A Novel Zinc Finger Protein Zfp637 Behaves as a Repressive Regulator in Myogenic Cellular Differentiation

Kai Li,[1] Jie Zhang,[1] Jing-jing Ren,[1] Qi Wang,[1] Kai-yong Yang,[2] Zhu-juan Xiong,[1] Yong-qiu Mao,[3] Yan-yu Qi,[1] Xiao-wei Chen,[1] Feng Lan,[1] Xiu-jie Wang,[1] Heng-yi Xiao,[1] Ping Lin,[1]* and Yu-quan Wei[3]

[1]*Division of Geriatrics, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, People's Republic of China*

[2]*Department of Neurosurgery, West China Hospital, Sichuan University, People's Republic of China*

[3]*Division of Cancer Biotherapy, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, People's Republic of China*

ABSTRACT

Zinc finger proteins have been implicated as transcription factors in the differentiation and development of cells and tissues in higher organisms. The classical C2H2 zinc finger motif is one main type of motif of zinc finger proteins. Our previous studies have shown that Zfp637, which comprises six consecutively typical and one atypical C2H2 zinc finger motifs, is highly expressed in undifferentiated or poorly differentiated cell lines, but is moderately or slightly expressed in normal tissues. We have also demonstrated that Zfp637 can promote cell proliferation. However, its role in the regulation of cell differentiation remains unknown. We report here that endogenous Zfp637 as well as mTERT is expressed in proliferating C2C12 myoblasts and that their expression is downregulated during myogenic differentiation. Constitutive expression of Zfp637 in C2C12 myoblasts increased mTERT expression and telomerase activity, and promoted the progression of the cell cycle and cell proliferation. By contrast, endogenous repression of Zfp637 expression by RNA interference downregulated the mTERT gene and the activity of telomerase, and markedly reduced cell proliferation. Overexpression of Zfp637 also inhibited the expression of myogenic differentiation-specific genes such as MyoD and myogenin, and prevented C2C12 myoblast differentiation. Our results suggest that Zfp637 inhibits muscle differentiation through a defect in the cell cycle exit by potentially regulating mTERT expression in C2C12 myoblasts. This may provide a new research line for studying muscle differentiation. J. Cell. Biochem. 110: 352–362, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ZINC FINGER PROTEIN; C2C12 MUSCLE CELL; CELL DIFFERENTIATION; mTERT; CELL CYCLE

A significant number of transcription factors contain evolutionarily conserved zinc finger motifs. The classical C2H2 zinc finger motif, which employs two cysteine and two histidine residues to coordinate a single zinc ion, is a main type of zinc finger protein. Many of the C2H2 zinc finger proteins identified have been shown to be transcription factors that play important roles in the differentiation and development of cells and tissues in higher organisms. The human ZNF268 gene plays a role in the differentiation of blood cells during early human embryonic development and the pathogenesis of leukemia [Guo et al., 2006]. HZF1 plays important roles in erythroid and megakaryocytic

differentiation [Peng et al., 2006]. THAP1 promotes endothelial cells proliferation and G1/S cell cycle progression [Cayrol et al., 2007]. Zfp423 controls the proliferation and differentiation of neural precursors in cerebellar vermis formation [Alcaraz et al., 2006].

The ends of linear eukaryotic chromosomes are capped by specialized DNA-protein structures, called telomeres, which are composed of tandem hexanucleotide repeats (TTAGGG)n [Greider, 1996; Blackburn, 2001]. Telomeres are maintained by a specialized ribonucleoprotein enzyme, called telomerase, which adds motif-specific nucleotides using its RNA subunit as a template [Katsura et al., 2001]. In somatic cells, telomeric DNA is lost with every cell

division, and telomere length may serve as a mitotic clock in regulating cell division. When telomeres are shortened to such a critical point that they cannot stabilize the chromosome ends, most of the cells exit from the cell cycle and are destroyed by apoptosis [Hastie et al., 1990; Blackburn, 2000; Hackett et al., 2001]. Telomerase is a ribonucleoprotein enzyme that adds telomeric repeats to the chromosome ends. In the presence of telomerase, the telomere length is extended or maintained, thus avoiding replicative senescence [Bodnar et al., 1998; Vaziri and Benchimol, 1998; Natesan, 2005].

Recent studies show that telomerase comprises three major subunits: telomerase RNA component, telomerase-associated protein (TEP1) and telomerase reverse transcriptase (TERT). TERT is the most important component responsible for the catalytic activity of telomerase, which plays a central role in telomerase activity [Nakamura et al., 1997; Martin-Rivera et al., 1998]. Most telomerase-positive cells, including most malignant tumors and germ cells, are highly regenerative or immortal. Among normal tissues in adult humans, telomerase activity is almost undetectable. By contrast, telomerase activity in the normal mouse differs from that in the adult human [Broccoli et al., 1995; Hiyama et al., 1995; Wright et al., 1996; Roger et al., 1998]: in the normal mouse, telomerase activity exists in the colon, liver, ovary, and testis but not in the brain, heart, stomach, and muscle [Chadeneau et al., 1995; Prowse and Greider, 1995]. Telomerase activity decreases during cell differentiation, such as in embryonic stem cell differentiation, during placental differentiation and in C2C12 myoblast cells [Sharma et al., 1995; Holt et al., 1996; Kruk et al., 1996; Armstrong et al., 2000; Rama et al., 2001; Lopatina et al., 2003; Miura et al., 2004]. Many studies have shown that mTERT can be regulated by a number of inducible transcription factors, including AP1, Myc, and NF-κB [Takakura et al., 1999; Kyo et al., 2000; Yin et al., 2000; Masahiro et al., 2005; Flores et al., 2006; Natarajan et al., 2008; Bazarov et al., 2009]. These results suggest that, during differentiation, the reduced telomerase activity may result from downregulation of TERT by several transcription factors so that the cells cease to proliferate.

Zfp637, a novel zinc finger protein, is located in chromosome 6F1 of Mus musculus, and the full-length cDNA is 1,114 bp, which encodes 272 amino acids (GenBank GeneID: 232337) and comprises six consecutively typical and one atypical C2H2 zinc finger motifs. According to bioinformatics analysis, Zfp637 probably contains sites for protein kinase C phosphorylation, phosphotyrosine kinase phosphorylation, and N-terminal myristoylation, and an epidermal growth factor (EGF)-like structural domain, etc. However, the function of Zfp637 in cell differentiation has not been reported.

In this study, we used C2C12 myoblast as a model to examine the role of Zfp637 in regulating cell differentiation. We show that both endogenous Zfp637 and mTERT expression are downregulated during muscle differentiation. The constitutive overexpression of Zfp637 in C2C12 myoblasts inhibited their differentiation and significantly promoted cell proliferation compared with control C2C12 cells. We also have found that the inhibition of muscle differentiation by Zfp637 is dependent on its effects on cell cycle progression and that Zfp637 regulates myogenesis by potential promoting mTERT expression.

MATERIALS AND METHODS

CELL CULTURE AND SUBCELLULAR LOCALIZATION OF pEGFP-Zfp637 FUSION PROTEIN DURING C2C12 MYOBLAST DIFFERENTIATION

Mouse C2C12 myoblasts were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMRM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (growth medium, GM). Cells were sustained at 37°C in a humidified 95% air and 5% CO_2 atmosphere. When the C2C12 cells were 80% confluent, differentiation of C2C12 cells was initiated by shifting to the DMEM supplemented with 2% heat-inactivated horse serum (differentiation medium, DM) as described previously [Watanabe et al., 2007]. Differentiation media was replaced every 2 days.

C2C12 cells were transfected with the Zfp637 expression plasmid (pEGFP-Zfp637) by a liposome-mediated method using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were shifted to DM for 6 days. Differentiation and subcellular localization of the fusion protein were checked by cell morphology under phase contrast and fluorescence microscopy (ECLIPSE TE2000-U, Nikon, Japan).

RNA ISOLATION AND QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (REAL–TIME PCR) ANALYSIS

At the indicated times, total RNA was isolated using RNAiso plus (TAKARA, Japan) from cells that had been cultured in DM or GM according to the manufacturer's instructions. Samples of 10 μg of total RNA were separated by electrophoresis on a 1% agarose gel for quantitation. The quantity and purity of total RNA were detected using a Biophotometer (Eppendorf, Germany). Real-time PCR was performed and analyzed using an iCycler iQ™ Multicolor Real-Time Detection System (Bio-Rad, Japan). The real-time PCR primers sequences were as follows. Zfp637: forward, 5′-GCC TTT TTC AAT GTG ATG ACA GA-3′ and reverse, 5′-TCC CAC ATT CCT GGC AAT C-3′; mTERT: forward, 5′-GTT CTG TGA CTA CGC AGG TTA TGC C-3′ and reverse, 5′-TCA GAC TGT CGG GAG GCT ATT CAC-3′; MyoD: forward, 5′-GAC GGC TCT CTC TGC TCC TT-3′ and reverse, 5′-AGT AGG GAA GTG TGC GTG CT-3′; myogenin: forward, 5′-GCT GCC TAA AGT GGA GAT CCT-3′ and reverse, 5′-GCG CTG TGG GAG TTG CAT-3′; GAPDH: forward, 5′-CAT GGA GAA GGC TGG GGC TC-3′ and reverse, 5′-CAC TGA CAC GTT GGC AGT GG-3′. Each 1 μg of total RNA derived from different cells was subjected to reverse transcription with a PrimeScript™RT reagent kit (TAKARA) according to the manufacturer's instructions. The PCR reaction mixtures (25 μl) comprised 2 μl of reverse-transcribed products, 1 μl of each Forward and Reverse primer, and 12.5 μl of 2 × SYBR Green Buffer. Amplification involved initial denaturation at 95°C for 10 s followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 45 s. Fluorescence data were acquired at 60°C during each cycle. To determine the specificity of PCR reactions, melt curve analysis was performed after amplification by slow heating from 55 to 95°C, with fluorescence acquisition at 1°C intervals and a 5 s hold at each increment. The expression of the housekeeping gene glyceraldehyde phosphate dehydrogenase

(GAPDH) was used to normalize for transcription and a "no template" sample was used as a negative control. The relative expression unit was determined by using Gene Expression Macro Version 1.1 software (Bio-Rad Laboratories, Inc.). Samples were analyzed in triplicate.

MEASUREMENT OF TELOMERASE ACTIVITY

Cell telomerase activity was detected by a modification of the protocol as described previously [Lin et al., 2003]. Briefly, total cellular extracts were made according to the protocol of the telomerase PCR-enzyme-linked immunosorbent assay (ELISA) kit

(Roche Diagnostics, Mannheim, Germany). The telomerase activity of extracts was measured using the telomerase PCR-ELISA according to the manufacturer's instructions. PCR products were detected using a photometric enzyme immunoassay and the absorbance of samples was read at 450 nm on a Model 680 microplate reader (Bio-Rad). All assays were performed in triplicate. In the telomerase PCR-ELISA, the level of telomerase activity in the positive control cell extract supplied in the kit was set to 100%, and the relative specific telomerase activity (RTA) of each extract was expressed as a percentage of the positive control standard (mean ± SD)
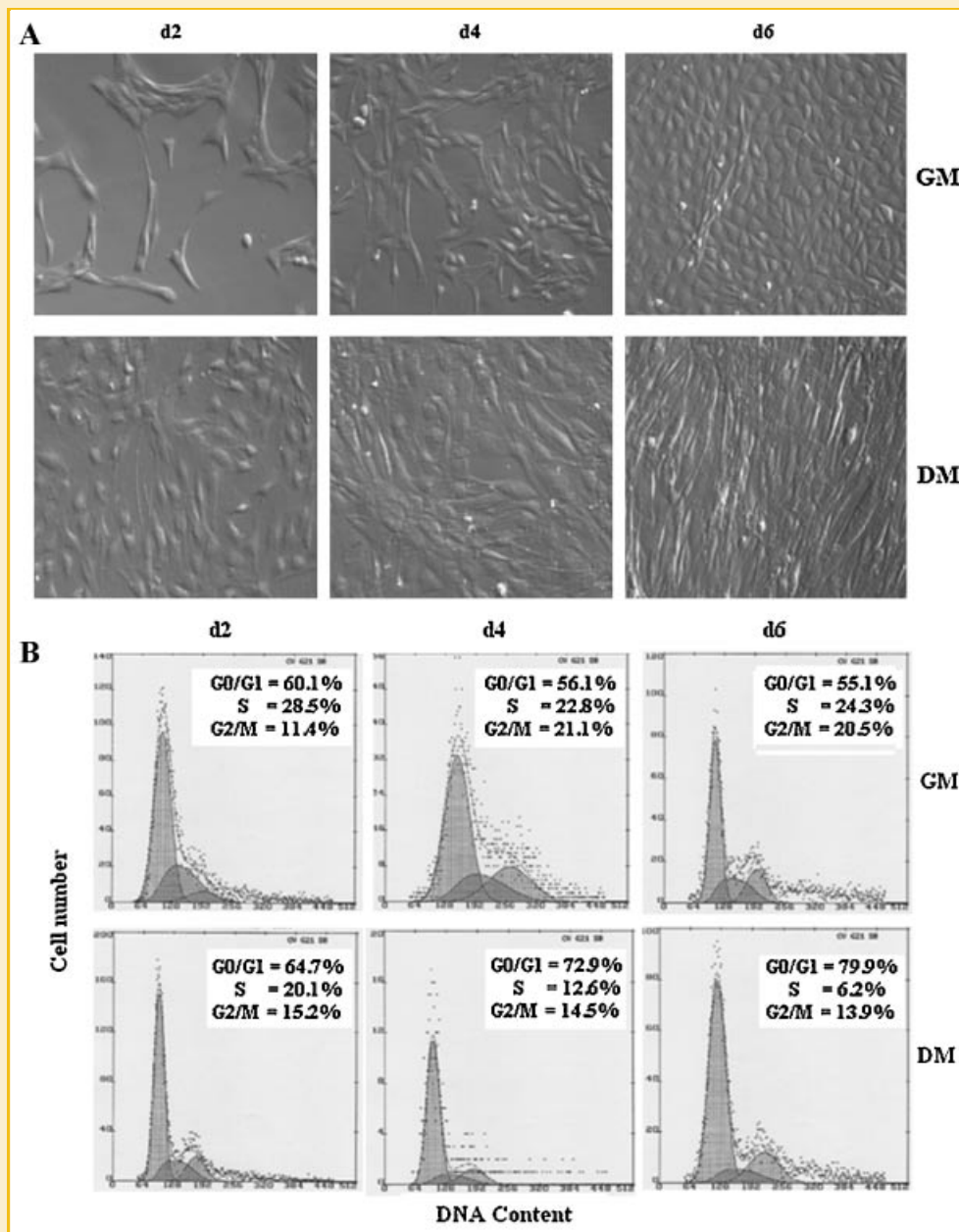

Fig. 1. Assessment of C2C12 cell myogenic differentiation. A: Cellular morphology analysis during C2C12 cell differentiation (magnification, 10×). B: C2C12 cell cycle phase distribution analysis. Cells were analyzed 2 days, 4 days, and 6 days after differentiation. The micrographs represent three independent experiments.

TRANSIENT TRANSFECTION OF siRNAs AND GENERATION OF STABLE C2C12 CELL LINES OVEREXPRESSING Zfp637

siRNA targeting Zfp637 was synthesized by GenePharma Co., Ltd (Shanghai, China) with the following sense and antisense sequences: siRNA-Zfp637, 5'-CCA GCG AAG CUG CUC ACA AdTdT-3' (sense) and 5'-UUG UGA GCA GCU UCG CUG GdTdT-3' (antisense). Fifty-thousand C2C12 cells per well were plated in a six-well plate, and 20 pmol of siRNA-Zfp637 was transfected into C2C12 cells by using Lipofectamine™ 2000. Cells were maintained for 48 h in GM before inducing differentiation.

To obtain stable transfectants overexpressing Zfp637, C2C12 cells were transfected with an expression constructor for Zfp637 (pcDNA3.1-Zfp637). Forty-eight hours after transfection, cells were selected in screening medium (GM containing 600 µg/ml G418). After 14–21 days, G418-resistant clones were isolated, expanded, and screened by real-time PCR and Western blot analysis.

CELL PROLIFERATION ASSAY

The same amount of cells was seeded into a 96-well plate and cultured in DM or GM, 50 µg/well of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solutions (Sigma, St. Louis,

MO) in PBS was added, and the plate was incubated for additional 4 h at 37°C. Thereafter, 150 µl of DMSO was added to each well to dissolve the dye crystal formazan, and the plate was shaken on a microplate shaker for 15 min to dissolve all of the purple crystals. Absorbance was measured at 570 nm on a Model 680 microplate reader (Bio-Rad).

WESTERN BLOT ANALYSIS

The cells were collected and washed three times with PBS and then lysed with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml aprotinin) for 30 min on ice. The protein content was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratory, Inc., Hercules, CA), and equal amounts of extracted proteins (100 µg) were loaded, separated by 10% or 12% sodium dodecyl sulfate–polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking with TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl) containing 5% skim milk for 1 h at 37°C, the membrane was incubated with primary antibodies at 4°C overnight. Monoclonal antibodies to MyoD, myogenin and GAPDH were purchased from Santa Cruz Biotechnology
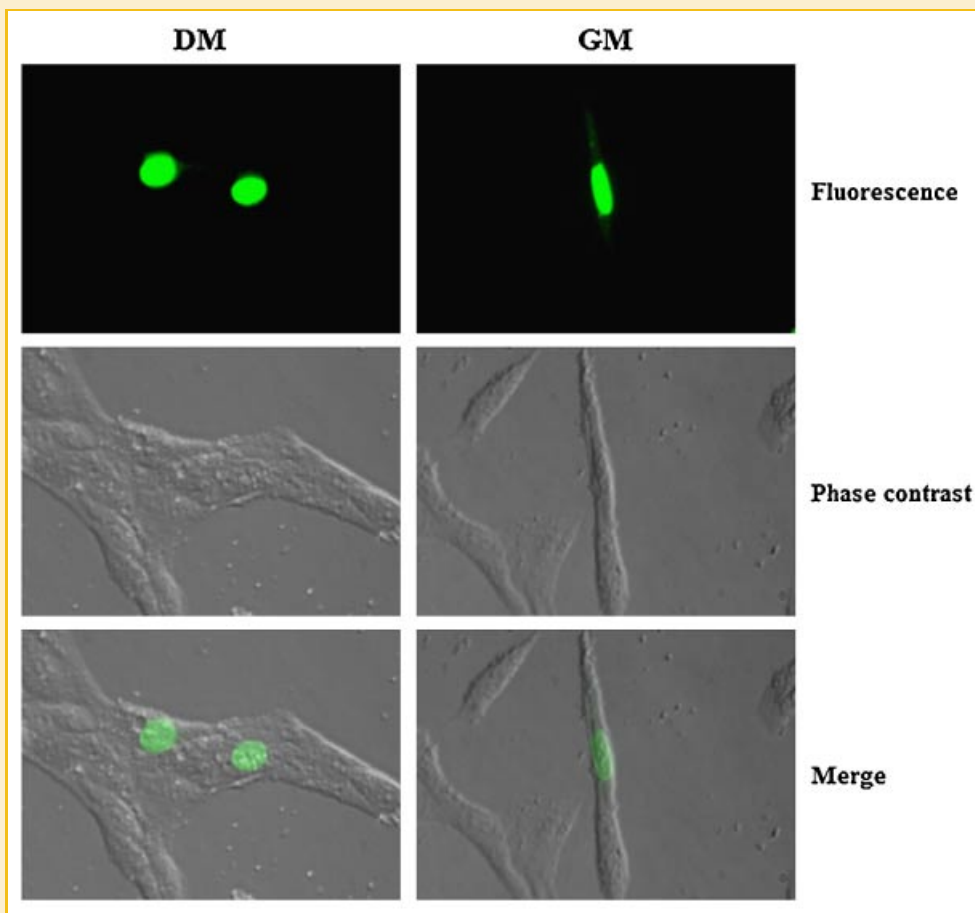

Fig. 2. Subcellular localization of pEGFP–Zfp637 fusion protein during C2C12 cell myogenic differentiation. C2C12 cells were transiently transfected with pEGFP–Zfp637, and 48 h later, the cells were shifted to DM for 4 days. The micrographs represent three separate experiments (magnification, 40×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Santa Cruz, CA), and the polyclonal antibody to Zfp637 was purchased from by Huaan Biotechnology Co., Ltd (Hangzhou, China). After incubation with primary antibody, the membranes were washed in TBST (TBS with 0.1% Tween-20) and then incubated with secondary antibody conjugated to horseradish peroxidase (Sigma) for 1 h. After three washes with TBST, the films were developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Protein levels were quantified by scanning blots on a Typhoon scanner (GE Healthcare) and analyzed with ImageQuant software (GE Healthcare).

FLOW CYTOMETRY

To measure the DNA content and study the cell cycle, the cells were harvested and fixed in 70% ice-cold ethanol at $-20°C$ overnight. After fixation, cells were washed with PBS, resuspended in 1 ml PBS containing 1 mg/ml RNase (Sigma) and 50 μg/ml propidium iodide (PI) (Sigma), and incubated at 37°C for 30 min in the dark. At least 10^5 cells were analyzed using an EPICS® Elite ESP flow cytometer (Beckman Coulter, USA). Cell cycle phase distributions were analyzed using MultiCycle software (Beckman Coulter).

STATISTICAL ANALYSIS

All data are expressed as means ± SD. To evaluate the significant differences between two groups, the means were compared using Student's t test. Multiple group comparisons were performed using one-way analysis of variance. The data were analyzed using SPSS statistical software 13.0. $P ≤ 0.05$ was considered significant.

RESULTS

CELLULAR LOCALIZATION OF Zfp637 FUSION PROTEINS DURING C2C12 CELL MYOGENIC DIFFERENTIATION

The time course of conversion of C2C12 myoblasts into myotubes was monitored by observing the cell morphology and by flow cytometry. Replacing GM with DM led to the formation of myotubes within 4 days; more than 90% of C2C12 cells had fused to form multinucleated myotubes by 6 days (Fig. 1A). DM induced a clearly visible change in cell shape, and flow cytometry showed an increase in the G0/G1 cell fraction (Fig. 1B). Both analyses indicated that C2C12 cells switched from the proliferating to the postmitotic differentiated state.

To localize intracellular Zfp637 fusion proteins during myogenic differentiation, Zfp637 fusion proteins were localized microscopically in C2C12 cells transiently transfected with pEGFP-Zfp637 plasmid when cultured in GM or DM. The subcellular localization of Zfp637 fusion protein in transfected C2C12 cells cultured in GM or DM was analyzed 4 days after differentiation. As shown in Figure 2, Zfp637 fusion protein was localized exclusively in the cell nuclei in both the proliferating myoblasts and the differentiating myotubes, suggesting that Zfp637 functions in the nucleus as a potential transcription factor.

DECREASED EXPRESSION OF Zfp637 DURING MUSCLE CELLS DIFFERENTIATION

To explore the functions of Zfp637 in skeletal muscle, we examined its expression in the myoblast cell line C2C12. C2C12 cells cultured
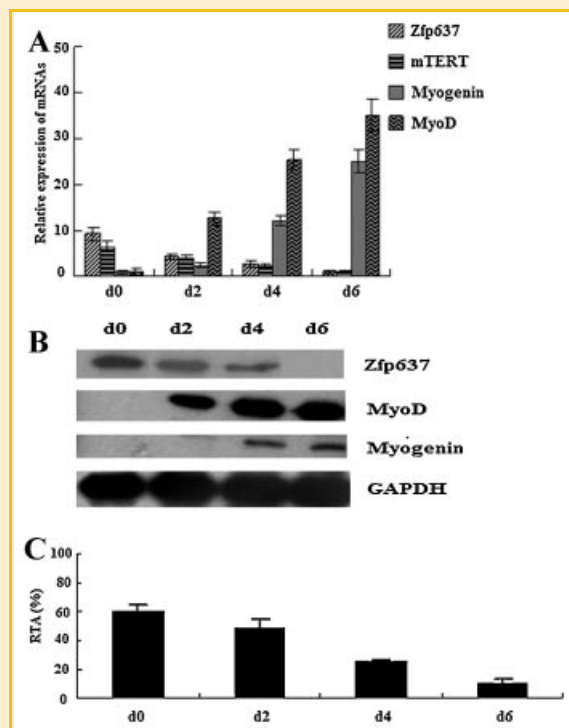

Fig. 3. Expression of Zfp637 during myoblast differentiation. C2C12 cells were grown to confluence in GM (day 0) and then shifted to DM for 6 days. Samples were harvested at the time points indicated. A: Transcripts for Zfp637, mTERT, myogenin, and MyoD were analyzed 4 days after differentiation by real-time PCR. The mRNA levels were normalized to GAPDH expression. B: Western blot analysis of zfp637, myogenin, and MyoD expression during C2C12 myoblast differentiation. C: PCR–ELISA was used to measure the telomerase activity in the same samples. The results are representative of three independent experiments. Western immunoblotting was performed with monoclonal antibodies to MyoD and myogenin, and polyclonal antibody to Zfp637. GAPDH was the internal control.

in GM and DM for up to 6 days were harvested and analyzed for Zfp637 expression by real-time PCR and Western blot analysis. Figure 3 shows a typical time course analysis of the transcripts accumulation during C2C12 differentiation. Zfp637 was relatively abundant in proliferating myoblasts cultured in growth medium, but decreased markedly in association with the induction of myotube differentiation. The mRNAs and proteins for the muscle-specific genes MyoD and myogenin increased markedly during differentiation (Fig. 3A,B). Interestingly, during the culture in DM, mTERT mRNA levels and telomerase activity also decreased gradually (Fig. 3A,C), in agreement with previous reports [Roger et al., 1998; Katsura et al., 2001]. These results suggest a relationship between mTERT and Zfp637 expression during muscle cell differentiation.

CONSTITUTIVE OVEREXPRESSION OF Zfp637 INHIBITS C2C12 MYOBLAST DIFFERENTIATION

To investigate the potential role of Zfp637 in myogenic differentiation, C2C12 cells were first stably transfected with the Zfp637 expression constructor pcDNA3.1-Zfp637. Cells were selected by G418 and several independent colonies (C2C12-Zfp637) were selected. The overexpression of Zfp637 in these cells was analyzed
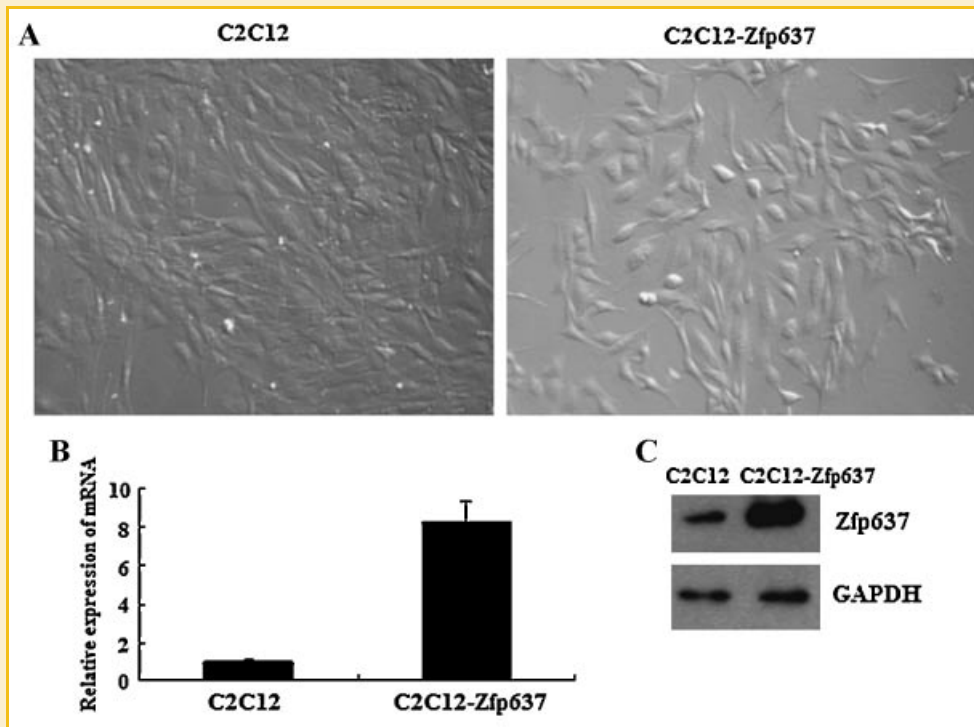
Fig. 4. Effect of zfp637 overexpression on myogenesis in C2C12 cells. A: Control C2C12 cells and C2C12–Zfp637 cells were induced to differentiate in DM for 4 days and analyzed morphologically for myotube formation (magnification, 10×). B: Zfp637 transcripts in C2C12 and C2C12–zfp637 cells were analyzed by real–time PCR. The mRNA levels were normalized to GAPDH expression. C: Expression of zfp637 protein in control C2C12 and C2C12–zfp637 cells was analyzed by Western blot analysis. The results are representative of three independent experiments. Western immunoblotting was performed with polyclonal anti–Zfp637 antibody. GAPDH was used as the internal control.

by real-time PCR and Western blot analysis. Figure 4A,B shows 8.2-fold higher mRNA levels of the exogenously transfected Zfp637 and 4.4-fold higher protein levels than for the endogenous gene. The normal C2C12 cells cultured in DM ceased to proliferate and fused to form multinucleated fibers within 4 days (Fig. 1). By contrast, myotube formation was blocked almost completely in the C2C12-Zfp637 cells, suggesting that overexpression of Zfp637 inhibits morphological differentiation of C2C12 myoblasts (Fig. 4A). More-over, overexpression of Zfp637 suppressed the expression of the muscle-specific genes MyoD and myogenin in C2C12-Zfp637 cells even when cultured in DM, whereas DM enhanced the expression of these two (Fig. 5A,B). In addition, overexpression of Zfp637 in C2C12 cells cultured in GM or DM increased the expression of the mTERT transcript and telomerase activity compared with those of control C2C12 cells cultured in GM or DM (Fig. 5A,C). Because the regulation of telomerase is tightly linked to the process of immortalization, (i.e., high telomerase activity occurs frequently in immortalized cells and cancers [Kim et al., 1994]), we measured the proliferation of C2C12 cells and C2C12-Zfp637 cells using an MTT assay. Similar to the gene expression data, the cell number increased in C2C12-Zfp637 cells compared with control C2C12 cells; 2 days after differentiation, cell number increased by 51% and 25% in cells grown in DM and GM, respectively (Fig. 5D). Taken together, these results indicate that overexpression of Zfp637 promotes cell proliferation by regulating mTERT directly or indirectly and that Zfp637 blocks C2C12 cell differentiation.

EFFECT OF siRNA TARGETING Zfp637 ON THE DIFFERENTIATION OF C2C12 CELLS

To study further the endogenous effects of Zfp637 knockdown in the differentiation of C2C12 cells, a siRNA-mediated gene-silencing method was used. The specific siRNA targeting Zfp637 was transfected into C2C12 cells by the lipofection method. Introducing the Zfp637-targeting siRNA downregulated Zfp637 expression (Fig. 6A,B). Forty-eight hours after transfection, the C2C12-siRNAZfp637 cells were subjected to myotube induction by DM, and the effect of Zfp637 knockdown on the C2C12 cells differentiation was examined by real-time PCR and Western blot analysis. Depression of Zfp637 suppressed the expression of mTERT and telomerase activity, but did not affect the expression of the differentiation-specific genes MyoD and myogenin (Fig. 6C–E). The MTT assay showed that transfection of Zfp637 siRNA, had a significant inhibitory effect on C2C12 cell proliferation compared with control C2C12 cells regardless of whether the cells were cultured in DM or GM (Fig. 6F). These results show that the endogenous depression of Zfp637 expression inhibits cell prolife-ration even when C2C12 cells are cultured in GM, this effect coincides with the overexpression of Zfp637.

EFFECT OF Zfp637 ON CELL CYCLE DISTRIBUTION DURING C2C12 DIFFERENTIATION STUDIED BY FLOW CYTOMETRY

To examine whether the inability of the C2C12-Zfp637 cells to differentiate occurs because of defects in cell cycle progression exit,
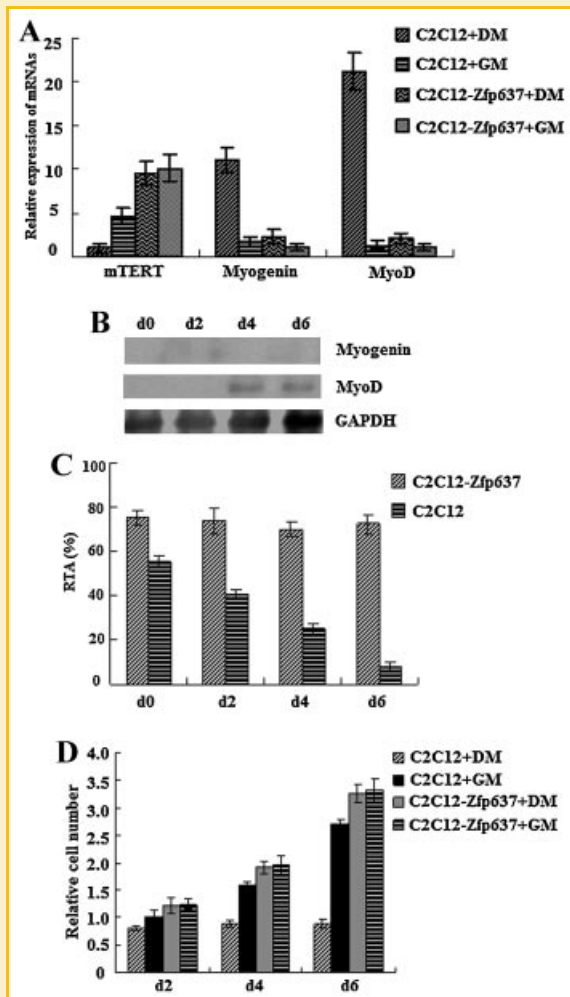
Fig. 5. Inhibition of the differentiation of C2C12 cells by overexpression of Zfp637. C2C12 and C2C12–Zfp637 cells were cultured in GM or DM for 6 days, and samples were harvested at the time points indicated. A: The expression of mTERT, MyoD, and myogenin was analyzed 4 days after differentiation by real-time PCR. The mRNA levels were normalized to GAPDH expression. B: The expression of mTERT, MyoD, and myogenin protein was analyzed by Western blot analysis in C2C12–Zfp637 cells cultured in DM at the times indicated. C: PCR–ELISA was used to measure telomerase activity in control C2C12 and C2C12–zfp637 cells. D: An MTT assay was performed in the samples at the times indicated. The absorbance was measured at 570 nm with a microplate reader. Data are expressed as means ± SEM for at least five separate determinations. The absorbance of C2C12–GM on day 2 was set as the reference standard, and the absorbance in each group was compared with this reference. Experiments at each time point were performed in triplicate, and the graph is representative of three independent experiments. Western immunoblotting was performed with monoclonal antibodies to MyoD and myogenin. GAPDH was used as the internal control.

the effects of Zfp637 on the cell cycle were analyzed by flow cytometry. C2C12 cells, C2C12-Zfp637 cells, and C2C12-siRNA cells, were cultured in GM or DM, and harvested and stained by PI 4 days after differentiation. As shown in Figure 7, regardless of whether cells were cultured in GM or in DM, in C2C12-Zfp637 cells, the percentage of cells in the S phase increased significantly and in the G1 phase decreased significantly. By contrast, in C2C12-siRNA cells, the percentage of C2C12 cells in the S phase decreased in both the GM and DM cultures. These results suggest that Zfp637 enhances DNA synthesis and that siRNA-Zfp637 induces cell cycle arrest at the G0/G1 phase and that the overexpression of Zfp637 resulting from the blocking of C2C12 cell differentiation by promoting cell proliferation occurs because of a failure to exit the cell cycle progression.

DISCUSSION

Members of the C2H2 zinc finger protein of transcription factors have been shown to play critical roles in cellular differentiation, growth, and apoptosis. In our previous study, we demonstrated that Zfp637 can promote cell proliferation in vivo and in vitro (data in preparation for publication). In our current study, we found that expression of Zfp637 mRNA and protein decrease during C2C12 muscle cell differentiation and displayed a time dependence. We also found that overexpression of Zfp637 can stop C2C12 differentiation and inhibit the expression of the muscle-specific genes MyoD and myogenin. Our data suggest that Zfp637 plays a role in differentiation. Based on the structural characterization of Zfp637 and its location in the nucleus (Fig. 2), we deduce that Zfp637 may function as a DNA-binding protein to regulate gene transcription during cell differentiation. The amino acid composition of the Zfp637 protein N-terminal sequence also supports possible roles of Zfp637 in gene regulation [Sigler, 1988; Mitchell and Tjian, 1989] Thus, Zfp637 may affect cell differentiation by regulating some genes.

The telomere plays an essential role in stabilizing the chromosome ends and in preventing end-to-end fusion, and its expression correlates with cell proliferation in many different types of cells [Harley et al., 1990]. Thus, telomerase activation is important in determining the proliferative capacity of cells and in counteracting telomere loss. The National Center for Biotechnology Information (NCBI) UniGene analysis of human Znf32, which has substantial sequence homology to the Zfp637, indicated that there is a relationship between Znf32 and hTERT, even though it has not been proven experimentally [Hiyama et al., 2005]. Thus, we hypothesized that Zfp637 affects cell differentiation by regulating the expression of mTERT. Zfp637 is expressed highly in all tumor cell lines, spermatids, spleen, and liver but slightly in the heart, bone marrow, and skeletal muscle of the normal mouse. The different expression levels of Zfp637 are consistent with the tissue-specific expression of telomerase activity in the adult mouse [Horikawa et al., 2005]. Telomerase activity is not detected in the heart, stomach, or muscle in the mouse [Chadeneau et al., 1995]. Hence, we postulated that there is a relationship between Zfp637 and mTERT expression in cell differentiation. Here, we demonstrated clearly that the expression of Zfp637 and mTERT, and telomerase activity decreased during C2C12 muscle cell differentiation, as shown previously [Bestilny et al., 1996]. Overexpression of Zfp637 increased the mTERT expression and telomerase activity, and silencing Zfp637 had the opposite effects. These results show that Zfp637 can regulate mTERT expression during C2C12 myoblasts differentiation.
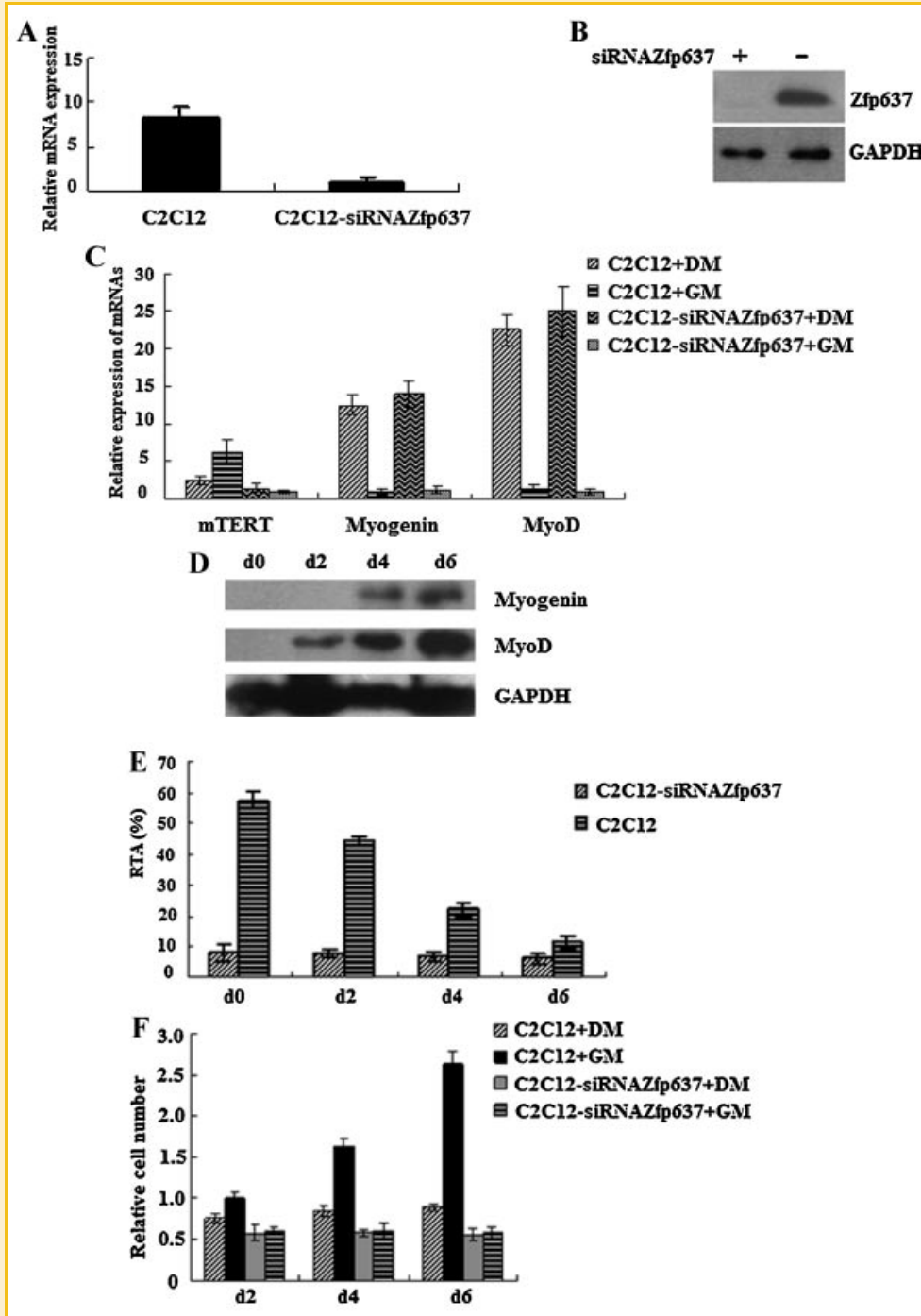
Fig. 6. Effect of zfp637 knockdown on myogenesis in C2C12 cells. A: C2C12 cells were transfected with siRNA–Zfp637, and 48 h later, the zfp637 transcripts in C2C12 and C2C12–siRNAZfp637 cells were analyzed by real–time PCR. The mRNA levels were normalized to GAPDH expression. B: Expression of zfp637 protein was analyzed by Western blot analysis on the same samples. C: The expression of mTERT, MyoD, and myogenin was analyzed in C2C12 and C2C12–siRNAZfp637 cells cultured in GM or DM on day 4 by real–time PCR. D: The expression of MyoD and myogenin protein was analyzed by Western blot analysis in C2C12–siRNAZfp637 cells cultured in DM at the times indicated. E: PCR–ELISA was used to measure telomerase activity in C2C12 and C2C12–siRNAZfp637 cells cultured in DM at the times indicated. F: An MTT assay was performed at the times indicated. Absorbance was read at 570 nm on a microplate reader. Data are expressed as means ± SEM for at least five separate determinations. The absorbance of C2C12–GM on day 2 was set as the reference standard, and absorbance in each group was compared with this value. Experiments at each time point were performed in triplicate, and the graph is representative of three independent experiments. Western immunoblotting was performed with polyclonal anti–Zfp637 and monoclonal antibodies to MyoD and myogenin. GAPDH was used as the internal control.
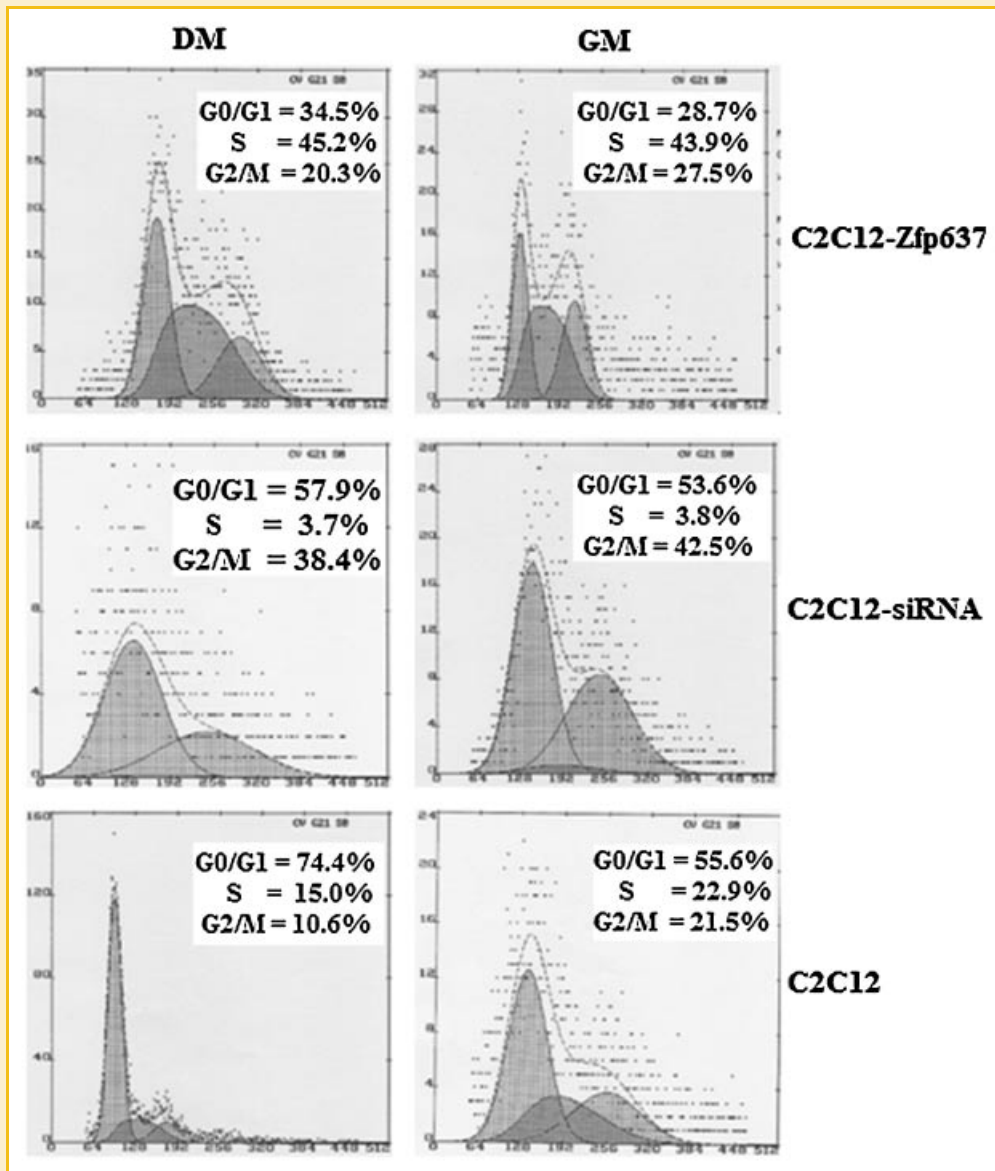
Fig. 7. Effect of zfp637 on the cell cycle distribution during C2C12 myotube differentiation. C2C12 cells, C2C12–zfp637 cells, and C2C12–siRNA cells were cultured in GM or DM, harvested, and stained with PI 4 days after differentiation. Experiments at the times indicated were performed in triplicate, and the graph is representative of three independent experiments.

Differentiation of skeletal muscle can be divided into two major steps: (1) withdrawal of myoblasts from the cell cycle and (2) expression of muscle differentiation genes [Sameena et al., 2004]. To determine the mechanism by which Zfp637 inhibits myogenesis, we analyzed the effect of Zfp637 on the cell cycle and on markers for terminal differentiation. The effects on cell cycle progression and cell proliferation were examined by flow cytometry and MTT assay respectively. Interestingly, Zfp637-overexpressing C2C12 cells were increased at S phase and decreased at G1 phase. In contrast, knockdown of Zfp637 expression depressed S phase and augmented G1 phase. Consistent with these observations, the MTT assay showed an increased cell number in Zfp637-overexpressing C2C12 cells compared with the controls. In addition, overexpression of Zfp637 could reduce the expression of myogenin, which is the marker of

terminal differentiation and critical for differentiation of skeletal muscle [Hasty et al., 1993; Nabeshima et al., 1993]. Thus, the differentiation block induced by Zfp637 is due to a defect in cell cycle exit. Like oncogenes that inhibit differentiation by promoting proliferation and preventing cell cycle exit [Olson, 1992; Hasan et al., 2005; Ye et al., 2007], the inhibitory effects of Zfp637 on differentiation can be linked to its effects on the cell cycle.

Overexpression of Zfp637 intensified the expression of mTERT and telomerase activity, and maintained cells at a higher proliferative ability than that of normal C2C12 cells. Even when Zfp637-overexpressing cells were shifted from GM to DM, the cells did not differentiate. By contrast, Zfp637 knockdown suppressed the cell proliferation and induced cell cycle arrest at the G0/G1 phase so that the cells were retained in the resting state, and it was easier to

induce differentiation with DM in these cells than in untransfected control cells. A murine mesenchymal stem cell line (MSC) transfected with mTERT maintains the typical MSC self-renewal capacity, continuously expresses the MSC phenotype, and retains the functional features of freshly isolated MSC in culture without evidence of senescence or spontaneous differentiation events [Forte et al., 2009]. Telomerase expression has been shown to be linked tightly to the cell cycle [Zhu et al., 1996]. On the basis of these findings, we infer that Zfp637 affects the cell cycle by regulating mTERT expression.

Our studies provide experimental support for the regulation of mTERT by Zfp637 in cell differentiation, but there is insufficient mechanistic evidence to explain the correlation between Zfp637 and mTERT in C2C12 cells. We are currently performing follow-up experiments, such as chromatin immunoprecipitation and promoter regulation of mTERT by Zfp637. Other groups have shown that telomerase activity is downregulated during the differentiation of muscle cells as a consequence of decreased mTERT mRNA [Katsura et al., 2001]. Both Sp1 and Sp3 directly regulate the expression of mTERT in C2C12 cells by interacting with the core promoter region of the mTERT gene, and they are downregulated in myogenesis. Zfp637 may play a direct role in increasing mTERT gene transcription by enhancing the promoter of mTERT or an indirect role by promoting mTERT gene transcription in vivo, that is, by regulating or synergistically cooperating with Sp1 and Sp3.

In conclusion, our data provide evidence that Zfp637 is a negative regulator of muscle differentiation. Consistent with its inhibitory role, Zfp637 is expressed at higher levels in proliferating myoblasts, and its expression is downregulated during differentiation. Over-expression of Zfp637 in C2C12 myoblasts inhibits muscle differentiation by causing a defect in cell cycle exit, and Zfp637 regulates myogenesis by potential promoting mTERT expression. Future studies aimed at evaluating its role in vivo will help elucidate more precisely the role of Zfp637 in cell proliferation and differentiation.

ACKNOWLEDGMENTS

REFERENCES

Alcaraz WA, Gold DA, Raponi E, Gent PM, Concepcion D, Hamilton BA. 2006. Zfp423 controls proliferation and differentiation of neural precursors in cerebellar vermis formation. Proc Natl Acad Sci 103:19424–19429.

Armstrong L, Lako M, Lincoln J, Cairns PM, Hole N. 2000. mTert Expression correlates with telomerase activity during the differentiation of murine embryonic stem cells. Mech Dev 97:109–116.

Bazarov AV, Hines WC, Mukhopadhyay R, Beliveau A, Melodyev S, Zaslavsky Y, Yaswen P. 2009. Telomerase activation by c-Myc in human mammary epithelial cells. Cell Cycle 8:3373–3378.

Bestilny LJ, Brown CB, Miura Y, Robertson LD, Riabowol KT. 1996. Selective inhibition of telomerase activity during terminal differentiation of immortal cell lines. Cancer Res 56:3796–3802.

Blackburn EH. 2000. Telomere states and cell fates. Nature 408:53–56.

Blackburn EH. 2001. Switching and signaling at the telomere. Cell 106:661–673.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.

Broccoli D, Young JW, de Lange T. 1995. Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci USA 92:9082–9086.

Cayrol C, Lacroix C, Mathe C, Ecochard V, Ceribelli M, Loreau E, Lazar V, Dessen P, Mantovani R, Aguilar L, Girard JP. 2007. The THAP-zinc finger protein THAP1 regulates endothelial cell proliferation through modulation of pRBE2F cell-cycle target genes. Blood 109:584–594.

Chadeneau C, Siegel P, Harley CB, Muller WJ, Bacchetti S. 1995. Telomerase activity in normal and malignant murine tissues. Oncogene 11:893–898.

Flores I, Evan G, Blasco MA. 2006. Genetic analysis of myc and telomerase interactions in vivo. Mol Cell Biol 26:6130–6138.

Forte G, Franzese O, Pagliari S, Pagliari F, Di Francesco AM, Cossa P, Laudisi A, Fiaccavento R, Minieri M, Bonmassar E, Di Nardo P. 2009. Interfacing Sca-1pos mesenchymal stem cells with biocompatible scaffolds with different chemical composition and geometry. J Biomed Biotechnol 2009:910610.

Greider CW. 1996. Telomere length regulation. Annu Rev Biochem 65:337–365.

Guo MX, Wang D, Shao HJ, Qiu HL, Xue L, Zhao ZZ, Zhu CG, Shi YB, Li WX. 2006. Transcription of human zinc finger ZNF268 gene requires an intragenic promoter element. J Biol Chem 281:24623–24636.

Hackett JA, Feldser DM, Greider CW. 2001. Telomere dysfunction increases mutation rate and genomic instability. Cell 106:275–286.

Harley CB, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. Nature 345:458–460.

Hasan UA, Trinchieri G, Vlach J. 2005. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. J Biol Chem 280:20620–20627.

Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC. 1990. Telomere reduction in human colorectal carcinoma and with ageing. Nature 346:866–868.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501–506.

Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. 1995. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol 155:3711–3715.

Hiyama K, Otani K, Ohtaki M, Satoh K, Kumazaki T, Takahashi T, Mitsui Y, Okazaki Y, Hayashizaki Y, Omatsu H, Noguchi T, Tanimoto K, Nishiyama M. 2005. Differentially expressed genes throughout the cellular immortalization processes are quite different between normal human fibroblasts and endothelial cells. Int J Oncol 27:87–95.

Holt SE, Wright WE, Shay JW. 1996. Regulation of telomerase activity in immortal cell lines. Mol Cell Biol 16:2932–2939.

Horikawa I, Chiang YJ, Patterson T, Feigenbaum L, Leem SH, Michishita E, Larionov V, Hodes RJ, Barrett JC. 2005. Differential cis-regulation of human versus mouse TERT gene expression in vivo: Identification of a human-specific repressive element. Proc Natl Acad Sci USA 102:18437–18442.

Katsura N, Kayoko M, Ken-ichi I. 2001. Mechanism for the reduction of telomerase expression during muscle cell differentiation. J Biol Chem 276:22016–22023.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015.

Kruk PA, Balajee AS, Rao KS, Bohr VA. 1996. Telomere reduction and telomerase inactivation during neuronal cell differentiation. Biochem Biophys Res Commun 224:487–492.

Kyo S, Takakura M, Taira T, Kanaya T, Itoh H, Yutsudo M, Ariga H, Inoue M. 2000. Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). Nucleic Acids Res 28:669–677.

Lin P, Azusa A, Aki O, Kenichi I, Hideo N. 2003. Dramatic increase of telomerase activity during dendritic cell differentiation and maturation. J Leukoc Biol 74:270–276.

Lopatina NG, Poole JC, Saldanha SN, Hansen NJ, Key JS, Pita MA, Andrews LG, Tollefsbol TO. 2003. Control mechanisms in the regulation of telomerase reverse transcriptase expression in differentiating human teratocarcinoma cells. Biochem Biophys Res Commun 306:650–659.

Martin-Rivera L, Herrera E, Albar JP, Blasco MA. 1998. Expression of mouse telomerase catalytic subunit in embryos and adult tissues. Proc Natl Acad Sci USA 95:10471–10476.

Masahiro T, Satoru K, Masaki I, Woodring EW, Jerry WS. 2005. Function of AP-1 in transcription of the telomerase reverse transcriptase gene (TERT) in human and mouse cells. Mol Cell Biol 25:8037–8043.

Mitchell PJ, Tjian R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371–378.

Miura T, Mattson MP, Rao MS. 2004. Cellular lifespan and senescence signaling in embryonic stem cells. Aging Cell 3:333–343.

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I, Nabeshima Y. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364:532–535.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR. 1997. Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955–959.

Natarajan M, Mohan S, Konopinski R, Otto RA, Herman TS. 2008. Induced telomerase activity in primary aortic endothelial cells by low-LET gamma-radiation is mediated through NF-kappaB activation. Br J Radiol 81:711–720.

Natesan S. 2005. Telomerase extends a helping hand to progenitor cells. Trends Biotechnol 23:1–3.

Olson EN. 1992. Proto-oncogenes in the regulatory circuit for myogenesis. Semin Cell Biol 3:127–136.

Peng H, Du ZW, Zhang JW. 2006. Identification and characterization of a novel zinc finger protein (HZF1) gene and its function in erythroid and megakaryocytic differentiation of K562 cells. Leukemia 20:1109–1116.

Prowse KR, Greider CW. 1995. Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc Natl Acad Sci USA 92:4818–4822.

Rama S, Suresh Y, Rao AJ. 2001. Regulation of telomerase during human placental differentiation: A role for TGFbeta1. Mol Cell Endocrinol 182:233–248.

Roger AG, Richard CA, Lynda C, Gregg BM, Ronald AD. 1998. Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation. Oncogene 16:1723–1730.

Sameena A, Anne O, Reshma T. 2004. Sharp-1/DEC2 inhibits skeletal muscle differentiation through repression of myogenic transcription factors. J Biol Chem 279:52643–52652.

Sharma HW, Sokoloski JA, Perez JR, Maltese JY, Sartorelli AC, Stein CA, Nichols G, Khaled Z, Telang NT, Narayanan R. 1995. Differentiation of immortal cells inhibits telomerase activity. Proc Natl Acad Sci USA 92: 12343–12346.

Sigler PB. 1988. Transcriptional activation acid blobs and negative noodles. Nature 333:210–212.

Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M. 1999. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res 59:551–557.

Vaziri H, Benchimol S. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol 8:279–282.

Watanabe S, Kondo S, Hayasaka M, Hanaoka K. 2007. Functional analysis of homeodomain-containing transcription factor Lbx1 in satellite cells of mouse skeletal muscle. J Cell Sci 120:4178–4187.

Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. 1996. Telomerase activity in human germline and embryonic tissues and cells. Dev Genet 18: 173–179.

Ye X, Zerlanko B, Zhang R, Somaiah N, Lipinski M, Salomoni P, Adams PD. 2007. Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Mol Cell Biol 27:2452–2465.

Yin L, Hubbard AK, Giardina C. 2000. NF-kappa B regulates transcription of the mouse telomerase catalytic subunit. J Biol Chem 275:36671–36675.

Zhu X, Kumar R, Mandal M, Sharma HW, Dhingra U, Sokoloski JA, Hsiao R, Narayanan R. 1996. Cell cycle-dependent modulation of telomerase activity in tumor cells. Proc Natl Acad Sci USA 93:6091–6095.