (Fig. 5c).

FoxO1 inhibits calcineurin phosphatase activity

The calcineurin/NFAT pathway is important for the formation of slow oxidative fibers [20, 35]. The authors

during the differentiation of C2C12 myoblasts transduced with empty pcDNA3.1 and FoxO1-A3 constructs. **g** Densitometric analyses showed MyoD and myogenin proteins significantly increased in the early differentiation time (D1) and then decreased both in the late differentiation stage (D3) in control cells. **h** MyoD expression had a little reduction and an almost 40% decrease in myogenin protein in FoxO1-expressing cells at 3 days differentiation (D3). Results are expressed as mean \pm SEM from triplicate samples within the same experiment. * $P < 0.05$, ** $P < 0.01$

examined whether FoxO1-mediated myofiber switch was associated with calcineurin/NFAT signaling. In this study, relative to empty vector-infected control cells, cells expressing FoxO1-A3 showed a significant decrease in endogenous calcineurin phosphatase activity (Fig. 6a). Figure 6b shows that FoxO1 activation significantly decreased the mRNA abundance of modulatory calcineurin interacting protein, exon 4 isoform (MCIP1.4), a direct target of the calcineurin/NFAT pathway [36, 37].

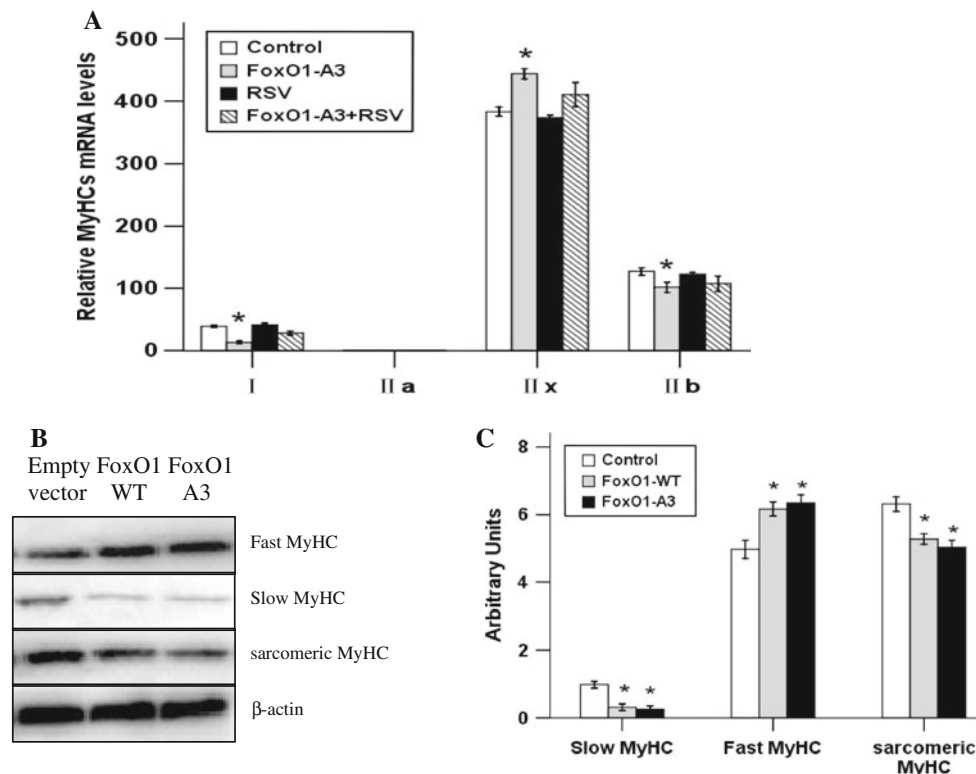


Fig. 4 FoxO1 induces the formation of fast-twitch fiber. Proliferating C2C12 cells were infected for 2 days in PM and followed by 3 day in DM (D3). **a** Real-time PCR was performed to quantify the relative levels of MyHC transcripts in C2C12 myotubes at day 3 of differentiation (D3). FoxO1 overexpression in C2C12 cells up-regulated *MyHCIIx* but down-regulated *MyHC I*, *MyHC IIa*, and *MyHC IIb*. Inhibition FoxO1 by resveratrol (RSV, 100 μ M) did not

switch the fiber from fast to slow twitch but could partial reverse FoxO1-induced muscle fiber transition. **b** Western blot detected fast MyHC, slow MyHC and total sarcomeric MyHC protein expression in control and FoxO1-infected cells. **c** Densitometric analyses showed FoxO1 enhanced fast MyHC protein but suppressed slow MyHC protein. * $P < 0.05$ relative to control

The effect of RSV treatment on calcineurin activity and found no changes (Fig. 6a) was tested. However, using a more sensitive assay, quantitative real-time PCR showed that inhibition of FoxO1 activity by RSV markedly increased the mRNA abundance of *MC1P1.4* (Fig. 6b). Furthermore, RSV antagonized FoxO1-induced down-regulation of *MC1P1.4* expression (Fig. 6b), coincident with previous study by the authors on increased muscle oxidative capacity (Fig. 5b).

Discussion

FoxO1 is a transcription factor that has been shown to be a negative regulator of muscle growth [38, 39] and involved in metabolism and mitochondrial biogenesis [9]. In this study, the authors describe that FoxO1 induces a slow-to-fast fiber-type switch and suppresses muscle oxidative capacity at least in part through inhibition of the calcineurin pathway.

These specialized myofibers are highly plastic, and under several physiological and pathological circumstances, the

metabolic and enzymatic nature of the fiber can be altered [6]. The most notable is exercise training, and an endurance exercise protocol could increase muscle oxidative capacity and trigger transformation of glycolytic fibers into oxidative ones [5, 7]. Previous study discovered *FoxO1* gene increased by 5.2-fold at 3 h, but returned to baseline by 48 h during recovery from acute training in human skeletal [40], consistent with other studies [41]. In addition, acute exercise improved insulin signaling, increasing insulin-stimulated Akt, and FoxO1 phosphorylation [42]. However, FoxO1 expression pattern after endurance exercise is still unclarified. Here, Swimming training to mimic endurance exercise-induced adaptation was adopted [25] and FoxO1 expression change in this process was detected. Concomitant with the increase in slow fiber type (Fig. 1), the authors also found a significant reduction of FoxO1 mRNA contents both in slow and fast muscles compared to untrained mice (Fig. 2b,c). Surprisingly, total FoxO1 protein has no change; however, the level of FoxO1 phosphorylation at Ser256 was increased (Fig. 2b, d). These results reflect that FoxO1 inactive appears to influence muscle fiber-type composition.

Fig. 5 Inhibition of FoxO1 increases myotube oxidative capacity. **a** RSV (100 μ M) inhibited the expression level of the FoxO1 target gene *Gadd45a*. **b** The real-time PCR showed FoxO1 largely blocked RSV-induced the mRNA abundance of oxidative fiber markers. **c** Western blots and densitometric analyses showed effects of RSV on muscle fiber transition. Enhanced fast MyHC expression by FoxO1-A3 was inhibited in the presence of RSV whereas suppressed slow MyHC was abrogated. * $P < 0.05$, ** $P < 0.01$

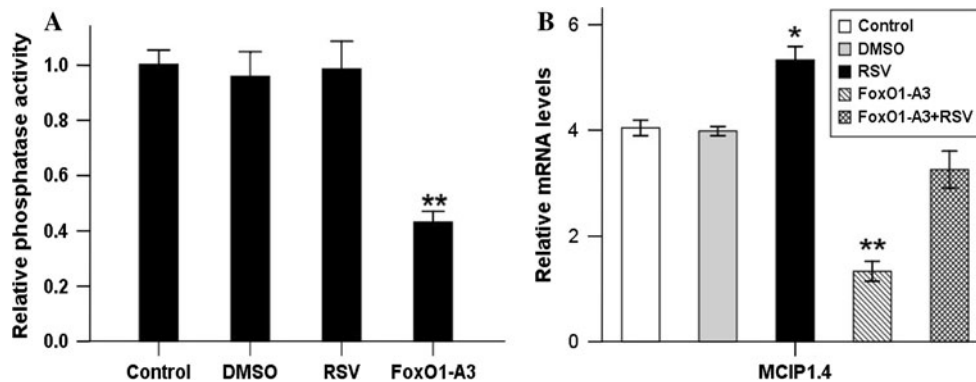
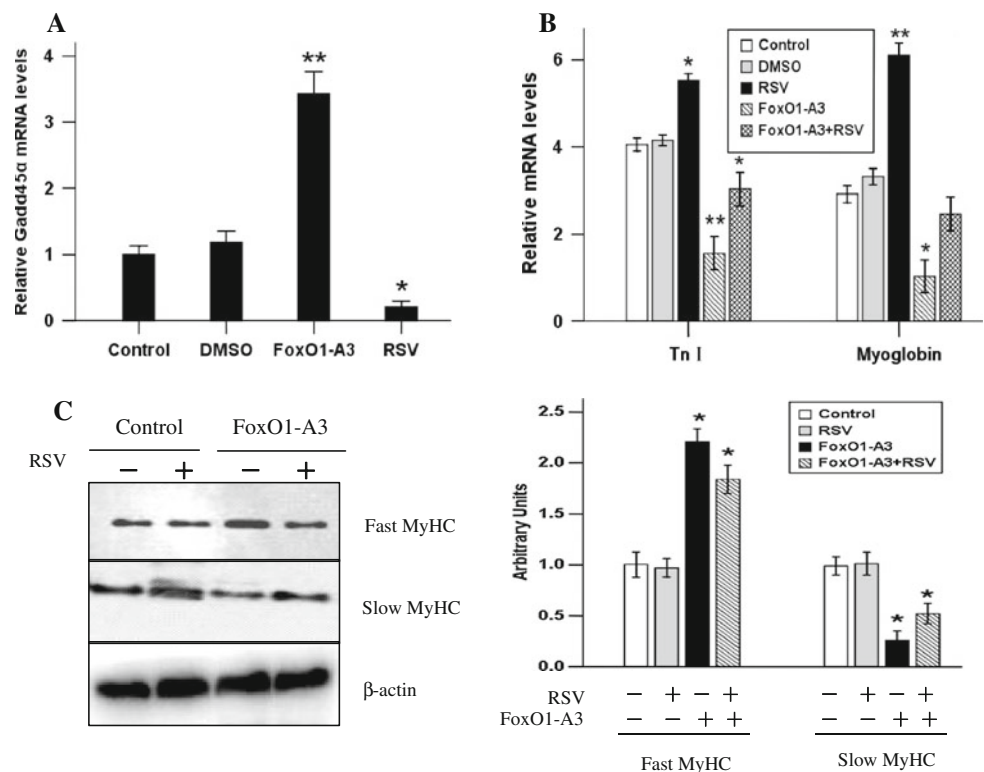


Fig. 6 FoxO1 decreases calcineurin phosphatase activity. **a** Relative phosphatase activity was normalized to protein content. FoxO1 inhibited calcineurin phosphatase activity, and RSV (100 μ M) was not sufficient to induce the clearly increased calcineurin activity. **b** Overexpression of FoxO1 decreased calcineurin/NFAT pathway

target gene *MCIP1.4*, while RSV (100 μ M) antagonized the activity of FoxO1 factor. Results are graphed as mean \pm SEM of a representative experiment with quadruplicate samples. * $P < 0.05$, ** $P < 0.01$

Myogenic regulation factors are important regulators of myogenesis and, recently have been shown to have a role in the regulation of *MyHC* gene expression. MyoD and myogenin mRNAs are preferentially expressed in adult fast-glycolytic and slow-oxidative muscle fibers, respectively [29]. Overexpression MyoD or Myf-5 significantly increased MyHC IIB promoter activity [43]. In this study, the authors have clearly shown that overexpression FoxO1 leads to decreased myogenin transcription factor (Fig. 3f, h). It is noteworthy that FoxO1 activation also up-regulates

MyHCIIx but down-regulates the other three type *MyHC* genes, viz., *MyHC I*, *MyHC IIa*, and *MyHC IIb* (Fig. 4a). Thus, in FoxO1-infected myotubes, the changes in MyHC isoforms tend to follow a general scheme of sequential and reversible transitions: I \leftrightarrow IIa \leftrightarrow IIx \leftrightarrow IIb [1, 6]. Furthermore, the rise in fast MyHC protein and the reduction of MyHC slow protein were revealed. These findings suggest that FoxO1 promotes the formation of fast-twitch fiber at the expense of slow-twitch fiber. Interestingly, the authors use MF-20 monoclonal antibody specific for sarcomeric MyHC

and find that the expression of the total MyHC protein was also suppressed in FoxO1-expressing myotubes (Fig. 4b), which probably connect with FoxO1-caused muscle atrophy [38, 39].

Even though an increase in fast twitch fiber's composition was noted in FoxO1-infected cells, inhibition of endogenous FoxO activity by RSV was not sufficient to change the ratio of oxidative to glycolytic type (Figs. 4a, 5c). However, the expression of oxidative fibers-related gene *troponin I* and *myoglobin* was increased by RSV inhibition of FoxO1 (Fig. 5b). Moreover, the active form of FoxO1-mutant mediated the transformation from slow-to-fast fiber type was partially reversed by addition of RSV (Figs. 4a, 5c). Therefore, FoxO1 functions as a negative factor in oxidative metabolism. These findings further support the notion that FoxO1 induces slow-to-fast fiber transition. On the other hand, RSV increases the activities of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) [44, 45], both of which are required for mitochondrial biogenesis and could improve the oxidative activity of skeletal muscle [46, 47]. Also, PGC-1 α is a principal factor regulating muscle fiber-type determination, which is known to be preferentially expressed in slow muscle and drives the formation of slow-twitch muscle fibers [48]. As the FoxO1 protein can interact with the PGC-1 α protein [49] and transfection of PGC-1 α into adult fibers reduced the capacity of FoxO3 [50], FoxO1 may counteract the function of PGC-1 α in this process. Thus, further studies are required to examine this possibility.

Several signaling pathways are involved in mediating fiber type-specific gene expression. To the best of knowledge, calcineurin is a chief regulatory pathway of slow fiber-selective gene expression [21]. Overexpression of FoxO1 provokes a remarkable decrease in calcineurin phosphatase activity (Fig. 6a) and reduces the basal mRNA level of the MCIP1.4 (Fig. 6b), a direct target of the calcineurin pathway, consistent with previous reports on FoxO1 in heart [22, 23]. Interestingly, the authors detected no changes in calcineurin phosphatase activity triggered by RSV (Fig. 6a). However, RSV treatment significantly increased the mRNA abundance of MCIP1.4 in FoxO1-infected cells (Fig. 6b), consistent with the enhanced expression of oxidative-related gene (Fig. 5b). The data so far available show inhibition of FoxO1 could promote muscle oxidative capacity at least in part through inhibition of the calcineurin pathway.

In summary, the authors have established the relationship between skeletal muscle fiber-type specification and a function of FoxO1 expression. FoxO1 promotes slow-to-fast fiber-type switch and attenuates muscle oxidative metabolism at least, in part, through inhibiting calcineurin pathway.

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