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Positive feedback control between STIM1 and NFATc3 is required for C2C12 myoblast differentiation

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ABSTRACT

Up-regulation of STIM1-mediated store-operated Ca^{2+} entry (SOCE) and Ca^{2+}-dependent NFAT signaling is important for myogenic differentiation. However, the molecular mechanisms for differentiation specific up-regulation of STIM1/SOCE-mediated signaling are poorly understood. This study explored whether functional crosstalk between STIM1 and a member of NFAT transcription factor is important for C2C12 myoblast differentiation. Transient increase of NFATc3 expression was observed in the initial phase of differentiation, and the increased activity of NFATc3 isoform was correlated with up-regulation of STIM1 expression. Overexpression of NFATc3 increased STIM1 expression, SOCE activity, and myotube formation, whereas NFATc3 knockdown showed the opposite effects. Overexpression of STIM1 increased the activity and expression level of NFATc3, and enhanced myotube formation, whereas STIM1 knockdown resulted in the opposite effects. Taken together, our findings suggest that a positive feedback control between STIM1/SOCE and NFATc3 is required for efficient induction and progression of myoblast differentiation.

1. Introduction

Myoblast differentiation involves a cell cycle exit, the expression of pro-myogenic genes, myoblast fusion, and myotube formation. Augmented activation of intracellular Ca^{2+} signaling has been known to be pivotal for the initiation and progression of myogenic differentiation [1,2]. Ca^{2+} signaling pathways modulate the activity of a number of Ca^{2+}-dependent enzymes that regulate the activity of muscle specific transcription factors, including the nuclear factor of activated T cells (NFAT). Ca^{2+} activation of calcineurin dephosphorylates NFAT and allows for its nuclear translocation, where it activates transcription of a number of muscle specific genes [3,4]. Expression of the member of NFAT isoforms (NFATc1–4) in skeletal muscles was identified, and the roles of individual NFAT isoforms in different stages of myogenesis and muscle fiber type have been demonstrated [5,6]. However the molecular mechanism of NFAT isoform-specific signaling pathways and target molecules are not fully understood in myogenic differentiation.

Stromal interaction molecule 1 (STIM1) is an ER membrane protein that senses changes in luminal Ca^{2+} contents of ER. In response to ER Ca^{2+} stores depletion, STIM1 proteins translocate into specialized ER-plasma membrane junctions and directly bind to plasma membrane Orai1 Ca^{2+} channels. This STIM1-mediated opening of Orai1 channels creates store-operated Ca^{2+} entry (SOCE) that produce the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), replenishes ER Ca^{2+} stores, and activates a number of Ca^{2+}-dependent signaling by which diverse transcriptional and post-transcriptional processes are regulated [7]. Recently, the importance of STIM1/SOCE-mediated signaling has been recognized in skeletal muscle development and physiology [8]. It has been revealed that STIM1 is highly expressed in mammalian skeletal muscle and ablation of STIM1 strongly impairs skeletal muscle development and contractile function [9]. STIM1-deficient mice exhibited impaired neonatal muscle growth and differentiation, perinatal lethality, smaller body size and weight gain, skeletal myopathies, and impaired excitation–contraction coupling [9,10]. Up-regulation and the promyogenic role of STIM1 have been demonstrated in cultured human myoblast, where STIM1 deficiency impaired myoblast differentiation [11]. Furthermore, human patients with loss of function mutations in STIM1 showed symptoms of a congenital skeletal myopathy and hypotonia [12].

Taken together, it is clear that enhanced Ca^{2+}-dependent signaling by the up-regulation of STIM1-mediated SOCE and downstream NFAT trans-activation are required for skeletal myogenesis. However, the control mechanism of STIM1 expression and downstream signaling pathways are not fully understood in myogenesis. In this study, we therefore aimed to investigate the molecular mechanism of STIM up-regulation and the modes of

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interaction between STIM1 activation and NFAT trans-activation in differentiating C2C12 myoblast.

2. Materials and methods

2.1. C2C12 myoblast culture and differentiation

Murine C2C12 myoblasts were purchased from ATCC and cultured in high glucose DMEM (Welgen) growth medium (GM) supplemented with 15% fetal bovine serum, 1% penicillin and streptomycin at 37 °C with 5% CO_2. C2C12 myoblasts were cultured in 60 mm dish until reached >90% confluence, and then differentiation was induced by replacing GM with differentiation medium (DM; DMEM supplemented with 2% horse serum) for 3 days. To quantify myotube formation, the cells were fixed with 4% cold paraformandehyde for 10 min, and then permeabilized with cold methanol containing 0.3% H_2O_2 for 10 min. 5% horse serum solution was used to block the samples for 30 min. Fixed samples were incubated with anti-MyHC Ab at 4 °C overnight, and then incubated with biotylated anti-mouse Ab (1:500, Jackson Immunoresearch) at room temperature for 2 h. After that, the cells were incubated with HRP-conjugated streptoavidin (1:1000, Jackson Immunoresearch) at room temperature for 2 h. Finally, the samples were developed with 3,3′ diaminobenzidine chromogen (Dako). Myotubes formation was assayed by counting the number of cells with mono-nucleus and multi-nuclei MyHC-positive ($MyHC^+$) myotubes from five selected areas of three to five independent samples. Data were presented as percentage of the cells that contained nucleus number of 1–2, 3–7, or $\geqslant 8$ $MyHC^+$ myotubes, as previously described [13] with slightly modification.

2.2. Quantitative real time-PCR (qRT-PCR)

Total RNA from C2C12 cells was isolated with Trizol reagent (Invitrogen). cDNA was synthesized with random hexamer primers and SuperScript III RNase H-Reverse Transcriptase (Invitrogen), then was amplified with the premix cyber green (Takara; 94 °C for 5 min then 95 °C for 5 s, 60 °C for 30 s for 40 cycles). The PCR amplification was done using TP800 real time-PCR System (Takara). The primers using to quantify specific mRNA levels were designed by a primer designing tool (NCBI) and were synthesized by Cosmo Genentech, Korea. The detailed DNA sequences were described in Table 1. The expression of the genes was normalized with internal control (tubulin). The relative mRNA value was calculated from Ct value by using the $2^{-\Delta\Delta CT}$ method for relative gene expression analysis.

2.3. Overexpression and knockdown studies

To generate C2C12 cells that stably overexpression STIM1, a human pIRES-STIM1-GFP was cloned from pCMV6-STIM1 (purchased from Origene) and transfected into the myoblasts by lipofectamine™2000 (Invitrogen) for 48 h, and then the transfected cells were selected by treating 200 μg/ml G418 for a week. To construct C2C12 cells that stably knockdown STIM1, a shRNA target sequence for mouse STIM1 (accession number: BC021644.1), 5′-GCT GCT GCT GTC ACA TCT T-3′, was selected using the Invitrogen shRNA design tool. shSTIM1 (sense), 5′-gatccc GCTGCTGCTGTCACATCTT ttcaagaga AAGATGTGACAGCAGCAGC ttttttggaaa-3′; shSTIM1 (antisense), 5′-agcttttccaaaaaa GCTGCTGCTGTCACATCTT tctcttgaa AAGATGTGACAGCAGCAGC gg-3′. Oligonucleotides were cloned into p-Super.puro vector (Clontech). C2C12 myoblasts were transfected with shSTIM1 or p-Super.puro vector using lipofectamine™2000 (Invitrogen) for 48 h and then puromycin (1 μg/ml)-resistant colonies were selected for a week. Overexpression or knockdown of STIM1 was confirmed by quantitative real time-PCR and Western blot analysis. For NFAT overexpression studies, C2C12 myoblasts were transiently transfected with 1.5 μg cDNA of individual NFATc construct (NFATc1, c2, c3, and c4) by lipofectamin™2000 for 48 h. After that, cells were harvested to check overexpression of NFAT isoforms by quantitative real time-PCR and Western blot analysis. When it is necessary, C2C12 cells with NFAT overexpression were induced to differentiation. The mouse NFATc1 cDNA was kindly gifted by Dr. Jin-Hyun Ahn (Sungkyunkwan University, Korea). The cDNA of mouse NFATc2 and human NFATc3 (Addgene plasmid #11100 and #21664, respectively) were provided by Dr. Anjana Rao. Human NFATc4 cDNA was generously gifted by Dr. Sébastien Jauliac (INSERM, French National Institute of Health and Medical Research, France). To generate stable knockdown of NFATc3, shRNA with the following sequences were designed and cloned into p-Super.puro vector. A shRNA target sequence for mouse NFATc3 (accession number: NM_010901), 5′-GCC ACT TCT CCC TGT GGT AA-3′, was selected using the Invitrogen shRNA design tool. The following forward and reverse oligonucleotides were synthesized: shNFATc3 (sense), 5′-gatccc GCCACTTCTCCCTGTGGTAA ttcaagaga TTACCACAGGGAGAAGTGGC ttttttggaaa-3′; shNFATc3 (antisense), 5′-agcttttccaaaaaa GCCACTTCTCCCTGTGGTAA tctcttgaa TTACCACAGGGAGAAGTGGC gg-3′. C2C12 cells were transfected with shNFATc3 or p-Super.puro vector using lipofectamine™2000 (Invitrogen) for 48 h, and then puromycin-resistant colonies were selected for a week. Knockdown of NFATc3 was confirmed by quantitative RT-PCR and Western blot analysis.

Table 1
DNA sequences of the primers used for quantitative RT-PCR, shSTIM1, and shNFATc3

Identity	Nucleotide sequences	Product size (bp)
mSTIM1	Forward: 5′-TGCAGCTTACTGGCCACGCC-3′ Reverse: 5′-GGCAAACCAGCAGCCACCCA-3′	221
mOrai1	Forward: 5′-TCCACGGTCATCGGGACGCT-3′ Reverse: 5′-GTCGCTGTGGTTGGCGACGA-3′	144
mNFATc1	Forward: 5′-ACGCAAGCCCGAAGCTCCAC-3′ Reverse: 5′-GGCTGCTCAGGCGAGTTGGG-3′	166
mNFATc2	Forward: 5′-TCCGCGTGCCCGTGAAAGTC-3′ Reverse: 5′-ACTCGGCCAGCATTGGGTGC-3′	201
mNFATc3	Forward: 5′-ACCAAAGCCTGGCCACACCC-3′ Reverse: 5′-GCTTTCACTGCCCCTCGGCT-3′	165
mNFATc4	Forward: 5′-ACAATGAGGTGGGGCCAGGCT-3′ Reverse: 5′-CTCACTCACTTCCTCCAGGGTGAT-3′	132
ß-Tubulin	Forward: 5′-CCCTTCTACAACCAGCACCAT-3′ Reverse: 5′-CTAGGATGGCCCGAGGTACA-3′	215

2.4. Western blotting and antibodies

Cell extracts were prepared by washing the C2C12 cells with PBS and then proteins were extracted with cold lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Trition X-100, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 μg/ml Leupeptin, 2 μg/ml Aprotinin, 1 mM PMSF, 3 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF. The same amounts of total cell lysates were resolved by 6–10% SDS–PAGE and then transferred to polymer of vinylidene fluoride (PVDF) membranes, which were incubated in a blocking solution containing of 5% skim milk in TBS-T for 1 h at room temperature and then immunoblotted with indicated antibody (Ab). Immunoblotted bands were visualized by using an enhanced chemiluminescence (ECL) kit (Amersham). Anti-STIM1, anti-Orai1, anti-NFATc1, and anti-NFATc2 Ab were obtained from ProSci Company. Anti-NFATc4 and anti-β-actin Ab were from Sigma Company. Anti-NFATc3, anti-p-NFATc3, anti-MyHC, and anti-myogenin Ab were

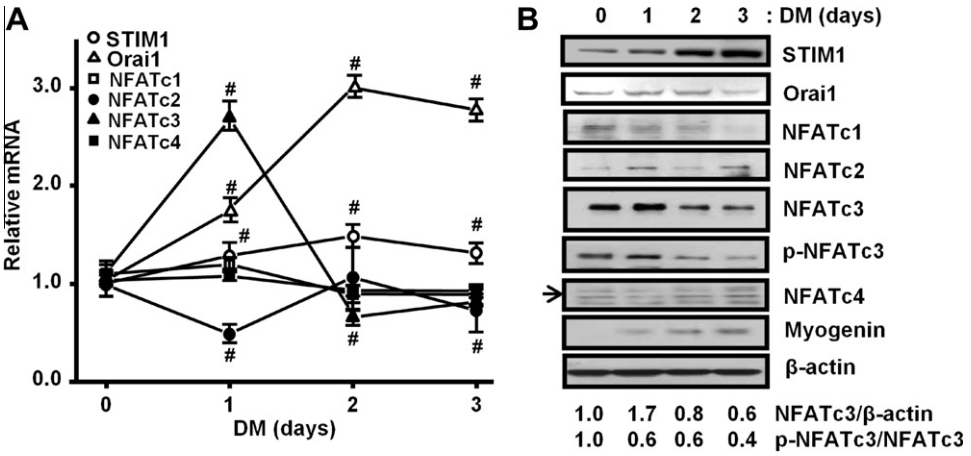

Fig. 1. Expression patterns of STIM1, Orai1, and NFAT isoforms during differentiation of C2C12 myoblast. (A) Changes of the mRNA expression levels were quantified by qRT-PCR during 3 days of differentiation. Relative mRNA expression levels were plotted against that of individual gene on DM0. Data are presented as means ± SD of two independent experiments (n = 4–6, $^{*}P < 0.05$, $^{\#}P < 0.01$). (B) Changes of the protein expression levels during differentiation were analyzed by Western blotting. Protein levels of STIM1, Orai1, four members of NFAT isoforms (NFATc1-c4), and phosphorylated form of NFATc3 (p-NFATc3) were plotted together with myogenic marker protein myogenin. The amounts of NFATc3 or p-NFATc3 proteins were quantified and normalized against loading control ß-actin, and then calculated p-NFATc3/NFATc3 ratio values were described under the plot. Representative results from 3–5 independent experiments are shown.

purchased from Santa Cruz Biotechnology. Otherwise stated, all chemicals used in this study were purchased from Sigma.

2.5. Luciferase assay

Luciferase reporter plasmids NFAT-Luc was kindly gifted by Dr. Jong-Sun Kang (Sungkyunkwan University, Korea), a firefly luciferase reporter with a defined promoter, and *renilla* luciferase control plasmid were co-transfected into C2C12 myoblasts (control, STIM1-overexpressed, or shSTIM-transfected C2C12 cells). After 48 h of transfection, C2C12 myoblasts were harvested and lysed in a lysis buffer and then NFAT-Luc and *renilla* luciferase activities were assayed with the dual luciferase assay kit (Promega).

2.6. $[Ca^{2+}]_i$ measurements

C2C12 cells were loaded with 3 μM Fura-2-AM (Molecular Probes) in experiment solution that contained (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose, and 20 HEPES (pH 7.4). Changes of $[Ca^{2+}]_i$ were measured in a 1 ml temperature-controlled cuvette system (DeltaScan, PTI). Emitted Fura-2 fluorescence ratios (F_{340}/F_{380}) were obtained and calibrated into the $[Ca^{2+}]_i$ by treating the cells with ionomycin (Biomol) and EGTA at the end of each measurement. To activate SOCE, ER Ca^{2+} stores were depleted by 1 μM thapsigargin (Tg) in extracellular Ca^{2+}-free condition, and then 2 mM Ca^{2+} was added back to the extracellular solution to evoke the SOCE activity. Resting $[Ca^{2+}]_i$ in 2 mM Ca^{2+}-containing solution,

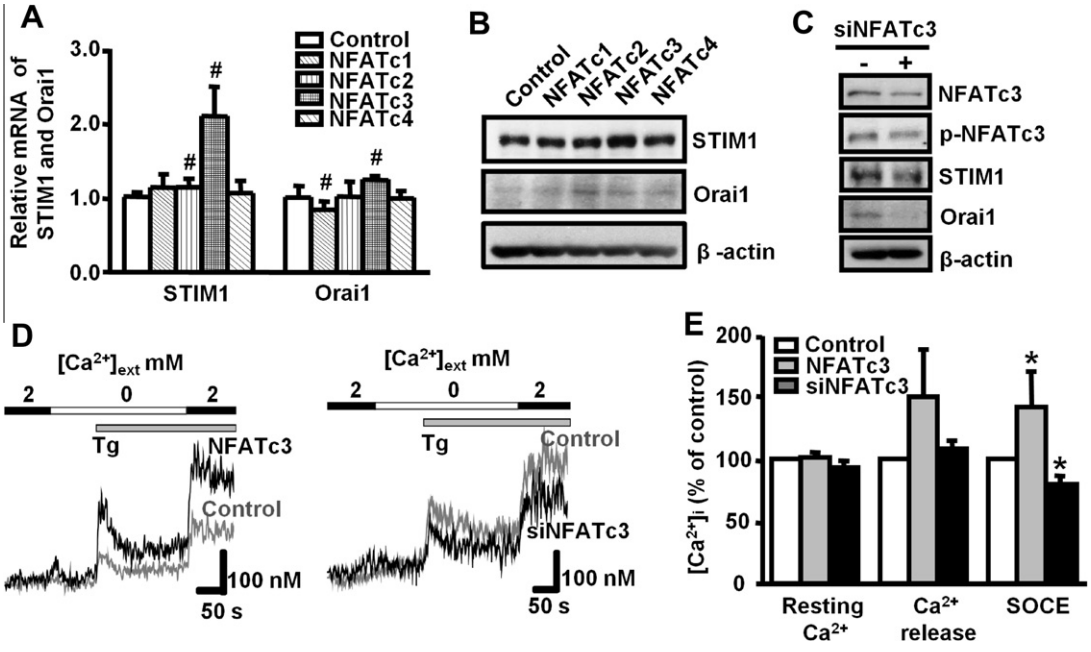

Fig. 2. NFATc3 regulates the expression levels of STIM1 and Orai1, and the activity of SOCE in C2C12 myoblast. (A) The change of mRNA levels of STIM1 and Orai1 were quantified by qRT-PCR in C2C12 cells with overexpression of individual NFAT isoforms. Shown are means ± SD from two independent experiments (n = 4–6, $^{\#}P < 0.01$). (B) Expression levels of STIM1 and Orai1 proteins were quantified by Western blotting in C2C12 myoblasts with overexpression of individual NFAT isoforms. (C) NFATc3 knockdown (siNFATc3) reduced protein expression of STIM1 and Orai1. Representative Western blotting results from three independent experiments are shown in (B) and (C). (D) Representative $[Ca^{2+}]_i$ traces of SOCE measurement in NFATc3-overexpression (left) and siNFATc3 (right) myoblasts are plotted. (E) Obtained peak $[Ca^{2+}]_i$ values were normalized to and compared with that of control (n = 7–8, $^{*}P < 0.05$).

the magnitudes of the rise in $[Ca^{2+}]_i$ following Tg (ER Ca^{2+} release) and reintroduction of Ca^{2+} (SOCE) were compared among groups.

2.7. Statistics

Data were presented as mean ± SD in qRT-PCR experiments and as mean ± SEM in other experiments with the indicated numbers of experiments for each study. Data were analyzed using the Student's *t*-test, and *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Expression patterns of STIM1 and NFATc3 during myoblast differentiation

The expression patterns of SOCE constituents (STIM1 and Orai1) and four members of NFAT (NFATc1–c4) were compared during 3-days of myoblast differentiation. In agreement with previous reports [8,9], we confirmed the marked increase of mRNA and protein levels of STIM1 and Orai1 during differentiation (Fig. 1A and B). Among NFAT isoforms, NFATc3 mRNA and protein levels were up-regulated transiently at 1-day of differentiation (DM1) and down regulated thereafter (Fig. 1A and B). Total NFATc3 protein level increased 1.7-fold at DM1, and then decreased to 80% (DM2) and 60% (DM3) of control. The amounts of inactive, phosphorylated NFATc3 (p-NFATc3) slightly increased (10%) at DM1, and declined thereafter to 50% (DM2) and 25% (DM3) of control. Therefore, NFATc3 activity calculated by p-NFATc3/NFATc3 ratios (0.6, 0.6, and 0.4 at DM1, DM2, and DM3, respectively) was increased continuously during differentiation (Fig. 1B). NFATc1 protein levels continuously decreased during differentiation, and NFATc2 and NFATc4 levels were not greatly changed in differentiating C2C12 cells (Fig. 1A and B). In summary, transient increase of NFATc3 expression was observed in the early phase of differentiation, and up-regulation of STIM1 expression was correlated with increasing NFATc3 activity during differentiation. And therefore, we next questioned whether NFATc3 up-regulates STIM1 expression level.

3.2. NFATc3 enhances SOCE activity by increasing STIM1 and Orai1 expression

We overexpressed individual NFAT isoforms in C2C12 myoblast, and the changes in expression levels of STIM1 and Orai1 were evaluated. Among NFAT isoforms transfected, NFATc3 showed the strongest effects on the expression levels of STIM1 and Orai1.

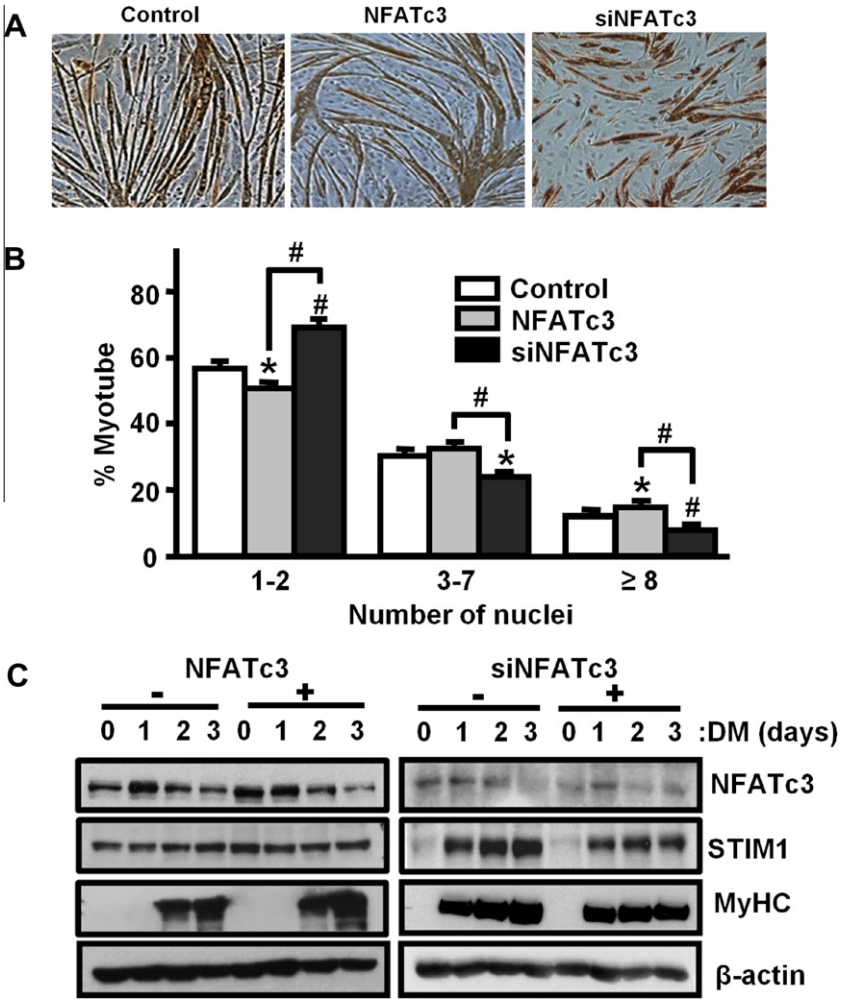

Fig. 3. NFATc3 regulates STIM1 expression and differentiation of C2C12 myoblast. (A) Representative images taken from the $MyHC^+$ myotubes with NFATc3-overexpression or siNFATc3 are presented. (B) Degrees of myotube formation were analyzed by counting nuclei number from $MyHC^+$ myotubes. Shown are means ± SEM from five independent experiments ($^*P < 0.05$, $^\#P < 0.01$). (C) Changes in protein expression levels of NFATc3 and STIM1 during myoblast differentiation were analyzed by Western blotting. The amounts of STIM1 protein and myogenic marker protein (MyHC) were increased or decreased in NFATc3-overexpressed or NFATc3-silenced cells, respectively. Representative results from three independent experiments are shown.

Overexpression of NFATc3 increased the mRNA levels of STIM1 (210 ± 39%) and Orai1 (126 ± 1.4%), while other NFAT isoforms increased STIM1 mRNA by only 8–15% (Fig. 2A). NFATc3 overexpression resulted in ~2-fold increase of STIM1 and Orai1 protein levels (Fig. 2B). NFATc3 knockdown decreased the protein levels of STIM1 and Orai1 to 70 ± 2% and 21 ± 5% of control, respectively (Fig. 2C). As a consequence, SOCE activities quantified with $[Ca^{2+}]_i$ measurement were increased (142 ± 31%) or decreased (79 ± 7%) by overexpression or knockdown of NFATc3, respectively (Fig. 2D and E). These results substantiate that, NFATc3 is the dominant NFAT isoform that determines SOCE activity through the expression regulation of STIM1 and Orai1 in C2C12 cells.

3.3. NFATc3-mediated regulation of STIM1expression and myoblast differentiation

We investigated whether the overexpression or knockdown of NFTATc3 affects C2C12 myoblast differentiation. Overexpression of NFATc3 enhanced myotube formation with 1.6-fold higher number of larger MyHC$^+$ myotubes containing ⩾8 nuclei (14.4 ± 1.2% *vs*. control 9.2 ± 1.6%). Knockdown of NFATc3 inhibited myotube formation, with a lower percentage of MyHC$^+$ myotubes containing ⩾8 nuclei (5.0 ± 0.8%) and a higher percentage of cells containing 1–2 nucleus (71 ± 1.8% *vs*. control 56 ± 1.6%) (Fig. 3A and B). The effects of NFATc3 expression levels on myotube formation were further confirmed by immunoblotting of myogenic marker protein MyHC, where MyHC levels were increased or decreased by overexpression or knockdown of NFATc3 (Fig. 3C). In terms of NFATc3-mediated STIM1 regulation, we found that differentiation specific up-regulation of STIM1 was strongly attenuated in NFATc3 knockdown cells (Fig. 3C), which correlated well with defects in myoblast differentiation.

3.4. STIM1-mediated regulation of NFATc3 expression and myoblast differentiation

We next questioned whether STIM1 expression levels determine NFATc3 expression level and activity. As shown in Figs. 4A–C, overexpression or knockdown of STIM1 in C2C12 myoblast increased or decreased the mRNA, protein levels, and transcriptional activity of NFATc3, respectively. Overexpression of STIM1 increased NFATc3 protein level by 80% and enhanced its activity (p-NFATc3/NFATc3 = 0.8), while siSTIM1 decreased NFATc3 protein level by 40% and reduced its activity (p-NFATc3/NFATc3 = 2.6) (Fig. 4B). Basal transcriptional activities of NFATc3 that were evaluated by luciferase assay were remarkably increased (141 ± 4%) or decreased (46.26 ± 6.63%) by the overexpression or knockdown of STIM1, respectively (Fig. 4C). We finally addressed whether the expression levels of STIM1 determines NFATc3 expression during differentiation. As shown in Fig. 4D–F, overexpression of STIM1 en-

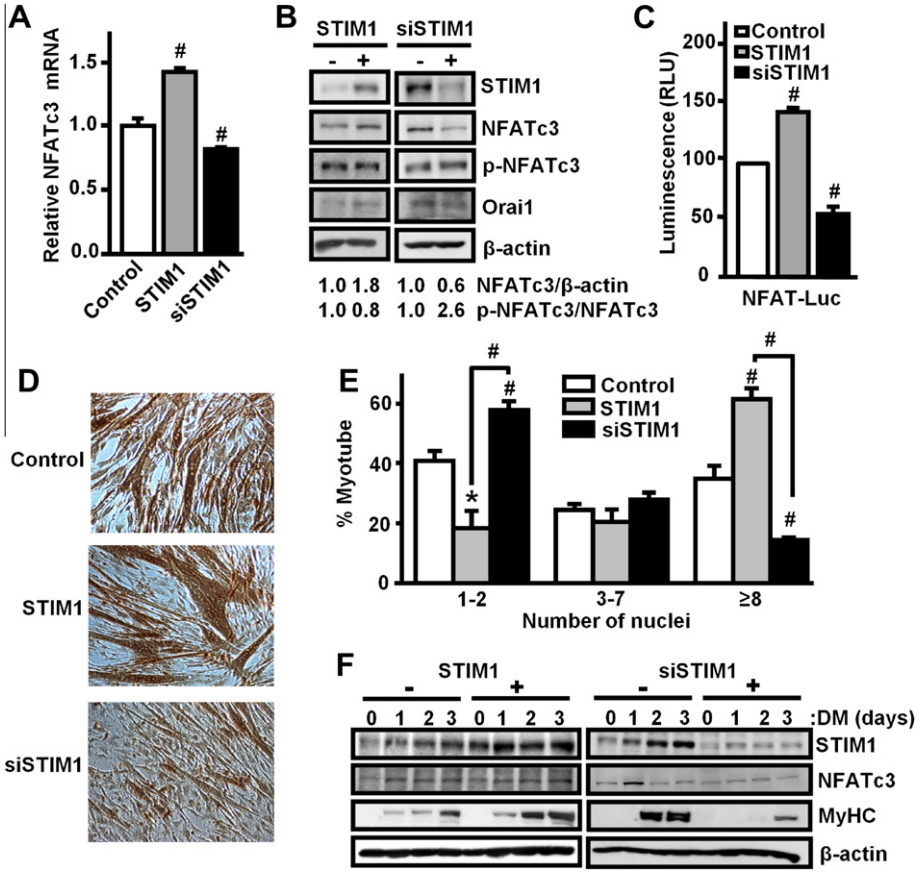

Fig. 4. STIM1-mediated regulation of NFATc3 expression and myoblast differentiation. (A) Changes of NFATc3 mRNA expression levels in STIM1-overexpressed or knockdown (siSTIM1) C2C12 myoblasts were quantified by qRT-PCR. Shown are means ± SD from two independent experiments (n = 4–6, $^{\#}P < 0.01$). (B) Protein expression levels of STIM1, NFATc3, and p-NFATc3 were analyzed in STIM1-overexpressed or siSTIM1 myoblasts. Normalized NFATc3 protein levels and p-NFATc3/NFATc3 ratio values, which reflect NFATc3 activity, are described under the Western blotting plots. (C) Luciferase assay of transcriptional activity of NFAT in STIM1-overexpressed or siSTIM1 myoblasts (three independent experiments, n = 6, $^{\#}P < 0.01$). (D) Representative images taken from the MyHC$^+$ myotubes with STIM1-overexpression or siSTIM1 are presented. (E) Myotube formation was analyzed by counting nuclei number of MyHC$^+$ myotubes (3 independent experiments, n = 3, $^{*}P < 0.05$, $^{\#}P < 0.01$). (F) Western blot analysis on the protein expression levels of STIM1, NFATc3, and myogenic marker MyHC showed that the NFATc3 protein expression during differentiation and myotube formation are markedly suppressed in STIM1 knockdown cells. Representative results from three independent experiments are shown.

hanced myotube formation with 1.8-fold higher number of larger MyHC$^+$ myotubes containing $\geqslant$8 nuclei (61.4 ± 3.6% *vs*. control 34.7 ± 4.4%), with a lower percentage of cells with 1–2 nucleus (18.4 ± 5.7% *vs*. control 40.8 ± 3.3%). Knockdown of STIM1 strongly inhibited myotube formation and resulted in a lower percentage of myotubes with $\geqslant$8 nuclei (14.3 ± 0.8%), with higher percentage of cells with 1–2 nucleus (57.8 ± 3.1%) (Fig. 4E). These effects of STIM1 on myotube formation were further confirmed by expression levels of myogenic marker protein MyHC (Fig. 4F). In terms of STIM1-mediated NFATc3 regulation, it was demonstrated that overall NFATc3 expression was reduced in differentiating cells and differentiation specific transient up-regulation of NFATc3 was completely impaired in STIM1 knockdown cells, which correlated well with defects in myoblast differentiation (Fig. 4F).

4. Discussion

We have demonstrated here that, among the members of NFAT expressed in C2C12 cells, NFATc3 expression (mRNA and protein) was transiently up-regulated in the early phase of myoblast differentiation (Fig. 1). When it was overexpressed, NFATc3 enhanced SOCE activity by increasing STIM1 protein level (Fig. 2). Since NFATc3 increased both the mRNA and protein levels of STIM1, we assumed that STIM1 is transcriptionally regulated by NFATc3. We have also demonstrated that, enhanced STIM1/SOCE-mediated signaling increases transcriptional activity of NFAT, not only by the classical Ca^{2+}/calcineurin-mediated dephosphorylation of NFAT but also by the increase of NFATc3 protein level. Taken together, it seems reasonable to propose that there is a functional crosstalk between NFATc3 and STIM1 and it permits a positive feedback loop directly or indirectly for efficient induction and progress of myoblast differentiation. Hence, future study on the NFATc3 binding site(s) in the STIM1 promoter will provide direct evidence for NFATc3-mediated transcriptional activation of STIM1.

Knowledge on the transcriptional control of STIM1 is limited. Recent work revealed that Early Growth Response 1 (EGR1) zinc finger transcription factor drives STIM1 expression, while EGR-related transcription factor Wilms Tumor Suppressor 1 (WT1) inhibits its expression [14]. Stimulation of Insulin-like Growth Factor-1 Receptor (IGF-1R) or Epidermal Growth Factor Receptor (EGFR) is known to lead EGR-1 trans-activation in other type of cells. However, it has not been revealed yet whether EGR1 and WT1 are related with differentiation specific expression of STIM1 in skeletal muscle. Recent observation demonstrated that transcriptional factor NFκB up-regulates expression of STIM1 and Orai1, and consequent SOCE activity [15]. Furthermore, it is evident that SGK1 up-regulates and AMPK down-regulates NFκB and thus synthesis of STIM1 and Orai1 proteins [15,16]. NFκB also can be activated by Ca^{2+} entered via SOCE, thus NFκB-mediated up-regulation of STIM1 and Orai1 can generate a positive feedback into SOCE activation of NFκB. However, we assumed that NFκB does not mediate the up-regulation of STIM1 in differentiating myoblast, because it is generally accepted that NFκB activity is inversely related to myogenesis but is required for myoblast proliferation [17]. The negative role of NFκB in myogenesis was further supported by the evidence that TNFα-mediated inhibition of myogenic differentiation requires transcriptional activation of NFκB [18]. It has been known that AMPK inhibits myogenic differentiation [19]. Hence, STIM1 up-regulation during myoblast differentiation cannot be explained by AMPK-mediated down-regulation of NFκB [16].

It has been suggested that individual NFAT-mediated transcription is restricted to a particular stage of myogenesis or fiber type of skeletal muscle [5,6]. In response to raised $[Ca^{2+}]_i$, NFATc3 trans-activation occurred only in myoblasts, and with NFATc1 and NFATc2 it occurred only in myotubes. Since NFATc2 trans-activation was restricted to newly formed myotubes, they suggested that NFATc2 contributed to the growth of myotubes [5]. In our study in C2C12 cells, however, NFATc2 expression is relatively weak and NFATc2 mRNA levels were markedly down-regulated during the initial phase (DM1) of differentiation (Fig. 1A). Hence, we suppose that the roles of NFATc2 in C2C12 myoblast differentiation are limited, at least in our experimental conditions. In the present study, transient NFATc3 up-regulation during the initial phase of differentiation was observed (Fig. 1). Our findings are in accordance with previous study on the signaling transduction mechanism of IGF-1-stimulated C2C12 myoblast differentiation [6]. They showed that calcineurin activation and subsequent NFATc3 trans-activation was transiently increased during the initiation of myoblast differentiation. We showed that transient NFATc3 up-regulation is ablated by STIM1 knockdown (Fig. 4F). Our results might suggest that STIM1-mediated NFATc3 up-regulation is a plausible explanation for the IGF-1-stimulated myoblast differentiation [6]. We suppose that impact of the initial FATc3 up-regulation has a relatively long-lasting effect on differentiation, because NFAT-induced gene products that can initiate subsequent waves of gene expression which can ultimately result in myoblast differentiation [20].

In the present study, the underlying mechanism of STIM-mediated induction of NFATc3 proteins is not fully understood. Since the mRNA and protein levels of NFATc3 are up-regulated by STIM1 expression (Fig. 4), we suppose that it is mediated by a transcriptional control by unknown molecules. The identity of this transcriptional factor(s) remains to be elucidated. Presumably, multiple Ca^{2+}-dependent transcription factors that are activated by STIM1/SOCE-mediated Ca^{2+} entry would be the possible candidates for this induction. Among the known SOCE-activated gene expression systems, we could exclude NFκB as described previously [17]. Other plausible mechanisms include the enhanced NFATc3 protein stability or the auto-transcriptional regulation by activated NFATc3.

Acknowledgments

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