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MyoD Overexpressed Equine Adipose-Derived Stem Cells Enhanced Myogenic Differentiation Potential

Soo-Eun Sung,*† Meeyul Hwang,*† Ah-Young Kim,*† Eun-Mi Lee,*† Eun-Joo Lee,*† Su-Kyeong Hwang,‡ Shin-Yoon Kim,§ Hong-Kyun Kim,¶ and Kyu-Shik Jeong*†

*Department of Pathology, College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea
†Stem Cell Therapeutic Research Institute, Kyungpook National University, Daegu, Republic of Korea
‡Department of Pediatrics, Kyungpook National University Hospital, Daegu, Republic of Korea
§Skeletal Diseases Genome Research Center, Kyungpook National University Hospital, Daegu, Republic of Korea
¶Department of Ophthalmology, Kyungpook National University Hospital, Daegu, Republic of Korea

Mesenchymal stem cells could potentially be used in the clinical treatment of muscle disorders and muscle regeneration. Adipose-derived stem cells (ADSCs) can be easily isolated from adipose tissue, as opposed to stem cells of other tissues. We believe that cell therapy using ADSCs could be applied to muscle disorders in horses and other species. We sought to improve the myogenic differentiation potential of equine ADSCs (eqADSCs) using a MyoD lentiviral vector. MyoD lentiviruses were transduced into eqADSCs and selected using puromycin. Cells were cultured in differentiation media containing 5% horse serum, and after 5 days the MyoD-transduced cells differentiated into myogenic cells (MyoD-eqADSCs). Using green fluorescent protein (GFP), MyoD-eqADSCs were purified and transplanted into the tibialis anterior muscles of mice after they were injured with the myotoxin notexin. The mice were sacrificed to examine any regeneration in the tibialis anterior muscle 4 weeks after the MyoD-eqADSCs were injected. The MyoD-eqADSCs cultured in growth media expressed murine and equine MyoD; however, they did not express late differentiation markers such as myogenin (MYOG). When cells were grown in differentiation media, the expression of MYOG was clearly observed. According to our reverse transcription polymerase chain reaction and immunocytochemistry results, MyoD-eqADSCs expressed terminal myogenic phase genes, such as those encoding dystrophin, myosin heavy chain, and troponin I. The MyoD-eqADSCs fused to each other, and the formation of myotube-like cells from myoblasts in differentiation media occurred between days 5 and 14 postplating. In mice, we observed GFP-positive myofibers, which had differentiated from the injected MyoD-eqADSCs. Our approaches improved the myogenic differentiation of eqADSCs through the forced expression of murine MyoD. Our findings suggest that limitations in the treatment of equine muscle disorders could be overcome using ADSCs.

Key words: Adipose-derived stem cells (ADSCs); MyoD; Myogenic differentiation; Muscle regeneration therapy

INTRODUCTION

Equine muscle disorders, like rhabdomyolysis, are classified into two classes based on their cause: nonexertional and exertional myopathies. Nonexertional myopathies can be due to nutritional deficiencies, such as vitamin E and selenium, and metabolic system problems such as glycogen branching enzyme deficiency, and can also be a result of the inflammatory response to bacterial, viral, or parasitic infections. Exertional myopathies are induced sporadically by a lack of training or overexertion, as well as chronic factors such as dietary imbalances or polysaccharide storage myopathy[1]. Some of these causes are similar in humans and other species, so the cure for these muscle disorders can have clinical applications. Many types of cell therapy have been researched as possible methods for these muscle disorders[2].

An mdx mouse model for Duchenne muscular dystrophy (DMD), an X-linked muscular dystrophy caused by a genetic mutation, was used in this study. DMD fatally inhibits muscle regeneration and causes progressive muscle weakness[3]. Cell transplantation has been broadly used to research this disorder; for example, muscle cells

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Address correspondence to Kyu-Shik Jeong, D.V.M., Ph.D., 201, Department of Pathology, College of Veterinary Medicine, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu, Republic of Korea. Tel: +82-53-950-5975; Fax: +82-53-955-5955; E-mail: jeongks@knu.ac.kr *or* Hong-Kyun Kim, M.D., Ph.D., Department of Ophthalmology, Kyungpook National University Hospital 130 Dongdeok-ro, Jung-gu, Daegu, Republic of Korea. Tel: +82-53-420-5806; Fax: +82-53-426-6552; E-mail: okeye@knu.ac.kr

have been transplanted into DMD patients and adipose-derived stem cells (ADSCs) have been transplanted into mdx mice[4].

Mesenchymal stem cells (MSCs) have the potential to differentiate into cells of multiple lineages, including adipocytes, chondrocytes, osteocytes, and myocytes[5]. MSCs have been isolated from the bone marrow, umbilical cord blood, periosteum, and adipose tissue[6]. Large quantities of ADSCs can be easily isolated from the adipose tissue. The cell surface markers of ADSCs are similar to those of MSCs from other tissues[7]. The role of ADSCs in vitro and in vivo in the field of regenerative medicine has been widely investigated. While ADSCs have the potential for spontaneous myogenic differentiation, they have a low efficiency of myogenic differentiation on their own.

The process by which muscles are formed is known as myogenesis. During embryonic myogenesis, muscle fibers are first generated from mesoderm-derived structures, with additional fibers generated from template fibers. Once the muscle has matured, progenitors enter a quiescent state and are referred to as satellite cells. Satellite cells play a role in repair when muscle injuries occur; therefore, satellite cells are essential for myogenesis, as they have the capacity to differentiate into new muscle fibers[8].

Several myogenic regulatory factors (MRFs) are associated with myogenesis, including myogenic determination 1 (MyoD), Myf5, myogenin (MYOG), and MRF4[9]. Myf5 and MyoD play a role in determining myogenic populations in the myotome, while MYOG and MRF4 are responsible for the terminal differentiation and homeostasis of myofibers[10].

The myogenic differentiation of ADSCs is facilitated by low serum conditions or through coculture with skeletal muscle cells. The coculture of ADSCs with human DMD myoblasts promotes the formation of myotubes[11]. In a recent study, myogenic markers, such as dystrophin and myosin heavy chain (MHC), were detected in a coculture condition with mouse C2C12 myoblasts and human ADSCs[12].

Lentiviral vectors are capable of the conversion of equine ADSCs (eqADSCs) with high efficiency and can permanently integrate the exogenous transgene into the host genome[13]. Human ADSCs are easy to access from the adipose tissue and have high lentiviral transduction efficiency; thus, the forced expression of MyoD results in the enhancement of myogenic potential[14]. However, eqADSCs are not known to show MyoD-induced myogenesis. In this study, we induced myogenic differentiation by transducing ADSCs to express upregulated MyoD by using lentiviral vectors. The generation of muscle fibers from this research will be helpful for clinical applications in therapy for muscle disorders in horses and potentially in humans.

MATERIALS AND METHODS

Isolation of eqADSCs

We isolated eqADSCs from the abdominal adipose tissue of an 8-month-old, male horse. Handling and experimental procedures for isolating cells from the horse were approved by the Kyungpook National University Institutional Animal Care and Use Committee (IACUC; Approval No. KNU 2013-0082). The adipose tissue was washed with povidone-iodine (SF Co., Ansan, South Korea) in 70% ethanol (Duksan Science, Seoul, South Korea) and was then washed with phosphate-buffered saline (PBS; WelGENE, Gyeongsan, South Korea). It was mechanically homogenized and then digested with 2 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) for 5–7 min at 37°C. Digested samples were filtered through a 70-μm nylon mesh with low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; PAA Laboratories, Dartmouth, MA, USA) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) and 2% antibiotics-antimycotics (Gibco, Gaithersburg, MD, USA). Samples were centrifuged (1,258×*g*, 3 min), and the supernatants were aspirated. The cell pellets were resuspended in growth media and seeded into culture dishes.

Staining of Multilineage Differentiated Cells

Chondrogenic, osteogenic, and adipogenic differentiation media were all from the StemPro Differentiation Kit (Gibco). We stained each of the differentiated cells. Cells that were cultured in adipogenic differentiation media for 7 days were stained with Oil red O stain. The Oil red O powder (Sigma-Aldrich, St. Louis, MO, USA) working solution was made with a 6:4 ratio of propylene glycol (Sigma-Aldrich) and isopropanol (Sigma-Aldrich). The cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) and were then stained for 30 min. After a wash, the cells were stained with hematoxylin (Sigma-Aldrich) for a nuclei stain, and were finally rinsed with distilled water. Cells that were cultured in chondrogenic differentiation media for 14 days were stained for 2 h with 1% Alcian blue (Sigma-Aldrich) stain. Cells that were cultured in osteogenic differentiation media for 21 days were stained with 2% Alizarin red S (Sigma-Aldrich) solution for 20 min and then rinsed three times with distilled water after each step.

Cell Culture Conditions

The isolated eqADSCs were maintained in growth medium containing DMEM-LG, 10% FBS, and 1% penicillin–streptomycin (WelGENE), which was refreshed every 2 days. To subculture, cells were trypsinized with 0.05% trypsin-EDTA (Gibco) when cultures were 80–90% confluent. To test the multipotency of eqADSCs,

such as adipogenesis, osteogenesis, and chondrogenesis, a StemPro Differentiation Kit (Gibco) was used. For myogenic differentiation, cells were cultured for up to 2 weeks in differentiation media with DMEM-LG, 5% horse serum (Gibco), and 1% penicillin–streptomycin (WelGENE).

Preparation of Plasmids and Lentiviruses

The open reading frame encoding MyoD (1,785 bp) was digested from a polymerase chain reaction (PCR) product using *Nhe*I (Takara Bio Inc., Shiga, Japan) and *Eco*RI (Takara), and then subcloned into the pLJM lentiviral vector (Addgene, Cambridge, MA, USA). The resulting pLJM-MyoD vector had a puromycin selective marker. Plasmids were amplified in *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). Vectors were transfected into 293FT cells (Invitrogen) using Lipofectamine 2000 (Invitrogen). Briefly, 3 μg of constructed pLJM-MyoD and 9 μg of packaging plasmid (Invitrogen) in 1 ml of Opti-MEM (Invitrogen) were added to 90% confluent cultures of 293FT cells in 100-mm plates. Supernatants were collected at 48 and 72 h posttransfection, and filtered through a 0.45-μm filter (Merck Millipore, Darmstadt, Germany). Aliquots were prepared and stored at −80°C until needed. Viral titers were determined using Lenti-X GoStix (Clontech, Mountain View, CA, USA), and only lentiviruses with a minimum titer of 5×10^5 infectious units (IFU)/ml were used for reprogramming experiments.

Transduction of eqADSCs

Isolated eqADSCs (4×10^5 cells) were seeded into 60-mm culture dishes. Cells were transduced with 6 μg/ml Polybrene (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and lentiviruses at a multiplicity of infection (MOI) of 2.3. At 2–3 days posttransduction, MyoD-eqADSCs were selected using puromycin (Gibco).

Reverse Transcription PCR (RT-PCR) Assays

Total RNA was isolated using a TRIzol reagent (Invitrogen) as recommended by the manufacturer. Total RNA was purified from DNA and proteins using chloroform (Sigma-Aldrich) and was then precipitated with isopropanol (Sigma-Aldrich). The concentration of RNA in the samples was determined using a Quant-iT RNA Assay Kit (Invitrogen) and a Qubit 3.0 Fluorometer (Invitrogen). To synthesize cDNA, total RNA (500 ng) and Molony murine leukemia virus (M-MLV) reverse transcriptase (Bioneer, Daejeon, South Korea) were used. The thermal cycling conditions involved an initial denaturation step at 94°C for 3 min, followed by 35 amplification cycles [94°C for 30 s, and the appropriate annealing temperature (see below) for 30 s, 72°C for 30 s], with a final extension step at 72°C for 7 min. Specific primers and annealing temperatures were used to amplify murine MyoD (5′-TAC AGT GGC GAC TCA GAT GC-3′ and 5′-CTG GGT TCC CTG TTC TGT GT-3′, 53°C), equine MyoD (5′-GTC GAG GAC AGT CGG GTG TA-3′ and 5′-AAG TCG TCC GCT GTA GCA AA-3′, 55°C), *MYOG* (5′-GCT TAG AGG GGC TCA GGT TT-3′ and 5′-ACA ATG GAG GTG AGC GAG TG-3′, 55°C), desmin (5′-ATC CCT TCT CGG CAT CCA CT-3′ and 5′-GGA ACG CGA TTT CCT CGT TG-3′), dystrophin (5′-TCC AGT GGA GAT CAC GCA AC-3′ and 5′-CGG CTT TTT CTC GCT CGA TG-3′, 56°C), MyHC (5′-GGG AGG TTG AGA GTG AGA A-3′ and 5′-TCA TTC CAT AGC GTG AAG GC-3′, 56°C), troponin I (5′-ACG TGG GTG ATT GGA GGA AG-3′ and 5′-CAC GGG GCT TGG AAT CCT T-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-CCC CTA ACG TGT CAG TCG TG-3′ and 5′-TTA GGG GGT CAA GTT GGG AC-3′, 55°C). Amplified PCR products were visualized using 1.5% (w/v) agarose gel electrophoresis.

Immunostaining

We seeded eqADSCs on four-well chamber slides or 35-mm tissue culture dishes. Cells were fixed with 4% PFA or 10% formaldehyde for 15 min. Cells were covered with ice-cold methanol (Duksan Science) for 10 min at −20°C prior to staining for MyoD and MYOG. Cells were blocked with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (Sigma-Aldrich) containing 0.05% Tween 20 (Generay Biotech, Shanghai, P.R. China) and then incubated at 4°C overnight with the appropriate primary antibody. We used mouse and rabbit antibodies against MyoD (1:100 dilution; Santa Cruz Biotechnology), MYOG (Dako, Carpinteria, CA, USA), troponin I (1:100; Santa Cruz Biotechnology), and Myh2 (dilution 1:100; Santa Cruz Biotechnology). The secondary antibodies that we used were FITC-conjugated anti-mouse immunoglobulin G (IgG) (Invitrogen), tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (Invitrogen), and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Invitrogen). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Leiden, The Netherlands).

Flow Cytometry and Cell Sorting

To analyze cell surface markers such as CD44, cells were trypsinized with 0.05% trypsin-EDTA (Gibco) and centrifuged at $1{,}258 \times g$ for 3 min at room temperature. The cell pellets were resuspended in 1 ml of DMEM and quantified. Cells (1.5×10^4) were then suspended in 200 μl of 1% BSA in PBS and incubated for >1 h with antibodies. The antibodies used for flow cytometry were FITC-conjugated rat anti-mouse CD44 (clone IM7; BD Pharmingen, San Diego, CA, USA), FITC-conjugated rat anti-mouse CD34 (clone RAM34; BD Pharmingen), FITC-conjugated rat anti-mouse CD90.2 (clone 30-H12;

BD Pharmingen), FITC-conjugated rat anti-mouse CD106 [clone MVCAM A(429); Bio-Rad Laboratories, Hercules, CA, USA], human/equine integrin β_1/CD29 (clone 419127; R&D Systems, Minneapolis, MN, USA), and human/equine 5′-nucleotidase/CD73 (clone 606112; R&D Systems). The cells were then washed three times with PBS containing 2% FBS. If appropriate, the cells were incubated with FITC-conjugated rat IgG2b (clone A95-1; BD Pharmingen) for >30 min. The samples were centrifuged (400 × *g*, 5 min, 4°C), the supernatants were aspirated, and the cell pellets were washed with either PBS containing 1% BSA or PBS containing 2% FBS. The samples were centrifuged again (400 × *g*, 5 min, 4°C), the supernatants were aspirated, and the cell pellets were resuspended in PBS supplemented with 1% BSA. Analysis of fluorescent signals and the determination of green fluorescent protein (GFP)-positive cells was conducted using FACSAria III (BD Biosciences, San Jose, CA, USA) and FACSDiva 6.1.3 software (BD Biosciences).

Transplantation of Transduced Cells Into Mice

Transplanted MyoD-eqADSCs were identified through GFP expression. Following puromycin selection, cells were sorted with FACSAria III, and GFP-positive cells were collected. The GFP-positive MyoD-eqADSCs were injected into BALB/c-nu/nu-SIc mice (Central Lab Animal Inc., Seoul, South Korea) (2–3 months old, male, 24–26 g, in a specific pathogen-free condition) that were intraperitoneally anesthetized with a mixture of Rompun (Bayer, Leverkusen, Germany) and Zoletil (Virbac, Carros cedex, France). To induce muscle injury, about 25 µl of 2 µg/ml of notexin (Latoxan, Valence, France) was injected into the tibialis anterior (TA) muscle of both legs. After 1 day, 1×10^6 cells were resuspended in 40 µl of PBS and injected using an insulin syringe into the left and right TA muscles[15,16]. At 4 weeks after cell transplantation, TA muscle tissue was collected and fixed with 4% PFA at 4°C overnight. The tissue was dehydrated in a 5% sucrose solution for 6 h at 4°C and soaked in 20% sucrose solution at 4°C overnight. The TA muscles were then embedded in freezing medium (Leica, Wetzlar, Germany) and sectioned (8- to 10-µm thickness) on a cryostat.

For immunocytochemistry, muscle tissue sections were fixed in methanol for 3 min at room temperature. Muscle sections were then incubated in blocking solution (PBS containing Tween 20 with 10% horse serum) overnight at 4°C. To detect dystrophin and GFP, sections were incubated with antibodies against dystrophin (1:100; Abcam, Cambridge, MA, USA) and GFP (1:100; Abcam) overnight at 4°C. After washing (3 × 3 min), the sections were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Thermo Fisher Scientific, Rockford, IL, USA) and FITC-conjugated goat anti-rabbit IgG (1:100; Invitrogen) overnight at 4°C. The sections were then washed (3 × 3 min) and stained with DAPI (Molecular Probes) for 10 min. The sections were washed three times with PBST, and coverslips were mounted using mounting medium (Sigma-Aldrich)[17]. The animal experiments were performed in accordance with the National Institutes of Health (NIH; Bethesda, MD, USA) Guidelines for the Care and Use of Laboratory Animals and were approved by the IACUC of Kyungpook National University (Approval No. KNU 2013-0033-2).

Statistical Analysis

All data are presented as the means ± standard deviation (SD). A Student's *t*-test was used for all statistical analyses, and values of $p < 0.05$ were considered statistically significant. The statistical data were analyzed by Microsoft Excel (Microsoft, Redmond, WA, USA).

RESULTS

Characterization of eqADSCs

Using flow cytometry, we analyzed typical MSC surface markers to determine if they were also present on eqADSCs. We observed a strong expression of CD44 and CD29. Other cell surface markers, such as CD90.2, CD106, and CD73, were weakly expressed. The CD34 protein was not expressed in eqADSCs (Fig. 1A)[18]. Our results suggest that the isolated eqADSCs were similar in nature to MSCs. In adipogenic media, eqADSCs differentiated into adipocytes over 7 days, as determined by Oil red O staining. In the chondrogenic media, eqADSCs formed high-density clusters over 14 days. When eqADSCs were cultured in osteogenic media for 21 days, the cells differentiated into osteoblasts, as shown by Alizarin red S staining (Fig. 1B). It was difficult to differentiate myogenic cells in the differentiation media, with the isolated eqADSCs mainly proliferating instead of differentiating (Fig. 1C).

Overexpression of MyoD in eqADSCs

We transfected pLJM1-MyoD and pLJM1-GFP into 293FT cells to produce lentiviruses (Fig. 2A). In addition, the pLJM1-GFP plasmid on its own was used as a negative control vector. The GFP and MyoD lentiviruses were harvested at 48 and 72 h posttransfection, respectively, and transduced into eqADSCs at an MOI of 2.3. Transduced eqADSCs were selected for using 2.5–5 µg/µl of puromycin (Fig. 2B), such that only MyoD-eqADSCs and GFP-eqADSCs remained. The GFP-eqADSCs did not express MyoD (Fig. 2C and D), but they did express GFP (Fig. 2E). In growth media, GFP-eqADSCs and MyoD-eqADSCs proliferated, but their doubling time was slower than that of normal eqADSCs. The MyoD-eqADSCs were unable to form myogenic tubes for more than 7 days when cultured in growth media (Fig. 2B).

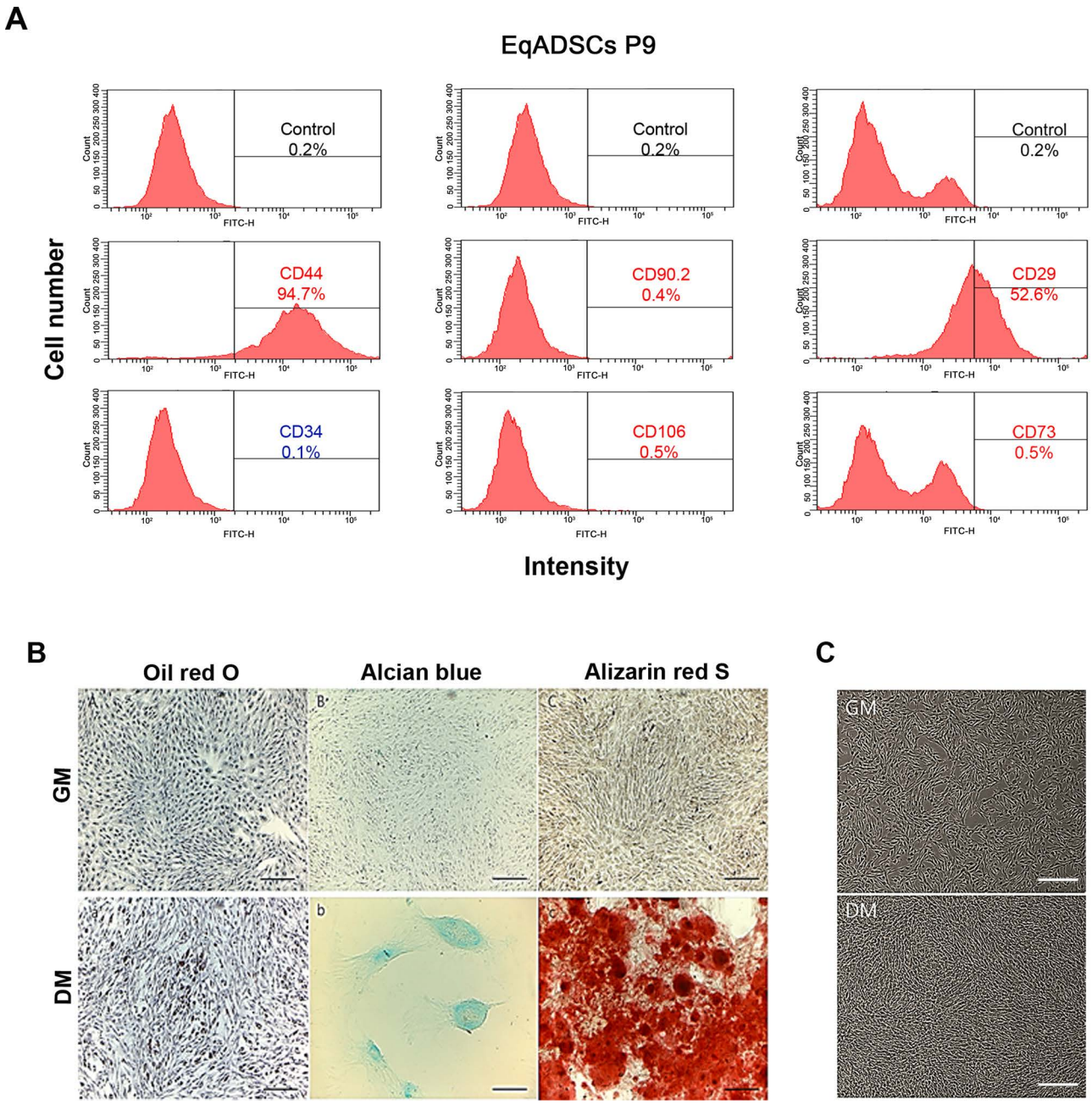


Figure 1. Characterization of equine adipose-derived stem cells. (A) Flow cytometry analysis results. Lines 1 and 2 show directly stained FACS, and line 3 shows indirectly stained FACS data. The *x*-axis is fluorescence intensity, and *y*-axis is cell number. (B) Multipotency test. Equine adipose-derived stem cells (eqADSCs) cultured in growth media (GM), differentiation media (DM). (C) (upper) eqADSCs in the GM condition; (lower) eqADSCs in DM conditions for 10 days. Original magnification: 40× (scale bars: 200 μm).

Formation of Myotube-Like Cells In Vitro

The MyoD-eqADSCs did not spontaneously differentiate into myogenic cells when cultured in growth media. The use of DMEM-LG with 5% horse serum as a differentiation medium potentiated the differentiation of myotube-like cells. After 5 days in the differentiation medium, spindle-like cells were observed among circular cells. After 10 days in the differentiation medium, we observed many multinucleated myotube-like cells. After 2 weeks in the differentiation medium, MyoD-eqADSCs were stable at the myogenic stage. The majority of MyoD-eqADSCs had differentiated into myotube-like cells (Fig. 3A). The expression levels of certain mRNAs in MyoD-eqADSCs were assessed after 14 days in the

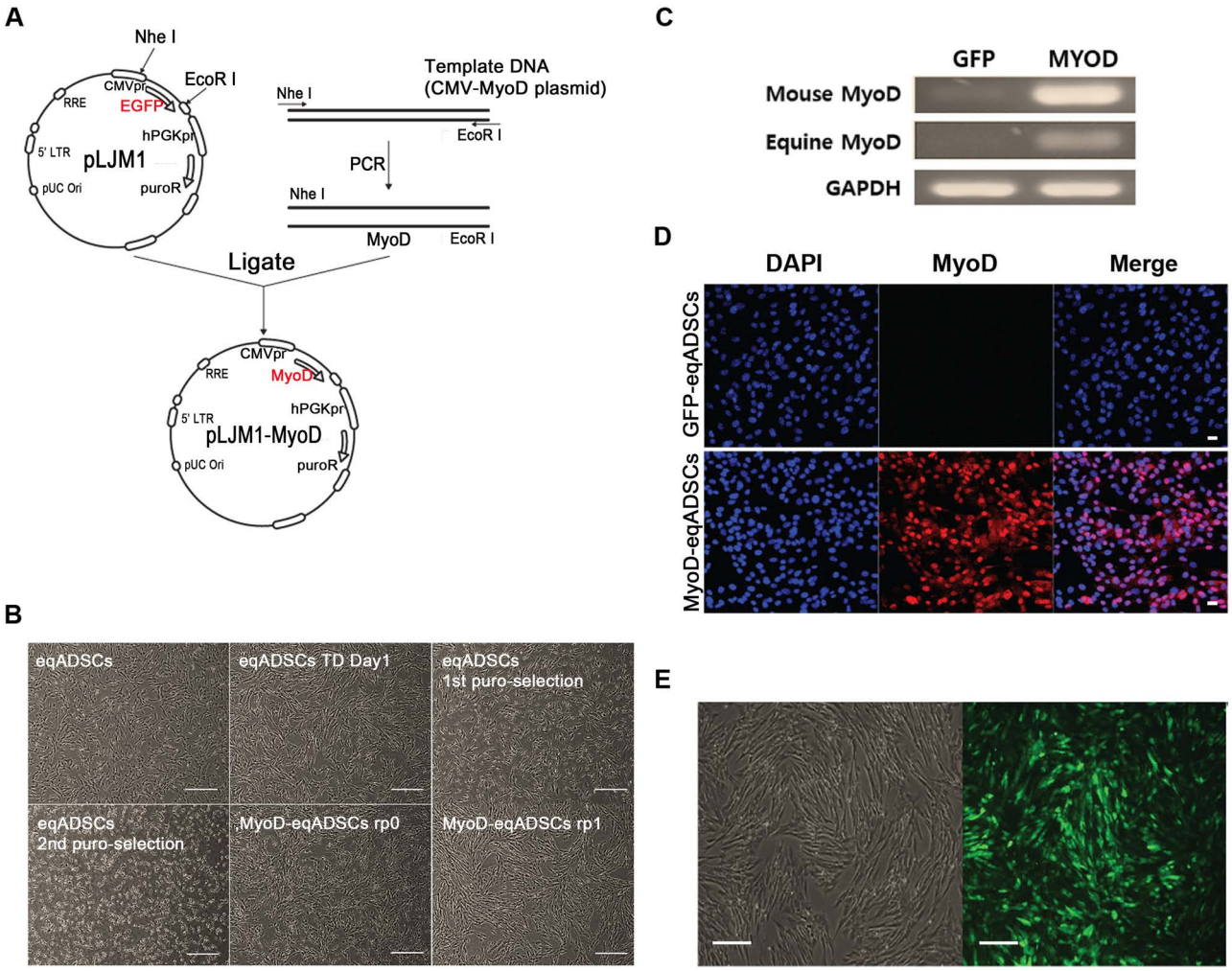


Figure 2. Transduction of MYOD into eqADSCs. (A) The constructed plasmid, pLJM1-MyoD, expressed the MyoD gene by the CMV promoter and the puromycin resistance region by the hPGK promoter. (B) Cell morphologies of MyoD-eqADSCs. (C, D) Expression of MyoD. Green fluorescent protein (GFP)-eqADSCs did not express MyoD, while MyoD-eqADSCs expressed MyoD at the RNA and protein levels. Original magnification: 200× (scale bars: 20 μm). (E) GFP expressed in GFP-eqADSCs. Original magnification: 40× (scale bars: 200 μm).

differentiation medium and were compared with those in MyoD-eqADSCs after 14 days in growth media. Equine MyoD was expressed in MyoD-eqADSCs cultured in growth and differentiation media. The late myogenic differentiation stage marker, MYOG, was only expressed in MyoD-eqADSCs cultured in differentiation media, as were MHC, troponin I, and dystrophin (Fig. 3B). Our immunofluorescence data supported our RT-PCR results. The troponin I protein is a marker of skeletal and cardiac muscle tissues and is expressed in the cytoplasm of muscle cells. We simultaneously stained MyoD-eqADSCs for troponin I and MyoD and observed the formation of myotubes, with troponin I evident throughout the cytoplasm. Of the troponin I-stained cells, their nuclei were positive for MyoD (Fig. 3C). We also detected MYOG and MHC following the formation of myotubes. Our results indicate that MyoD promotes the terminal myogenic differentiation of eqADSCs.

Myogenic Differentiation of MyoD-eqADSCs In Vivo

To distinguish injected eqADSCs from original muscle tissue, GFP lentiviruses were transduced into MyoD-eqADSCs. However, this resulted in a critical decrease in the viability of MyoD-eqADSCs. Therefore, GFP and MyoD lentiviruses were simultaneously transduced into eqADSCs. Cells were selected using puromycin and sorted by flow cytometry. When using eqADSCs as a GFP control, 15% of MyoD- and GFP-eqADSCs were GFP positive (Fig. 4A). Sorted MyoD- and GFP-eqADSCs were cultured for 2 days in a cell culture dish. Notexin was used to induce damage in the TA muscles of mice. We then injected MyoD and GFP-eqADSCs into the TA muscles of the mice. The mice were

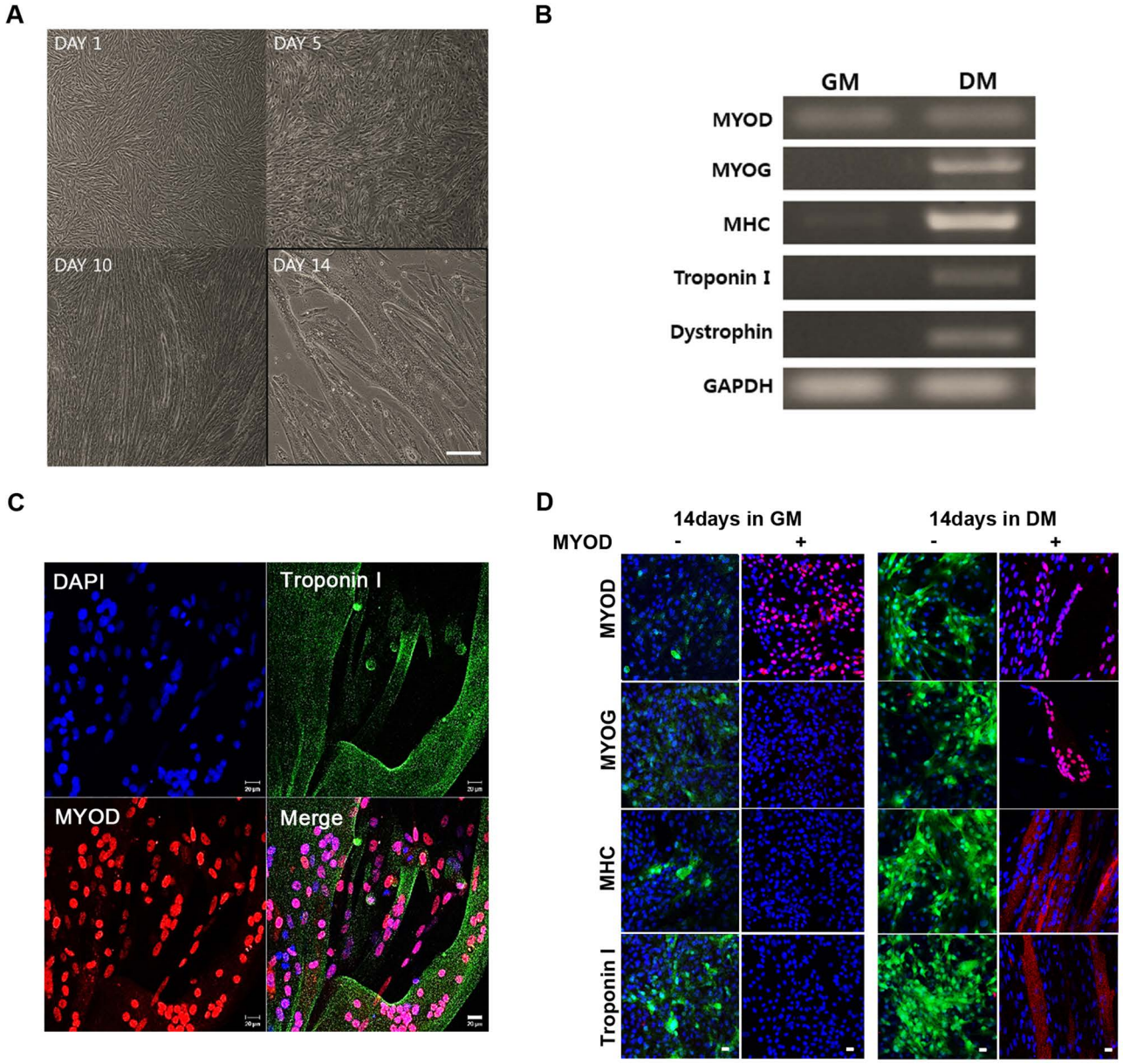


Figure 3. Effects of culturing in DM. (A) Cell morphologies during differentiation conditions on days 1, 5, 10, and 14. Original magnification: 40× (scale bar: 200 μm). (B) mRNA expression of myogenic genes. (C) MyoD and troponin I were double stained at 14 days. Original magnification: 200× (scale bars: 20 μm). (D) Comparison of GM and DM by MyoD transduction at 14 days. Original magnification: 200× (scale bars: 20 μm).

euthanized after 4 weeks, and the TA muscles were examined. According to our immunofluorescence results with an anti-GFP antibody, a single myofiber was positive in the group treated with MyoD- and GFP-eqADSCs (Fig. 4B). The GFP-positive myofiber had many peripheral nuclei, but there was no central nucleus. Centrally nucleated myofibers are indicative of fibrosis and regenerative processes. So these results show that MyoD- and GFP-eqADSCs helped the regeneration of injured muscles. Therefore, we observed that GFP-positive myofibers were indicative of MyoD- and GFP-eqADSC-induced myogenic differentiation. Also, we calculated the average size of the centrally nucleated myofiber and found that it was 1,643 and 2,134 μm^2 for the control and MyoD- and GFP-eqADSC-injected groups, respectively (Fig. 4C).

DISCUSSION

MSCs can be differentiated into muscle cells given that they possess the potential to differentiate into multiple lineages[19,20]. MSCs can be isolated from many tissues,

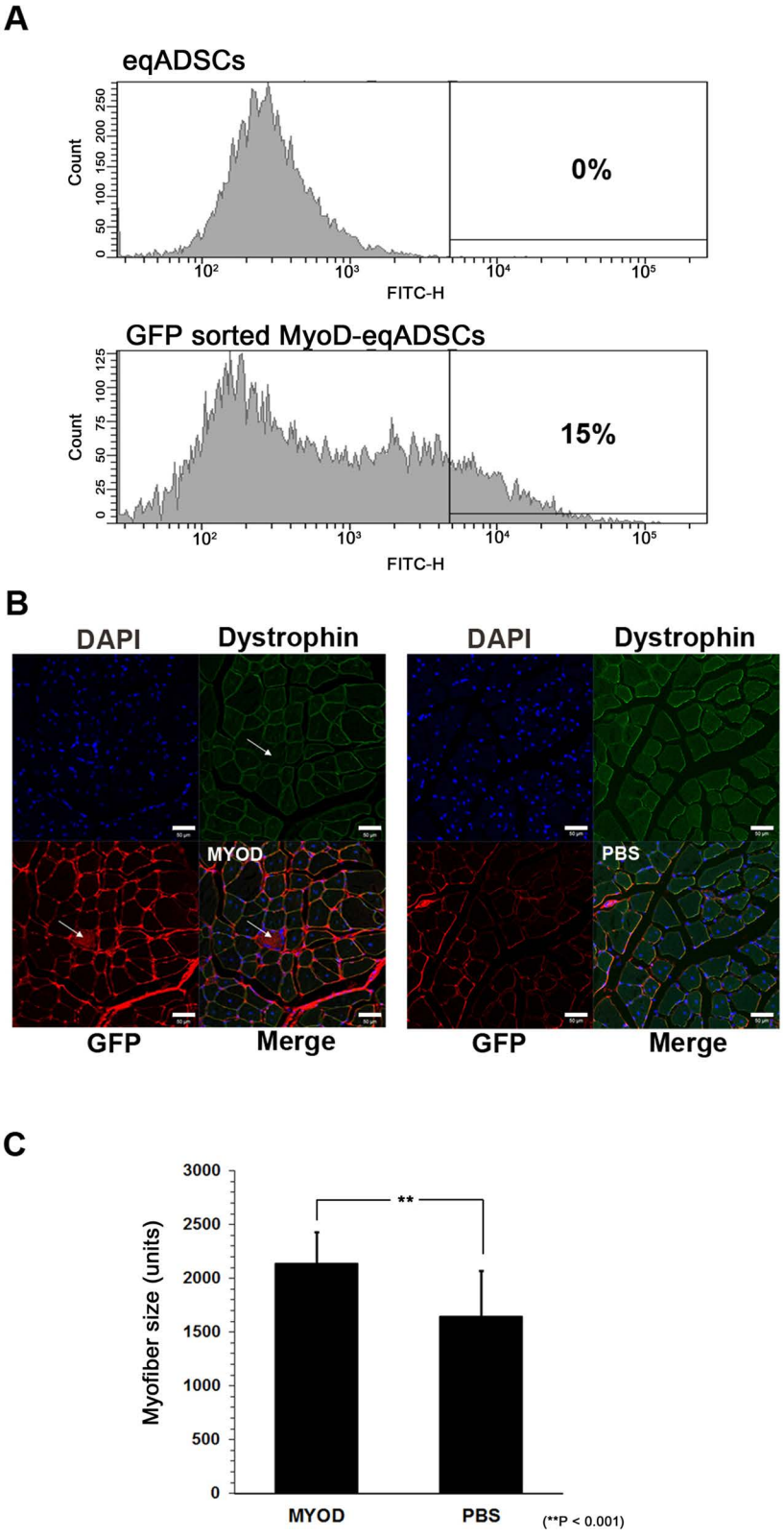


Figure 4. Effectiveness of injecting MyoD-eqADSCs in TA muscles. (A) GFP-labeled cell sorting according to FITC expression level by FACS. (B) GFP and dystrophin are expressed by immunofluorescence in TA muscles. (Left) MyoD-eqADSCs were injected in the TA muscle. (Right) PBS was injected in the TA muscle (scale bars: 50 μm). (C) The size of myofibers, which have centrally located nuclei, was calculated using ImageJ (National Institutes of Health) (**$p < 0.001$).

such as bone marrow, umbilical cord blood, amniotic fluid, and adipose tissue[21,22]. ADSCs can be easily isolated, as opposed to stem cells from other tissues.

Equine MSC surface and intracellular markers are similar to those of human MSCs. In general, MSCs are positive for CD29, CD44, CD73, CD90, CD105, CD106, CD166, and MHCI, and negative for CD11B, CD14, CD19, CD34, CD45, CD79α, and MHCII[18,23–25]. CD44 and CD29 (β_1 integrin) are stromal cell-associated markers that are strongly expressed by eqADSCs. Other stromal cell-associated markers, such as CD90.2 and CD73 (5′-nucleotidase), were weakly expressed[25]. The expression of CD106 (VCAM-1) was also weak. The CD34 protein was not expressed on the surface of isolated eqADSCs. Our findings indicate that the isolated eqADSCs exhibited the same characteristics as MSCs. Lentiviral vectors are used for gene therapy because they are specific and efficient and can be safely used to infect host cells[26]. Transduced cells were selected by puromycin treatment.

In general, the culture medium used for myoblast cell lines contains a low concentration of serum (2–5% horse or calf serum) and other essential components, such as sericin, insulin, transferrin, dexamethasone, and cortisol. These components help to drive myogenic differentiation[11,14,27,28]. To differentiate MyoD-eqADSCs into muscle cells, various media were used. The most suitable differentiation medium for eqADSCs was DMEM-LG supplemented with 5% horse serum and 1% penicillin–streptomycin.

Our RT-PCR results revealed that MyoD-eqADSCs differentiated into myoblasts, as they expressed early differentiation phase myogenic factors. The MyoD-eqADSCs expressed equine and murine MyoD, but not MYOG, which is a marker of myocytes[10]. Therefore, MyoD-eqADSCs were similar to myoblasts according to our RT-PCR results. However, when MyoD-eqADSCs were differentiated in DMEM-LG with 5% horse serum, cells fused to each other and formed myotube-like cells. The analysis of mRNA expression levels in these cells during differentiation revealed that several late and terminal myogenic differentiation markers were expressed. We showed that murine MyoD promoted the myogenic differentiation of eqADSCs. The GFP-eqADSCs failed to induce the expression of any myogenic markers. The GFP-eqADSCs cultured in differentiation and growth media did not express any myogenic factors. For the MyoD-eqADSCs, MyoD was expressed in growth media. In the differentiation medium, MyoD-eqADSCs expressed MyoD, MYOG, troponin I, and MHC. These results correspond with our RT-PCR results.

During in vivo cell transplantation, cells overexpressed GFP simultaneously with MyoD. We transduced eqADSCs with a MyoD lentivirus followed by a GFP lentivirus; however, cell viability was significantly reduced. Therefore, we modified eqADSCs by transducing them with both lentiviruses at once to produce MyoD- and GFP-eqADSCs. These cells were transplanted into the TA muscles of mice that had been injured with notexin. Notexin is derived from the venom of the Australian tiger snake and has myotoxic activities. After 12–24 h following notexin injection, the majority of muscle fibers are destroyed due to the neurotoxicity of phospholipase A_2[29]. Notexin can effectively treat skeletal muscle injuries and is used in regeneration experiments[16]. In our experiments, we found that it took around 4 weeks for full regeneration of TA muscles to occur following treatment with notexin. During regeneration, central nuclei appeared, as did dystrophin-positive fibers[30]. Our in vivo results show that GFP-positive myofibers were apparent when MyoD- and GFP-eqADSCs were injected into the TA muscle and that the myofibers were dystrophin negative. The nuclei in the myofiber were located at the periphery, instead of centrally. Centrally nucleated fibers were counted to determine the regenerating fibers[20]. For the results of equine cell transplantation into the mouse, injected MyoD- and GFP-eqADSCs and mouse myofibers were fused[31]. If injected cells did not fuse with host myofibers, the cells were located near host mouse myofibers without any fusion[22]. However, our results show that injected cells did, in fact, fuse with host mouse myofibers. Although ADSCs have the potential for myogenic differentiation, they did not differentiate in this study (Fig. 1C). Our results show that MyoD-eqADSCs enhanced myogenic differentiation of endogenous muscle cell precursors; this conclusion is based on our observation of GFP-positive myofibers (Fig. 4B). Therefore, from these data, MyoD-overexpressed eqADSCs will be helpful to overcome many muscle disorders.

CONCLUSIONS

In summary, we concluded that overexpression of MyoD promotes myogenic differentiation when cells are cultured in medium containing a low concentration of horse serum. In this differentiation medium, MyoD-eqADSCs expressed MyoD and late myogenic differentiation markers. In addition, MyoD-eqADSCs cultured in differentiation medium tended to fuse to each other and form myotube-like cells in vitro. We also investigated the possibility that MyoD-eqADSCs could be used in vivo for effective muscle regeneration. Research using ADSCs could help in the development of treatments for muscle disorders in horses and other species.

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