Transgene expression and differentiation of baculovirus-transduced adipose-derived stem cells from dystrophin-utrophin double knock-out mouse☆

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Abstract

In this study, recombinant baculovirus carrying the microdystrophin and β-catenin genes was used to infect adipose-derived stem cells from a dystrophin-utrophin double knock-out mouse. Results showed that, after baculovirus transgene infection, microdystrophin and β-catenin genes were effectively expressed in adipose-derived stem cells from the dystrophin-utrophin double knock-out mouse. Furthermore, this transgenic expression promoted adipose-derived stem cell differentiation into muscle cells, but inhibited adipogenic differentiation. In addition, protein expression related to the microdystrophin and Wnt/β-catenin signaling pathway was upregulated. Our experimental findings indicate that baculovirus can successfully deliver the microdystrophin and β-catenin genes into adipose-derived stem cells, and the microdystrophin and Wnt/β-catenin signaling pathway plays an important role in myogenesis of adipose-derived stem cells in the dystrophin-utrophin double knock-out mouse.

Key Words

baculovirus; adipose-derived stem cells; Duchenne muscular dystrophy; microdystrophin; β-catenin; myogenesis; gene therapy; neural regeneration

Research Highlights

(1) Recombinant baculovirus carrying the microdystrophin and β-catenin genes was used to infect adipose-derived stem cells from the dystrophin-utrophin double knock-out mouse.
(2) Dystrophin gene and dystrophin-related proteins contribute to induce adipose-derived stem cell differentiation into myoblasts, in a broader attempt to promote autologous stem cell transplantation for Duchenne muscular dystrophy.

Abbreviations

microdys, microdystrophin; β-cat, beta-catenin; dko, dystrophin-utrophin double knock-out; MyoD, myogenic differentiation antigen; MHC, myosin heavy chain; MSCs, mesenchymal stem cells

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INTRODUCTION

Duchenne muscular dystrophy is the most common and lethal genetic muscular disorder in children[1]. Although the pathogenesis of duchenne muscular dystrophy is clear, no efficient pharmacological treatments currently exist. Stem cell transplantation offers hope for

duchenne muscular dystrophy patients. Several stem cell lines have been used to study the treatment for duchenne muscular dystrophy[2-5]. Vieira *et al*[6] co-cultured muscle cells from duchenne muscular dystrophy patients and adipose-derived stem cells from patients' precursors, and the results showed that adipose-derived stem cells interacted with dystrophic muscle cells and restored dystrophin expression in duchenne muscular dystrophy cells *in vitro*. However, stem cell transplantation cannot correct the gene defect in duchenne muscular dystrophy patients. For this to succeed, gene therapy is required in combination with an applicable vector. Since it was reported that baculovirus effectively transduced hepatic cells, a growing number of cells, including CHO, HeLa[7], human fibroblasts, keratinocytes[8], neural cells , fish cells[9], rat articular chondrocytes and human bone marrow mesenchymal stem cells[10], have also been reported to be permissive to baculovirus transduction. Baculovirus is reported not to cause visible cytopathic effects and uncontrolled replication in mammalian cells. Furthermore, a large genome (130 kb) confers the capacity of baculovirus to accept multiple or large genes up to 38 kb. However, some hematopoietic cells are not effectively transduced by baculovirus. To our knowledge, there is little evidence regarding baculovirus-transduced adipose-derived stem cells from the dystrophin-utrophin double knock-out (dko) mouse (dko-adipose-derived stem cells).

The dystrophin gene is responsible for duchenne muscular dystrophy and plays a central role in organizing a multiprotein complex at the sarcolemma and in linking cytoskeletal proteins to extracellular matrix proteins. The 3.75 kb microdystrophin (microdys) gene is a functional fragment of the dystrophin gene[11]. The beta-catenin (β-cat) gene is an important positive regulatory factor in the Wnt/β-cat signaling pathway, which plays an important role in embryonic myogenesis[12].

It is reasonable to expect encouraging data from on-going trials that combine cytotherapy and gene therapy for muscular dystrophies. In the present study, we aimed to demonstrate the potential of baculovirus as an alternative vector for gene delivery into dko-adipose-derived stem cells and to prove that the microdys and β-cat genes can promote differentiation of dko-adipose-derived stem cells into muscle cells, thus providing reliable evidence for the treatment of duchenne muscular dystrophy.
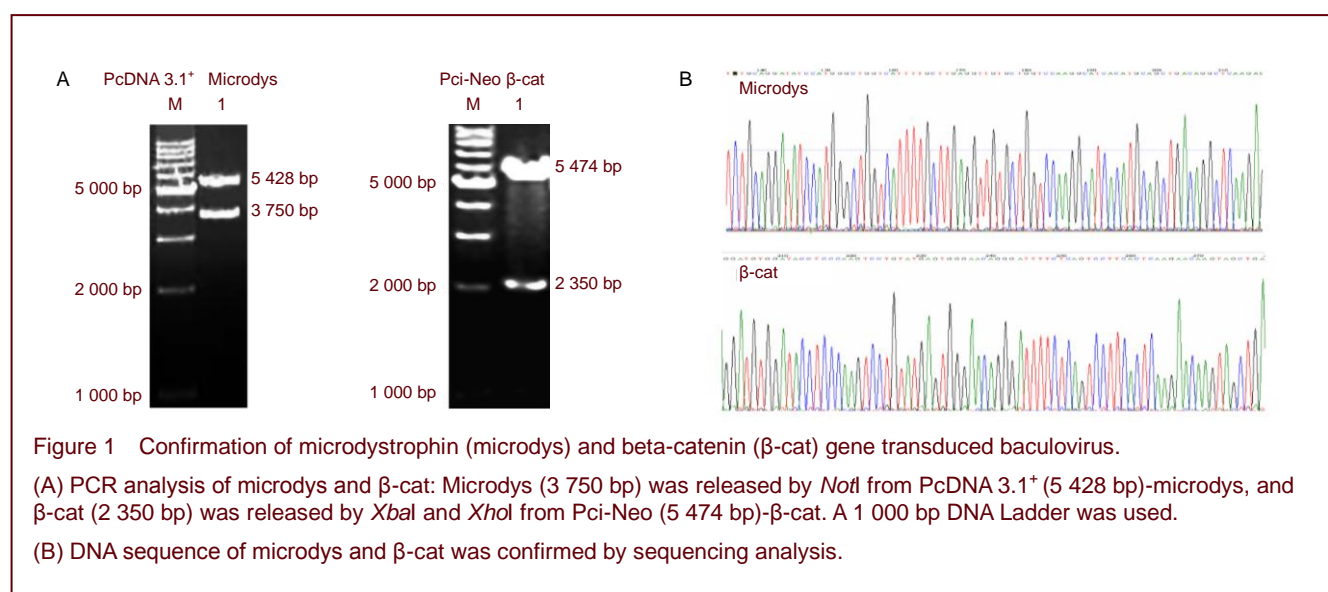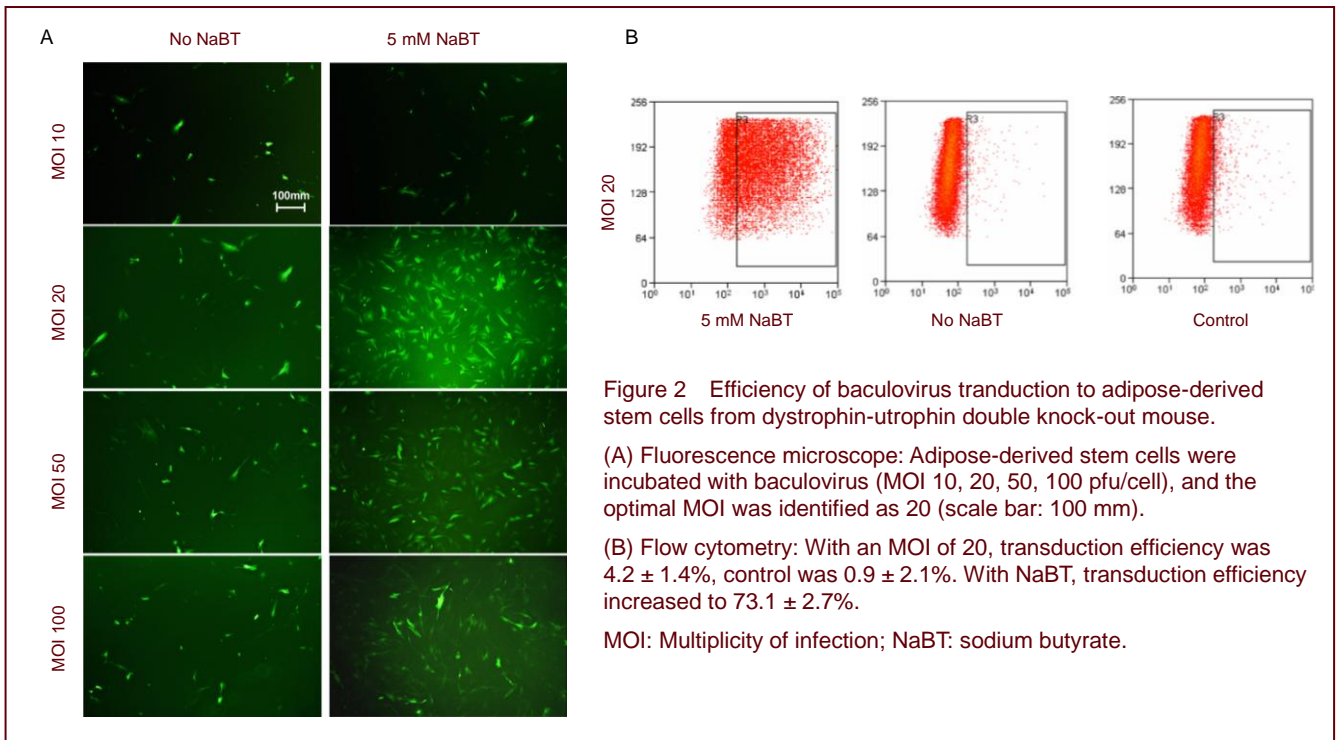
RESULTS

Confirmation of microdys and β-cat gene sequences
The microdys gene was released by NotI as a 3.75 kb gene from the plasmid pcDNA 3.1[+]-micrody. The β-cat gene was released by *Xba*I and *Xho*I as a 2.4 kb gene (Figure 1A). The microdys and β-cat genes were confirmed by sequencing (Figure 1B).

Optimal condition and efficiency of baculovirus transduction of dko-adipose-derived stem cells
The concentrated virus titer was 5×10^8 pfu/mL. In this study, we observed that baculovirus effectively infected dko-adipose-derived stem cells with little harm. Larger multiplicity of infection (MOI) caused higher efficiency of transduction. However, when MOI was greater than 20, the efficiency of transduction failed to increased, with increased cell death. MOI 20 was the optimal virus dose (Figure 2A). Following baculovirus infection of dko-adipose-derived stem cells (MOI = 20), the percentage of GFP^{+} cells was less than 5%, while the control was only 0.9%. In the 5 mM NaBT group, the GFP[+] rate increased to 73.1% (Figure 2B).

Figure 1   Confirmation of microdystrophin (microdys) and beta-catenin (β-cat) gene transduced baculovirus.

(A) PCR analysis of microdys and β-cat: Microdys (3 750 bp) was released by *Not*I from PcDNA 3.1[+] (5 428 bp)-microdys, and β-cat (2 350 bp) was released by *Xba*I and *Xho*I from Pci-Neo (5 474 bp)-β-cat. A 1 000 bp DNA Ladder was used.

(B) DNA sequence of microdys and β-cat was confirmed by sequencing analysis.

Figure 2 Efficiency of baculovirus tranduction to adipose-derived stem cells from dystrophin-utrophin double knock-out mouse.

(A) Fluorescence microscope: Adipose-derived stem cells were incubated with baculovirus (MOI 10, 20, 50, 100 pfu/cell), and the optimal MOI was identified as 20 (scale bar: 100 mm).

(B) Flow cytometry: With an MOI of 20, transduction efficiency was 4.2 ± 1.4%, control was 0.9 ± 2.1%. With NaBT, transduction efficiency increased to 73.1 ± 2.7%.

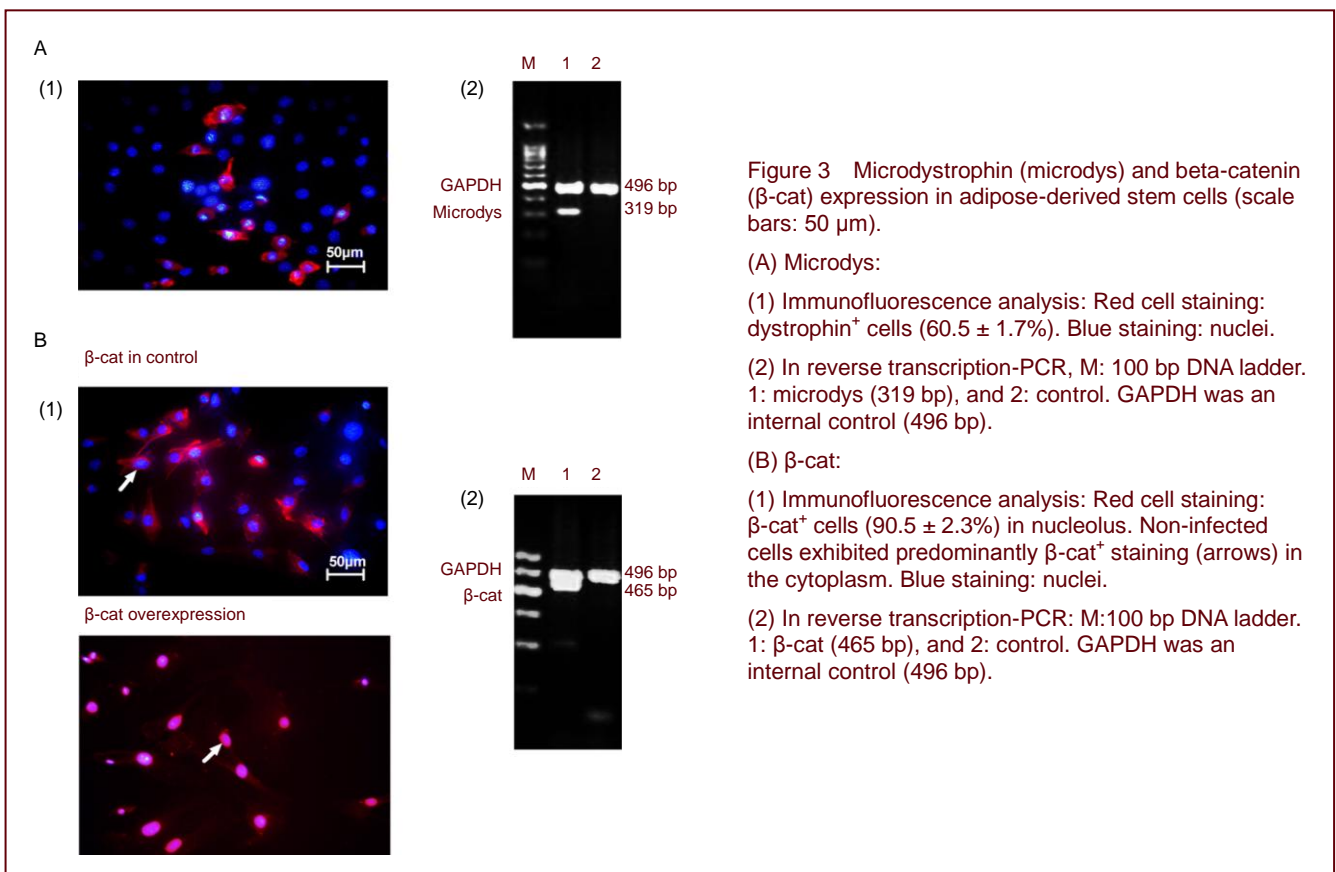MOI: Multiplicity of infection; NaBT: sodium butyrate.

Microdys and β-cat expression in transgenic adipose-derived stem cells

In dko-microdys-adipose-derived stem cells, reverse transcription-PCR identified a 309 bp gene and immunofluorescence microscopy identified dystrophin$^+$ cells (60.5 ± 1.7%). Conversely, non-infected cells were negative for both (Figure 3A).

In dko-microdys-β-cat-adipose-derived stem cells, reverse transcription-PCR identified a 391 bp gene and immunoflurorescence microscopy identified nuclear β-cat$^+$ in cells (90.5 ± 2.3%). Conversely, non-infected cells were predominant for cytoplasmic β-cat$^+$ (Figure 3B).

Figure 3 Microdystrophin (microdys) and beta-catenin (β-cat) expression in adipose-derived stem cells (scale bars: 50 μm).

(A) Microdys:

(1) Immunofluorescence analysis: Red cell staining: dystrophin$^+$ cells (60.5 ± 1.7%). Blue staining: nuclei.

(2) In reverse transcription-PCR, M: 100 bp DNA ladder. 1: microdys (319 bp), and 2: control. GAPDH was an internal control (496 bp).

(B) β-cat:

(1) Immunofluorescence analysis: Red cell staining: β-cat$^+$ cells (90.5 ± 2.3%) in nucleolus. Non-infected cells exhibited predominantly β-cat$^+$ staining (arrows) in the cytoplasm. Blue staining: nuclei.

(2) In reverse transcription-PCR: M:100 bp DNA ladder. 1: β-cat (465 bp), and 2: control. GAPDH was an internal control (496 bp).

Microdys and β-cat promoted myogenesis of adipose-derived stem cells and inhibited adipogenesis

For myogenesis, dko-adipose-derived stem cells, dko-microdys-adipose-derived stem cells and dko-microdys-β-cat-adipose-derived stem cells exhibited different outcomes. Dko-adipose-derived stem cells presented fibroblast-like growth in absence of myotubes and only showed transcription and expression of myogenic differentiation antigen (MyoD). In dko-microdys-adipose-derived stem cells and dko-microdys-β-cat-adipose-derived stem cells, myotubes appeared by day 28, and the majority of myotubes were observed in the latter. Diverse myogenic proteins were detected using two different methods. Immunofluorescence analysis was used to examine expression of MyoD, myogenin, desmin and myosin heavy chain (MHC). (1) For MyoD, the percentage of MyoD$^+$ cells in dko-microdys-β-cat- adipose-derived stem cells was significantly higher than that in dko-adipose-derived stem cells and dko-microdys-adipose-derived stem cells ($P < 0.05$). However, there was no significant difference between the latter two ($P > 0.05$). (2) For myogenin, the percentage of myogenin$^+$ cells in dko-microdys-adipose-derived stem cells and dko-microdys-β-cat- adipose-derived stem cells showed no significant difference ($P > 0.05$). (3) For desmin, dko-microdys-β-cat-adipose-derived stem cells had significantly more desmin$^+$ than dko-microdys-adipose-derived stem cells. (4) For MHC, both dko-microdys-adipose-derived stem cells and dko-microdys-β-cat-adipose-derived stem cells showed myotubes under the optical microscope. The number of MHC$^+$ cells in the latter was significantly greater than the former ($P < 0.05$) (Figure 4A). Reverse transcription-PCR showed the transcription of MyoD, myogenin, desmin, MHC, pax7, pax3, Mif4 and Mif5 in both groups of transduced dko-adipose-derived stem cells. However, only transcription of MyoD was found in dko-adipose-derived stem cells (Figure 4B). This is evidence that microdys and β-cat promote myogenesis of adipose-derived stem cells (supplementary Video 1 online).

For adipogenesis, microdys alone or in combination with β-cat were able to reduce the number of adipocytes and lipid droplets in dko-adipose-derived stem cells. In the two transduced dko-adipose-derived stem cells groups, there were less lipid droplets scattered in the cytoplasm. A greater number of Oil red O$^+$ cells were observed in dko-adipose-derived stem cells. The percentage of Oil red O$^+$ cells in dko-microdys-adipose-derived stem cells was in-between the other two groups. The number of Oil red O$^+$ cells in dko-microdys-β-cat-adipose-derived stem cells was significantlly less than in the dko-adipose-derived stem cells and dko-microdys-adipose-derived stem cells ($P < 0.05$; Figure 4C). Our findings indicate that microdys and β-cat inhibited adipogenesis of adipose-derived stem cells.
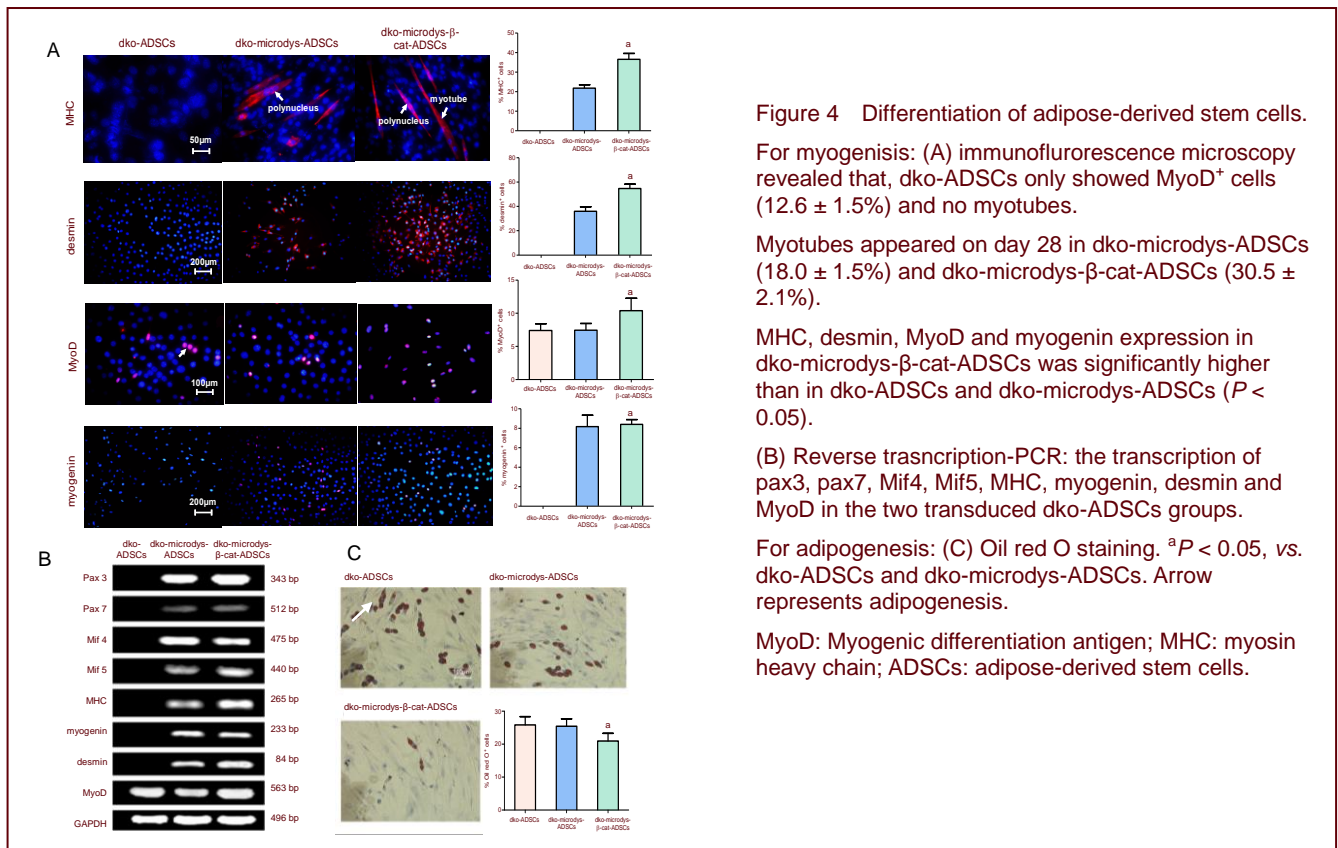

Figure 4   Differentiation of adipose-derived stem cells.

For myogenisis: (A) immunofluororescence microscopy revealed that, dko-ADSCs only showed MyoD$^+$ cells (12.6 ± 1.5%) and no myotubes.

Myotubes appeared on day 28 in dko-microdys-ADSCs (18.0 ± 1.5%) and dko-microdys-β-cat-ADSCs (30.5 ± 2.1%).

MHC, desmin, MyoD and myogenin expression in dko-microdys-β-cat-ADSCs was significantly higher than in dko-ADSCs and dko-microdys-ADSCs ($P < 0.05$).

(B) Reverse trasncription-PCR: the transcription of pax3, pax7, Mif4, Mif5, MHC, myogenin, desmin and MyoD in the two transduced dko-ADSCs groups.

For adipogenesis: (C) Oil red O staining. $^a P < 0.05$, *vs.* dko-ADSCs and dko-microdys-ADSCs. Arrow represents adipogenesis.

MyoD: Myogenic differentiation antigen; MHC: myosin heavy chain; ADSCs: adipose-derived stem cells.

DISCUSSION

Baculovirus is an alternative gene vector with the advantage of reduced toxicity to many mammalian cells[13-16]. In this study, when MOI was greater than 20, the efficiency of transduction showed little increase and when MOI was greater than 100, significant damage was observed. We found that 5 mM NaBT can efficiently break the gene silence and promote protein expression with less damage.

After transplantation, mesenchymal stem cells (MSCs) migrate to muscle lesions to participate in muscle regeneration. Li et al [17] found that bone marrow MSCs from mdx mice could not be induced into muscle cells, likely due to the lack of dystrophin and CD34-. Therefore, we speculated that dko-adipose-derived stem cells cannot differentiate into muscle cells.

Dystrophin plays an important role in myogenesis. At later time points, dystrophin may induce adipose-derived stem cell differentiation into muscle cells by resisting myotasis to protect myolemma and myocytes. In this study, only MyoD could be detected in dko-adipose-derived stem cells. However, in the other two groups, adipose-derived stem cells could differentiate into muscle cells with MyoD, myogenin, desmin and MHC. These results confirm our earlier hypothesis that dystrophin and β-cat can faciliate myogenesis of adipose-derived stem cells.

Activation of the Wnt signaling pathway was confirmed with accumulation of β-cat and its transfer from the cytoplasm to the nucleus. In the cytoplasm, β-cat is free for Wnt signal transduction or for cell adhesion. MyoD and Myf5 are necessary for muscle precursor cells to differentiate into muscle cells. Once precursors have differentiated into muscle cells, they generate integrated myotubes and muscle fibers. Myogenin plays an important role in the direction of myogenesis in late stage differentiation. The absence of Mif4 leads to defective cell integration and maintenance of muscle fibers on reaching terminal differentiation. Wnt/β-cat pathway activation has been observed during the process of damage and repair of muscle after birth[18]. Tajbakhsh et al [19] found that several proteins in the Wnt/β-cat pathway could activate grafted cells to express Mif5 and MyoD. Etheridge et al[20] found signal proteins of the Wnt/β-cat pathway in MSCs, which suggests MSCs may be its target cells.

In this study, the β-cat gene was delivered into dko-adipose-derived stem cells to activate the Wnt/β-cat pathway. We compared the myogenic effects of dko-microdys-adipose-derived stem cells with dko-microdys-β-cat-adipose-derived stem cells. The results showed overexpressed β-cat transferred from the cytoplasm to the nucleus. Besides MyoD, myogenin, desmin and MHC, the transcription of pax7, pax3, Mif4

and Mif5 were detected in transduced dko-adipose-derived stem cells using reverse transcription-PCR *in vitro*. The microdys-β-cat gene promoted adipose-derived stem cells to differentiate into myocytes to a greater extent than microdys alone. The results showed that the Wnt/β-cat pathway can activate myogenic regulatory factor genes to initiate myogenesis of dko-adipose-derived stem cells. This regulatory path was similar to skeletal muscle formation in embryonic development, confirming our initial hypothesis.

MSCs can differentiate into adipocyte. Gesta et al [21] reported that overexpression of a secreted Wnt/β-cat pathway protein, could significantly improve the volume of visceral adipose tissue. Singh et al[22] found that β-cat could combine with androgen receptor and receive stimulation from androgen to transfer into the nucleus in 3T3-L1 cells. Once in the nucleus, β-cat inhibited stem cells from differentiating into adipocytes. These studies indicate that the Wnt/β-cat pathway and its downstream molecules may participate in the regulation of adipogenesis by interacting with other factors.

In this study, we compared adipogenesis between dko-β-cat-adipose-derived stem cells and dko-adipose-derived stem cells and controls. Results showed that in the former, there were significantly fewer differentiated adipocytes, with delayed formation of lipid droplets. Our results confirm that activation of the Wnt/β-cat pathway could inhibit differentiation of dko-adipose-derived stem cells into fat cells[23].

We draw conclusions that baculovirus can effectively deliver genes to dko-adipose-derived stem cells, and that microdys and the Wnt/β-cat pathway play an important role in myogenesis of dko-adipose-derived stem cells.

MATERIALS AND METHODS

Design
A comparative observation on the cytology.

Time and setting
The study was performed in May 2011 at the laboratory of the Department of Neurology, the First Affiliated Hospital of Sun Yat-Sen University in China.

Materials
Animals
Thirty-two 8-week-old Dko mice, female and male, of specific pathogen free grade were used. Mice were offspring to Utrophin^{+/-} mdx mated mice which were seperated by gene identification. Utrophin [+/-] mdx mice were obtained from the Department of Physiology, Anatomy and Genetics, Oxford University, UK. The mice were housed in identical cages with access to water and a standard rodent diet named Shuliang. All experimental

disposal of animals was in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[24].

Plasmid and virus

Plasmid Pci-Neo-β-catenin (kindly provided by Huazhong University of Science and Technology, China) was digested with *Xho*I and *Xba*I. PBSK-microdys plasmid, containing the full-length dys gene N-terminal region, three rod repeats (R1, R2, R24), three hinge regions (H1, H2, H4), with a short gene length of 3.75 kb, was constructed by the Department of Neurology of Washington University School of Medicine, USA Baculovirus was purchased from Invitrogen (Carlsbad, NY, USA).

Methods

Isolation and culture of adipose-derived stem cells

After anesthesia and sterilization, subcutaneous adipose tissue was isolated from the pars inguinalis of the Dko mouse[4]. Briefly, tissue was minced into 1 mm^3 pieces, digested in 1% collagenase type I (Millipore, Bedford, MA, USA) at 37°C for 30 minutes, centrifuged and resuspended twice. After the cells were counted, 1×10^6 nucleated cells were seeded into 25 cm^2 culture flasks in Dulbecco's minimum essential medium/F12 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco; complete culture medium). On reaching 80% confluence, adipose-derived stem cells were trypsinized, resuspended in complete medium and split at a ratio of 1:3. Cells at passage 3−5 were seeded into 6- or 12-well culture dishes and allowed to recover for 1 day.

Recombinant baculovirus preparation and transduction

pcDNA3.1⁺ microdys (containing the CMV-IE1 promoter and enhancer) plasmids were cloned with pBSK-microdys and stored by our laboratory. The microdys gene (ΔR 4−R 23/ΔCT) is a truncated version of the full-length dystrophin cDNA which was generated by introducing deletions encoding repeats 4 though to 23 within the rod domain and the c-terminal domain[25]. The 3.75 kb microdys gene was released from the plasmid pcDNA 3.1⁺-microdys by NotI. About 2.4 kb of the β-cat gene was released from the plasmid Pci-Neo-β-cat by *Xho*I and *Xba*I. The two genes were amplified and identified by PCR and sequencing.

Recombinant baculovirus integrating EGFP under the CMV-IE promoter, was constructed with the Bac-to-Bac baculovirus expression system (Invitrogen) and pIRES 2 co-expression system (Clontech, Mountain View, CA, USA; baculovirus-microdys, baculovirus-β-cat; unpublished). The viruses were propagated by infecting Sf-9 cells and were harvested on day 4 post-infection.

Viral titers were determined by the end-point dilution method using Sf-9 cells as the host[26].

Before transduction, the virus was concentrated by sucrose-cushioned ultracentrifugation (80 000 × *g*, 90 minutes) and was resuspended in PBS. For transduction, passage 3 dko-adipose-derived stem cells were seeded at 4×10^3/cm^2 in flasks to achieve 80% confluence. The virus dose was adjusted so that the MOI was 10, 20, 50 or 100. PBS was used to adjust the final volume to 500 µL. Flasks were shaken for 4 hours at room temperature[27-28]. The virus solutions with cells transduced were added to 5 mM sodium butyrate (NaBT) and were replaced by complete medium by 24 hours. In the course of experiments, the transduced adipose-derived stem cells were observed and photographed using a fluorescence microscope (Nikon, Japan) equipped with a digital camera (CoolSNAP, Media Cybernetics, USA).

Percentage of cells emitting fluorescence detected by flow cytometric analysis

Forty-eight hours after transduction, the transduced adipose-derived stem cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA). The efficiency of transduction was detected by the percentage of cells emitting fluorescence (% GFP⁺ cells) using non-transduced cells as the background and analyzed using CellQuest software (Becton Dickinson, San Jose).

Differentiation of adipose-derived stem cells

For adipogenesis, 5×10^3 adipose-derived stem cells per well were incubated in complete culture medium supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 µg/mL insulin. Medium was changed twice per week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes and stained with 0.5% Oil red O in methanol for 20 minutes at room temperature[28].

For myogenesis, 5×10^3 adipose-derived stem cells per well were incubated in 6-well plates in complete culture medium, and were treated for 24 hours with 10 µM 5-azacytidine. The following day, medium was replaced with 5% horse serum in DMEM. Medium was changed twice per week for 4 weeks[17].

Immunofluorescence analysis of MyoD, myogenin, MHC, desmin, microdys and β-cat

MyoD, myogenin, desmin, MHC, microdys and β-cat were analyzed by immunofluorescence analysis on day 28. The following primary antibodies were used: MyoD, myogenin, MHC, desmin (MyoD, myogenin, anti-mouse monoclonal antibody; MHC, desmin, rabbit multiple antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-cat (goat multiple antibody, R&D Systems

Inc. Basel, Switzerland; 1:200 in PBS), and dystrophin (Abcam; 1:400 in PBS). After three washes with PBS, cells were incubated for further 1 hour at room temperature with fluorescein-conjugated antibody (CY3, Sigma, St. Louis, MO, USA; 1:200 in PBS). Nuclear localization of immunostain was confirmed by counterstaining with DAPI (Sigma; 1:5 000 in PBS). For negative controls, we omitted the primary antibody. Cells were examined by fluorescence microscopy (Olympus DP 70 scope, Japan). For each slide, the percentage of total positive cells was calculated based on the average number of positive cells counted in five different fields of view. To avoid bias in cell counting due to the presence of multinucleated cells, the percentage of positive cells was estimated by assessing the number of nuclei.

Reverse transcription-PCR analysis

The transcription of MyoD, myogenin, desmin, MHC, pax3, pax7, Mif4, Mif5, microdys and β-cat were analyzed by reverse transcription-PCR. Total cellular RNA was extracted with Trizol (Gibco-BRL, Life Technologies, MD, USA). Reverse transcription was performed using 2 µg total RNA for 60 minutes at 42°C with the RT System (MBI Formentas Inc, Burlingtonl, ON, USA). Specific cDNA was detected by PCR. Each reaction contained equal amounts of cDNA, 1 × PCR buffer with $MgCl_2$, 1.0 µM primer, 0.2 µM of each dNTP and 1 U Taq DNA polymerase (MBI Formentas Inc). The sequences of PCR primers are presented as follows:

Primer	Sequence	Product size (bp)
MyoD	F: 5'-CTA CAG CGG CGA CTC AGA CG-3' R: 5'-TTG GGG CCG GAT GTA GGA-3'	563
Myogenin	F: 5'-ACT ACC CAC CGT CCA TTC AC-3' R: 5'-TCG GGG CAC TCA CTG TCT CT-3'	233
Desmin	F: 5'-CGA CGC TGT GAA CCA GGA GT-3' R: 5'-TAG TTG GCG AAG CGG TCA T-3'	84
MHC	F: 5'-CTC AGG CTT CAA GAT TTG GTG G-3' R: 5'-TTG TGC CTC TCT TCG GTC ATT C-3'	265
Pax3	F: 5'-GGC TTT CAA CCA TCT CAT TC-3' R: 5'-GTT GGT CAG AAG TCC CAT TAC-3'	343
Pax7	F: 5'-TTC GGG AAG AAA GAG GAC G-3' R: 5'-ATG GTT GAT GGC GGA AGG-3'	512
Myf4	F: 5'-TTA GAA GTG GCA GAG GGC TC-3' R: 5'-AGG TGC GCA GGA AAT CCG CA-3'	475
Myf5	F: 5'-GAG CCA AGA GTA GCA GCC TTC G-3' R: 5'-GTT CTT TCG GGA CCA GAC AGG G-3'	440
β-cat	F: 5'-GCT CTT CGT CAT CTG ACC AGC C-3' R: 5'-GAG CAA GTT CAC AGA GGA CCC-3'	465
Microdys	F: 5'-GCT GGG TCC GAC AAT C-3' R: 5'-ATG GCT TCA ATG CTC ACT-3'	319
GAPDH	F: 5'-ACC ACA GTC CAT GCC ATC AC-3' R: 5'-TCC ACC ACC CTG TTG CTG TA-3'	496

F: Forward; R: reverse.

GAPDH was used as an internal control. MyoD, myogenin, and MHC PCR were performed at 94°C for 45 seconds, 62°C for 20 seconds and 72°C for 30 seconds for 45 cycles, followed by a final amplification

step of 72°C for 10 minutes. Desmin, microdys, β-cat, pax3, pax7, Myf4 and Myf5 PCR were performed at 94°C for 45 seconds, 58°C for 45 seconds and 72°C for 1 minute for 35 cycles, followed by a final amplification step of 72°C for 10 minutes. Amplification conditions of the other 5 primers are as follows: 25 cycles of 94°C for 30 seconds; 55°C for 60 seconds; and 72°C for 1 minute, followed by a 72°C incubation for 10 minutes. The PCR products were detected after electrophoresis on a 1.5% agarose gel in Tris borate EDTA buffer, stained with ethidium bromide and photographed using the Gel Doc2000 Gel imaging analysis system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were presented as mean ± SD. Inter-group differences were analyzed using analysis of variance and $P < 0.05$ was considered statistically significant. The experiments were repeated three times, and data from representative experiments were used for final analysis.

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