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Effective myotube formation in human adipose tissue-derived stem cells expressing dystrophin and myosin heavy chain by cellular fusion with mouse C2C12 myoblasts

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ABSTRACT

Stem cell therapy for muscular dystrophies requires stem cells that are able to participate in the formation of new muscle fibers. However, the differentiation steps that are the most critical for this process are not clear. We investigated the myogenic phases of human adipose tissue-derived stem cells (hASCs) step by step and the capability of myotube formation according to the differentiation phase by cellular fusion with mouse myoblast C2C12 cells. In hASCs treated with 5-azacytidine and fibroblast growth factor-2 (FGF-2) for 1 day, the early differentiation step to express MyoD and myogenin was induced by FGF-2 treatment for 6 days. Dystrophin and myosin heavy chain (MyHC) expression was induced by hASC conditioned medium in the late differentiation step. Myotubes were observed only in hASCs undergoing the late differentiation step by cellular fusion with C2C12 cells. In contrast, hASCs that were normal or in the early stage were not involved in myotube formation. Our results indicate that stem cells expressing dystrophin and MyHC are more suitable for myotube formation by co-culture with myoblasts than normal or early differentiated stem cells expressing MyoD and myogenin.

1. Introduction

Muscular dystrophies are heritable, heterogeneous neuromuscular disorders that include Duchenne and Becker muscular dystrophies. Duchenne muscular dystrophy (DMD) is a lethal X-linked genetic disorder caused by loss of dystrophin, a protein essential for maintenance of muscle fiber integrity. Dystrophin is a key molecule of the dystrophin glycoprotein complex (DGC), which is involved in stabilizing interactions between the sarcolemma, the cytoskeleton, and the extracellular matrix of skeletal and cardiac muscles [1].

Currently, there are no effective therapies for DMD. Three main therapeutic approaches are being pursued to develop strategies for DMD. These consist of (i) molecular therapy by introducing the dystrophin gene via viral or non-viral vectors [2,3] or repairing the genetic disorder via exon skipping or premature stop codon suppression [4,5], (ii) cell therapy by transplantation of myoblasts or stem cells [6–8], and (iii) pharmacological therapy by up-regulation of utrophin gene expression [9,10]. Cell therapy using myoblasts or stem cells has been attempted to improve muscle function and pathology by muscle regeneration. Partridge et al. demonstrated that myoblasts could be transplanted into dystrophic muscle and give rise to dystrophin-expressing myofibers [6]. However, in the early 1990s, several clinical trials demonstrated that intramuscular injections of allogenic myoblasts had no functional benefit. Failure usually resulted due to massive cell death of myoblasts, limited migratory ability of myoblasts, or possibly immune responses to donor myoblasts [11]. As an alternative to myoblasts, stem cells were proposed to be preferable for therapeutic applications. Stem cells such as bone marrow-derived cells, mesoangioblasts, $CD133^+$ cells, multipotent adult progenitor cells (MAPCs), and mesenchymal stem cells (MSCs) have been evaluated for muscular dystrophy cell therapy in preclinical animal models [12]. To use stem cells for muscular dystrophy, theoretically, stem cells should fulfill the following criteria: (1) be expandable in vitro, (2) be immune-privileged, (3) be systemically-deriverable, (4) be capable of expressing dystrophin, etc. [13]. Because MSCs have several properties such as proliferation, differentiation, anti-inflammation and immuno-suppression [14–16], MSCs is one of the ideal cell types to treat muscular dystrophies. Although MSCs reside in various adult tissues, such as bone marrow, adipose

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tissue, umbilical cord blood, peripheral blood, brain, lung, liver, dermis, and skeletal muscle [17–19], adipose tissue can be obtained with less invasive procedures compared to other tissues and, more importantly, adipose tissue-derived stem cells (ASCs) can be recovered in high quantities because adipose tissues are an abundant reservoir of MSCs, containing approximately $\geqslant$ 100-fold higher MSCs than the bone marrow [20]. Several groups have demonstrated that ASCs are differentiated into muscle cells by procedures summarized in the following two methods: (1) culture of ASCs with horse serum and/or under reduced serum conditions [21–23] and (2) co-culture with skeletal myoblasts [21,22]. Despite the differentiation potential of ASCs into muscle cells, the frequency of myotube formation was very low [21,22]. Di Rocco et al. reported that approximately 1% of in vitro expanded ASCs gave rise to myotubes when ASCs were co-cultured with primary myoblasts.

However, because there is still unknown whether cells that differentiated into muscle are more effective contributors to the formation of myotubes in co-culture systems, we investigated the frequency of myotube formation according as the myogenic differentiation steps. It is well known that myogenesis is initiated by myogenic regulatory factors (MRFs), and that these transcription factors control the activation of skeletal muscle genes, which leads to the formation of differentiated muscle. Based on the expression of early (MyoD and myogenin, which are expressed in initiation step of myogenesis) and late (dystrophin and MyHC, which are expressed in mature fibers) skeletal-muscle-specific markers, the process was divided into an early and late differentiation step. Then ASCs were evaluated based on the capability of myotube formation according to the differentiation step by cellular fusion with mouse myoblast C2C12 cells.

2. Materials and methods

2.1. Materials

Human skeletal muscle mRNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The human rhabdomyosarcoma (RD) cell line was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Type IA collagenase, 5-azacytidine, and FGF-2 were obtained from Sigma. pBOS-H2BGFP was purchased from BD Biosciences (San Diego, CA, USA).

2.2. Isolation of adipose tissue-derived stem cells

Human adipose tissue (AT) from three healthy donors (age 21–40 years) was obtained from Park's Cosmetic and Plastic Surgery (Seoul, Korea) by elective liposuction procedures under anesthesia according to procedures approved by the Institutional Review Board of Ajou University Hospital. Informed consent was obtained from all donors. Mononuclear cells were isolated using a modified protocol described by Zuk et al. [23]. Briefly, lipoaspirates were extensively washed with phosphate-buffered saline (PBS) to remove contaminating blood cells and local anesthetics. Lipoaspirates were enzymatically digested at 37 °C for 40 min with 0.075% type IA collagenase (Sigma) in PBS. Mononuclear cells were harvested by centrifugation at 1200 g for 5 min, resuspended with low glucose Dulbecco's minimal essential medium (LG-DMEM) containing 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin), and passed through a 100-μm mesh filter (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris. Cells were plated in 100-mm culture dishes at a density of 5×10^6 mononuclear cells with LG-DMEM containing 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin). After 2 days, the medium was changed to remove nonadherent cells. The medium was changed twice weekly, and cells were passaged with 0.25% trypsin/0.1% EDTA (GibcoBRL) upon reaching 90% confluency.

2.3. Myogenic differentiation

hASCs were plated at 1×10^3 cells/cm^2 in 6-well plates. After 24 h, cells were preinduced for one day with LG-DMEM supplemented with 10% FBS, 3 μM 5-azacytidine (Sigma), and 10 ng/ml FGF-2 (Sigma). The medium was then changed with early differentiation medium (10% FBS and 10 ng/ml FGF-2 in LG-DMEM) [24] and finally, cells were supplied with the filtered supernatant of hASC culture (conditioned medium). The conditioned medium was prepared as described previously [24]. Briefly, hASCs were subcultured at 20,000 cells/cm^2 for 5 days, and the conditioned medium was collected and then filtered.

For myogenic differentiation of C2C12 cells, cells were seeded at a density of 3.4×10^4 cells per well in high glucose Dulbecco's modified Eagle's medium (HG-DMEM; Gibco BRL, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 100 μg/ml streptomycin (Gibco BRL) for 5 days. When cells reached 60–70% confluency, the medium was changed to differentiation medium consisting of HG-DMEM (Gibco BRL, NY, USA) containing 2% heat-inactivated horse serum (HS; Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 100 μg/ml streptomycin (Gibco BRL) to induce myotube formation for 3 days.

2.4. Immunoblotting

Cells were washed with cold DPBS and lysed in cold RIPA buffer (50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Sigma, USA). The cell lysate was centrifuged at 13,000*g* for 10 min at 4 °C. The supernatant was harvested, and its protein concentration was measured using a protein assay kit (Bio-Rad, USA). For electrophoresis, 30 μg protein was dissolved in sample buffer (60 mM Tris–HCl, pH 6.8, containing 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 5 min, and separated on a 10% SDS reducing gel. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, UK) using a trans-blot system (Bio-Rad, USA). Blots were blocked for 30 min in TBST (10 mM Tris, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20) containing 5% non-fat dry milk (Bio-Rad, USA) at room temperature, washed three times with TBST, and incubated at 4 °C overnight with primary antibody specific for dystrophin (1:500 dilution; Santa Cruz Biotech, USA) and MyHC (1:1000 dilution; Santa Cruz Biotech, USA) in TBST containing 5% non-fat dry milk. The next day, blots were washed three times with TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution; Santa Cruz Biotech, USA) in TBST containing 5% non-fat dry milk at room temperature. After washing blots three times with TBST, the proteins were visualized with an ECL detection system (Amersham Pharmacia Biotech, UK).

2.5. Immunocytochemistry

Cells were fixed in 10% formalin solution (Sigma), permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and blocked with 2% FBS in PBS for 30 min at room temperature. They were incubated with primary antibody specific for MyoD (1:100 dilution; Santa Cruz Biotech, USA), myogenin (1:100 dilution; Santa Cruz Biotech, USA), MyHC (1:100 dilution; Santa Cruz Biotech, USA) and dystrophin (1:100 dilution; Santa Cruz Biotech, USA) at 4 °C overnight. For fluorescence labeling, cells were

Table 1
RT-PCR primers for validation of gene expression.

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
GAPDH	CAAGGCTGAGAACGGGAAGC	AGGGGGCAGAGATGATGACC	194
MyoD	AATGTAGCAGGTGTAACCGT	GCCTTTATTTTGATCACCTG	230
Myogenin	CACTACTTCTGTAGCAGGGG	TCTCTCAAACCGTTTCACTT	305
Dystrophin	CAGTAGCCCCATCACATTTG	ATAACGCAATGGACAAGTGG	566
MyHC	ATAGGAACACCCAAGCCATC	TTTGCGTAGACCCTTGACAG	599

incubated with FITC- or rhodamine-conjugated secondary antibodies (1:100, Santa Cruz Biotech) for 1 h at room temperature after primary incubation. The cells were also stained with 1 μg/ml 4′,6-diamino-2-phenylindole (DAPI, Sigma, USA) to visualize their nuclei. The slides were observed and photographed under a fluorescent microscope (IX-71, Olympus, Japan).

2.6. Co-cultures

To obtain C2C12 cells expressing green fluorescent protein (GFP; C2C12-GFP) only in the nuclei, the C2C12 cells were transfected with an histone H2B-GFP plasmid (pBOS-H2BGFP; BD Biosciences) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and C2C12-GFP cells were isolated by serial dilution methods using 96-well plates. C2C12-GFP cells were seeded at a density of 3.4×10^4 cells per well of 6-well plates in high glucose Dulbecco's Modified Eagle's Medium (HG-DMEM; Gibco BRL, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) for 5 days. Then C2C12-GFP cells were supplied with HG-DMEM (Gibco BRL, NY, USA) containing 2% heat-inactivated horse serum (HS; Gibco BRL) followed by the addition of hASCs undergoing myogenesis for 0, 7, and 14 days at a density of 9.6×10^4 cells pre well. After 3 days, co-cultures were washed and fixed in 4% paraformaldehyde for 10 min and dystrophin was stained for using anti-dystrophin primary antibody (1:100 dilution; Santa Cruz Biotech, USA) and rhodamine-conjugated secondary antibodies (1:100, Santa Cruz Biotech). Subsequently, co-cultures were counterstained with DAPI (Sigma).

2.7. RT-PCR

Total RNA was extracted from cells using the TRIzol Reagent (Gibco BRL, USA). A total of 2 μg of RNA was reverse-transcribed with AMV reverse transcriptase XL (TaKaRa Shuzo, Japan) for 60 min at 42 °C in the presence of oligo-dT primer. PCR was performed using Taq DNA polymerase (BioQuest, Seoul, Korea). Amplified products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under an ultraviolet light transilluminator (Bio-Rad, USA). The sequences of oligonucleotide primers used for RT-PCR and the expected transcript sizes are listed in Table 1.

3. Results

3.1. The myogenic differentiation steps of hASCs

To evaluate which myogenic differentiation steps play a key role in myotube formation, we induced differentiation of hASC by using two steps; early and late differentiation. They can be distinguished by the expression of skeletal-muscle-specific markers, MyoD and myogenin or dystrophin and MyHC. In the early differentiation step, hASCs were treated with 5-azacytidine and FGF-2 for 1 day, and then the medium was changed to one containing 10 ng/ml FGF-2 for an additional 6 days. Next, to induce high expression of dystrophin and MyHC, the medium was replaced with conditioned medium and cultured for three weeks (late differentiation step). Although long spindle-shaped myotubes were rarely observed on day 8, the frequency of myotube

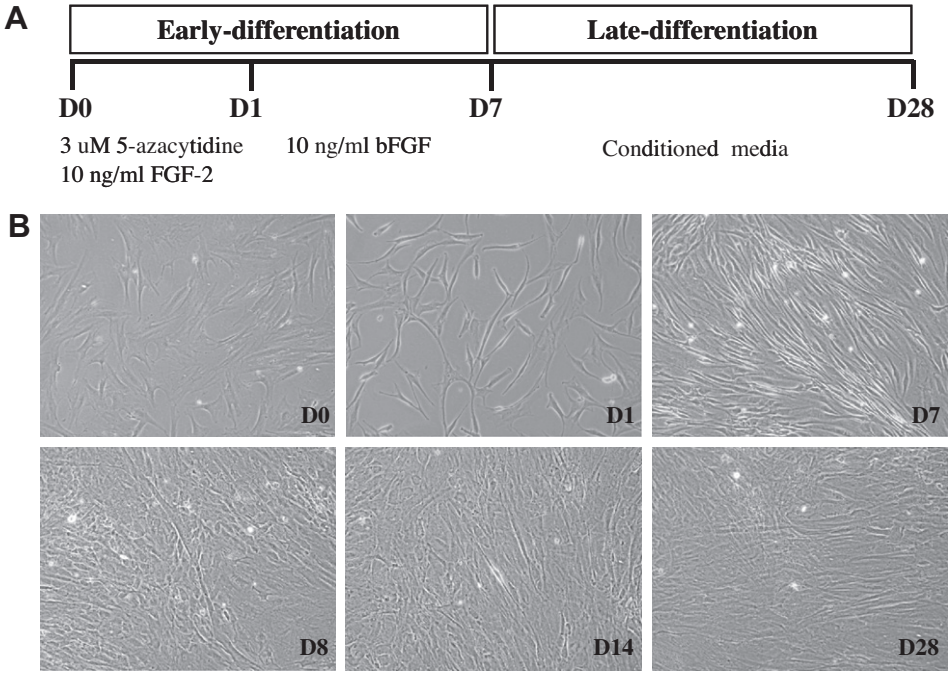

Fig. 1. Myogenic differentiation of hASCs. (A) Schematic diagram of the induction process. hASCs were treated with 5-azacytidine and FGF-2 for 1 day, FGF-2 for an additional 6 days, and then supernatant of hASC culture for 3 weeks. (B) Phase contrast microscopy of hASCs at the indicated time points.

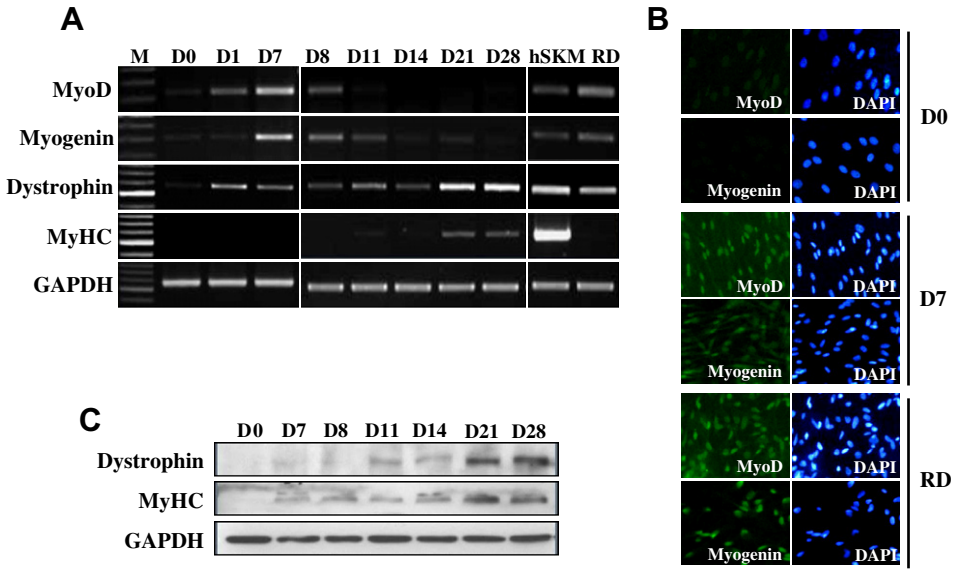

Fig. 2. Expression of skeletal muscle lineage cell markers. (A) Expression levels of mRNAs (MyoD, myogenin, dystrophin, and MyHC) were detected by RT-PCR. At the indicated time points, total RNAs was prepared and expression of several skeletal muscle lineage cell-related genes was investigated. Human skeletal muscle total RNA and RD cells were used for positive control of RT-PCR. (B) MyoD and myogenin immunostaining of hASCs at 0 and 7 days of culture in the early differentiation medium. Nuclei stained with 4,6-diamidino-2-phenylindole (DAPI; blue). (C) Expression levels of dystrophin and MyHC were detected by immunoblotting. At the indicated time points, total proteins were prepared with RIPA buffer, separated by SDS–PAGE, and visualized with an ECL detection system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

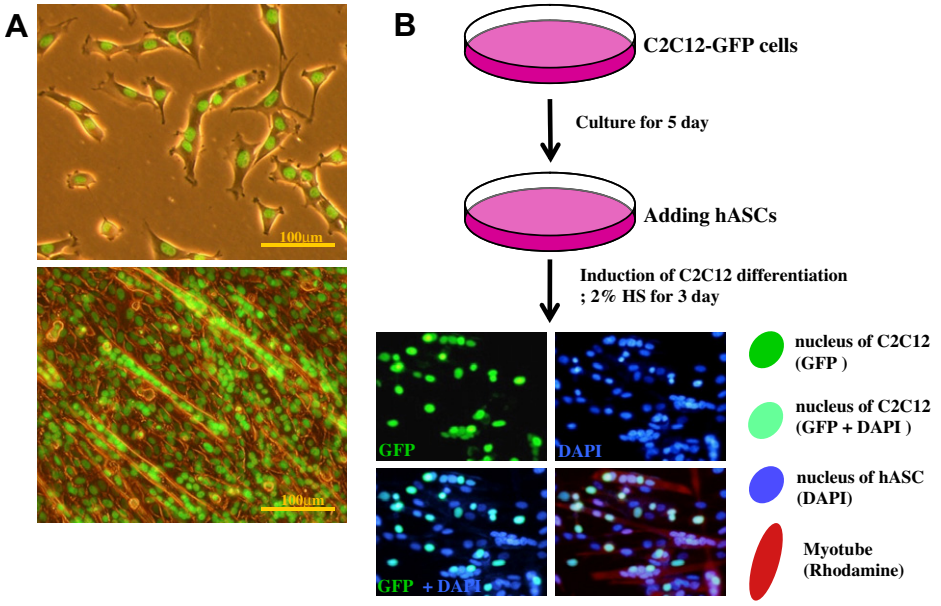

Fig. 3. Experimental protocol to investigate myotube formation by hASCs undergoing myogenic differentiation. (A) C2C12-GFP cells were differentiated into skeletal muscle cells by HG-DMEN containing 2% HS. C2C12-GFP cells were obtained by transfection with pBOS-H2BGFP in C2C12 cells. (B) Co-cultures of C2C12-GFP cells and hASCs undergoing myogenic differentiation were exposed to HG-DMEM containing 2% HS and then cells were fixed, immunostained with anti-dystrophin (red) and DAPI (blue), and analyzed by fluorescent microscopy. Dystrophin-positive and syncytia indicate the differentiation of C2C12 cells into myotubes (red). The participation of hASCs in the generation of myotubes through cellular fusion was distinguished with color of nuclei in syncytia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formation did not increase (Fig. 1). hASCs expressed MyoD and dystrophin from day 1 and the expression of MyoD and myogenin was highest at day 7 (Fig. 2A and B). Then, the expression of MyoD and myogenin decreased gradually, but dystrophin or MyHC did not increase (data not shown) under the condition of FGF-2 treatment for an additional 27 days. Thus, to elevate the dystrophin expression, the medium was changed to conditioned media at day 7 and cultured for an additional 21 days. mRNA of dystrophin and MyHC peaked at days 21 and 28 (Fig. 2A), and the level of expression of dystrophin and MyHC proteins peaked at days 21 and 28 (Fig. 2C). In conclusion, MyoD and myogenin were highly expressed at day 7 in early differentiation step, and the expression of dystrophin and MyHC peaked in the late differentiation step (Fig. 2). Although dystrophin was expressed from day 1, the expression level did not increase until day 21 by treatment with FGF-2.

3.2. Myotube formation in hASCs expressing dystrophin and MyHC

To evaluate the efficiency of myotube formation according to the myogenic differentiation phase, hASCs undergoing myogenesis for 0 (control), 7 (early differentiation), and 21 days (late differentiation) were co-cultured with mouse myoblast C2C12-GFP cells, which stably express GFP-tagged histone H2B. GFP proteins were detected only in the nuclei of C2C12 cells expressing GFP-tagged histone H2B (C2C12-GFP cells), and C2C12-GFP cells had similar phenotypes and differentiation potential compared with normal C2C12 cells (Fig. 3). Representative features of myotubes and nuclei in myotubes were observed under a fluorescein microscope and described in Fig. 3B. The nuclei of hASCs undergoing myogenesis for 0 or 7 days were infrequently observed in myotubes, but the nuclei of hASCs expressing dystrophin and MyHC were frequently detected in the myotubes (Fig. 4). The percentage of nuclei

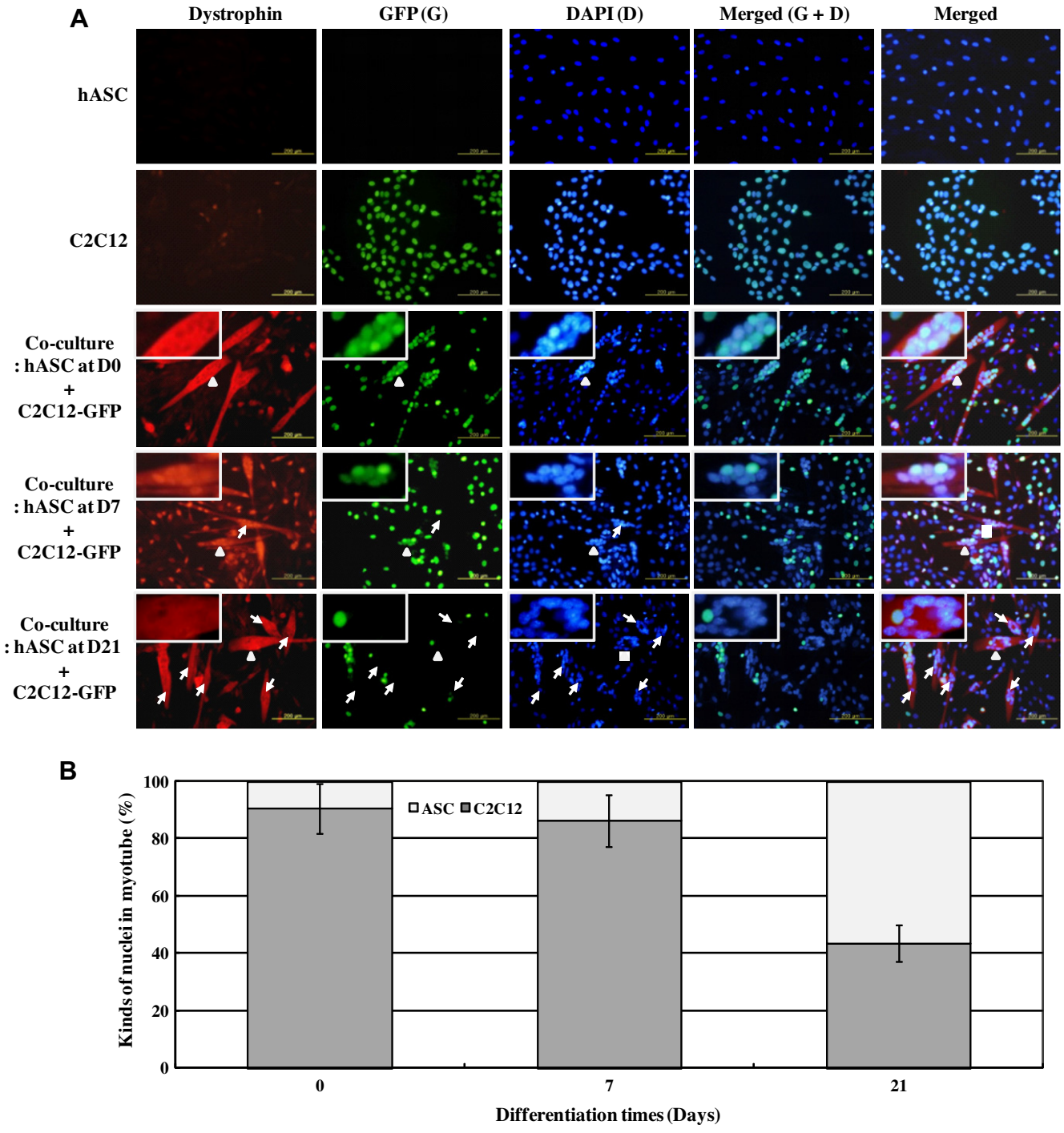

Fig. 4. Analysis of co-cultures of C2C12 cells and hASCs undergoing myogenesis. (A) Co-cultures were performed for 3 days, and the color of nuclei in syncytia was observed by fluorescence microscopy. hASC-derived nuclei were detected in syncytia (arrow). Scale bar is 200 μm. (B) Graph representing the percentage of nuclei derived from hASCs or C2C12 cells in syncytia. To distinguish the nuclei of myotubes derived from mouse C2C12 cells or hASCs or counting the nuclei, five representative pictures (100×) were analyzed and the percentage of nuclei was calculated among total nuclei in myotubes (>50 myotubes). Data are mean ± SE.

derived from hASCs undergoing myogenesis for 0, 7, and 21 days were 9.44 ± 8.84, 13.56 ± 9.05, and 56.32 ± 6.38, respectively (Fig. 4B).

4. Discussion

We reported that hASCs differentiate into muscle cells expressing MyoD, myogenin, dystrophin, and MyHC with 5-azacytidine, FGF-2, and conditioned medium. MyoD and myogenin were highest at day 7 on treatment with 5-azacytidine and FGF-2 for 1 day and then FGF-2 for an additional 6 days (early differentiation step). The expression of dystrophin and MyHC was increased with the use of conditioned media and their expression levels peaked at days 21 and 28 (late differentiation step). 2% horse serum, insulin-transferrin-selenite (ITS) serum-free medium, and the supernatant of the original MSCs could promote differentiation of myoblasts to myotubes [24]. Because horse serum is not appropriate for clinical usage, and cell survival and myotube formation is unsatisfactory in ITS serum-free medium, the supernatant (conditioned medium) of the MSCs is an alternative and an effective inducer for increasing the fusion index of myoblasts. We found that mRNA of growth factors such as HGF, IGF-1, and IGF-2, which influenced on muscle growth, metabolism, and repair was dramatically increased in hASCs for preparing the conditioned medium (data not shown) and hypothesize that these growth factors play roles in dystrophin and MyHC expression as well as in increasing the fusion index. hASCs expressing dystrophin and MyHC contribute to myotube formation by the high-frequency through cellular fusion with mouse myoblast C2C12 cells, while hASCs expressing MyoD and myogenin do not form myotubes effectively. Our results demonstrate that hASCs undergoing the late differentiation phase express dystrophin and MyHC and can dramatically contribute to myotube formation by the high-frequency. Interestingly, more than half of the nuclei in myotubes were derived from hASCs undergoing the late differentiation phase. In other words, hASCs expressing dystrophin and MyHC contribute significantly to myotube formation in co-culture with mouse myoblasts, suggesting that only hASCs undergoing the late differentiation step, and thereby expressing dystrophin and MyHC, are appropriate to use in stem cell therapy for muscular dystrophies.

Although several groups have already reported that ASCs contribute to myotube formation spontaneously or by co-culture with myoblasts, the frequency of myotube formation was very low [21,22]. Vieira et al. reported spontaneous fusion between ASCs in earlier passages at high density [22]. Di Rocco et al. [21] also reported that approximately 0.001% of adipose tissue-derived stromal vascular fraction (AT-SVF) cells demonstrated sporadic spontaneous myogenic differentiation, which was confirmed by the expression of troponin-T (TnT). In addition, AT-SVF cells from GFP mice can be incorporated into differentiating skeletal myotubes by co-culture with primary myoblasts. After a co-culture for 7 days in differentiation medium (DM), the number of myotubes expressing both GFP and TnT corresponded to approximately 0.2% of input cells. They also showed that approximately 1% of in vitro expanded ASCs gave rise to myotubes when ASCs were co-cultured with primary myoblasts.

Because MSCs are easy to isolate and expand, they have great potential as therapeutic agents. However, despite their potential for use in cellular therapy, practical application to muscular dystrophies depends on the ability to control their differentiation into functional skeletal muscle cells with high efficiency and purity. Several factors required for skeletal myogenesis of stem cells have been reported: 5-azacytidine, horse serum, connexin 43, galectin-1, Notch1, and Pax3. 5-azacytidine can induce DNA demethylation and reactivate the MRFs, followed by myogenesis in MSCs [25]. Two percent horse serum can induce multinucleated myotubes from myoblasts [26], and connexin 43 is important for the fusion of myoblasts into multinucleate myotubes [27]. Recently, human fetal MSCs and embryoid body-derived (EBD) cells were shown to undergo myogenesis in vitro and mature into dystrophin-positive myofibers upon treatment with human galectin-1 [28]. Although several groups have reported that myotubes were formed from stem cells, the efficiency was very low without ectopic expression of Notch1 or Pax3, despite expression of MyoD and myogenin [21,24,28]. In our study, although 5-azacytidine and horse serum alone could not induce multinucleate myotubes, and mRNA of galectin-1, Notch1, and Pax3 was not changed during myogenic differentiation (data not shown), we were able to incorporate hASC into differentiating mouse myotubes by co-culturing hASCs expressing dystrophin and MyHC with mouse myoblast C2C12 cells. These results suggested that the expression of dystrophin and MyHC play a key role in myotube formation in co-culture systems with myoblasts. Because hASCs expressing dystrophin and MyHC have a higher potential for myotube formation and are much smaller than myotubes in our systems, they are useful for the clinical treatment of muscular dystrophy. Further investigations are currently under way to confirm that hASCs expressing dystrophin and MyHC play a role in muscle regeneration in animal models of muscular dystrophy.

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