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KLF15 regulates slow myosin heavy chain expression through NFATc1 in C2C12 myotubes

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ABSTRACT

A comprehensive understanding of genetic and environmental factors that control skeletal muscle fiber type specification and transformation is essential not only in sports science, but also in myopathy and metabolic disorders. Krüppel-like factors (KLFs) are a subfamily of the zinc-finger class of transcription factors, which are involved in the development, homeostasis, and pathology of cardiovascular systems. Compared to cardiac and smooth muscles, the role of KLFs in skeletal muscle is much less understood. In this study, the endogenous expression of KLF15 was analyzed in differentiating C2C12 muscle cells and mouse skeletal muscle. Our data indicated that *Klf15* was upregulated during myogenic differentiation and higher levels of *Klf15* mRNA were detected in mouse slow, oxidative *soleus* muscle (SL) compared to that in fast, glycolytic *tibialis anterior* muscle (TA), indicating that KLF15 may play a role in myogenesis or myofiber typing. Additional studies revealed that KLF15 regulated the expression of MHC-β/slow rather than muscle cell differentiation. Gene silencing, overexpression, and luciferase reporter assay showed that KLF15 regulated MHC-β/slow by binding to *Nfatc1* promoter, inducing its activity, therefore mediating calcineurin/NFAT signaling. Our study contributed to the current knowledge on KLFs in skeletal muscle, and it indicated a need for further intensive studies on the redundant and divergent functions of KLFs.

1. Introduction

The skeletal muscle is composed of muscle fibers with coordinated expression of a distinct set of structural proteins and metabolic enzymes. Muscle fibers are classified into different types based on the predominantly expressed myosin heavy chain (MHC) isoform within cells. In the adult trunk and limb skeletal muscle, four major MHC isoforms, MHC-β/slow, MHC-2a, MHC-2x/d, and MHC-2b are expressed and muscle fibers are classified as type I/slow, IIa, IIx/d, and IIb [1,2]. Mammalian skeletal muscles are heterogeneous in nature. The relative proportion of the different fiber types varies between muscles, between species, and even between individuals. Since different muscle fiber types differ in metabolic, physiological, and biochemical properties, and since muscle performance is in part dictated by muscle fiber composition, a comprehensive understanding of genetic and environmental factors as well as signaling pathways that determine the fiber type profile and muscle remodeling is essential not only in sport science, but also in neuromuscular and metabolic diseases.

Krüppel-like factors (KLFs) are a large family of zinc finger-containing transcription factors that regulate diverse arrays of biological processes including cell stemness, proliferation, differentiation, apoptosis, and energy metabolism. Alterations in their functions have been associated with the pathobiology of several human diseases including cardiovascular disease, metabolic disorders, and cancer [3]. Currently, 17 mammalian KLFs have been identified. They are designated as KLF1–KLF17 considering the chronological order of identification. Despite the initial description of the *Drosophila* Krüppel protein as a critical determinant of the myogenic fate in the gestating fly [4], the role of KLFs in muscle biology, especially their role in skeletal muscle, only started to be understood [5]. So far, more than 10 KLFs are expressed in skeletal muscle cells. However, only few reports described their regulation and function in skeletal muscle [6–11]. KLF15 is among these members. It is robustly expressed in skeletal muscle, but its regulation and function are not well defined. In the heart, KLF15 has been reported to inhibit cardiac hypertrophy by inhibiting GATA4 and myocyte enhancer factor 2 (MEF2) function [12]. KLF15 has also been reported to strongly inhibit myocardin activity and inhibition of KLF15 by activated p38 MAPK is critical for the induction of left ventricular hypertrophy (LVH) [13]. In smooth muscle, KLF15 inhibits cell proliferation and migration, and regu-

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lates smooth muscle response to vascular injury [14]. In skeletal muscle, KLF15 has been reported to serve as a molecular link between myogenic factors and the D4Z4 enhancer, mediating facioscapulohumeral muscular dystrophy (FSHD)-related gene expression [15]. Since KLF15 is upregulated in myogenic differentiation induced by serum starvation or by overexpression of MyoD [15], it is likely that KLF15 plays a role in the transformation of myoblasts to myotubes. However, its functions in myogenesis, muscle growth and development, or muscle fiber type specification have not yet been reported.

To further our knowledge of KLF15 in skeletal muscle, its role in myogenic differentiation was investigated using C2C12 myoblasts. The data obtained showed that, although KLF15 expression was elevated during differentiation, it had no effect on myogenic differentiation of C2C12 cells. Analysis of fiber type specific gene expression revealed that KLF15 upregulated slow-twitch fiber gene *Myh7* (MHC-β/slow). Additional experiments on the underlying mechanism indicated that KLF15 promoted slow myosin heavy chain expression by direct targeting of NFATc1 (Nuclear factor of activated T-cells, cytoplasmic 1) promoter to activate its expression. Our data revealed that KLF15 coordinates muscle cell identity and functions by acting on both oxidative metabolism-related enzyme [16,17] and contractile protein expression.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 myoblasts were seeded in 35-mm or 60-mm culture dish in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin and streptomycin in 5% CO_2 at 37 °C. The cells were induced to differentiate in differentiation medium (DMEM containing 2% horse serum, Gibco) when they reached 80–90% confluence. The medium was then replaced daily for 4 days before analysis.

2.2. Small RNA interference (siRNA)

Once C2C12 myoblasts reached 80–90% confluence, they were transfected with 3 μL of scrambled sequence control or gene-specific siRNA (Genepharma, China) in OPTI-medium (Invitrogen, Carlsbad, CA) using 3-μL Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After transfection, the cells were induced to differentiate as described above. The siRNA sequences were 5′-GUACCAUCCUCCAACUUGATT-3′ (forward) and 5′-UCAAGUUGGAGGAUGGUACTT-3′ (reverse) for *Klf15*, and 5′-CCCGUCCAAGUCAGUUUCUAUTT-3′ (forward) and 5′-AUAGAAACUGACUUGGACGGGTT-3′ (reverse) for *Nfatc1*.

2.3. Overexpression of KLF15

The eukaryotic mouse KLF15 expressing plasmid (PcDNA3.1-KLF15) was a kind gift from Susan J. Gray (University of Massachusetts Medical School). When the cells reached 80–90% confluence, 3 μg of the control vector (PcDNA3.1) or expression vector were transfected into C2C12 myoblasts using the same protocol as described above for siRNA. After transfection, the cells were induced to differentiate in DMEM containing 2% horse serum and the medium was replaced daily.

2.4. RNA isolation and quantitative real time PCR (qPCR)

Total RNA from differentiated myotubes or mouse skeletal muscle was isolated using Trizol reagent (TaKaRa, Japan). The concentration of RNA was determined using a NanoDrop® ND-1000 (Thermo, Pittsburgh, PA). Five hundred nanograms of RNA was transcribed into single-stranded cDNA using the PrimerScriptTM RT reagent (TaKaRa, Japan), and used for PCR amplification. qPCR was performed using the SYBR premix ExTaq II (TaKaRa, Japan) and the IQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The sequences of the primers are listed in the Supplementary Table S1.

2.5. Western blotting

C2C12 myotubes were lysed in RIPA lysis buffer (Sangon, China) containing PMSF and collected. Collected cell lysates were centrifuged at 12,000*g*, 4 °C for 10 min. The supernatant was used for immunoblotting assay. Briefly, after separation by SDS–PAGE, the proteins were transferred to PVDF membranes (Millipore, Billerica, MA), blocked, and immunoblotted with the primary antibody overnight at 4 °C. The primary antibodies were mouse monoclonal antibodies against NFATc1 (DSHB, University of Iowa, USA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the primary antibody, the membranes were washed three times with TBS–Tween 20, then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti mouse IgG, Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were visualized by application of chemoluminescent substrate (Millipore) and quantified by using Quantity One software (Bio-Rad).

2.6. Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were then washed three times with PBS and permeabilized in 0.3% Triton X-100-PBS for 15 min. Cells were blocked in 2% goat serum in TBS for 1 h at room temperature and incubated with anti-MHC (sarcomeric myosin heavy chains) and anti-MyoG (myogenin) primary antibodies (1:200, DSHB) overnight at 4 °C. After three washes in TBST, the cells were incubated with the secondary antibodies, AlexaFluor-488 and AlexaFluor-568 conjugated mouse IgG1 and rabbit IgG2b (1:1000, Invitrogen) for 2 h at room temperature. To stain the nuclei, the specimen was incubated with DAPI for 15 min. Images were collected using a NIKON TE2000-U fluorescent microscope (Nikon, Japan). Total nuclei, myogenin-positive and MHC-positive nuclei were counted separately.

2.7. Luciferase reporter vector construct and activity assay

A 1887 bp (from −1554 bp to +333 bp) 5′-flanking region of the mouse *Nfatc1* gene was amplified by PCR from the genome DNA library generated from mouse skeletal muscle with a forward primer (*Xho I* + 5′-CCCTCGAGATCCTCTCAAAGCCAGAGGTAGA-3′) and a reverse primer (*Hind III* + 5′-CCAAGCTTAAGGGACTGGAAAGCTGGTACTT-3′). The primers used for cloning were designed to include restriction enzyme sites so that the PCR products could be easily cloned into the multicloning sites (*Xho I* and *Hind III*) of the vector pGL3-Basic (Promega, Madison, WI). The Renilla (sea pansy) luciferase vector pRltk was co-transfected into HEK293T cells as an internal control to normalize the transfection efficiency. Forty-eight hours after transfection, the cells were washed twice with cold Dulbecco's Phosphate-Buffered Saline (D-PBS) and lysed with 1× passive lysis buffer (Promega). Firefly luciferase activities and Renilla luciferase activities were measured sequentially using the Dual-luciferase reporter assay system (Promega). The luminescence signal was detected by VICTOR™ *X*5 multilabel plate reader (PerkinElmer, Waltham, MA). The firefly luciferase activity was

normalized to the Renilla luciferase activity and expressed as relative luciferase units (RLU) to reflect the promoter activity.

2.8. Statistical analysis

Statistically relative alterations of groups were analyzed by an unpaired Student's *t*-test using SPSS 18.0 software. Each experiment was repeated at least three times per group. All data are presented as mean ± SEM.

3. Results

3.1. Endogenous Klf15 gene expression in skeletal muscle and cultured muscle cells

To investigate the role of KLF15 in myogenesis, its transcription profile during myogenic differentiation was first analyzed using C2C12 cells. As shown in Fig. 1A, *Klf15* mRNA expression in C2C12 cells increased by about 2.4-fold after two days of differentiation ($P < 0.01$). This increase in *Klf15* expression persisted afterwards. The upregulation of *Klf15* expression in C2C12 muscle cells during myogenic differentiation indicated that it may play a role in myogenesis. At the same time, the endogenous expression of *Klf15* was analyzed in adult mouse skeletal muscle (Fig. 1B). *Klf15* mRNA level was 3-fold higher in the mouse slow, oxidative *soleus* muscle (SL) than in the fast, glycolytic *tibialis anterior* muscle (TA).

3.2. KLF15 regulates slow myosin heavy chain expression in C2C12 myotubes

To test if KLF15 regulates myogenic differentiation of muscle cells, *Klf15* in C2C12 myoblasts was silenced by siRNA and the cells were then induced to differentiate for 4 days. qPCR analysis showed that siRNA effectively downregulated *Klf15* in C2C12 cells (Supplementary Fig. S1A). Surprisingly, inhibition of *Klf15* did not alter the expression of the myogenic regulatory factor myogenin (Supplementary Fig. S1B), an early marker of myogenic differentiation, showing that upregulation of *Klf15* (Fig. 1A) was not necessary for myogenesis.

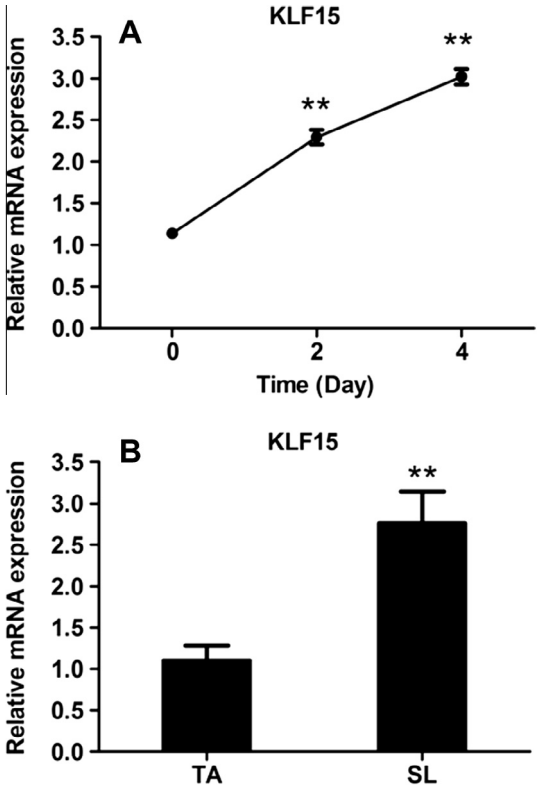

Fig. 1. Expression of endogenous *Klf15* gene. qPCR was used to analyze the *Klf15* mRNA expression during C2C12 myoblast differentiation (A), in mouse slow, oxidative *soleus* muscle (SL) and fast, glycolytic *tibialis anterior* muscle (TA) (B). Data are presented as mean ± SEM. $^{**}P < 0.01$; $n \geqslant 3$.

Additionally, C2C12 myotubes transfected with a scrambled sequence (mock) or *Klf15* siRNA were immunostained for myogenin and sarcomeric myosin heavy chains (MHC), a final differentiation marker for muscle cells. As shown in Supplementary Fig. S1C–E, the knockdown of *Klf15* had no effect on myogenic differentiation of C2C12 myoblasts as evidenced by the lack of difference in the number of myogenin- and MHC-positive nuclei between mock and *Klf15* knockout cells indicating that KLF15 may not regulate muscle cell differentiation.

Since knockdown of *Klf15* by siRNA had no effect on myogenic differentiation of C2C12 cells (Fig. S1) and that it is expressed in the slow, oxidative SL muscle (Fig. 1B), it is reasonable to hypothesize that KLF15 may play a role in muscle fiber specific gene expression and muscle fiber typing. To test this hypothesis, the expression of the four myosin heavy chain isoforms was analyzed by qPCR in C2C12 myotubes. The results obtained showed that knockdown of *Klf15* by siRNA significantly repressed the expression of slow myosin heavy chain (MHC-β/slow) ($P < 0.05$) with no change in fast myosin heavy chains of MHC-2a, 2x, and 2b, as well as the total sarcomeric myosin heavy chain (MHC-sar) (Fig. 2A–E). These results confirmed that KLF15 was not necessary for myogenic differentiation of C2C12 myoblasts.

Next, we evaluated the effect of KLF15 overexpression on slow myosin heavy chain expression. A eukaryotic plasmid expressing KLF15 (PcDNA3.1-KLF15) was transfected into C2C12 myoblasts. The cells were then induced to differentiate for 4 days by serum starvation. In comparison to the empty control vector, cells transfected with PcDNA3.1-KLF15 show a 4-fold increase in *Klf15* mRNA (Fig. 2F). Overexpression of KLF15 did not change the expression of sarcomeric myosin heavy chains (Fig. 2G) showing that KLF15 did not regulate myogenic differentiation. However, myotubes with KLF15 overexpression presented an increase in MHC-β/slow expression (Fig. 2H), supporting that KLF15 regulates slow myosin heavy chain expression.

3.3. KLF15 regulation of slow myosin heavy chain depends on NFAT signaling

Calcineurin/NFAT signaling is a well define signaling pathway that regulates slow myosin heavy chain expression in response to calcium stimulation. To explore the mechanism by which KLF15 upregulates slow myosin heavy chain, NFAT signaling was analyzed in both *Klf15* knocked down and KLF15 overexpressing C2C12 myotubes by measuring the expression of *Nfatc1* target gene, modulatory calcineurin interacting protein exon 4 isoform (*Mcip1.4*). The data obtained showed that silencing of *Klf15* by siRNA and overexpression of KLF15 significantly decreased and increased *Mcip1.4* expression in C2C12 myotubes, respectively ($P < 0.05$) (Fig. 3A and B). Analysis also revealed that KLF15 regulated *Nfatc1* gene expression at both the mRNA and protein levels (Fig. 3C–F), showing that NFATC1 was downstream of KLF15. To test if NFATc1 was involved in KLF15 regulation of slow myosin heavy chain, NFATc1 was knocked down by siRNA in C2C12 cells (Fig. 3G) and the expression of MHC-β/slow was analyzed. As shown in Fig. 3H and I, inhibition of NFATc1 abolished KLF15 overexpression induced upregulation of MHC-β/slow showing that KLF15-mediated upregulation of MHC-β/slow is dependent on NFATc1 signaling.

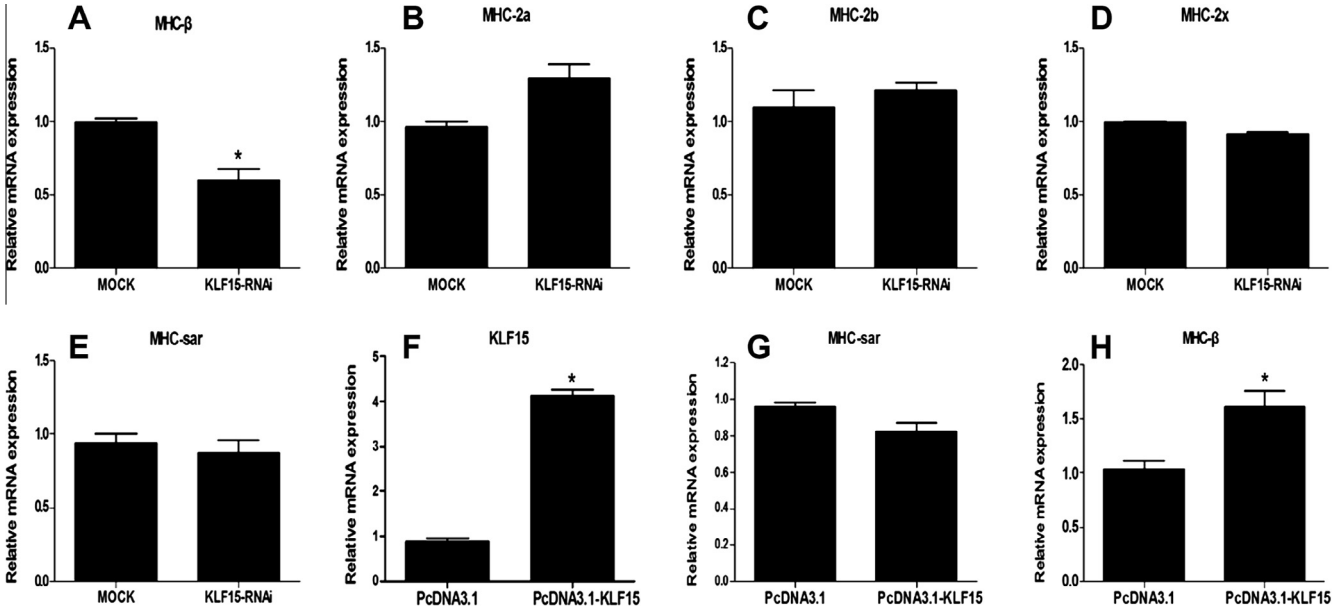

Fig. 2. KLF15 regulates the expression of MHC-β/slow in C2C12 myotubes C2C12 myoblasts were transfected with *Klf15* siRNA or KLF15-expressing eukaryotic plasmid PcDNA3.1-KLF15 when they reached 80–90% confluence and induced to differentiate for 4 days before analysis. (A–E) qPCR analysis of the expression of the different isoforms of myosin heavy chains and the total sarcomeric myosin heavy chain in control and *Klf15* knockout C2C12 myotubes. (F–H) Expression of *Klf15*, sarcomeric MHC, and MHC-β/slow in control and KLF15 overexpressing C2C12 myotubes. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus control; $n \geqslant 3$.

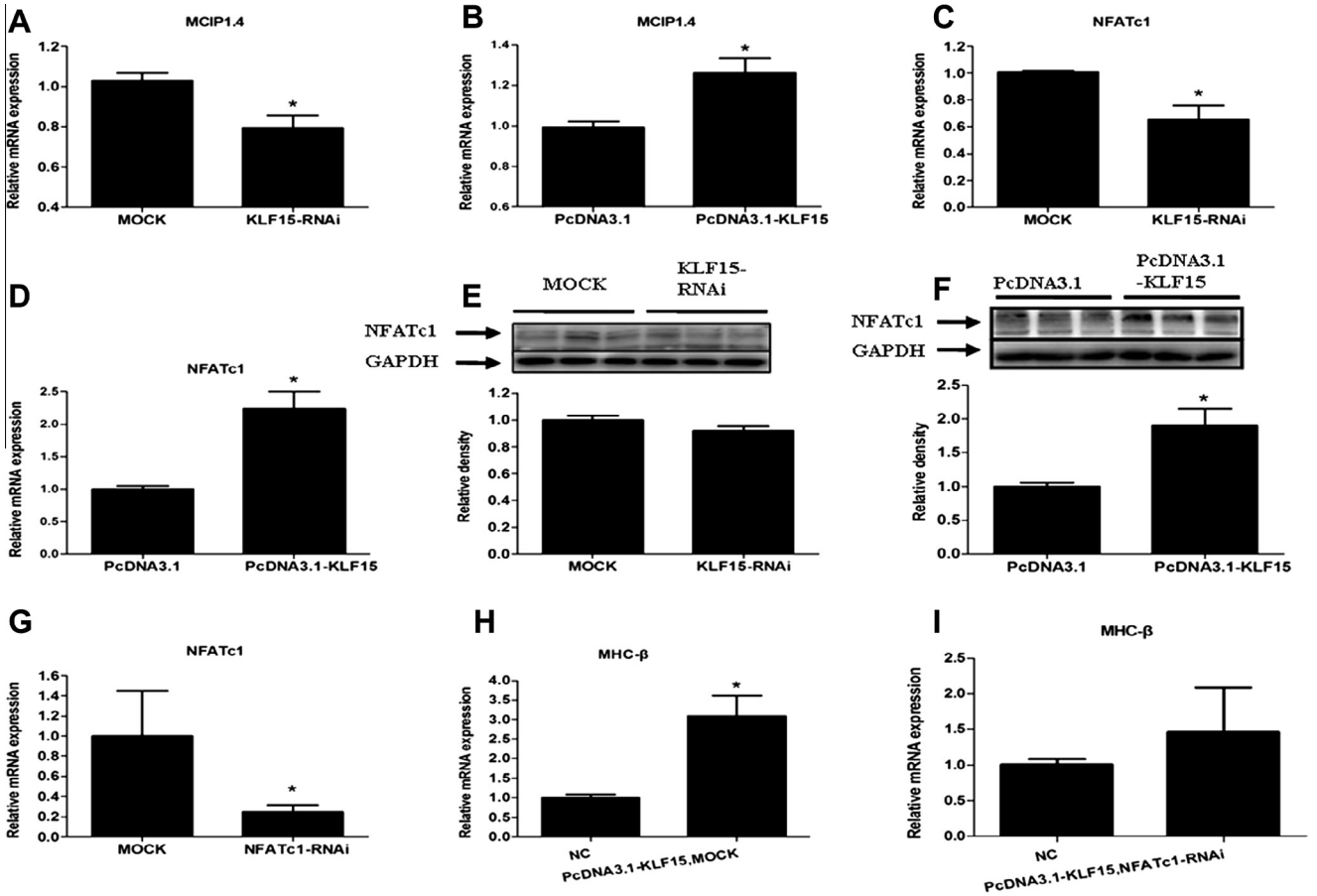

Fig. 3. KLF15 regulates the NFAT signaling by regulating NFATc1 protein expression C2C12 cells were cultured, transfected and induced to differentiate as in Fig. 2. qPCR was used to analyze the mRNA expression of *Mcip1.4* (A, B) and *Nfatc1* (C, D) in C2C12 myotubes. Immunoblot analysis was used to analyze the NFATc1 protein expression in C2C12 myotubes (E, F). qPCR analysis showed that the siRNA induced an 80% downregulation of *Nfatc1* (G). Knockdown of NFATc1 abolished KLF15 overexpression induced upregulation of MHC-β/slow (H, I). Data are presented as mean ± SEM. $^{*}P < 0.05$; $n \geqslant 3$.

3.4. KLF15 regulates NFATc1 expression by direct targeting of the promoter region

To explore the mechanism of regulation of NFATc1 by KFL15, the promoter structure of *Nfatc1* gene (−1554 to +333) was analyzed and nine potential KLF15 binding sites were identified (Fig. 4A). A luciferase reporter vector using the *Nfatc1* promoter (pGL3-Basic-Nfatc1) was constructed and co-transfected with PcDNA3.1 (Control) or PcDNA3.1-KLF15 into HEK293T cells for 48 h. As shown in Fig. 4B, co-transfection of the luciferase reporter vector with PcDNA3.1-KLF15 induced a significant 4-fold increase in the cell lysate luciferase activity ($P < 0.01$) when compared to the control showing that KLF15 upregulates NFATc1 through direct activation of its promoter activity.

4. Discussion

KLFs are a subfamily of the zinc-finger class of transcription factors, which are involved in the development, homeostasis, and pathology of numerous organ systems, including the cardiovascular, digestive, respiratory, hematological, and immune systems [3]. KLFs are characterized by three C_2H_2 zinc fingers motifs at the extreme C-terminus of the protein which allow KLFs to bind GC-rich sequences, with a preference for the 5′-CACCC-3′ core motif, in the promoter and enhancer regions of the genes they regulate [18,19]. In contrast to the zinc finger regions, the amino-terminal sequences of KLFs are highly divergent, providing unique regions for interaction with specific binding partners to modulate transactivation and trans-repression, and often mediate protein–protein interactions.

Numerous reports have described the involvement of KLFs in cardiovascular development, function, and pathology of diseases. Compared to cardiac and smooth muscles, the role of KLFs in skeletal muscle is far less understood. Currently, about 12 KLFs have been reported to be expressed in muscle cells, which include KLF2, KLF3, KLF4, KLF5, KLF6, KLF7, KLF9, KLF10, KLF12, KLF13, KLF15, and KLF16 [7,10,20]. However, only KLF2, KLF3, KLF4, KLF 9, and KLF10 have been reported to participate in the regulation of myoblast proliferation and differentiation. KLF2 and KLF4 have been reported to be involved in the ERK5 regulation of muscle cell fusion [10]. KLF3 regulates muscle cell differentiation by binding to the promoters of several muscle genes, including MCK and α-actin. KLF9 exerts bimodal actions on fibroblast growth factor receptor 1 promoter activity during myogenesis [20]. KLF10 regulates cell cycle progression and myoblasts proliferation [11,21]. KLF15 is highly expressed in skeletal muscle [7]. Several reports described its function in glucose uptake [7], amino acid catabolism and gluconeogenesis [22], and lipid metabolism [16,17]. However, no study reported its role in myoblast proliferation, differentiation, or myofiber specific gene expression and muscle fiber identification.

Since KLF15 expression is upregulated in differentiating muscle cells ([15] and Fig. 1A) and higher levels of *Klf15* mRNA were detected in slow, oxidative SL muscle compared to fast, glycolytic TA muscle (Fig. 1B), we first evaluated its effect on myogenesis using C2C12 cells. The data showed that knockdown of KLF15 by siRNA had no effect on myogenic differentiation of C2C12 myoblasts (Fig. S1), indicating that KLF15 may not act on myogenesis. Analysis of different isoforms of myosin heavy chains was carried out in both gene silencing and overexpression of KLF15 studies, and indicated that KLF15 regulated MHC-β/slow expression (Fig. 2). Additional experiments on the mechanism of regulation of MHC-β/slow expression by KLF15 revealed that KLF15 acted through direct binding to the *Nfatc1* promoter inducing the promoter activity (Figs. 3 and 4).

Calcineurin/NFAT signaling pathway is a well-studied mechanism that regulates myosin heavy chain expression in response to motor neuron activity. Calcineurin is activated by calcium/calmodulin in response to slow motor neuron activity and dephosphorylates NFAT inducing its translocation to the nucleus where NFAT activates the slow gene program and blocks the fast gene program [1]. The NFAT gene family is composed of five members, four of which (NFATc1 NFATc2, NFATc3, and NFATc4), are activated by calcineurin and expressed in skeletal muscle. In this study, KLF15 regulation of MHC-β/slow expression was mediated by

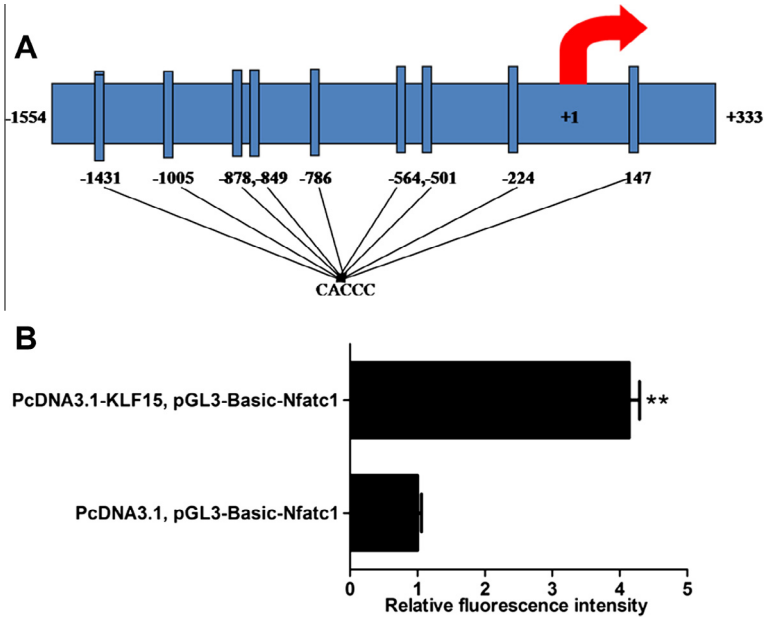

Fig. 4. KLF15 binds to and activates *Nfatc1* promoter activity. Structure analysis of the *Nfatc1* promoter (from −1554 bp to +333 bp) identified 9 potential binding sites (5′-CACCC-3′) for KLF15 (A). Luciferase reporter assay showed that KLF15 activated *Nfatc1* promoter activity (B). A luciferase reporter vector pGL3-Basic-Nfatc1 using *Nfatc1* promoter was constructed and transfected into HEK293T cells with a control plasmid PcDNA3.1 or PcDNA3.1-KLF15. Forty-eight hours after transfection, the cells were harvested and firefly luciferase activity was measured as described in Section 2. Data are presented as mean ± SEM. $^{**}P < 0.01$ versus control; $n \geqslant 3$.

NFATc1 signaling since knockdown or overexpression of KLF15 not only downregulated or upregulated MHC-β/slow, but also inhibited or promoted the expression of *Nfatc1* and its downstream target gene *Mcip1.4* (Fig. 3). Inhibition of NFATc1 expression by siRNA abolished the effect of KLF15 overexpression on MHC-β/slow expression (Fig. 3). Promoter sequence analysis and luciferase reporter assay further revealed that KLF15 regulated *Nfatc1* expression by direct binding to *Nfatc1* promoter inducing the promoter activity.

In contrast with our data, Haldar et al. report that *Klf15*$^{-/-}$ mice present no difference in muscle fiber type composition in both the *soleus* and *plantaris* muscles when compared to that of wild type mice [17]. This disagreement could be explained by the environmental complexity of the *in vivo* study since *in vivo* muscle fiber type specification is controlled by many factors, including cytokines, neurohormones, interactions of fiber typing regulating gene products and signaling pathways, and even muscle utilization. In consideration of all these factors, it is likely that the chronic knockout of *Klf15* in *Klf15*$^{-/-}$ mice is compensated by other redundant mechanisms. In our *in vitro* study, many environmental factors have been removed or minimized. Our *in vitro* study showed the acute and direct action of KLF15 on myosin heavy chain gene expression. In addition to redundant or compensatory functions of other proteins, structural homology among KLFs creates overlap in their transcriptional targets. For example, KLF2, 4, and 5 can all bind to and activate *Esrrb*, *Fbxo15*, *Nanog*, and *Tcl1* in ES cells [23]. Therefore, it is likely that the same mechanism exist in skeletal muscle cells. In fact, a downregulation in KLF6 expression was detected in our study when KLF15 was inhibited by siRNA (data not shown). Our study indicates a need for further studies on the redundant and specific functions of KLFs in skeletal muscle cells as well as a need for understanding the reciprocal regulation between KLF members.

To summarize, we reported that KLF15 regulates MHC-β/slow expression in skeletal muscle cells. This regulation is dependent on NFATc1. KLF15 regulates NFATc1 by direct binding and activation of the *Nfatc1* promoter activity. Our study not only brings new information on the role of KLFs in skeletal muscle cells, but also indicates the necessity for additional studies on the redundant and specific functions of KLFs in skeletal muscle biology.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.091.

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