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Restoration of muscle fibers and satellite cells after isogenic MSC transplantation with microdystrophin gene delivery

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ABSTRACT

Duchenne muscular dystrophy is the most prevalent inheritable muscle disease. Transplantation of autologous stem cells with gene direction is an ideal therapeutic approach for the disease. The current study aimed to investigate the restoration of myofibers in *mdx* mice after *mdx* bone marrow-derived mesenchymal stem cell (mMSC) transplantation with human microdystrophin delivery. Possible mechanisms of action were also studied. In our research, mMSCs were successfully transduced by retrovirus carrying a functional human microdystrophin gene. Transplantation of transduced mMSCs enabled persistent dystrophin restoration in the skeletal muscle of mdx mice up to the 12th week after transplantation. Simultaneous coexpression of human microdystrophin and desmin showed that implanted mMSCs are capable of long-term survival as muscle satellite cells.

1. Introduction

Duchenne muscular dystrophy (DMD), the most common genetic muscular disorder, is caused by mutations of the dystrophin gene. The dystrophin gene codes a 427 kDa membrane-associated protein that plays important roles in maintaining the myofiber structure. The absence of dystrophin often results in instability of myofibers, which further results in muscular degenerative pathology [1]. Degeneration often activates satellite cell proliferation, which then differentiates into new muscle fibers. Cycles of myofiber necrosis and regeneration could worsen the symptoms of patients or, in the present case, *mdx* mice [2,3]. Although the majority of satellite cells terminally form muscle fibers, some return to quiescence adjacent to myofibers as satellite cells for future cycles of muscle regeneration [4].

At present, no effective therapy for DMD is available as for the *mdx* mouse model, which has the same pathology as DMD patients [5]. Gene therapy using both viral vectors and stem cells has been shown to ameliorate dystrophic pathology [6–8]. Bone marrow-derived mesenchymal stem cells (mMSCs), which are able to differentiate into myocytes in vitro and in vivo, have been considered potential solutions [9–11]. Because exogenous MSC transplantation exhibits immunological rejection, transplantation of isogenic MSC carrying fully functional dystrophin genes is ideal. Retroviral vectors have also been proven to have low immunogenicity and stable transgene expression in the skeletal muscle [13]. However the dystrophin cDNA is too large to be delivered by retroviral vectors, truncated microdystrophin [a (3.5–4.5) kb gene], which can also translate a functionally truncated microdystrophin protein to ameliorate symptoms, has been considered [12].

In the current study, a retrovirus capable of human microdystrophin delivery (3.75 kb) was used to transduce mMSCs. Transduced mMSCs were transplanted into *mdx* mice through the tail vein. Restoration of myofibers was investigated via the detection of dystrophy expression in the gastrocnemius muscles of the mice after mMSC transplantation. Furthermore, how infused mMSCs participated in the restoration of skeletal muscles of *mdx* mice was also explored.

2. Materials and methods

2.1. Animals

All animal experimentation protocols were approved by the local ethics committee. All animals used in this experiment were *mdx* mice (The Jackson Laboratory, MA, USA). The mice were housed in the animal center of Sun Yat-sen University and fed with standard

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cube diet and tap water. A diurnal cycle was regulated by natural lighting. mMSCs were isolated from (7–9) week-old *mdx* mice, and only male *mdx* mice were used for transplantation. Mice were randomly divided into two groups: the control group, composed of 6 (7–9) week-old *mdx* mice transplanted with Hanks' balanced salt solution (HBSS) (Invitrogen, USA) by tail vein injection, and the transplantation group, composed of 24 (7–9) week-old *mdx* mice transplanted with genetically engineered mMSCs by tail vein injection.

2.2. Retrovirus construction

The plasmid PBSK-MICRO (presented by Prof. J.S. Chamberlain of University of Washington School of Medicine, USA), carrying human microdystrophin, and the retrovirus vector pLNCX2 (Clontech Laboratories Inc., CA, USA) were digested with the restriction enzyme NotI. Using T4 ligase, the plasmid pLNCX2–microdystrophin was obtained. The new construct was tested through the restriction enzyme Hind III and DNA sequencing. Recombinant retrovirus plasmid pLNCX2–microdystrophin (2 mg) was transfected into PA317 cell (Clontech Laboratories, Inc.) with 5 μL Lipofect™ 2000 (Invitrogen). Positive clones were selected and amplified by G418 (400 μg/mL). After 4 weeks of selection, the medium containing the virus was collected and filtered using a 0.45 μm cellulose acetate membrane. The collected retrovirus solutions (dystrophin retrovirus) were frozen at −80 °C until use.

2.3. Culture, differentiation, and flow cytometry of mMSCs

The normal culture medium (NM) consisted of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (both from Invitrogen). No antibiotics were added to the NM. The bone marrow was obtained from (7–9) week-old *mdx* mice. mMSCs were cultured and subcultured as described in a previous study [14]. Passage 7 (P7) mMSCs were used for the following experiments.

To identify the differentiation potential of mMSCs, osteogenic and adipogenic differentiation were induced in differentiation medium for up to 4 weeks. For osteogenic differentiation, NM was supplemented with 10^{-8} mol/L dexamethasone, 5 μg/mL ascorbic acid 2-phosphate, and 10 mmol/L β-glycerophosphate. To observe calcium deposition, cultures were washed once with phosphate buffered saline (PBS) and stained for 5 min at room temperature (RT) with Alizarin Red S stain. To induce adipogenic differentiation, NM was supplemented with 10^{-8} mol/L dexamethasone and 5 μg/mL insulin. To confirm the forming of adipocytes, cells were fixed with 4% paraformaldehyde in PBS for 1 h at RT, and stained with Oil Red O (all from Sigma–Aldrich, MO, USA) solution for 5 min at RT.

To analyze the expression of surface antigens, P7 mMSCs were trypsinized, collected, and incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated antibodies against murine Sca-1, CD11b, CD45, CD34, CD29, and CD44. Excess antibody was removed by washing. The samples were incubated with gentle shaking at RT for 20 min. The cells were pelleted, washed twice with HBSS, and analyzed by flow cytometry.

2.4. Retroviral transduction and detection of dystrophin

When P7 mMSCs grew to less than 50% confluence, 2.5×10^5 cells were plated in a 6-well plate, respectively. The following day, 400 μL dystrophin retrovirus and 8 μg polybrene were diluted to a total volume of 1.2 mL in proliferation medium and added to the cells for transduction. After 48 h of infection, the expression of microdystrophin was tested through immunofluorescence staining and reverse transcription-polymerase chain reaction (RT-PCR). Moreover, the myogenic ability of the transduced mMSCs was determined, and that of mMSCs with no transduction was also detected as control.

2.5. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 min, rinsed twice with PBS, and blocked by 2% goat serum for 1 h. Then, the cells were incubated overnight at 4 °C with the primary antibody dystrophin-MAB1690 (Chemicon, CA, USA), which only specifically reacted with human dystrophin but not with mouse dystrophin. After several rinses with PBS, Cy3-conjugated anti-mouse IgG was added as secondary antibody. The cells were incubated for 1 h at RT. Finally, after rinsing all cells three times with PBS, dystrophin expression was tested. Staining without the primary antibodies was performed as a negative control.

2.6. RT-PCR

mMSCs were grown in 6-well plates. The total RNA was isolated using 1 mL of TRIzol reagent (Invitrogen) per well, following the manufacturer's instructions. Reverse transcription reactions were performed using the First Strand cDNA Synthesis Kit and random primers (Fermentas, Vilnius, LTU), again, following the manufacturer's instructions. cDNA was used for microdystrophin and GAPDH PCR analysis. The primer sequences for human microdystrophin were as follows: sense, 5′-GCA AAC TGT ATT CAC TCC A-3′ and anti-sense, 5′-GTC TTT CAA GAT CCA CAG-3′; for mouse GAPDH, the primers were: sense, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and anti-sense, 5′-TCC ACC ACC CTG TTG CTG TA-3′. Reaction conditions included a 94 °C denaturing step (5 min), 30 cycles of 94 °C (30 s), 63 °C (GAPDH) or 50 °C (dystrophin) (50 s), 72 °C (60 s), and a final extension step of 72 °C (10 min).

2.7. Myogenic differentiation

To detect the myogenic differentiation of transduced mMSCs, cells were cultured in myogenic medium consisting of L-DMEM, 2% horse serum, and 10 mM 5-azacytidine. The medium was changed every (3–4) days. The cells were analyzed for expression of muscle-specific markers desmin (Sigma, USA) after up to 10 days in culture. mMSCs without transduction were as control.

2.8. Cell transplantation

The transduced mMSCs were trypsinized and resuspended in HBSS. A total of 1.2×10^7 cells were injected into *mdx* mice through the tail vein. Control injections were performed using non-transduced mMSCs.

2.9. Collection of samples

To investigate the persistence of transgene expression, recipient *mdx* mice were sacrificed (4, 8, and 12) weeks post-transplantation with an overdose of pentobarbital. Gastrocnemius muscles were taken for RNA analysis and dystrophin expression.

2.10. Dystrophin expression in recipient mdx mice

Dystrophin immunofluorescence was performed on gastrocnemius muscle sections to determine the presence of dystrophin positive fibers in recipient *mdx* mice. Gastrocnemius muscles of *mdx* mice receiving non-transduced mMSCs were used as negative controls. The sections were pre-incubated for 1 h at RT with goat serum and then incubated overnight with dystrophin-MAB1690 (1:40 dilution), followed by 1 h of incubation with goat anti-mouse IgG-FITC (1:200, Santa Cruz, CA, USA). The sections were viewed

using Leica DMCB fluorescent microscope. Dystrophin-positive myofibers were counted in sections from the muscle section that showed the highest number of dystrophin-positive myofibers. The average number was obtained by counting the dystrophin-positive myofibers in all the muscle sections in a given group.

Protein exacts were derived from the gastrocnemius muscle of recipient *mdx* mice. Soluble protein concentrations were determined by Bradford assays. For dystrophin detection, protein aliquots (10 μg) were electrophoresed on 6% SDS–PAGE gel and transferred to PVDF membrane. The blots were blocked with 5% nonfat milk and 5% goat serum in Tris-buffered saline-Tween. The primary antibodies were the same as those used in immunofluorescence staining. Horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000, Chemicon) was used as the secondary antibody. Chemiluminescent signals were detected with ECL reagents (Santa Cruz). Immunoblotting for GAPDH, using anti-GAPDH antibody (Sigma), was performed to control for protein loading. Band densitometry was analyzed from digital images of exposed films (MCID, Imaging Research Inc., Ontario, Canada). The ratios of dystrophin to GAPDH in both transplantation and control groups were computed.

2.11. Dystrophin and desmin expression of muscle mononuclear cells

After 12 weeks, the gastrocnemius muscles of *mdx* mice from both control and transplantation groups were excised, minced, and digested in PBS with 0.1% collagenase II (Chemican Corp., Shanghai, China) at 37 °C for 45 min, and then with 0.25% trypsin at 37 °C for 30 min. Finally, the solution was filtered through 40 μm nylon filters (Chemican Corp., Shanghai, China). Mononuclear cells were cultured on a 6-well plate in DMEM/F12 containing 10% fetal bovine serum. On day 7, differentiation was induced by 1.5% horse serum and 6 mg/mL insulin for 48 h, followed by refeeding cultures with F10C and 15% horse serum and insulin. On day 14, cells were fixed with 2% paraformaldehyde and 0.5% sucrose and then incubated with dystrophin MAB1690 (1:40 dilution, Chemicon) at RT for 1 h, followed by 1 h of incubation with goat anti-mouse IgG-CY3 (1:200, Santa Cruz, CA, USA). Then, cells were incubated overnight with purified mouse anti-desmin Abs (1:100 dilution, Chemicon) at 4 °C, followed by 1 h of incubation with goat anti-rabbit IgG-FITC (1:200, Santa Cruz, CA, USA). Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI). The cells were examined under a fluorescence microscope (Olympus BX51, Japan).

2.12. Statistics analysis

Data were expressed as means ± SED. Statistical comparisons were performed using analysis of variance, followed by Fisher's protected least significant difference test. A *P*-value of <0.05 was considered statistically significant.

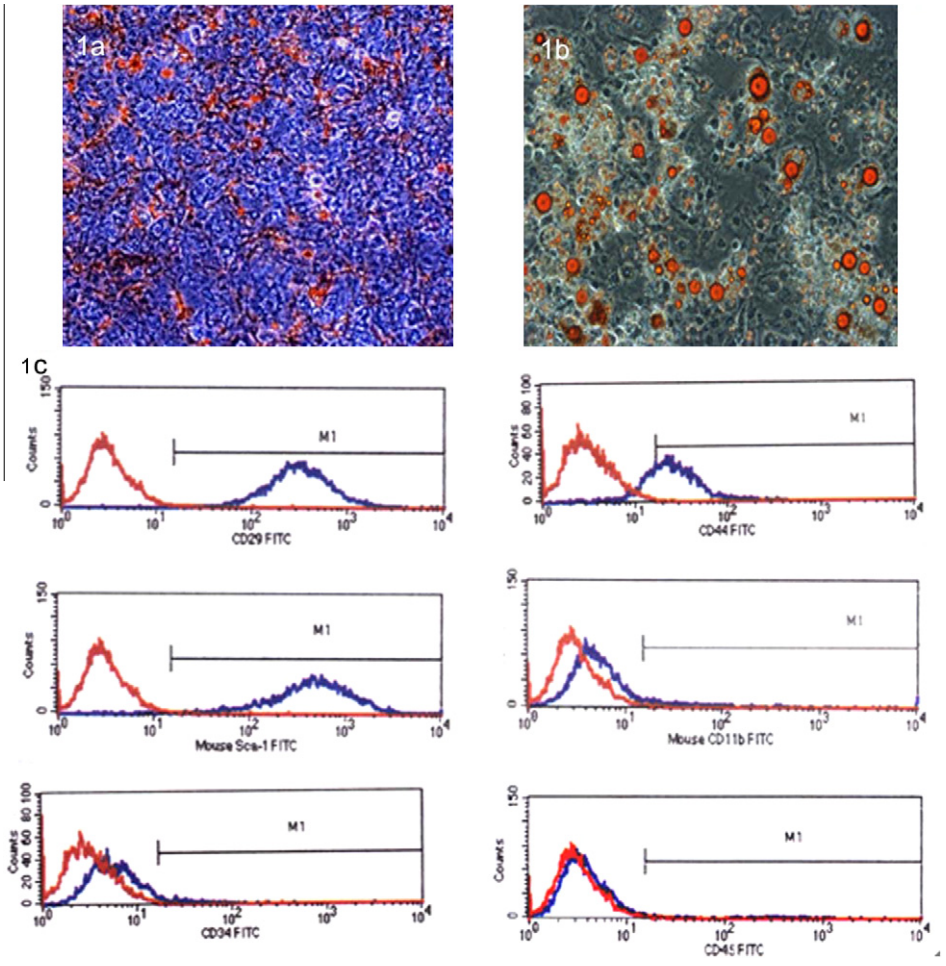

Fig. 1. Characterization of cultured mouse MSCs. (A) Osteogenic differentiation of MSC revealed formation of calcium deposits by Alizarin Red staining. (B) Adipogenic differentiation of MSC revealed formation of lipid vacuoles by Oil Red O staining. (C) FACS analysis of mouse MSCs revealed that MSCs were positive for CD29, CD44, and Sca-1 but negative for CD11b, CD34, and CD45 (magnification: 200×).

3. Results

3.1. Characterization of BM-derived mMSCs

MSCs are defined based on cell surface antigens and in vitro differentiation assays. MSCs express certain non-hematopoietic markers and can differentiate along the adipocytic and osteocytic lineages depending on specific cultures conditions [9–11]. The mMSCs employed in the present experiment fulfilled the defining criteria of MSCs (Fig. 1). First, the mMSCs can differentiate into adipocytes and osteoblasts [15]. Secondly, the surface antigen profile of the cells, as analyzed by flow cytometry, matched the immunophenotype of MSCs. mMSCs expressed high levels of the hyaluronate receptor (CD44) and ECM receptor (CD29). More than 90% of the cells were strongly stained by monoclonal antibodies directed against Sca-1, which is a hallmark of hematopoietic and mesenchymal stem/progenitor cells. Significantly, these cells did not express the hematopoietic markers CD11b, CD34, and CD45.

3.2. mMSC transduction by the retrovirus

Human microdystrophin mRNA was detected in retrovirally transduced mMSCs but not in non-transduced mMSCs (Fig. 2A and B). The transduced MSCs were characterized by a fibroblast-like morphology (Fig. 2C). When examined by immunofluorescence staining, more than 95% of the transduced mMSCs could stably express dystrophin (Fig. 2D). The negative controls, in which the dystrophin antibody was omitted, revealed no staining. When exposed to myogenic medium, the cells expressed muscle-specific marker desmin (Fig. 2E), just as that of non-transduced mMSCs.

3.3. Dystrophin expression in skeletal muscle

The recipient *mdx* mice were sacrificed at different time points (4, 8, and 12 weeks post-injection). Dystrophin expression was detected at each time point by immunofluorescence. No dystrophin positive myofibers were found in the recipient mdx with the transplantation of HBSS (Fig. 3A). On the 4th week after transduced mMSC transplantation, less than 1% of the myofibers expressed dystrophin (Fig. 3B). On the 8th and 12th weeks after transplantation, approximately (6 ± 1.2)% and (10 ± 1.6)% of the dystrophin positive fibers of the muscle were respectively found within mice (Fig. 3C and D). Using Western blot analysis, a 137 kD band was detected after retrovirus-transduced mMSC transplantation using the antibody MAB1690 (Fig. 3E). Compared with the control group, therapeutic *mdx* mice expressed dystrophin, which increased over time. On the 4th week, the ratio of dystrophin to GAPDH was approximately 0.1 ± 0.04, and on the 12th week, the ratio reached 0.45 ± 0.06.

3.4. Dystrophin expression in muscle satellite cells derived from transplanted transduced-mMSCs

Cultured satellite cells expressed desmin, and approximately (1.6 ± 1.4)% of all cells coexpressed dystrophin. In contrast, cells from the control group did not express dystrophin (Fig. 4).

4. Discussion

Based on previous studies, MSCs can deliver normal dystrophin to the skeletal and heart muscles of *mdx* mice after transplantation [16]. And a great number of studies have focused on either rat stem cell transplantation or primary murine MSC transplantation because of the difficulty in culturing murine MSCs, [17,18]. However, the both methods resulted in immunological rejection. So such methods required radiation or chemotherapy, ablating the bone marrow niche before transplantation. In the current study, MSCs were successfully cultured from the total BM of *mdx* mice using standard tissue culture techniques [14]. The resulting cell population exhibited a homogeneous, distinctive morphology that

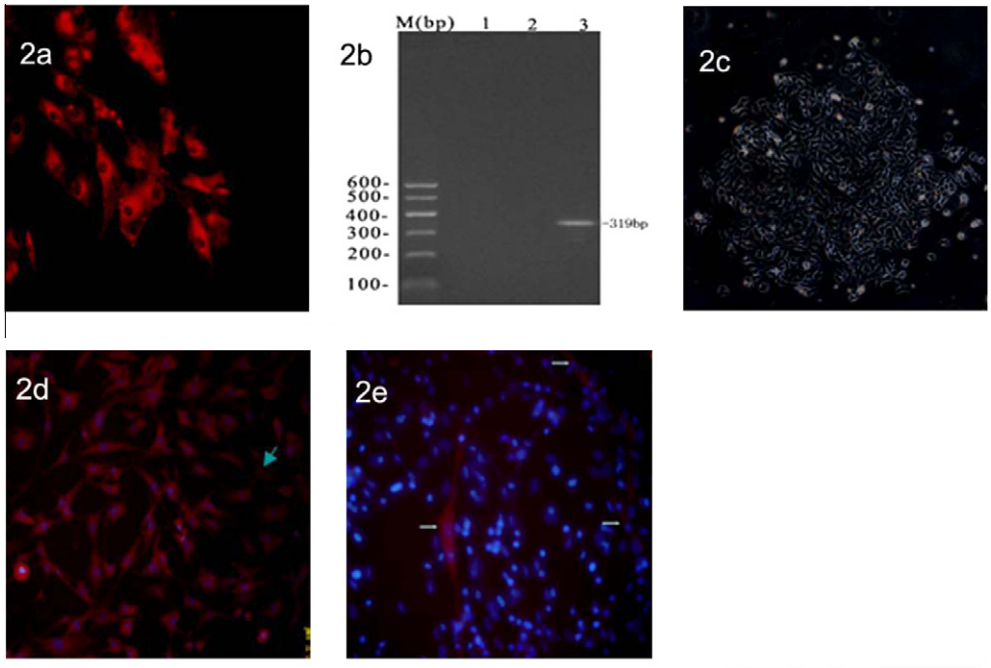

Fig. 2. Characterization of transduced MSCs. (A) Human microdystrophin expression in transduced MSCs was detected by immunofluorescence staining (red cytoplasm). (B) Human microdystrophin expression in transduced MSCs was detected by RT-PCR; only the retrovirally transduced mMSC group showed positive results (1: non-transduced group; 2: pLNCX2-transduced group; 3: retrovirally transduced mMSCs). (C) Transduced cells were selected by G418 and colonies appeared. The transduced MSCs were characterized with fibroblast-like morphology. (D) Transduced mMSCs in colonies consistently expressed human microdystrophin. (E) Tranduced mMSCs expressed the muscle-specific marker desmin upon exposure to myogenic medium (magnification: 200×).

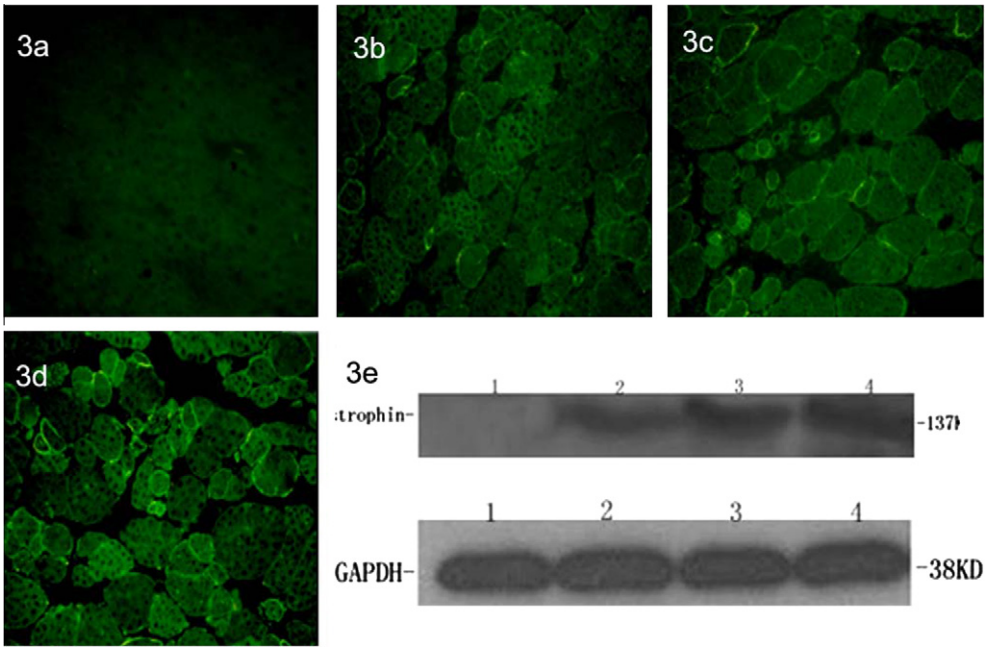

Fig. 3. Immunofluorescence and Western blot analysis of dystrophin expression after transduced MSC transplantation. (A) Untreated *mdx* mice. (B) Four weeks after MSC transplantation. (C) Eight weeks after MSC transplantation. (D) Twelve weeks after MSC transplantation. (E) Western blot detection of dystrophin and GAPDH expression after MSC transplantation (1: control *mdx* mice; 2: *mdx* mice 4 weeks after transplantation; 3: *mdx* mice 8 weeks after transplantation; 4: *mdx* mice 12 weeks after transplantation). The two figures show increasing dystrophin expression after MSC transplantation (magnification: 200×).

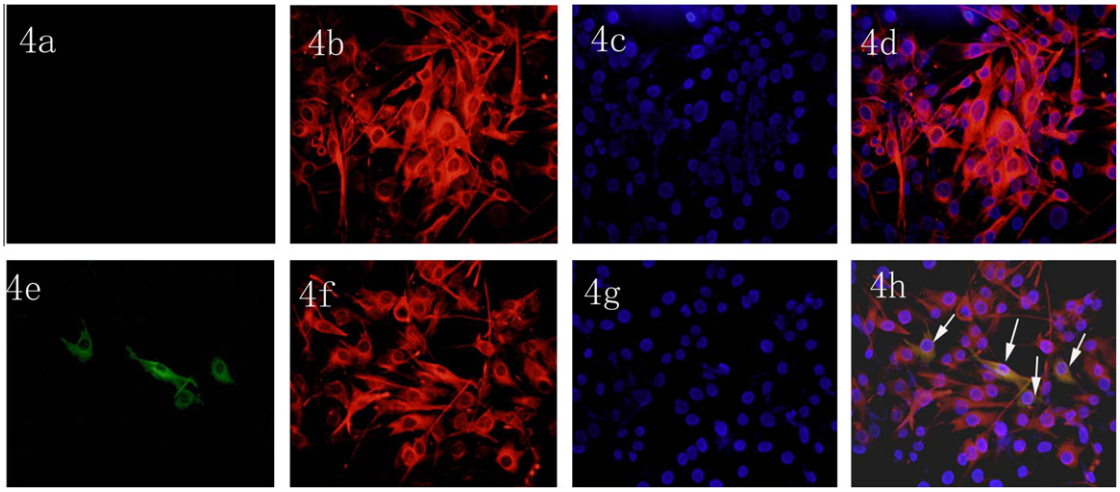

Fig. 4. Double immunostaining of microdystrophin and desmin expression in muscle satellite cells derived from transplanted transduced-mMSCs. (A–D) Control group. (E–H) Transplanted group. (A and E) Human microdystrophin expression with FITC-conjugation: (A) expressed negative cells while some cells in (E) were positive. (B and F) Desmin expression with Cy3-conjugation. (C and G) DAPI nuclear staining. (D and H) Merged images. Twelve weeks after transplantation, several mMSCs with transduced microdystrophin lived as satellite cells (magnification: 200×).

could be expanded for over 15 passages without losing differentiation potential.

Furthermore, the present study investigated the efficacy of ex vivo gene transfer by a retrovirus vector carrying a functional human microdystrophin gene into isogenic mMSCs from *mdx* mice. Since the previous shape and myogenic differentiation of the genes were preserved, it can be concluded that the retroviral vectors could efficiently transduce mMSCs. Although relatively low, the number of dystrophin-positive myofibers in the mdx muscle consistently increased up to 12 weeks post-injection. On the 12th week, the dystrophin-positive fibers accounted for approximately 12% of all the muscle fibers. Although slightly greater than in previous reports [19], the amount of dystrophin detected was still insufficient to alleviate muscle weakness. This may be attributed to the poor recruitment of bone-marrow cells into the dystrophic muscle, as well as their low differential ability. A recent study that used intra-arterial delivery of a different stem cell population reported donor-cell engraftment levels approaching 50% in transplanted host muscle [20].

Researchers generally assume that transplanted MSCs contribute to the restoration of damaged muscles by either forming new muscle fibers in donor cells or fusing with existing dystrophin-negative fibers. Similarly, in the current study, a number of injected cells survived as satellite cells. The persistence of dystrophin-positive myofibers could be related to the ongoing regeneration mediated by the activation and subsequent proliferation of

quiescent satellite cells [21,22]. Although satellite cells derived from transplanted MSCs were a few, these cells may provide seeds for the restoration of myofibers.

The mMSCs in the present experiment were expected not to generate an immune response when injected into *mdx* mice because they are derived from *mdx* mice. However, the retrovirus may trigger an immune response. Eventually, this response may lead to the decrease in number of dystrophin-positive myofibers [23,24]. Muscle-specific promoters, such as muscle creatine kinase, which drives transgene expression only in differentiated muscle cells [25], may present an alternative approach to prevent the rejection of genetically engineered mMSCs before fusion into myotubes and myofibers. Future studies are recommended to supplement the limitations of the present study.

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