Biochemical and Biophysical Research Communications 430 (2013) 664–669

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

TFE3 inhibits myoblast differentiation in C2C12 cells via down-regulating gene expression of myogenin

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ARTICLE INFO

Article history:
Received 8 October 2012
Available online 2 December 2012

Keywords:
Transcription factor E3
Myoblast differentiation
Myogenic regulatory factors
Myogenin

ABSTRACT

Transcription factor E3 (TFE3) belongs to a basic helix–loop–helix family, and is involved in the biology of osteoclasts, melanocytes and their malignancies. We previously reported the metabolic effects of TFE3 on insulin in the liver and skeletal muscles in animal models. In the present study, we explored a novel role for TFE3 in a skeletal muscle cell line. When TFE3 was overexpressed in C2C12 myoblasts by adenovirus before induction of differentiation, myogenic differentiation of C2C12 cells was significantly inhibited. Adenovirus-mediated TFE3 overexpression also suppressed the gene expression of muscle regulatory factors (MRFs), such as MyoD and myogenin, during C2C12 differentiation. In contrast, knockdown of TFE3 using adenovirus encoding short-hairpin RNAi specific for TFE3 dramatically promoted myoblast differentiation associated with significantly increased expression of MRFs. Consistent with these findings, promoter analyses via luciferase reporter assay and electrophoretic mobility shift assay suggested that TFE3 negatively regulated myogenin promoter activity by direct binding to an E-box, E2, in the myogenin promoter. These findings indicated that TFE3 has a regulatory role in myoblast differentiation, and that transcriptional suppression of myogenin expression may be part of the mechanism of action.

1. Introduction

The leucine zipper-containing bHLH protein TFE3 (Transcription factor E3, encoded by Tcfe3) was originally identified as a transcription factor that binds to the mu-E3 motif of the immunoglobulin heavy-chain enhancer [1–3]. TFE3 is a member of the MITF–TFE family of transcription factors encoding four family members: microphthalmia-associated transcription factor (MITF), TFE3, TFEB, and TFEC, and is involved in the biology of osteoclasts, melanocytes and their malignancies [4,5]. TFE3 is a ubiquitous protein that regulates the transcription of target genes through binding to the E-box, a consensus DNA sequence (CANNTG) for bHLH proteins. Although many genes contain E-box sequences in their promoter, the role of TFE3 in transcription is largely unknown.

Recently, we identified a novel TFE3 function in metabolic regulation. Previously, we demonstrated that TFE3 transactivated several metabolic genes that are regulated through an E-box in their promoters [6]. Adenovirus-mediated overexpression of TFE3 in liver led to the improvement of insulin sensitivity by upregulation of several metabolic genes and activation of various insulin signaling molecules [6]. Similar metabolic effects of TFE3 were also confirmed in skeletal muscle [7]. In addition, our current study suggests that TFE3 controls the adipogenic differentiation program of 3T3-L1 preadipocytes (unpublished data). These findings suggested a number of critical roles for TFE3 in a wide range of functions to maintain cell viability, such as proliferation, survival, differentiation and metabolism.

Recent studies observed that MITF, a member of the MITF–TFE family, was significantly expressed during myogenesis, and was required for efficient myotube formation [8]. Skeletal muscle development requires a highly ordered cascade of expression and activation of myogenic regulatory factors (MRFs), such as MyoD, myogenin, and Myf5, which contain E-boxes in their promoter regions. Myf5 and MyoD are required for the determination of myogenic lineage, whereas myogenin is important for the terminal differentiation of skeletal muscle [9–11]. Consistent with our previous findings, these data suggest that TFE3 may influence muscle differentiation by regulating MRF expression.

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http://dx.doi.org/10.1016/j.bbrc.2012.11.094

To test this hypothesis, we examined the effect of TFE3 on the myogenic differentiation program of C2C12 cells, a mouse myoblast cell line, using adenovirus-mediated overexpression and knockdown technologies.

2. Methods

2.1. Cell culture and C2C12 myoblast differentiation

C2C12 myoblasts were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. For the differentiation study, 24 h after subconfluence C2C12 cells were infected with adenovirus (300 multiplicity of infection) for 12 h, then culture medium was changed to differentiation medium (low glucose DMEM containing 2% horse serum). The medium was changed every 1 or 2 days.

2.2. Preparation of adenovirus

To generate the recombinant adenovirus plasmid (Ad-TFE3 or Ad-GFP), mouse TFE3 encoding cDNAs or green fluorescent protein (GFP) cDNAs were subcloned into the pShuttle-CMV vector and transferred into the adenovirus plasmid AdEasy-1 (Life Technologies, Carlsbad, CA) by homologous recombination. TFE3-specific RNA interference (RNAi) constructs (Ad-TFE3 RNAi) were generated with a BLOCK-iT™ U6 RNAi Entry Vector Kit and a BLOCK-iT™ Adenoviral RNAi Expression System (Life Technologies, Carlsbad, CA) using the following sequence: 5'-gtgtctggaaatctacttgatgt-3'. Ad-LacZ RNAi was used as a control [12]. Each adenovirus vector was propagated in 293A cells and purified by cesium chloride density centrifugation [6].

2.3. Real-time PCR

Total RNA was prepared from C2C12 cells using Trizol Reagent (Life Technologies, Carlsbad, CA). For gene expression analysis, total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) and subjected to real-time PCR using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara, Shiga, Japan). The following primers were used for analysis: TFE3 Fwd. 5'-cctgaaggcatctgtggatt-3' and, TFE3 Rv. 5'-tgtaggtccagaagggcatc-3'; Myf5 Fwd. 5'-gagggaacaggtggagaacta tta-3' and, Myf5 Rv. 5'-cgctggtcgctggagag-3'; MyoD Fwd. 5'-ccccg gcggcagaatggctacg-3' and, MyoD Rv. 5'-ggtctgggttccctgttctgtgt-3'; myogenin Fwd. 5'-gggcccctggaagaaaag-3' and, myogenin Rv. 5'-aggaggcgctgtgggagtt; Gapdh Fwd. 5'-gtcgtggatctgacgtgcc-3' and, Gapdh Rv. 5'-atgcctgcttcaccaccttc. Gene expression was normalized to Gapdh expression. Data were analyzed using the comparative CT method.

2.4. Immunoblotting

Tissues were homogenized in lysis buffer (1% Triton X-100, 10% glycerol, 100 mM NaF, 0.45% sodium pyrophosphate, 1 mM Na_3VO_4, protease inhibitor cocktail, 9.2 mM HEPES (pH 8.0), 11.5 mM NaCl and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was used as the total cell lysate. Tissue lysate protein (50 μg per lane) was resolved by 7.5–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and was transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA). The membranes were blocked with 5% BSA for 30 min at room temperature and incubated overnight at 4 °C with each of following antibodies: anti-TFE3 (BD Pharmingen), anti-MHC (MF-20; DSHB), and anti-β-actin (Santa Cruz Biotechnology). Bands were visualized using the enhanced chemiluminescence system (Western Lightning™ Plus-ECL; Perkin Elmer, Waltham, MA).

2.5. Plasmids

The TFE3 expression plasmid was generated by subcloning mouse TFE3 cDNA into the mammalian expression plasmid pcDNA3 (Life Technologies, Carlsbad, CA). For promoter analysis, 400 base pairs (bp) of mouse myogenin promoter (bp −363 to + 38, relative to transcriptional start site) were amplified by PCR and subcloned into pGL3-basic luciferase vector (Promega, Madison, WI). TFE3-specific RNA interference in the promoter assay was achieved using the plasmid vector based on BLOCK-iT™ U6 RNAi Entry Vector (described in Section 2.2).

2.6. Transfection and luciferase assays

C2C12 cells were plated on 24-well culture dishes at 80,000 cells/well. The next day, cells were transfected with 250 ng of luciferase reporter, 250 ng of the indicated expression plasmid or RNAi plasmid and 25 ng of pRL-SV40 vector as a reference (Promega, Madison, WI), using FuGENE6 transfection reagent (Promega, Madison, WI). After transfection, cells were cultured in growth medium for 24 h and the medium was changed to differentiation medium. After 48 h incubation, the amount of firefly luciferase activity was measured and normalized to the amount of renilla luciferase activity.

2.7. Electrophoretic mobility shift assay (EMSA)

TFE3 protein was generated from TFE3 expression plasmid using a coupled *in vitro* reticulocyte transcription–translation system (Promega, Madison, WI). Double-stranded ^{32}P-labeled oligonucleotides used in EMSA were directed against E-boxes of the myogenin promoter, 5'-gcacccagcagttggtgtgagggg-3' for E1, and 5'-gaaggggaatcacatgtaatcca-3' for E2 [13]. *In vitro* synthetic protein lysate was incubated in a reaction mixture as previously described [14]. Increasing amounts of unlabeled double-stranded oligonucleotides were added as competitor DNA. For supershift experiments, the anti-TFE3 antibody (Santa Cruz Biotechnology) was added to the mixture. DNA–protein complexes were resolved on a 3% polyacrylamide gel and visualized by an image analyzer (BAS2500; Fuji Photo Film, Tokyo, Japan).

2.8. Statistical analysis

Values are means + SE. Data between groups were compared using unpaired Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. The expression pattern of TFE3 during myoblast differentiation in C2C12 cell line

To explore a novel role for TFE3 in skeletal muscle, we characterized the expression pattern of TFE3 during myoblast differentiation in C2C12 cells. Real-time PCR analysis showed that TFE3 expression was significantly increased in the initial phase (day 1) of induction of differentiation, and thereafter there was a gradual decrease following terminal differentiation (Fig. 1A).

3.2. TFE3 inhibits C2C12 myoblast differentiation

To determine the role of TFE3 in C2C12 myoblast differentiation, overexpression and knockdown of TFE3 were performed

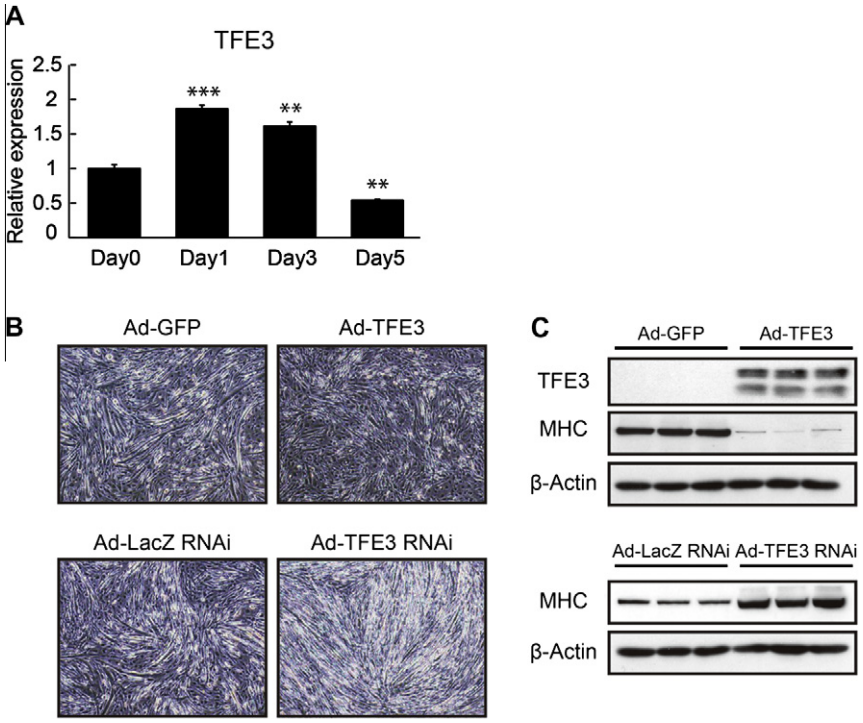

Fig. 1. TFE3 inhibits C2C12 myoblast differentiation. (A) Quantitative analysis of TFE3 expression during C2C12 myoblast differentiation. Gene expression of TFE3 at the indicated time points of differentiation was analyzed by real-time PCR analysis ($n = 3$ per group), $^{**}p < 0.01$, $^{***}p < 0.001$ vs. day 0. (B and C) Microscopic images and myosin heavy chain (MHC) protein content of adenovirus (Ad) infected C2C12 cells on day 5 of differentiation. C2C12 cells were infected with Ad-GFP or Ad-TFE3 (*upper panel*), Ad-LacZ RNAi or Ad-TFE3 RNAi (*lower panel*) for 12 h and then maintained in differentiation medium for 5 days. The microscopic images of cells (B), and protein expression levels assessed by immunoblotting (C) are shown. β-actin was used as an internal standard for protein loading.

using adenovirus, just before initiation of differentiation. Morphological analysis demonstrated that TFE3-overexpression with Ad-TFE3 markedly inhibited myotube formation in C2C12 cells (Fig. 1B, *upper panel*). In contrast, myoblast differentiation was greatly promoted in cells infected with Ad-TFE3 RNAi compared with Ad-lacZ RNAi control (Fig. 1B, *lower panel*). Concomitant with these morphological changes, myosin heavy chain (MHC) protein, which is expressed in terminally differentiated muscle cells, was decreased by overexpression of TFE3 and significantly increased in TFE3 knockdown cells, implying a suppressive effect of TFE3 on myoblast differentiation (Fig. 1C). We also confirmed increased amounts of TFE3 protein in the overexpressing TFE3 cells by immunoblotting (Fig. 1C, *upper panel*).

3.3. TFE3-induced inhibition of gene expression related to myoblast differentiation

To clarify the molecular mechanism of the observed effects of TFE3 on C2C12 myogenesis, gene expression of MRFs, Myf5, MyoD, and myogenin was examined. MyoD and myogenin were evaluated over the cell differentiation time course of 5 days, and Myf5, a very early marker of muscle differentiation [9,10], was analyzed on day 1. We determined the efficiency of overexpression and RNA interference and confirmed that adenovirus-mediated overexpression and knockdown were well preserved throughout the course of myoblast differentiation (Figs. 2A and 3A). Although TFE3 had no effect on Myf5 expression on day 1 of differentiation (Figs. 2B and 3B), the expression of MyoD and myogenin were suppressed by TFE3 overexpression as expected (Fig. 2C and D). The opposite results were partly observed by TFE3 knockdown. Interestingly, myogenin expression was significantly increased in TFE3 knockdown cells (Fig. 3D) while induction of MyoD expression was observed only on day 1. Contrary to our expectation, MyoD expression on Day 5 of knockdown experiment was significantly decreased as compared with control (Fig. 3C). This suggested that TFE3 controlled the myogenic program by affecting the expression of MRFs, especially myogenin.

3.4. TFE3 directly inhibits myogenin transcriptional activity

As TFE3 functions as a transcription factor, we expected that TFE3 was involved in myogenesis through the transcriptional regulation of the myogenin gene. Therefore, we performed a luciferase reporter assay. We confirmed that the isolated myogenin promoter (Fig. 4A) had sufficient activity in C2C12 myoblasts as compared to the promoterless control pGL3-basic (Fig. 4B). When the reporter vector was co-transfected with TFE3 expression vector, promoter activity was significantly inhibited by one-third compared to the control pcDNA3 empty vector (Fig. 4C). In contrast, TFE3-specific RNA interference resulted in the significant induction of myogenin promoter activity, approximately 3-fold of the control (Fig. 4D).

As previously described, the myogenin proximal upstream region contains two E boxes at −15 to −10 (E1) and −141 to −136 (E2) (Fig. 4A) [13]. EMSA was used to determine whether there is direct binding between TFE3 and E-boxes. Only oligonucleotides containing an E2 site produced a positive signal by binding to synthesized TFE3 protein. This band was supershifted by TFE3 specific antibody (Fig. 4E). These results confirmed that E2 is a specific binding site for TFE3 binding and transactivation.

4. Discussion

In the present study, we elucidated a novel role for TFE3 as a regulator of skeletal muscle differentiation, using a C2C12 cell line that is suitable for an *in vitro* model of muscle differentiation [15]. TFE3 negatively regulated myoblast differentiation by suppressing gene expression of MRFs such as myogenin.

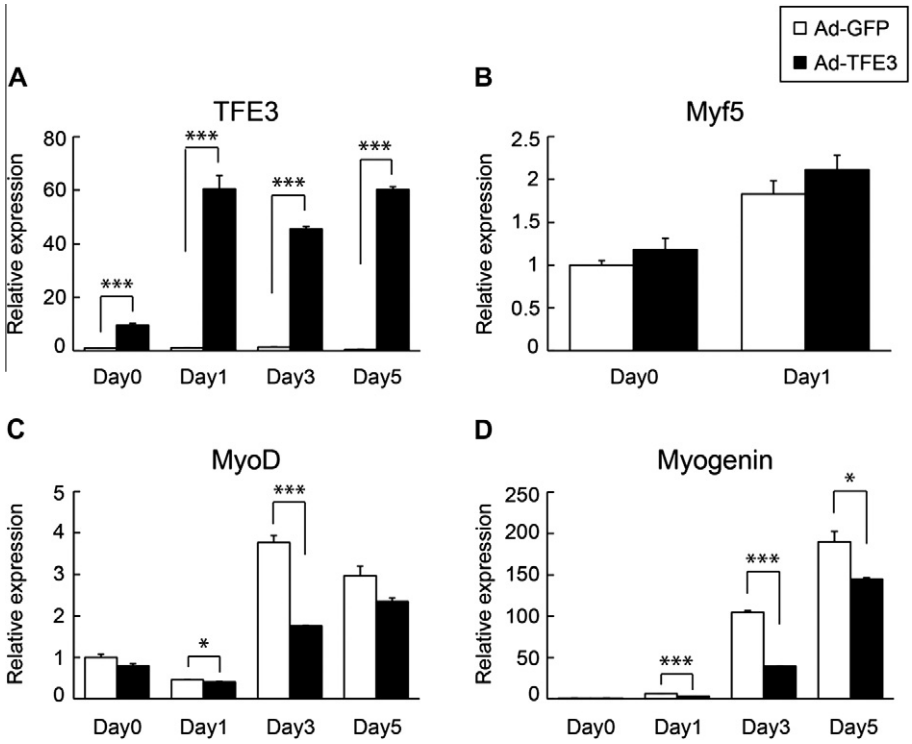

Fig. 2. Overexpression of TFE3 suppresses expression of myogenic regulatory factors during myoblast differentiation. C2C12 cells were infected with Ad-GFP or Ad-TFE3 for 12 h and allowed to differentiate. Expression levels of TFE3 (A), Myf5 (B), MyoD (C) and myogenin (D) at the indicated time points during differentiation were analyzed by real-time PCR ($n = 3$ per group). $^{*}p < 0.05$, $^{***}p < 0.001$.

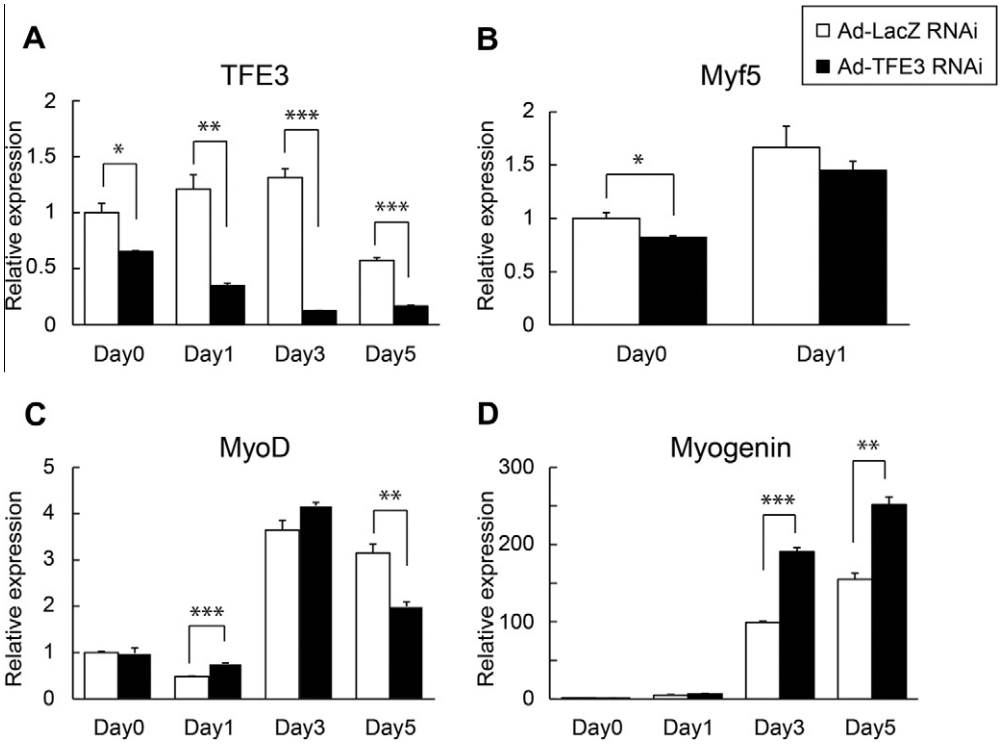

Fig. 3. Knockdown of TFE3 enhanced expression of myogenic regulatory factors during myoblast differentiation. C2C12 cells were infected with Ad-LacZ RNAi or Ad-TFE3 RNAi for 12 h and allowed to differentiate. Expression levels of TFE3 (A), Myf5 (B), MyoD (C) and myogenin (D) at the indicated time points during differentiation were analyzed by real-time PCR analysis ($n = 3$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TFE3 and its family members, MITF–TFE transcription factors, are ubiquitously expressed in various tissues and cells with varied expression levels [4,5,16]. However, the essential roles of TFE3 and other family members in a number of tissues remain unclear. Re-

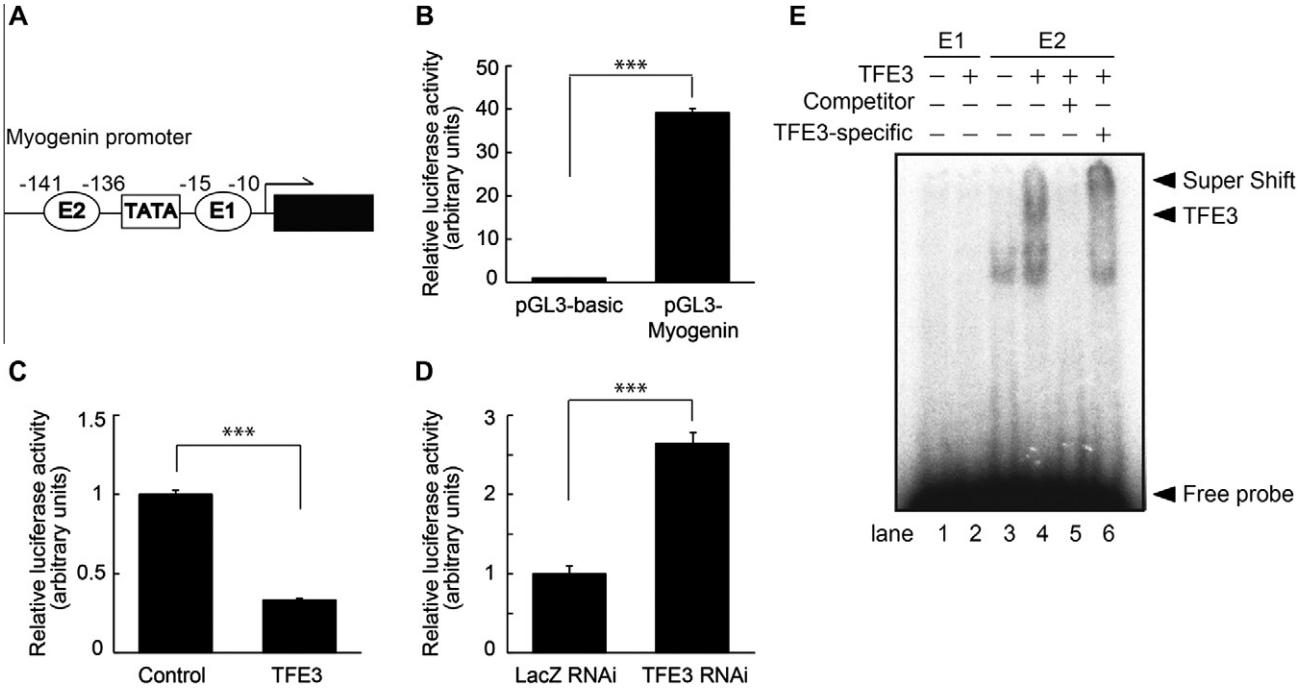

Fig. 4. TFE3 directly inhibits myogenin transcriptional activity. (A) Structure of mouse myogenin promoter used in the luciferase assay. The promoter contains two E-box binding sites (E1 and E2). (B–D) Myogenin promoter activity in C2C12 cells, as estimated by luciferase reporter assay. C2C12 cells were transfected with pGL3-myogenin reporter or pGL3 basic vector (B), pGL3-myogenin reporter and the indicated expression plasmid (C), or pGL3-myogenin reporter and the indicated RNA interference plasmid (D), and then incubated in differentiation medium for 48 h. Luciferase reporter activity was measured and normalized to data from pRL-SV40 ($n = 4$ per group). $^{***}p < 0.001$. (E) The binding of TFE3 with myogenin E-box shown in EMSA. ^{32}P-labeled oligonucleotide probes containing E1 (*lane 1, 2*) and E2 (*lane 3–6*) were incubated with TFE3 protein (*lanes 2, 4–6*) or control reticulocyte lysate (*lane 1, 3*). The oligonucleotide and protein complex was electrophoresed on a 3% polyacrylamide gel. The samples in *lanes 5 and 6* were incubated with the competitor oligonucleotide and the anti-TFE3 antibody respectively.

cently, it was reported that MITF, a member of the MITF–TFE family, increased and positively regulated the course of C2C12 differentiation [8]. Interestingly, TFE3 expression was increased only in the early differentiation stage and gradually decreased to lower than basal levels of undifferentiated cells following terminal differentiation (Fig. 1A). Concomitant with the observation that continuous overexpression of TFE3 resulted in suppression of myogenic differentiation (Fig. 1B and C), this expression pattern suggested that stage-specific limited expression of TFE3 in the initial stages are required for the differentiation program of C2C12 myoblasts. In support of this, knockdown of TFE3 expression abnormally promoted C2C12 myoblast differentiation (Fig. 1B and C). These findings imply that TFE3 and the MITF–TFE family may play a reciprocal role in the orchestration of transcriptional regulation of muscle development.

The present study suggested that TFE3 is involved in myogenesis through transcriptional activation of myogenic genes. It is noteworthy that myogenin expression was significantly suppressed in TFE3-overexpressed cells (Fig. 2D). In contrast it was significantly induced in TFE3-knockdown cells (Fig. 3D) throughout the course of differentiation. The expression of MyoD was also suppressed by TFE3 overexpression (Fig. 2C), but in the case of TFE3 knockdown, consistent changes throughout the course of differentiation were not clearly observed (Fig. 3C). Therefore, we hypothesize that myogenin is a candidate for TFE3-mediated regulation.

Promoter analysis suggested that TFE3 regulated myogenin at the transcriptional level. Myogenin is an important regulator of skeletal muscle development [17]. Ablation of the myogenin gene has causes severe muscle abnormality at birth [18]. Mouse myogenin gene has two E-boxes (E1 and E2) in its promoter [13,19], and as a consensus, the E1 is more important for its promoter activity [13,20]. However, contrary to our expectations, TFE3 had potent ability to bind to E2. Parker et al. previously described that the E1 and E2 E-boxes have different roles in the regulation of myogenin promoter activity [21]. Upon induction of differentiation, a MyoD–HEB complex activates transcription by binding to E1, and MyoD–HEB synergy was enhanced in the absence of E2. It was suggested that an unidentified complex negatively regulated the activity of E1 through binding to E2. From these observations, TFE3 may be considered a potential factor that negatively regulates the myogenin promoter through E2.

TFE3 functions not only as a single transcriptional regulator, but also as a partner of other transcriptional regulators such as E2F, PU-1, Max and Smads in the regulation of a variety of genes [22–25]. For example, TFE3 synergistically activates transforming growth factor-β (TGF-β) induced gene expression in cooperation with Smad proteins [24]. TGF-β and its intracellular effector, Smad3, inhibit myogenic differentiation [26]. Thus, it is important to clarify the precise mechanism of TFE3 in the inhibition of muscle differentiation taking into account the relationship between TFE3 and other factors.

In summary, TFE3 inhibited myoblast differentiation by regulating myogenin expression through direct binding with the E-box in its promoter region. Skeletal muscle development is achieved by the highly ordered expression of a series of transcription factors. Although the some transcription factors have been well characterized, many aspects of other factors remain unclear. The present study may present a new view of the mechanisms involved in myogenic differentiation.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research, a Grant-in-Aid for JSPS Fellows (22-1029) from the Ministry of Education, Culture, Sports, Science and Technology, a Grant from NOVARTIS Foundation (Japan) for the Promotion of Science, a

Grant from Kowa Life Science Foundation, a Grant from Mochida Memorial Foundation for Medical and Pharmaceutical Research, a Grant from The Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical Care, a Grant from Astellas Foundation for Research on Metabolic Disorders and a Grant from The Sumitomo Foundation and a Grant from The Nakatomi Foundation.

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