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CD36 is required for myoblast fusion during myogenic differentiation

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ABSTRACT

Recently, CD36 has been found to be involved in the cytokine-induced fusion of macrophage. Myoblast fusion to form multinucleated myotubes is required for myogenesis and muscle regeneration. Because a search of gene expression database revealed the attenuation of CD36 expression in the muscles of muscular dystrophy patients, the possibility that CD36 could be required for myoblast fusion was investigated. CD36 expression was markedly up-regulated during myoblast differentiation and localized in multinucleated myotubes. Knockdown of endogenous CD36 significantly decreased the expression of myogenic markers as well as myotube formation. These results support the notion that CD36 plays an important role in cell fusion during myogenic differentiation. Our finding will aid the elucidation of the common mechanism governing cell-to-cell fusion in various fusion models.

1. Introduction

Myoblast fusion is required for skeletal muscle formation during regeneration and growth after muscle injury and during myogenesis. Skeletal muscle consists of multinucleated myofibers formed by the fusion of mononucleated myoblasts in a process involving cell migration, alignment, recognition, adhesion, and membrane fusion [1,2]. Many molecules, such as cytokines, soluble proteins, transcription factors, and membrane receptors, have been implicated in myoblast fusion during myogenic differentiation [3,4]. However, the molecular mechanisms governing myoblast fusion remain to be investigated.

CD36 is a multifunctional protein that is involved in metabolism, immunity, angiogenesis, and behavior [5,6], and is also known to mediate apoptotic cell clearance via the recognition of phosphatidylserine (PS) on apoptotic cell surface [7,8]. Although the externalization of PS is known as a marker of apoptotic cells and critical for cell corpse clearance, accumulating data show that PS is also important for cell-to-cell fusion [9–13], which suggests that PS-binding proteins play a crucial role during muscle cell fusion. Recently, the exposure of PS on cell surfaces and lipid recognition by CD36 were found to be required for the cytokine-induced fusion of macrophages, but not for osteoclast fusion [14], which suggests that CD36 is selectively involved in cell-to-cell fusion of a specific cell type. Interestingly, a search of the GEO Profiles database revealed that CD36 expression is attenuated in the muscles of muscular dystrophy patients (GEO accession numbers GDS262 and GDS265) [15]. These observations led us to speculate that CD36 might be required for muscle cell fusion during myogenic differentiation. Here, we present evidence that CD36 is involved in myoblast fusion during myogenic differentiation.

2. Materials and methods

2.1. Antibodies

Goat polyclonal anti-CD36 antibody was purchased from R&D system, anti-myogenin antibody from Santa Cruz Biotechnology, monoclonal anti-MyHC antibody (clone MF20) from the Developmental Studies Hybridoma Bank, anti-actin antibody from Sigma Aldrich, and Alexa Fluor 488-conjugated anti-goat IgG and Alexa Fluor 647-conjugated anti-mouse IgG were purchased from Molecular Probes.

2.2. Cell culture

C2C12 myocytes were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM (high glucose) medium containing 10% (v/v) fetal bovine serum (FBS) and antibiotics. To induce myogenic differentiation, cells were grown to 100% confluence in maintenance medium and then switched to differentiation medium (DMEM supplemented with 2% horse serum).

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2.3. Western blotting

C2C12 cells were lysed in cold lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and proteinase inhibitors] for 30 min on ice. Identical amounts (20 μg of protein) of total cell lysates were resolved by 6–10% SDS–PAGE and then transferred to nitrocellulose membranes, which were incubated in blocking solution consisting of 5% skim milk in TBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature and then immunoblotted with anti-CD36 (R&D system), anti-MHC (Hybridoma bank), anti-myogenin (Santa Cruz), or anti-actin antibody (Sigma). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham).

2.4. Real-time quantitative PCR

Total RNA was purified from C2C12 cells using Trizol reagent, in accordance with the manufacturer's instructions (Invitrogen). Reverse transcription was performed with M-MLV reverse transcriptase (Promega) using 2 μg of total RNA for 50 min at 42 °C followed by 3 min at 95 °C. Real time PCR amplification was carried out in a LightCycler 480 (Roche Applied Science) using SYBR green master mix (Roche Applied Science) as follows: initial denaturation at 95 °C for 5 min followed by 45 amplification cycles (denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s). The primers used in this study are detained in Table 1. qRT-PCR results were analyzed as previously described [16,17]. Briefly, the comparative cycle threshold (C_T) method was used to analyze data by generating relative values for the amounts of target cDNA. Relative quantitations for given genes (expressed as fold induction over control) were calculated after determining the difference between the C_T of the target gene A and that of the reference gene B (β-actin) in C2C12/shCont cells ($\Delta C_{T1} = \Delta C_{T1A} - \Delta C_{T1B}$) and C2C12/shCD36 cells ($\Delta C_{T0} = \Delta C_{T0A} - \Delta C_{T0B}$) using the formula $2^{-\Delta\Delta C_{T(1-0)}}$.

2.5. Immunofluorescent staining

C2C12 cells were seeded onto 8-well chamber slides (Nunc), incubated in differentiation medium for the indicated times, fixed in 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.03% Triton X-100 for 5 min. Non-specific binding was then minimized by incubating the cells in PBS containing 10% goat serum for 2 h. After three washes with PBS, slides were incubated for 1 h at room temperature with a polyclonal anti-CD36 antibody and/or a monoclonal anti-MHC antibody. The slides were washed three times for 5 min each with PBS at room temperature and then incubated with Alexa Fluor 488-conjugated anti-goat IgG and Alexa Fluor 647-conjugated anti-mouse IgG (Molecular Probes) for 1 h at room temperature, followed by staining DAPI. The slides were washed five times with PBS for 5 min each and then treated with a solution of SlowFade (Molecular Probes). Slides were viewed under a fluorescence microscope (Leica).

Table 1
DNA sequences of the primers used for RT-PCR and quantitative real-time PCR.

Identity	Nucleotide sequences	Product size
CD36	Forward: 5′-GGTCTATCTACGCTGTGTTCG-3′ Reverse: 5′-ATCTAAGTATGTCCTATGCTC-3′	299
Myogenin	Forward: 5′-GTAAGGTGTGTAAGAGGAAG-3′ Reverse: 5′-TGTGGGAGTTGCATTCACTG-3′	288
MyoD	Forward: 5′-ATGGCATGATGGATTACAGCG-3′ Reverse: 5′-TCCCTGTTCTGTGTCGCTTAG-3′	260
Myf5	Forward: 5′-ATGCCATCCGCTACATTGAG -3′ Reverse: 5′-GGGTAGCAGGCTGTGAGTTG-3′	353
MRF4	Forward: 5′-CTACATTGAGCGTCTACAGG-3′ Reverse: 5′-CTTAGCAGTTATCACGAGGC-3′	190
GAPDH	Forward: 5′-AACATCAAATGGGGTGAGGCC-3′ Reverse: 5′-GTTGTCATGGATGACCTTGGC-3′	252
β-Actin	Forward: 5′-TCACCCACACTGTGCCCATCTACGA-3′ Reverse: 5′-GGATGCCACAGGATTCCATACCCA-3′	348

2.6. Knockdown of CD36 in C2C12 cells

A shRNA target sequence for mouse CD36 (accession number: NM_007643), 5′-GCU CAA GAA UGU CCG CAU AGA-3′, was selected using the Invitrogen shRNA design tool. For control shRNA, a scrambled sequence was used. The following forward and reverse oligonucleotides (sense-loop-antisense) were synthesized: shCD36 (sense), 5′-gatcccc GCTCAAGAATGTCCGCATAGA cgaa TCTATGCG GACATTCTTGAGC tttttggaaa-3′; shCD36 (antisense), 5′-agcttttc-caaaaa GCTCAAGAATGTCCGCATAGA ttcg TCTATGCGGACATTCTT-GAGC ggg-3′; scrambled shCont (sense), 5′-gatcccc GTACGATA CGACCCTTAATGT cgaa ACATTAAGGGTCGTATCGTAC tttttggaaa-3′; and scrambled shCont (antisense), 5′-agcttttccaaaaa GTACGATAC-GACCCTTAATGT ttcg ACATTAAGGGTCGTATCGTAC ggg-3′. Oligonucleotides were annealed, and cloned into the BglII/HindIII site of pSuper/neo vector (OligoEngine). C2C12 cells were then transfected with pSuper/shCD36 or pSuper/shCont vector using Lipofectamine 2000 (Invitrogen). At 24 post-transfection, cells were selected in the medium containing G418 (600 μg/ml). Individual G418-resistant colonies were isolated after 2 weeks of culture and designated as C2C12/shCD36. Negative control clones were randomly selected following transfection with pSuper/shCont vector (C2C12/shCont). The down-regulation of CD36 expression was verified by quantitative real-time PCR and Western blotting.

2.7. Fusion assays

After 3 and 5 days in differentiation medium (DM3 and 5), C2C12 cells were fixed and immunostained with antibody against myosin heavy chain (MF20, Developmental Studies Hybridoma Bank). The fusion index was calculated by dividing the number of nuclei in myotubes (⩾2 nuclei) by the total number of nuclei analyzed (1000–2000), as previously described [18]. At DM5, myoblast fusion was analyzed by calculating numbers of nuclei in MyHC+ cells, as previously described [19] with slightly modification. The number of nuclei in individual myotubes was counted for 100–200 myotubes in 10 random fields. MyHC+ cells were then grouped into: those with one nuclei, those with 2–4 nuclei, and those with ⩾5 nuclei. The percentages of nuclei in these three groups were expressed as percentages of total nuclei in MyHC+ cells.

2.8. Cell proliferation assay

Cell proliferation was analyzed using a CellTiter 96 AQueous One Solution cell proliferation assay kit (Promega), as previously described [20]. In brief, C2C12/shCD36 and C2C12/shCont cells were seeded at a density of 1000 cells per well into 96-well plates and incubated for 4 days under standard culture conditions. At the indicated time points, 20 μl of the AQueous One Solution reagent were added to each well and incubated for 1 h. Absorbance at 490 nm was then measured using a plate reader (BioRad).

2.9. Statistical analysis

Statistical significances were assessed using the student's *t*-test. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. CD36 expression was induced during myogenic differentiation

To investigate the involvement of CD36 in muscle cell fusion, we examined CD36 expression during differentiation in C2C12 cells, which develops the biochemical and morphological properties characteristic of skeletal muscle cells [21]. CD36 mRNA expression was found to be up-regulated in C2C12 cells during differentiation in a time-dependent manner (Fig. 1A), and this peaked after 3 days of treatment with differentiation medium (DM) and slightly declined thereafter (Fig. 1B). In agreement with this result, CD36 protein levels were also increased in C2C12 cells during differentiation (Fig. 1C), and were highly expressed at DM3–5 when large multinucleated myotubes were formed. We also found that expression of CD36 protein was increased in primary myoblasts during differentiation (Fig. 1D). To confirm the expression of CD36 during myogenic differentiation, C2C12 cells were immunofluorescent stained. CD36 was found to be rarely expressed in C2C12 myoblasts, but highly expressed in multinucleated myotubes, in which it was colocalized with myosin heavy chain (MyHC), a marker of muscle cell differentiation (Fig. 2). These results support the possibility that CD36 is involved in cell-to-cell fusion during myogenic differentiation.

3.2. CD36 was required for myogenic differentiation

To investigate the involvement of CD36 in skeletal muscle fusion, we generated CD36 knockdown C2C12 (C2C12/shCD36) and irrelevant knockdown C2C12 cells (C2C12/shCont) using vector expressing mouse CD36 shRNA or scrambled control shRNA, respectively (Fig. 3A). Quantitative real-time PCR analysis confirmed that CD36 mRNA level was significantly decreased in C2C12/shCD36 cells (Fig. 3B). In agreement with this mRNA result, CD36 protein expression was completely suppressed in C2C12/shCD36 cells at DM3 and DM5 (Fig. 3C). Because the formation of multinucleated myotubes is coordinated by myogenic transcription factors, such as Myogenin, Myf5, MRF4, and MyoD [22], we investigated the effect of CD36 knockdown on the expressions of myogenic transcription factors in C2C12 cells. As shown in Fig. 3D–G, C2C12/shCD36 cells showed significant reductions in the expressions of myogenic transcription factors as compared with C2C12/shCont cells. Furthermore, in C2C12/shCD36 cells, the expression of myosin heavy chain (MyHC) was reduced to 33% and 34% of those in C2C12/shCont cells on DM3 and DM5, respectively (Fig. 3H). These results suggest that CD36 is required for myogenic differentiation.

3.3. CD36 was required for muscle cell fusion

We next investigated the effects of CD36 down-regulation on cell proliferation and fusion. The proliferation rates of C2C12/shCD36 and C2C12/shCont cells were comparable (Supplemental Fig. 1). The morphology in maintenance medium did not also alter in both cells (data not shown). However, suppression of CD36 expression caused a significant decrease in myotube formation during differentiation (Fig. 4A). On DM5, C2C12/shCont cells formed large myotubes, whereas C2C12/shCD36 cells produced small myotubes (Fig. 4A). To quantify the cell-to-cell fusion, the fusion indices were calculated by expressing numbers of nuclei within MyHC+ cells with $\geqslant 2$ nuclei as a percentage of total nuclei in random fields. Parent C2C12 and C2C12/shCont cells share similar fusion indices of approximately 20% and 43% at DM3 and DM5, respectively, whereas fusion indices of C2C12/shCD36 cells at DM3 and DM5 were slightly lower with 4% and 12%, respectively (Fig. 4B). In C2C12/shCont cells, nuclei in MyHC+ cells were mainly observed in myotubes with $\geqslant 5$ nuclei at DM5, whereas in C2C12/shCD36 cells, nuclei in MHC+ cells were observed in cells containing 1–4 nuclei (Fig. 4C). Collectively, our results demonstrate that CD36 plays an important role in myoblast fusion for myogenic differentiation.

4. Discussion

Recently, it was reported that CD36 is involved in the cytokine-induced fusion of macrophages [14]. However, osteoclast fusion is

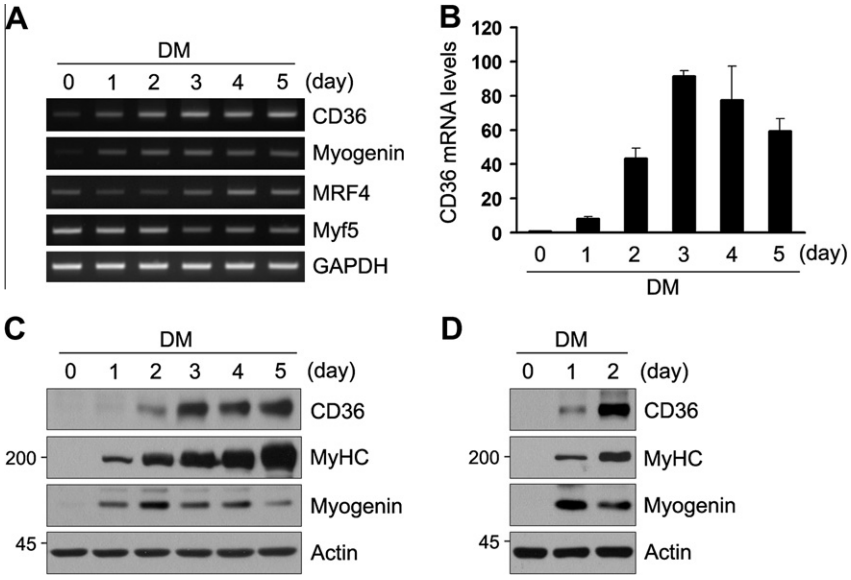

Fig. 1. CD36 expression was up-regulated during muscle cell differentiation. (A) Expression levels of CD36 and of myogenic markers were examined in C2C12 cells during differentiation by RT-PCR. A representative result from three independent experiments is shown. MyHC, myosin heavy chain; DM, differentiation medium. (B) Quantitation of CD36 mRNA expression during myogenic differentiation by quantitative real-time PCR. Relative expression levels were plotted against that of CD36 gene on DM0, which was set as 1. Results are expressed as means ± SD of three independent experiments. (C and D) CD36 protein expression was analyzed in C2C12 cells (C) and primary myoblasts (D) during differentiation by Western blotting. Representative results from three independent experiments are shown.

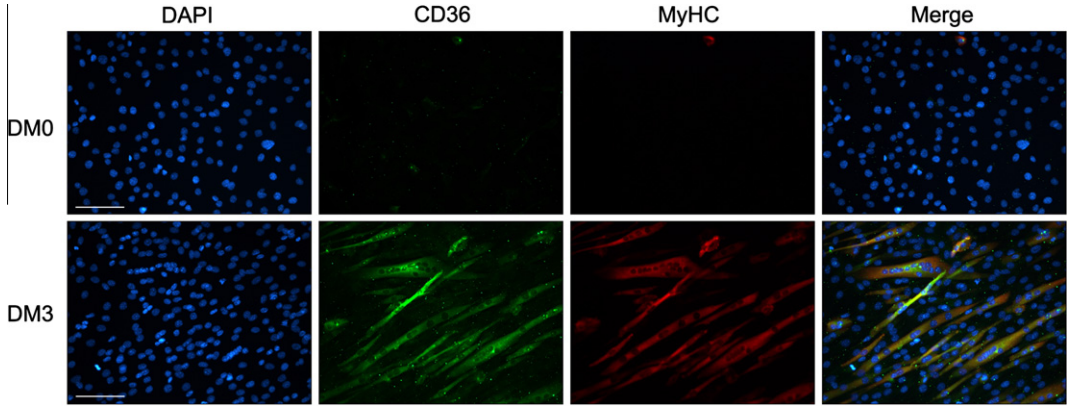

Fig. 2. CD36 was expressed in myotubes during myoblast fusion. C2C12 cells were induced to differentiate in DM, and CD36 protein expression was analyzed at DM0 and DM3 by immunostaining with anti-CD36 and anti-MyHC antibodies. CD36 is shown in green and MyHC (a myotube marker) in red. Yellow indicates colocalization of CD36 and MyHC. MyHC, myosin heavy chain. Scale bar, 100 μm.

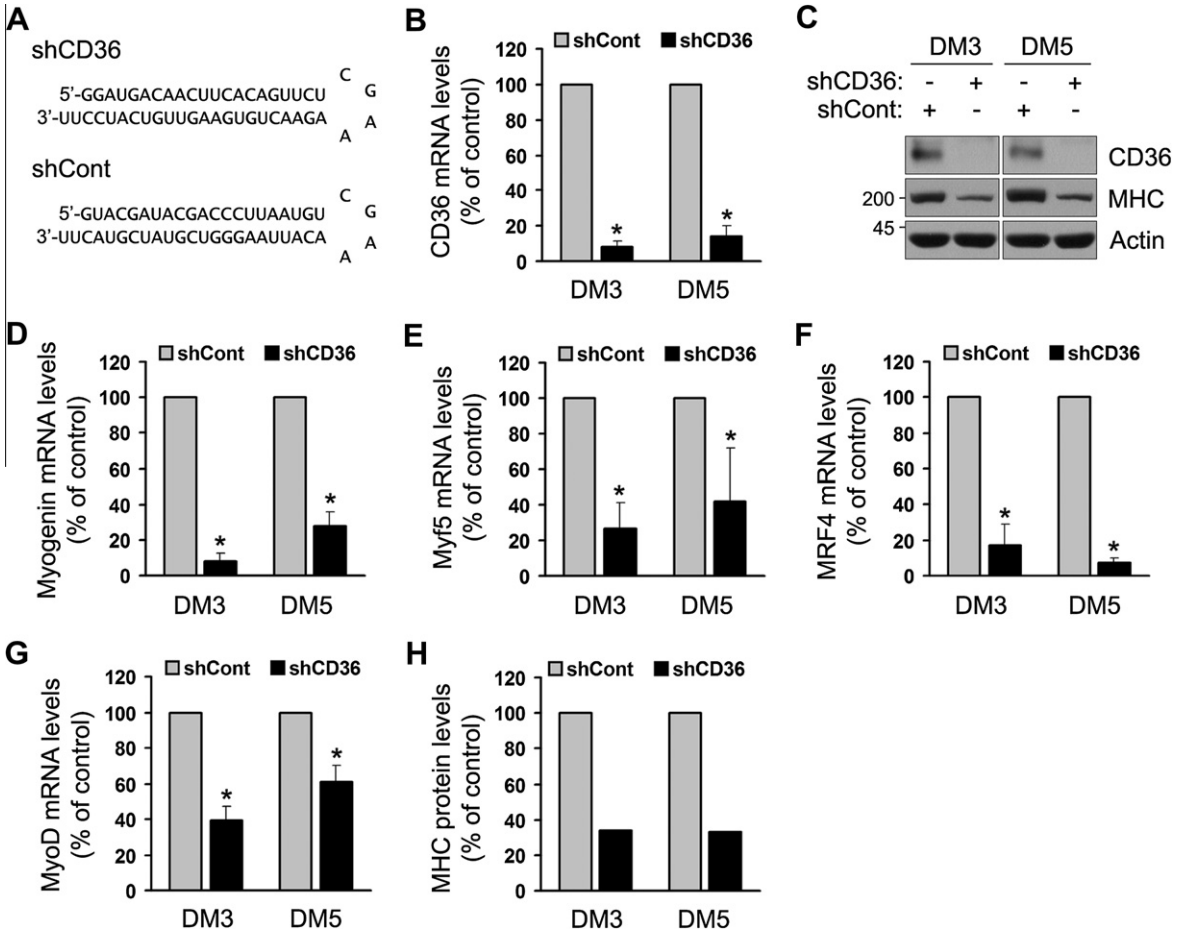

Fig. 3. Knockdown of CD36 inhibited the expression of myogenic markers in C2C12 cells during myogenic differentiation. (A) Schematic drawing of the shRNA used for knockdown of CD36 (shCD36) and its scrambled control shRNA (shCont). (B) C2C12 cells were stably transfected with a vector expressing shRNA against CD36 or scrambled control shRNA. On DM3 and 5, CD36 mRNA expressions were determined by quantitative real-time PCR. Relative expression levels were plotted against that of C2C12/shCont cells (set as 100%) at each time point. Results are expressed as means ± SD of three independent experiments. *t*-test: $^{*}P < 0.05$. (C) On DM3 and 5, CD36 and MyHC protein expressions were examined in C2C12/shCD36 and C2C12/shCont cells by Western blotting. A representative result from three independent experiments is shown. (D–G) C2C12/shCD36 and C2C12/shCont cells were induced to differentiate in DM, on DM3 and 5, the expression of Myogenin (D), Myf5 (E), MRF4 (F), and MyoD (G) were analyzed by quantitative real-time PCR. Relative expression levels were plotted against that of C2C12/shCont cells (set at 100%) at each time point. Results are expressed as means ± SD of three independent experiments. *t*-test: $^{*}P < 0.05$. (H) Immunoblot intensities for MyHC/Actin in C2C12/shCD36 and C2C12/shCont cells were quantitated by densitometry and expressed in arbitrary units. The intensity in C2C12/shCont cells was set to 100%. A representative result is shown.

independent of CD36 [14], which indicates that CD36 is selectively involved in various fusion processes. Four results obtained during the present study support the proposition that CD36 is involved in cell-to-cell fusion in skeletal muscle. First, CD36 expression was upregluated in C2C12 cells and primary myoblasts during myogenic differentiation. Second, CD36 protein expression was

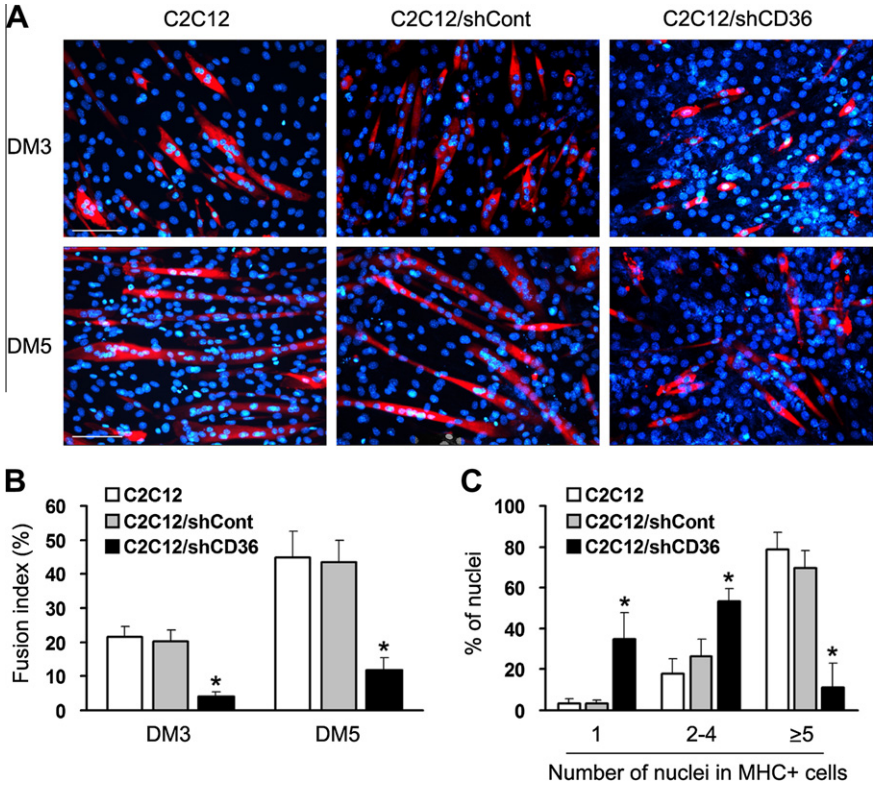

Fig. 4. Knockdown of CD36 inhibited the differentiation of C2C12 cells. (A) Representative images of myotube formation in C2C12, C2C12/shCont, and C2C12/shCD36 cells. Cells were induced to differentiate in DM and then were immunostained with anti-MyHC antibody at DM3 and 5. Scale bars, 100 μm. (B) At DM3 and 5, fusion indices were calculated for C2C12, C2C12/shCont, and C2C12/shCD36 cells, as described in Section 2. Results are expressed as means ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.05$. (C) C2C12, C2C12/shCont, and C2C12/shCD36 cells at DM5 were fixed and immunostained for MyHC, and then the percentage of nuclei present in MyHC+ cells with the indicated number of nuclei were determined. Results are expressed as means ± SD of at least three independent experiments. *t*-test: $^{*}P < 0.05$.

localized in multinucleated myotubes. Third, the expression of myogenic markers was downregulated in CD36 knocked down C2C12 cells. Fourth, the suppression of CD36 expression using shRNA significantly inhibited myotube formation during C2C12 differentiation. Thus, our results indicate that CD36 is involved in muscle cell fusion during myogenic differentiation, and given the known role of CD36 in macrophage fusion, it appears that CD36 contributes to two different types of multinucleated cell formation by macrophages and myoblasts.

Multinucleated myotubes in mammals are produced via two phases: During the first phase, myoblasts fuse with one another to generate small, nascent myotubes, and during the second, additional myoblasts fuse with nascent myotubes to form large, mature myotubes [4,23]. In the present study, CD36 mRNA and protein were highly expressed in myotubes on DM3–5, that is, when large multinucleated myotubes were formed. C2C12 and shCont-treated C2C12 cells formed large multinucleated myotubes on DM5, whereas shCD36-treated C2C12 cells formed small myotubes with 2–4 nuclei. These observations suggest the possibility that CD36 is involved in fusion step for the enlargement of myotubes in myogenesis.

Although we provide evidence that CD36 is involved in cell-to-cell fusion during myogenic differentiation, the mechanism by which CD36 mediates cell-to-cell fusion remains to be investigated. However, it is known that CD36 mediates apoptotic cell clearance via PS recognition [7,8], and recently, the exposure of PS on cell surfaces and lipid recognition by CD36 were found to be required for the cytokine-induced fusion of macrophages [14]. In addition, PS is known to be transiently expressed at cell-to-cell contact areas during myotube formation [10], and the treatment with Annexin V protein or anti-PS antibody significantly inhibits myotube formation during myogenic differentiation [10,11]. Indeed, in the present study, anti-PS antibody inhibited myogenic differentiation in C2C12 cells (data not shown). In a previous study, the recognition of PS activated signaling events within phagocytes that led to cytoskeletal reorganization allowing corpse internalization [24]. Actually, the alignment, adhesion, and fusion steps that occur during myotube formation also require cytoskeletal rearrangement [25], and myoblast and macrophages share molecular components that are involved in cytoskeletal rearrangement during cell fusion [26]. These observations suggest that interaction between PS and CD36 could induce signaling for cytoskeletal rearrangement during myogenesis. Although we did not provide direct evidence that CD36 recognizes PS during myogenic differentiation, it is possible that CD36 provides a link between an intercellular signaling pathway and phospholipid involvement in muscle cell fusion.

Summarizing, the present study shows that CD36 plays an important role in muscle cell fusion. Because it has been previously established the role of CD36 in macrophage fusion, we suggest that this finding will aid the elucidation of the common mechanism by which cell-to-cell fusion is regulated in various fusion models.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.119.

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