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Efficient myogenic differentiation of human adipose-derived stem cells by the transduction of engineered MyoD protein

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ABSTRACT

Human adipose-derived stem cells (hASCs) have great potential as cell sources for the treatment of muscle disorders. To provide a safe method for the myogenic differentiation of hASCs, we engineered the MyoD protein, a key transcription factor for myogenesis. The engineered MyoD (MyoD-IT) was designed to contain the TAT protein transduction domain for cell penetration and the membrane-disrupting INF7 peptide, which is an improved version of the HA2 peptide derived from influenza. MyoD-IT showed greatly improved nuclear targeting ability through an efficient endosomal escape induced by the pH-sensitive membrane disruption of the INF7 peptide. By applying MyoD-IT to a culture, hASCs were efficiently differentiated into long spindle-shaped myogenic cells expressing myosin heavy chains. Moreover, these cells differentiated by an application of MyoD-IT fused to myotubes with high efficiency through co-culturing with mouse C2C12 myoblasts. Because internalized proteins can be degraded in cells without altering the genome, the myogenic differentiation of hASCs using MyoD-IT would be a safe and clinically applicable method.

1. Introduction

Skeletal muscle has the ability to regenerate new muscle fibers depending on quiescent precursor cells called satellite cells. In response to injury, they become activated and then proliferate and differentiate into myoblasts while a minor subset of them self-renew. Myoblasts undergo terminal differentiation, which leads to their incorporation into damaged myofibers or to self-fusions to form new myotubes. Cell therapy using a transplantation of myoblasts or satellite cells has been explored as a treatment for muscle disorders including muscular dystrophy. However, the initial attempts were not successful due to poor cell survival, immune rejection and the restricted migration of the transplanted cells [1]. Moreover, satellite cells and myoblasts are associated with limited availability depending on a muscle biopsy and a very low capacity of *in vitro* expansion. Therefore, stem cells that are capable of myogenic differentiation have received increasing amounts of attentions as an alternative source of myogenic cells for cell therapies [2]. In particular, mesenchymal stem cells (MSCs) have advantageous features, including the ability to expand *in vitro*, immune-suppressive activity, and myogenic potential. While they can be obtained from various tissues including bone marrow and umbilical cord blood, adipose tissues have been highlighted as an abundant, accessible and attractive source of MSCs. Human adipose-derived stem cells (hASCs) as one type of MSCs have been reportedly differentiated into myogenic cells *in vitro* by a myogenesis-promoting culture condition [3,4] or by the forced expression of myogenic transcription factor MyoD [5]. In the myogenesis-promoting condition, 5-azacytidine, known as an inhibitor of DNA methylation, is usually applied to a culture containing horse serum (HS) or a reduced amount of bovine serum. However, its use in cell therapy should be restricted, as 5-azacytidine is toxic and mutagenic with the ability to incorporate into DNA [6]. An alternative myogenesis method using the forced expression of MyoD would also have a high risk of genome alteration due to the viral vectors used for efficient gene transfection. Therefore, the development of a safe myogenesis technology appropriate for clinical application is crucial.

The introduction of transcription factors into cells using protein transduction is considered safe because the applied proteins can be degraded in cells without altering the genome. The protein transduction of MyoD was utilized for the myogenic differentiation of myoblast cells [7,8]. Although a protein transduction domain (PTD) such as the TAT peptide of HIV is usually fused to a target protein to penetrate the cell membrane, Noda et al. reported that MyoD without an addition of PTD was efficiently internalized and induced the myogenic differentiation of myoblast cell line C2C12 [7]. It was speculated that the basic helix-loop-helix motif

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in MyoD facilitated protein transduction in a manner similar to that of PTD. On the other hand, Hidema et al. reported that MyoD containing PTD displayed better ability in myogenic differentiation compared to authentic MyoD protein [8]. These two groups induced myogenic differentiation of only C2C12 or primary myoblast cells, which can be voluntarily differentiated to myotube under favorable conditions such as a reduced serum condition. The myogenic differentiation of stem cells using the transduction of MyoD proteins has yet to be reported. This may be related to the low efficiency of MyoD proteins to induce myogenic differentiation, as explained by the endosome trapping of internalized MyoD proteins [7]. Although proteins containing PTD have been known to enter cells efficiently by endocytosis, most of them become trapped inside endosomes, leading to degradation by acidic hydrolases when they trafficked into lysosomes [9]. Therefore, in order to accomplish the biological function efficiently, an endosomal escape process is required for the protein to reach the cytosol and/or nucleus. Fusogenic peptides disrupting the endosomal membrane upon activation at a low pH have frequently been employed for efficient endosomal escape [9].

In the present study, we generated MyoD proteins containing both of PTD and an endosome-disruptive INF7 peptide which was a modified version of HA2 peptide derived from the influenza HA protein [10,11]. This engineered MyoD protein displayed enhanced nuclear targeting capabilities and successfully differentiated hASCs into myogenic cells.

2. Materials and methods

2.1. Construction of recombinant MyoD expression vectors

The human MyoD gene was amplified from the corresponding cDNA clone, which was purchased from OriGene (Rockville, MD, USA) by a polymerase chain reaction (PCR) using the sets of primers described in Supplementary Table S1. To construct the His-tagged MyoD expression vector, the gene was amplified using His-MyoD_F and His-MyoD_R primers. For the amplification of the His-tagged MyoD-IT gene, three successive PCRs were performed using His-MyoD_F as a forward primer and His-Cterm-IT_R1, His-Cterm-IT_R2, and His-Cterm-IT_R3 as reverse primers in a numerical order. The resulting DNA fragments for His-tagged MyoD and MyoD-IT genes were digested with *Nde*I and *Eco*RI and then inserted into *Nde*I-*Eco*RI-digested pET21a (Life Technologies, Carlsbad, CA, USA), generating pET21a-MyoD and pET21a-MyoD-IT, respectively. For the construction of the Halo-tagged protein expression vectors, the MyoD gene was amplified using Halo-MyoD_F and Halo-MyoD_R while the MyoD-IT gene was amplified from pET21a-MyoD-IT using Halo-MyoD_F and Halo-MyoD-IT-R. The amplified DNA fragments were digested with *Nco*I and *Pme*I and then inserted into *Nco*I-*Pme*I-digested pFN18A (Promega, Madison, WI, USA), yielding pFN18A-MyoD and pFN18A-MyoD-IT.

2.2. Expression and purification

The T7 promoter vectors pET21a-MyoD and pET21a-MyoD-IT expressing His-tagged MyoD proteins were transformed into the *Escherichia coli* BL21 (DE3) strain. An overnight culture of the transformants was re-inoculated in a Luria-Bertani broth medium containing ampicillin and grown at 37 °C. After the optical density at 600 nm (OD_{600}) reached 0.5, 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added for induction and this was further cultured at 16 °C overnight. The harvested cell pellet was lysed by sonication and centrifuged to remove the cell debris and insoluble fraction. The His-tagged MyoD proteins were purified by using a Ni-NTA column (Qiagen, Valencia, CA, USA) as previously described [12]. The Halo-tag vectors pFN18A-MyoD and pFN18A-MyoD-IT were transformed into the KRX strain (Promega), which allows tighter regulation of the T7 RNA polymerase gene under the rhamnose promoter. When the OD_{600} of the culture reached 0.5, 0.1% rhamnose was added for induction and this was further cultured at 16 °C overnight. MyoD proteins containing Halo-tag were purified according to the manufacturer's instructions (Promega). Briefly, the collected cell lysate supernatant was mixed well with HaloLink™ resin pre-equilibrated with a purification buffer. After the resulting resin was packed into the column, it was thoroughly washed. After a cleavage solution containing TEV protease was applied at room temperature for one hour, the MyoD proteins were eluted from the column by adding the purification buffer. The remaining TEV protease in the eluted solution was removed by using Ni-NTA column. The purified MyoD proteins were dialyzed against phosphate-buffered saline (PBS). Expression and purification processes were monitored and analyzed by 10% SDS–PAGE and Western blot using an anti-MyoD antibody (Abcam, Cambridge, MA, USA).

2.3. Cells, cultures and differentiation condition

The hASCs used in this study were purchased from Life Technologies and were expanded in the MesenPRO RS™ Basal Medium Kit also from Life Technologies according to manufacturer's instructions. For the stabilization before myogenic differentiation, the hASCs were seeded in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (GM) at 1×10^4 cells/well in μ-slide 8-well plates (Ibidi, Verona, WI, USA) and incubated in a 5% CO_2 incubator at 37 °C for one day. MyoD and MyoD-IT were applied at the indicated protein concentrations (0.2–10 μg/ml) in GM for one more day. The medium was then changed to DMEM containing 2% HS (DM) added at the same concentrations of the MyoD proteins and further cultured with exchanges of the same medium every 2–3 days for 12 days.

2.4. Evaluation of cell penetration and nuclear localization characteristics

Purified MyoD and MyoD-IT were labeled with the NHS-Rhodamine labeling kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. For the stabilization as described in the previous section, the hASCs were seeded in GM at 1×10^4 cells/well in μ-slide 8-well plates for one day. Then, Rhodamine-labeled MyoD proteins (0.5 μg/ml) were added to the cells in DM for 15 h. After incubation, the hASCs were fixed with 4% paraformaldehyde for one hour at room temperature and then stained with PKH67 (Sigma–Aldrich, St. Louis, MO, USA) for the plasma membranes and with 4′,6 diamidino-2-phenylindole (DAPI) (Life Technologies) for the nuclei of the cells. Images of triply-labeled cells were analyzed using a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.5. Myogenic differentiation analysis using immunofluorescent staining

After the differentiated cells were fixed with 4% paraformaldehyde in PBS for one hour, they were stained with PKH67. Then, they were permeabilized with 0.2% Triton X-100 at room temperature for 5 min, followed by blocking for one hour using 1% bovine serum albumin in PBS. The resulting cells were immunostained with an anti-myosin heavy chain (MHC) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS at 4 °C overnight, followed by a treatment with a secondary antibody conjugated with Texas Red (Vector Labs, Burlingame, CA, USA). After nuclei staining with DAPI, fluorescent images were obtained. The differentiation index

was calculated from the fraction of the nuclei in MHC-positive cells to the total number of nuclei.

2.6. Co-cultures of hASCs with C2C12 cells

The overall co-culturing scheme is graphically represented in Fig. 4A. Briefly, hASCs were seeded at a density of 1×10^5 cells per well in 6-well plates in GM for one day, followed by a 2 μg/ml MyoD-IT treatment and further culturing in the same medium for one day. Then, the medium was exchanged with DM containing the same concentration of MyoD-IT every 2–3 days for 5 days or 12 days. After staining with PKH26 (Sigma–Aldrich), 1×10^5 hASCs were added to C2C12 cells in 50 mm dishes (Ibidi), which were pre-grown in GM medium for 3 days with the seed of 1.5×10^5 cells. The co-culture media were then changed to DM and further cultured for 3 days, followed by fixation with 4% paraformaldehyde in PBS. They were immunostained with anti-MHC antibody and secondary antibody conjugated Alexa-488 (Life Technologies), as described in the previous section.

3. Results

3.1. Enhanced solubility and purification by Halo-tag fusion

First, two expression vectors were constructed to produce authentic and engineered MyoD proteins attached to N-terminal His-tag for protein purification. The engineered MyoD protein (MyoD-IT) was designed to contain the INF7 peptide (GLFEAIEGFIENGWEGMIDGWYG) for endosomal escape and TAT PTD (YGRKKRRQRRR) for cell penetration subsequently in C-terminal of MyoD protein (Fig. 1A). Here, INF7 peptide was used instead of the parent HA2 peptide due to its enhanced pH-sensitive membrane disruption capabilities [10,11]. When MyoD proteins containing the His-tag were expressed in *E. coli*, significant portions were found in the insoluble fraction, even at low-temperature induction (Fig. 1B). When MyoD protein was purified from the soluble fraction using a Ni-NTA column, the yield was low (~0.4 mg of protein purified from 200 ml of culture) and the resulting purity was poor (Fig. 1C). In order to solve the problems of poor solubility and a low purification yield, we constructed vectors expressing MyoD and MyoD-IT attached to Halo-tag instead of His-tag. The fusion of the soluble Halo-tag domain greatly increased the solubility of MyoD proteins and improved the purity when using a Halo-tag purification system (Fig. 1B and C). In this system, the Halo-tag of MyoD proteins was covalently attached to the HaloLink resin. After extensive washing to remove impurities, TEV protease cleaved the MyoD proteins from the Halo-tag which was still attached to the resin. Then, MyoD was eluted from the column and the remaining TEV protease containing His-tag was removed using a Ni-NTA column. A considerable amount of pure MyoD protein was prepared by this method (~1.7 mg of protein purified from 200 ml of culture) and was used for the subsequent experiments.

3.2. Comparison of the intracellular delivery of MyoD proteins

MyoD proteins were labeled with red fluorescent dye, rhodamine, for the observation of their localization in cells. After 15 h of incubation with hASCs, the localization characteristics of MyoD

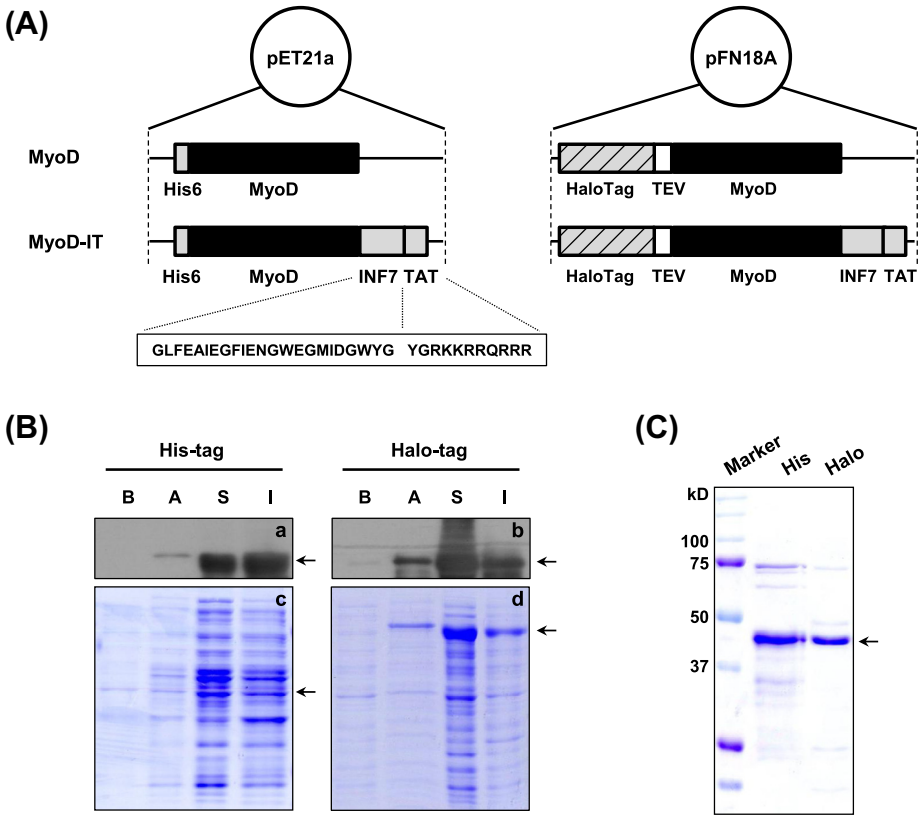

Fig. 1. Expression and purification of recombinant MyoD proteins. (A) Schematic representation of MyoD and MyoD-IT containing INF7 and TAT peptides, of which the amino acid sequences are also shown. They were expressed by pET21a- or pFN18A-based vectors, which added His-tag or Halo-tag followed by a TEV cleavage site to the N-terminal of the MyoD proteins, respectively. (B) Expressions of His- and Halo-tagged MyoD proteins were analyzed by Western blot analyses (a and b) using anti-MyoD antibody and SDS–PAGE (c and d). Here, B and A represent the cells obtained before and after induction, respectively. S and I represent the soluble and insoluble fractions after cell lysis. (C) The purity of MyoD purified by means of His- or Halo-tag affinity chromatography was checked using 10% SDS–PAGE.

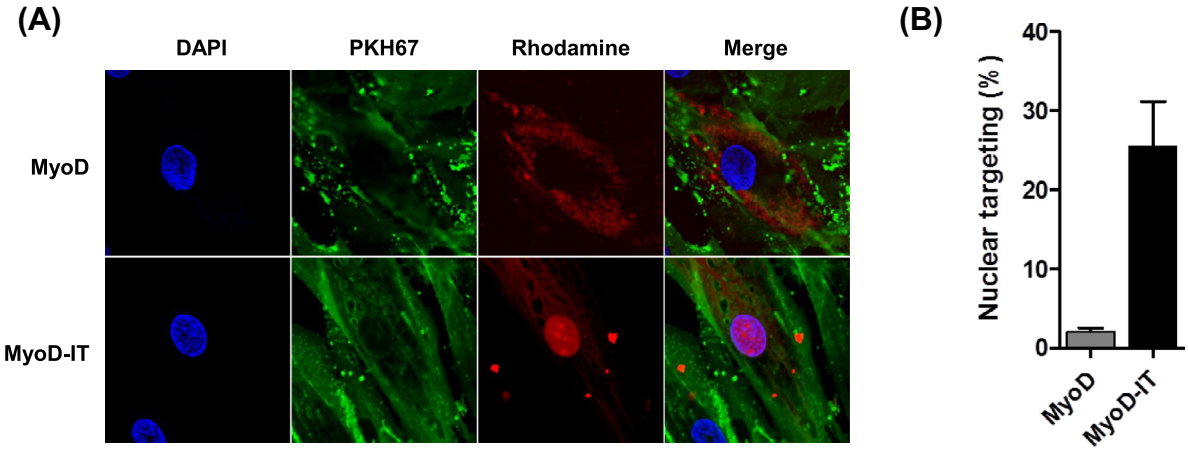

Fig. 2. Comparison of the nuclear localization characteristics of MyoD and MyoD-IT. (A) hASCs were incubated with 0.5 μg/ml MyoD or MyoD-IT labeled with NHS-Rhodamine (red). After the incubation, the cells were stained with PKH67 (green) for the cell membranes and DAPI (blue) for nuclei. (B) Ratios of nuclear-targeted MyoD and MyoD-IT proteins were obtained by calculating the red fluorescence intensity levels in nuclear areas against the total intensity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and MyoD-IT were compared (Fig. 2 and Supplementary Fig. S1). The overall intensity of red fluorescence was clearly higher when MyoD-IT was applied, suggesting its superior cell penetration ability. Moreover, the red fluorescence of MyoD-IT was much brighter in the nucleus region, as revealed by the blue fluorescence of DAPI, suggesting that MyoD-IT having an intrinsic nuclear localization sequence moved to the nucleus after an efficient endosomal escape induced by the INF7 peptide. In sharp contrast, that of MyoD was mostly found in the cytosol as dispersed spots, which was in accord with a previous result showing that most of the internalized MyoD remained in endosomes [7]. The nuclear targeting abilities of MyoD and MyoD-IT were quantitatively compared by obtaining the portions of red fluorescence overlapped with the blue fluorescence of nuclei (Fig. 2B). The nuclear targeted portion of the internalized MyoD-IT proteins was estimated as 25.4 ± 8.0% which was twelve times higher than that of MyoD (2.1 ± 0.6%). These results indicated that MyoD-IT was much more efficiently targeted to the nucleus due to the enhanced cell penetration and endosomal escape capabilities endowed by TAT and INF7 peptides.

3.3. Efficient myogenic differentiation of hASCs by the application of engineered MyoD

The myogenic differentiation of hASCs induced by MyoD proteins was evaluated by immunostaining using the anti-myosin heavy chain (MHC) antibody, a marker for late myogenic differentiation (Fig. 3). In this process, hASCs were much more efficiently differentiated into myogenic cells by the application of MyoD-IT, which was clearly verified by the longer spindle shapes of cells as well as by the stronger MHC immunostaining (Fig. 3A). For a quantitative comparison, myogenic differentiation index was calculated from the fraction of the nuclei in MHC-positive cells (Fig. 3B). MyoD-IT showed superior myogenesis-inducing activities on all of the tested concentrations (0.2–10 μg/ml) compared to

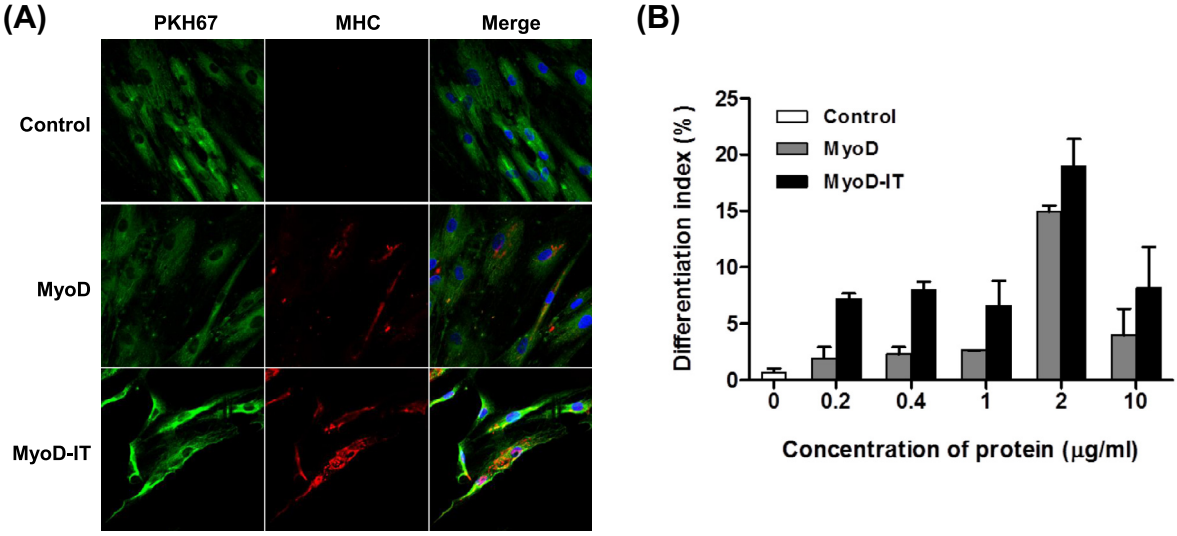

Fig. 3. Myogenic differentiation of hASCs by a treatment with recombinant MyoD proteins. (A) MyoD and MyoD-IT proteins were applied to hASCs for 14 days. The resulting cells were triple-stained with anti-MHC antibody (red) used as a myogenic differentiation marker and together with PKH67 (green) for the cell membranes and DAPI (blue) for the nucleus. (B) Differentiation indices of hASCs were obtained with variable amounts of applied MyoD protein. Here, the differentiation indices were calculated from the fraction of the nuclei in MHC-positive cells over the total number of nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MyoD. The myogenesis activity of both MyoD proteins was best at a dose 2 μg/ml, while apoptotic phenotypes were observed in some of the cells with 10 μg/ml dose, which may be related to the findings of a previous report stating that MyoD acts as a pro-apoptotic factor [13]. Notably, although hASCs were efficiently differentiated into spindle-shaped myogenic cells expressing MHC after the application of MyoD-IT, we did not observe multi-nucleated myotubes even with prolonged cultures (data not shown). This is in good agreement with a previous report stating that the final differentiation of hASCs to myotubes requires factors other than the expression of MyoD [3].

3.4. Myotube formation of hASCs by co-culturing with mouse C2C12 myoblasts

Eom et al. reported that myotubes only formed from hASCs in the late myogenic differentiation step by cellular fusion with C2C12 cells but not from hASCs in normal state or early differentiation stage [3]. Based on this observation, we tested whether hASCs differentiated by an application of MyoD-IT can be fused to myotubes through co-culturing with C2C12 cells. As schematically represented in Fig. 4A, hASCs were cultured for 7 or 14 days with or without an application of MyoD-IT and then stained with PKH26 (red) to identify hASCs in the subsequent co-cultures. The resulting fluorescently labeled hASCs were further co-cultured with C2C12 cells for 3 days, followed by immunostaining using an anti-MHC antibody (green) and nuclear staining using DAPI (blue). Fig. 4B shows that significant portions of hASCs differentiated by an application of MyoD-IT fused to multi-nucleated myotubes whereas hASCs without MyoD-IT were mono-nucleated. The co-fusion index of the hASCs was obtained by calculating the ratio of PKH26-stained multi-nucleated cells containing three or more nuclei to the total number of MHC-positive nuclei (Fig. 4C). The co-fusion index was higher in the 14-day culture (25.0 ± 3.4%) with

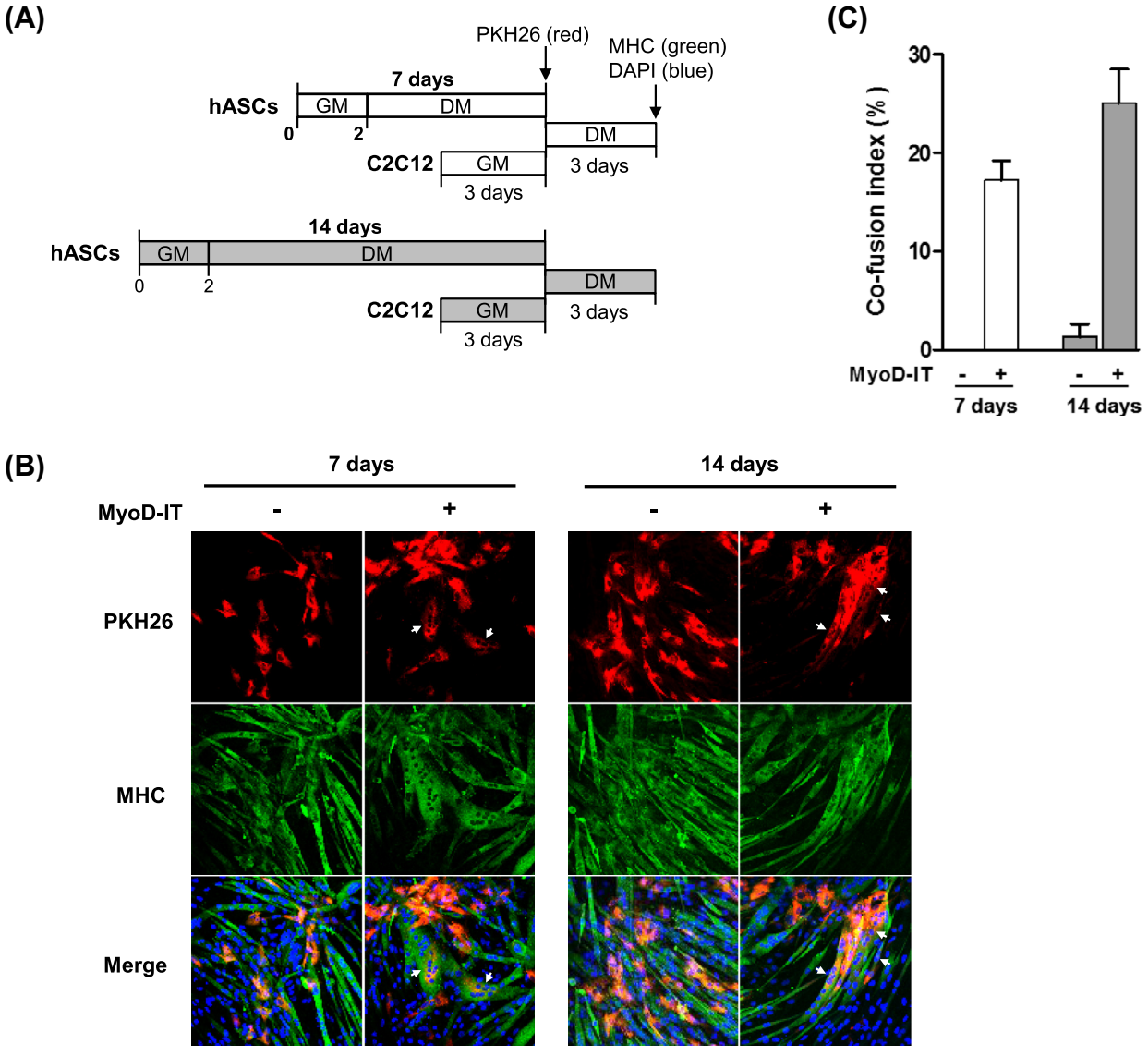

Fig. 4. Co-culture analysis of myogenic differentiated hASCs and C2C12 cells. (A) Overall scheme of the co-culture analysis of the myotube formation of hASCs by cellular fusion with C2C12. GM and DM represent the growth and differentiation media as described in Materials and methods, respectively. (B) Fluorescent images were obtained after the co-cultures of C2C12 and hASCs differentiated for 7 or 14 days with or without an application of MyoD-IT. Before the co-culturing process, hASCs were pre-stained with PKH26 (red) for identification. After the co-culturing process, myogenic differentiated cells and all nuclei were stained with anti-MHC antibody (green) and DAPI (blue), respectively. (C) Co-fusion indices were calculated from the ratio of PKH26-stained multi-nucleated cells to the total number of MHC positive nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

an application of MyoD-IT as compared to the 7-day culture (17.2 ± 2.0%), while both of the cultures without a MyoD applications showed a negligible result. This was also in good accord with a previous result showing that hASCs in a late myogenic differentiation step much more efficiently undergo final differentiation into multi-nucleated myotubes than those at earlier stages [3].

4. Discussion

We engineered human MyoD protein to have enhanced cell penetration and endosomal escape abilities by attaching TAT and INF7 peptides. It was solubly expressed in *E. coli* and purified to a high purity level through N-terminal Halo-tag fusion. The engineered protein, MyoD-IT, was much more efficiently targeted to the nucleus, compared to authentic MyoD containing intrinsic PTD [7]. The great improvement in nuclear targeting appears to result from the efficient endosomal escape induced by the pH-sensitive membrane disruption of the INF7 peptide of MyoD-IT, as most of the MyoD proteins was found in vesicles such as endosomes. Previous studies used MyoD or MyoD attached to only TAT PTD for myogenic differentiation [7,8]. However, these proteins were associated with low nuclear targeting efficiency and were only used for the myogenic differentiation of C2C12 and primary myoblast cells, which can be easily differentiated into myotubes. In the present study, we employed MyoD-IT with an enhanced ability to undergo endosomal escape for the successful myogenic differentiation of hASCs. This superior ability of MyoD-IT to induce myogenic differentiation was also observed when it was applied to MSCs derived from human bone marrow (Supplementary Fig. S2), suggesting that MyoD-IT can be used for the myogenic differentiation of various MSCs derived from different sources.

Currently, *in vitro* myogenic differentiations of hASCs are achieved mostly by a myogenesis-promoting condition using 5-azacytidine or by the forced expression of MyoD using a viral vector for gene transfection [3–5]. However, neither 5-azacytidine nor viral vectors is appropriate for clinical use due to the mutagenic potential via DNA incorporation and/or genome alteration. The myogenic differentiation of hASCs using MyoD-IT would be a safe alternative for clinical applications because internalized protein can be degraded in cells without altering the genome. It is noteworthy that a differentiation medium containing 2% HS, regarded as inappropriate for clinical use, was used together with the application of MyoD-IT in the present study. However, the use of HS may be easily avoided in further work when using the conditioned medium of MSCs [3] or by adding heparin to the medium [14].

Although the differentiation of hASCs into long spindle-shaped myogenic cells expressing the MHC marker was successfully achieved using MyoD-IT, the formation of multi-nucleated myotubes was not observed. Several groups reported that hASCs can contribute to myotube formation through a co-culturing with myoblasts although the frequency of myotube formation was very low [15,16]. Recently, Eom et al. greatly increased this efficiency using hASCs in late myogenic differentiation stages [3]. Therefore, we analyzed the myotube formation capabilities of hASCs with a MyoD-IT treatment when co-cultured with C2C12 myoblasts. As expected, myotube formation was observed only from a co-culture of hASCs with an application of MyoD-IT. Moreover, the efficiency was higher when MyoD-IT was applied for a longer period, indicating that hASCs in late myogenic differentiation stages can fuse to myotubes with higher efficiency. This finding was consistent with the previous results [3].

Due to useful advantages such as abundant cell sources and easy isolation, hASCs have great potential for use in cell therapy. However, they require the safe and precise control of myogenic differentiation before they can be used to help those with muscle disorders. The engineered MyoD protein was shown to induce the myogenic differentiation of hASCs efficiently without the concern over genome alteration, which can lead to the development of cell therapies which are clinically applicable for the treatment of muscle disorders.

Acknowledgments

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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.2013.06.058.

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