The transcripational co-repressor TLE3 regulates myogenic differentiation by repressing the activity of the MyoD transcription factor

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Satellite cells are skeletal muscle stem cells that provide myonuclei for postnatal muscle growth, maintenance, and repair/regeneration in adults. Normally, satellite cells are mitotically quiescent, but they are activated in response to muscle injury, in which case they proliferate extensively and exhibit up-regulated expression of the transcription factor MyoD, a master regulator of myogenesis. MyoD forms a heterodimer with E proteins through their basic helix-loop-helix domain, binds to E boxes in the genome, and thereby activates transcription at muscle-specific promoters. The central role of MyoD in muscle differentiation has increased interest in finding potential MyoD regulators. Here we identified transducin-like enhancer of split (TLE3), one of the Groucho/TLE family members, as a regulator of MyoD function during myogenesis. TLE3 was expressed in activated and proliferative satellite cells in which increased TLE3 levels suppressed myogenic differentiation, and, conversely, reduced TLE3 levels promoted myogenesis with a concomitant increase in proliferation. We found that, via its glutamine- and serine/proline-rich domains, TLE3 interferes with MyoD function by disrupting the association between the basic helix-loop-helix domain of MyoD and E proteins. Our findings indicate that TLE3 participates in skeletal muscle homeostasis by dampening satellite cell differentiation via repression of MyoD transcriptional activity.

Skeletal muscle consists of postmitotic multinucleated myofibers that are derived from skeletal muscle stem cells called satellite cells that reside beneath the basal lamina. Satellite cells are normally mitotically quiescent but are activated in response to stimulation, such as muscle injury, to become myoblasts and proliferate extensively (1, 2). Quiescent satellite cells express Pax7 in adult muscle and, upon stimulation, up-regulate expression of the master regulator of myogenesis MyoD (3–5). MyoD forms a heterodimer with E proteins, such as E12 and E47, through their basic helix-loop-helix (bHLH) domain and can bind E box genomic sequences, comprised of the nucleotides CANNTG, as heterodimers with E proteins and activate transcription at muscle-specific promoters (6). Along with three related proteins, Myf5 (7), Myogenin (8), and MRF4 (also known as Myf6 or herculin) (9–11), MyoD constitutes a family of transcription factors called myogenic regulatory factors (MRFs). MRFs display a certain degree of functional redundancy (12, 13) and share a highly conserved variant of the bHLH domain that confers their myogenic potential (14).

Groucho/TLE family members are transcriptional co-factors that do not bind DNA directly but play critical roles during development and cell differentiation events (15). Groucho/TLE proteins consist of a five-domain structure (16): a highly conserved Q domain, which is a glutamine-rich region predicted to form two coiled-coil motifs that facilitates oligomerization of Groucho/TLE molecules (17–19); a glycine/proline-rich (GP) domain, which is essential for interaction of Groucho/TLE proteins with histone deacetylases (18, 20, 21); a CcN

2 The abbreviations used are: bHLH, basic helix-loop-helix; MRF, myogenic regulatory factor; TLE, transducin-like enhancer; EDL, extensor digitorum longus; CTX, cardio-toxin; MCK, muscle creatine kinase; MHC, myosin heavy chain; luc, luciferase; qPCR, quantitative PCR; GM, growth medium; DM, differentiation medium; Q, glutamine-rich domain; SP, serine/proline-rich domain; WD, tryptophan/aspartic acid repeat domain.

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This article contains supplemental Figs. 1–10.

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domain, which contains a nuclear localization sequence and putative cdc2 and casein kinase II (CK2) phosphorylation sites; a serine/proline-rich (SP) domain, which is a region rich in serine/proline residues (16, 22–24); and a highly conserved WD40 domain, which contains multiple tryptophan and aspartic acid tandem repeats, has been shown by X-ray crystallography to form a propeller, and binds many kinds of transcriptional factors (15, 25).

The Groucho/TLE family member TLE3 is expressed in white adipose tissue. Recently, Villanueva et al. (26, 27) reported that TLE3 enhanced the transcriptional activity of PPAR (peroxisome proliferator-activated receptor γ) and induced adipogenesis in preadipocytes. We have also reported that TLE3 induces adipogenesis and suppresses osteoblastogenesis in bone marrow–derived mesenchymal stem cells (28, 29). Even though TLE3 is expressed in embryonic somites and somite-derived myoblasts with an expression pattern overlapping that of MyoD and Myf5 (30), the function of TLE3 in skeletal muscle tissue is still unknown. Thus, we sought to examine the possibility that TLE3 regulates myogenesis and found that TLE3 represses myogenic differentiation through a suppressive effect on MyoD activity.

Results

**TLE3 is up-regulated in activated satellite cells of skeletal muscle**

We took advantage of an ex vivo model wherein myofibers and associated satellite cells are isolated from the extensor digitorum longus (EDL) muscle of mice to characterize the expression of TLE3 during myogenesis. TLE3 protein was nearly undetectable in Pax7-positive, quiescent satellite cells on freshly isolated myofibers (day 0) (Fig. 1A). However, after culturing in mitogen-rich medium for 2 days, TLE3 was expressed in Pax7-positive and MyoD-positive satellite cells (Fig. 1A). These findings suggest that TLE3 expression correlates with myogenic status.

To extend these findings to the in vivo setting, we utilized cardiotoxin (CTX) to stimulate activation of satellite cells in vivo and found that the expression levels of TLE3 mRNA and protein in muscle were increased after CTX injection (Fig. 1, C and F). This expression occurred prior to the up-regulation of Cyclin A2, a proliferation marker (Fig. 1, D and F). In contrast,
the expression of Myogenin, a differentiation maker, was increased, followed by down-regulation of TLE3 (Fig. 1, E and F). Immunohistochemistry confirmed that all activated satellite cells expressed TLE3 in vivo (Fig. 1G). These data suggest that the expression of TLE3 protein links cell proliferation and/or cell differentiation of satellite cells.

**TLE3 stimulates cell proliferation and suppresses cell differentiation in primary satellite cells**

To examine the function of TLE3, endogenous TLE3 was knocked down by shRNA or siRNA in satellite cells (Fig. 2, A and G). Reduced TLE3 levels were associated with diminished levels of the proliferation makers Cyclin D1 and Cyclin A2 (Fig. 2, B, C, H, I, L, and M), and knockdown of TLE3 stimulated cell proliferation of plated satellite cells (Fig. 2O). In contrast, the mRNA levels of myogenic differentiation marker genes such as Myogenin, muscle creatine kinase (MCK), and myosin heavy chain 14 (Myh14) were increased in shTLE3- or siTLE3-transfected satellite cells under myogenic conditions (Fig. 2, D–F, J–L, and N), suggesting that TLE3 stimulates proliferation and inhibits myogenic differentiation of primary satellite cells.

**TLE3 suppresses myogenic differentiation in C2C12 cells**

To examine the mechanism(s) by which TLE3 regulates myogenic differentiation of satellite cells, we turned to the C2C12 cell model because these cells are satellite cell–derived (31) and express TLE3 in their nuclei (Fig. 3A). Under myogenic conditions, reducing TLE3 expression with shRNA against TLE3 in C2C12 cells increased the protein level of Myogenin and MHC (Fig. 3B and supplemental Fig. 1A) and promoted myoblast fusion (Fig. 3, C and D). This repressive effect of TLE3 shRNA on myogenic differentiation was confirmed by introduction of siRNA against TLE3 into cells (Fig. 3, E–H). Knockdown of endogenous TLE3 did not, however, affect the expression levels of MyoD, a master regulator of myogenic differentiation (Fig. 3B).

**TLE3 interacts with MyoD and represses MyoD transcriptional activity**

We next hypothesized that TLE3 regulates the activity of MyoD. Co-immunoprecipitation revealed that TLE3 interacts with MyoD endogenously in C2C12 cells (Fig. 4A). To further understand these findings, we turned to the C5H10T1/2 cell
TLE3 represses MyoD transactivation

Figure 3. TLE3 regulates myoblast differentiation in C2C12 cells. A, C2C12 myoblasts were immunostained with anti-TLE3 antibody or normal IgG together with phalloidin and DAPI. Scale bar = 20 μm. B–D, C2C12 cells were transfected with control shRNA (Mock) or shRNA against TLE3 (shTLE3) and then cultured in myogenic medium. Western blot analysis with anti-TLE3 antibody showed reduction of endogenous TLE3 by shTLE3 along with increased Myogenin on day 2 (B). C and D, C2C12 cells transfected with control (Mock) or shRNA against TLE3 (shTLE3) were cultured in myogenic medium for 5 days and immunostained with anti-MHC antibody. Scale bar = 50 μm (C). The number of MHC-positive fibers (Fusion Index) was quantified on day 5 (D). E–G, Cells were transfected with control siRNA (Mock) or siRNA against TLE3 (siTLE3) and cultured in myogenic medium. The mRNA levels of TLE3 (E), Myogenin (F), and Myh14 (G) were assessed by qPCR analysis on day 2. H, the protein levels of MHC, TLE3, Myogenin, and β-actin were measured by Western blotting analysis on day 3. Similar results were obtained in three independent experiments (A–C and H). The data are expressed as mean ± S.D. (n = 3). **, p < 0.01 versus mock-transfected cells (D and E–G).

model, which has myogenic potential but no expression of MyoD as well as low TLE3 expression levels compared with C2C12 cells (supplemental Fig. 2). Consistent with our prior results (Fig. 4A), physical interaction was observed between epitope-tagged versions of TLE3 and MyoD in C3H10T1/2 cells (Fig. 4B). Furthermore, overexpression of MyoD led to increased expression of Myogenin, MCK, and Myh14, which was then blunted by overexpression of TLE3 (Fig. 4, C–E). TLE3 also reduced the protein levels of Myogenin and MHC as well as the number of MHC-positive cells induced by MyoD (Fig. 4, F–H, and supplemental Fig. 1B). We employed luciferase reporters driven by MG185 and MCK0.8 promoters to confirm that this effect was due to TLE3 repressing MyoD-dependent transcriptional activity (Fig. 4, I and J). TLE3 also repressed the transcriptional activity of other MRFs such as Myf5, Myogenin, and MRF4 (supplemental Fig. 3). Furthermore, ChIP assays revealed that overexpression of TLE3 suppressed the binding capacity of MyoD to the myogenin promoter region (Fig. 4K).

Both the Q and SP domains of TLE3 are essential for MyoD interaction and repression

Truncation mutants of TLE3 were designed to examine the domain(s) of TLE3 involved in the interaction with and inhibition of MyoD (Fig. 5A). Prior studies indicate that the WD domain of Groucho/TLE plays a critical role in regulating transcription (32). However, the WD domain deletion mutant of TLE3 (1–461) still repressed MyoD transcriptional activity, as assessed by MG185-luciferase assay (Fig. 5B). Consistent with this, both full-length TLE3 (1–782) and WD domain deletion mutant TLE3 (1–461) physically interacted with MyoD (Fig. 5C). We also confirmed that, unlike full-length TLE3, the WD domain deletion TLE3 (1–461) failed to repress transcriptional activity of a known TLE3 target, Runx2 (28) (supplemental Fig. 4).

Having demonstrated that the WD domain is not necessary for interacting with MyoD, we generated additional deletion mutants of TLE3 and examined their ability to affect MyoD activity. This revealed that mutants lacking the SP domain in TLE3 (1–278) did not block MyoD activity, nor did they show physical interaction with MyoD (Fig. 5, B and C). An additional series of N-terminal mutants (Fig. 5D) demonstrated that mutants lacking the Q domain in TLE3 (141–782), TLE3 (209–782), and TLE3 (279–782) did not interact with MyoD (Fig. 5E) or repress the transcriptional activity of MyoD (Fig. 5F). Collectively, our results indicate that both the Q and SP domains of TLE3 are essential for interacting with and repressing MyoD.
The suppressive mechanism of TLE3 on MyoD involves an E protein

We next generated two deletion mutants of MyoD to determine which domains of MyoD were targeted by TLE3 (Fig. 6A). Although MyoD (1–99) failed to induce MG185-luc activity, presumably because this mutant lacked the bHLH domain (14), full-length MyoD (1–318) and C-terminal transactivation domain deletion MyoD (1–167) were able to induce MG185-luc activity. TLE3 firmly repressed these activities (Fig. 6B), indicating that the C-terminal transactivation domain is not required for interaction with TLE3. Subsequent immunoprecipitation assays revealed that only the bHLH domain of MyoD is required to interact with TLE3 (Fig. 6C).

Finally, given that the MyoD also interacts with E proteins such as E12 through its bHLH domain (33), we predicted that TLE3 competes with E12 for binding to MyoD. Overexpression of E12 alone only slightly increased MG185-luc activity (supplemental Fig. 5), whereas co-expression of E12 with MyoD significantly enhanced luciferase activity induced by MyoD (Fig. 7A). Overexpression of E12 rescued the suppressive effect of TLE3 on MyoD activity in a dose-dependent manner (Fig. 7A). TLE3 did not interact with E12 (Fig. 7B), but overexpression...
**Figure 5.** Both the Q and SP domain of TLE3 are essential for MyoD interaction and repression. 

A, schematic of the C-terminally truncated forms of the Myc-tagged TLE3 constructs used in these experiments. GP, glycine/proline-rich domain. 

B, C-terminally truncated forms of TLE3 or empty vector (Mock) were co-transfected with the MG185-luciferase reporter along with MyoD in C3H10T1/2 cells. Luciferase activity was determined on day 1. C3H10T1/2 cells were co-transfected with the FLAG-tagged MyoD and empty vector (Mock) or C-terminally truncated forms of Myc-tagged TLE3. C, whole-cell lysates were immunoprecipitated (IP) using anti-FLAG magnetic beads and immunoblotted (IB) with an anti-Myc antibody or anti-FLAG antibody. D, schematic of the N-terminally truncated forms of the Myc-tagged TLE3 constructs. C3H10T1/2 cells were co-transfected with the FLAG-tagged MyoD and empty vector (Mock) or N-terminally truncated forms of Myc-tagged TLE3. E, whole-cell lysates were immunoprecipitated using FLAG-tagged magnetic agarose and immunoblotted with an anti-Myc antibody or anti-FLAG antibody. Empty vector (Mock) or N-terminally truncated forms of TLE3 were transfected with MG185-luciferase reporter along with MyoD in C3H10T1/2 cells. F, luciferase activity was determined on day 1. Similar results were obtained in three independent experiments (C and E). The data are expressed as mean ± S.D. (n = 3). **, p < 0.01 versus mock-transfected cells (B and F).

**Figure 6.** TLE3 interacts with the bHLH domain of MyoD. A, schematic of the C-terminally truncated forms of the Myc-tagged MyoD constructs used in these experiments. N-TAD, N-terminal transactivation domain; C-TAD, C-terminal transactivation domain. B, Empty vector (Mock) or C-terminally truncated forms of MyoD were transfected with MG185-luciferase reporter with or without TLE3 in C3H10T1/2 cells. Luciferase activity was determined on day 1. The data are expressed as mean ± S.D. (n = 3). **, p < 0.01 versus mock-transfected cells. C3H10T1/2 cells were co-transfected with the FLAG-tagged TLE3 and empty vector (Mock) or C-terminally truncated forms of Myc-tagged MyoD. C, whole-cell lysates were immunoprecipitated (IP) using anti-FLAG magnetic beads and immunoblotted (IB) with an anti-Myc antibody or anti-FLAG antibody. Similar results were obtained in three independent experiments.
The suppression of TLE3 decreased the interaction between E12 and MyoD (Fig. 7C). This suggests that TLE3 interacts with MyoD and masks the E protein-binding region on MyoD, and thus TLE3 competitively interferes with MyoD interactions. In addition, although Mef2 also interacts with MyoD and enhances the transcriptional activity of MyoD (34), TLE3 did not inhibit the transcriptional synergy between MyoD and Mef2 (supplemental Fig. 6).

Discussion

In this study, we report that TLE3 represses MyoD activity, and, thus, myogenic differentiation, by a mechanism that involves, at least in part, disruption of MyoD and E protein association (Fig. 7D). Furthermore, the expression of TLE3 appears to be related to the proliferative status of the cell (Figs. 1 and 2), suggesting that TLE3 may orchestrate satellite cell proliferation and differentiation during myogenesis. However, TLE3 expression is not completely absent in Myogenin-positive cells (supplemental Fig. 7), and overexpression of TLE3 exerts little influence on myogenic differentiation of C2C12 cells (supplemental Fig. 8), suggesting that TLE3 amounts as well as the right timing, or the existence of unknown TLE3 regulators, may also be important for the regulation of myogenesis by TLE3.

It is also worth noting that a number of proteins have been identified to act as myogenic antagonists by directly binding to E proteins or MyoD family proteins and blocking their ability to bind E box sequences and/or activate transcription at muscle-specific promoters. Unlike TLE3, many of these inhibitors are themselves HLH domain proteins and include Id, Twist, MyoR, and Mist-1 (35). TLE3 interacts with MyoD independently of its WD domain, which is the most highly conserved portion of Groucho/TLE proteins. Instead, our results suggest that both the Q and SP domains of TLE3 are required to interact and repress MyoD (Fig. 5). It is interesting to note that both the Q and SP domains of Gro4 (murine TLE4) are also essential for its interaction with Pax5. However, the WD domain of Gro4 is still needed to repress the transcriptional activity of Pax5 (36). In contrast, a shorter Groucho/TLE family member lacking a WD domain, called AES, is able to repress NF-κB (37).

That said, our experiments cannot exclude the possibility that other factors are also involved in the suppressive effect of TLE3 on transactivation of MyoD. High levels of Id protein repress MyoD activity indirectly (38), and another Groucho/TLE family member, TLE4, up-regulates Id1 expression (39). Because overexpression of TLE3 increased endogenous Id1...
levels in C3H10T1/2 cells (supplemental Fig. 9), it is possible that TLE3 may regulate MyoD activity via direct and indirect mechanisms. Additionally, because HES-1 inhibits the transcriptional activity of MyoD (40), and Groucho/TLE family members can interact with HES proteins, it is also possible that TLE3 may cooperate with HES-1 during inhibition of myogenensis. We find this possibility unlikely, however, because the WD domain of TLE3 is required for interaction with the WRPW domain of HES proteins (23) and, in our hands, TLE3 mutants lacking the WD domain are capable of suppressing MyoD activity to a similar degree as full-length TLE3.

Understanding the mechanisms regulating how satellite cells differentiate into myoblasts is a central question in muscle pathophysiology (41, 42) because TLE3 is up-regulated in activated/proliferative satellite cells and suppresses myogenic differentiation (Fig. 2). TLE3 is also expressed in myoblasts derived from somites in the embryo (30), and the expression of TLE3 in embryonic hind limb skeletal muscle is higher than in adults (supplemental Fig. 10), suggesting that TLE3 may also play an important role in skeletal muscle development in addition to satellite cell physiology. This raises the possibility that TLE3 could be a potential target for stem cell–based therapies for muscle-wasting diseases such as Duchenne muscular dystrophy and age-related sarcopenia. It is unclear at present, however, exactly how the expression of TLE3 is regulated. In addition, our study was mostly restricted to in vitro and ex vivo models and used several different cell types. In vivo analyses using conditional knock-out mice will be required to elucidate the physiological function of TLE3.

Experimental procedures

Cell culture, skeletal muscle single-fiber culture, and transfection

C2C12 cells and C3H10T1/2 cells were cultured and maintained as described previously (43). To induce myogenic differentiation, C2C12 cells were grown to 90% confluence and then cultivated for 2–5 days in DMEM containing 5% horse serum (37). Single fibers of skeletal muscle and associated satellite cells were prepared from mouse EDL muscles (44, 45). Detailed experimental procedures can be found in the supplemental information.

Animal experiments

The tibial anterior muscle of 10-week-old male C57BL/6 mice was injected with 50 μl of 10 μM cardiotoxin (Sigma-Aldrich Chemicals, St. Louis, MO) using a 29-gauge 1/2 insulin syringe. Muscles were removed 1, 3, 5, 7, 10, and 14 days later and immediately frozen in isopentane cooled in liquid nitrogen. All studies were done in accordance with the guidelines of and approved by the Experimental Animal Care and Use Committee of Kyushu Dental University.

Immunohistochemistry and immunocytochemistry analysis

Isolated single fibers, C2C12 cells, and C3H10T1/2 cells were incubated with primary antibodies at 4 °C overnight following blocking/permeabilization with phosphate-buffered saline containing 0.3% Triton X-100 and 5% goat serum for 20 min at room temperature. The following antibodies were used: for immunohistochemistry and immunocytochemistry: polyclonal anti-TLE3 antibody (Proteintech, Chicago, IL), anti-TLE3 mouse monoclonal antibody (ab213596, Abcam, Cambridge, UK), anti-Ki67 (ab92742, Abcam) anti-Mycogenin mouse monoclonal antibody (F5D, Santa Cruz Biotechnology, Santa Cruz, CA), anti-MyoD mouse monoclonal antibody (5.8A, Santa Cruz Biotechnology), anti-Pax7 mouse monoclonal antibody (sc-81648, Santa Cruz Biotechnology), anti-MHC mouse monoclonal antibody (MF20, R&D Systems, Minneapolis, MN), and CyclinD1 mouse monoclonal antibody (72-13G, Santa Cruz Biotechnology). The target proteins were visualized using an Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen). A BZ-9000 (Keyence, Tokyo, Japan) microscope was used for analysis. To visualize cell nuclei, cells were mounted with Hard Set mounting medium with DAPI (Vector Laboratories, Burlingame, CA). To visualize the cytoskeleton, cells were stained with rhodamine phalloidin (Thermo Fisher Scientific, Waltham, MA).

Plasmids

Plasmids encoding murine TLE3 have been described previously (28). Myf5 (accession no. NM_008656), MRF4 (accession no. NM_008657), Myogenin (accession no. NM_0031189), E12 (accession no. NM_001164147), and Mef2 (accession no. NM_001170537) were obtained by PCR amplification of mouse cDNA using PrimeSTAR HS DNA polymerase (TaKaRa, Ohtsu, Japan) and cloned into a FLAG-tagged or Myc-tagged pcDEF3 expression vector (46). 0.8MCK-luc was constructed by subcloning a 1-kb fragment (−1.8kb to −0.8 kb relative to transcription start site) of the 5’-flanking region of the murine muscle creatine kinase gene (47) into the pGL4 promoter vector (pGL4.26). All constructs were confirmed by sequencing. The FLAG–MyoD expression plasmid was kindly provided by Dr. Takenobu Katagiri (Saitama Medical University). The pGL3MG-185 luciferase plasmid (48) was kindly provided by Dr. Kiyoshi Kawakami (Jichi Medical University).

shRNA plasmid

Short hairpin RNA against murine TLE3 constructs (shTLE3) was designed using the BLOCK-IT RNAi designer tool (Invitrogen), and sense and antisense oligos were annealed and cloned into pcDNA 6.2-GW/miR (28). The following shRNA oligos were used: control shRNA, TGCTGAATTGCTGATTGTTGATGTGTTTTGGCACCAGCTGAC-GACTACACATCGAGGATT; shRNA against TLE3, TGCTGTGCTGAGGCTGTCTTTTTTGTCTGTTTGCCACTGA-CTGACAAGAGAAACAGCCTCAGCA. Only sense strands are shown (28).

siRNA

siRNA against murine TLE3 (Stealth siRNA, MSS2385134, Thermo Fisher Scientific) was transfected into primary satellite cells or C2C12 cells according to the protocol of the manufacturer (49).
Cell proliferation assay

The proliferation of plated satellite cells was assessed using Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) according to the protocol of the manufacturer (50).

Chromatin immunoprecipitation assay

ChIP was performed with a ChIP assay kit (Cell Signaling Technology, Beverly, MA) according to the instructions of the manufacturer, using anti-FLAG antibody (Wako, Osaka, Japan) and normal mouse IgG (MBL, Aichi, Japan). The purified DNA was analyzed by PCR using primers that amplify sequences within the Myogenin promoter region, which harbors MyoD binding elements (48). The primer pairs for the MyoG promoter were 5-GGA CCA TGG AGG AGA GAG TA-3 (forward) and 5-CAT CAG GTC GGA AAA GGC TT-3 (reverse).

Luciferase assays

Luciferase assays were performed using MG185-luc, MCK 0.8-luc, or pHRL-SV40 (Promega, Madison, WI) with the Dual-Glo luciferase assay system (Promega) (51).

Reverse transcription and quantitative PCR (qPCR) analysis

Total RNA was isolated from murine skeletal muscle, murine satellite cells, C2C12 cells, or C3H10T1/2 cells using TRIzol (Invitrogen) and then reverse-transcribed into cDNA using Superscript IV (Invitrogen). The cDNA was amplified by PCR using specific primers. Detailed primer sequences can be found in the supplemental information. SYBR Green-based quantitative real-time PCR was performed in 96-well plates using Thunderbird qPCR Mix (Toyobo, Osaka, Japan) with a 7300 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). Values were normalized to β-actin using the 2-ΔΔCt method (52).

Immunoprecipitation and Western blot analysis

The following antibodies were used for immunoprecipitation and Western blotting: anti-Myogenin mouse monoclonal antibody (F5D, Santa Cruz Biotechnology), anti-MyoD polyclonal antibody (C-20, Santa Cruz Biotechnology), anti-β-actin mouse monoclonal antibody (Sigma-Aldrich Chemicals), anti-V5 mouse monoclonal antibody (Sigma-Aldrich Chemicals), anti-Myosin heavy chain mouse monoclonal antibody (MF20, R&D Systems), CyclinD1 mouse monoclonal antibody (72-13G, Santa Cruz Biotechnology), CyclinA2 rabbit polyclonal antibody (GTX103042, GeneTex, Irvine, CA), and normal rabbit IgG (MLB). The target proteins were immunoprecipitated for 1 h at 4 °C using anti-DDDDK (FLAG) tag mAb magnetic agarose (MBL) and anti-MyoD antibodies coupled to protein G-Sepharose 4 Fast Flow (GE Healthcare UK Ltd.). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology). The immunoreactive proteins were visualized using ECL (GE Healthcare UK Ltd.).

Statistical analysis

Comparisons were made using unpaired analysis of variance with Tukey-Kramer post hoc test and Wilcoxon’s signed-rank test. The results are shown as the mean ± S.D. The statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01.

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