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RESEARCH ARTICLE

Adipose-derived stem cells enhance myogenic differentiation in the mdx mouse model of muscular dystrophy *via* paracrine signaling

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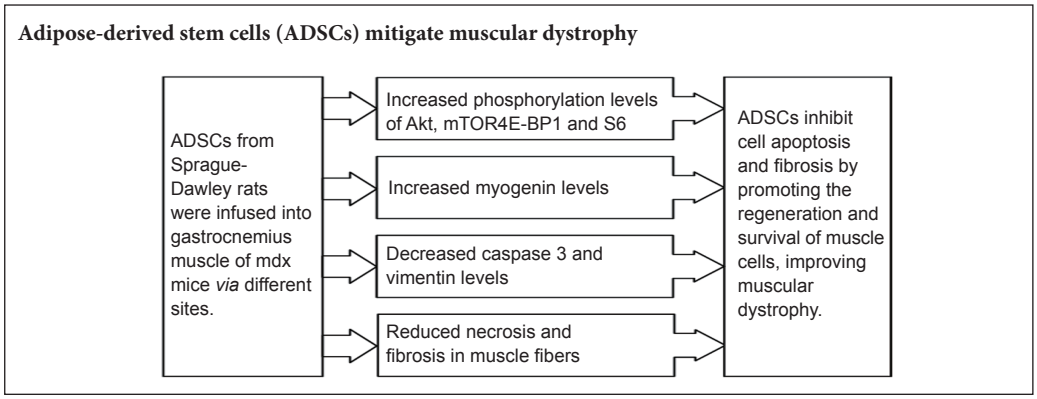
Graphical Abstract



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Abstract

Adipose-derived stem cells have been shown to promote peripheral nerve regeneration through the paracrine secretion of neurotrophic factors. However, it is unclear whether these cells can promote myogenic differentiation in muscular dystrophy. Adipose-derived stem cells (6×10^6) were injected into the gastrocnemius muscle of mdx mice at various sites. Dystrophin expression was found in the muscle fibers. Phosphorylation levels of Akt, mammalian target of rapamycin (mTOR), eIF-4E binding protein 1 and S6 kinase 1 were increased, and the Akt/mTOR pathway was activated. Simultaneously, myogenin levels were increased, whereas cleaved caspase 3 and vimentin levels were decreased. Necrosis and fibrosis were reduced in the muscle fibers. These findings suggest that adipose-derived stem cells promote the regeneration and survival of muscle cells by inhibiting apoptosis and fibrosis, thereby alleviating muscle damage in muscular dystrophy.

Key Words: *nerve regeneration; Duchenne muscular dystrophy; adipose-derived stem cells; myogenic differentiation; paracrine pathway; dystrophin; neural regeneration*

Introduction

Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy caused by a mutation in the dystrophin gene, with a relatively high incidence in male infants (Nallamilli et al., 2001). Currently, there is no known cure for this disease. Research on the pathogenetic mechanisms is crucial for developing effective therapeutic strategies for the disease. The mdx mouse has a muscle pathology similar to DMD patients, and is caused by a nonsense mutation in exon 23 of the mouse dystrophin gene, resulting in the absence of dystrophin expression in muscle cells (Graham et al., 2004). Because of similar genetics and muscular pathology, the mdx mouse has become a widely-used animal model of DMD.

Previous studies have suggested that adipose-derived stem cells (ADSCs) from subcutaneous adipose tissue can differentiate into myocytes *in vitro* and alleviate the dystrophic phenotype of mdx mice *in vivo* (Vieira et al., 2008; Goudenege et al., 2009). However, the muscular dystrophy and motor dysfunction are substantially improved despite a low rate of myogenesis, as shown by a previous study (Goudenege et al., 2009). This suggests that a process other than myogenic differentiation is involved in ameliorating the muscular dystrophy in mdx mice.

Recent studies demonstrate that ADSCs promote peripheral nerve regeneration at least in part through the paracrine secretion of trophic factors (Sowa et al., 2011). The paracrine factors produced by ADSCs protect murine myoblasts undergoing myostatin treatment (Gehmert et al., 2014). The cardioprotective effects of ADSCs in ischemic heart disease, including anti-apoptotic and angiogenic actions, are mediated by insulin-like growth factor-1 and vascular endothelial growth factor secreted by these cells (Sadat et al., 2007). Insulin-like growth factor-1 inhibits transforming growth factor-beta transcriptional responses that lead to muscle fibrosis *via* a PI3K/Akt/mTOR-dependent pathway (Song et al., 2003). The Akt/mTOR pathway is a downstream target of insulin-like growth factor and plays an important role in myogenesis and muscle regeneration (Bodine et al., 2001; Risson et al., 2009; Eghtesad et al., 2011). The Akt/mTOR pathway controls the phosphorylation of regulators of protein synthesis and cell growth, including Akt, mammalian target of rapamycin (mTOR), S6 kinase 1 (S6) and eIF-4E binding protein 1 (4E-BP1) (Song et al., 2006). Therefore, in this study, we investigated whether secretion of paracrine factors by ADSCs is involved in their ability to alleviate the muscular dystrophy in mdx mice.

Materials and Methods

ADSC culture, identification and infection of lentivirus containing green fluorescent protein (GFP)

All animal procedures were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Sun Yat-sen University of China, and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

The primary ADSCs from 4-week-old female Sprague-Dawley rats were harvested according to a previously published method (Zhang et al., 2015). When the cells were nearly confluent, the adherent cells were trypsinized (0.25% trypsin-ethylenediamine tetraacetic acid (EDTA), Invitrogen, Carlsbad, CA, USA), resuspended in complete medium, split at a 1:3 ratio, and seeded into fresh plates. The medium was replaced every 3–4 days. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. The third passage ADSCs were infected with lentivirus containing GFP (Shanghai GeneChem Co., Ltd., Shanghai, China) and confirmed for their capacity to differentiate into the adipogenic, neurogenic, osteogenic and myogenic lineages, as described in previously published reports (Zuk et al., 2002; Xiong et al., 2010). Flow cytometry was used to determine the purity of ADSCs. Cells at 80–90% subconfluence were incubated in phosphate-buffered saline containing CD29, CD34, CD44, CD45 and CD105 (Cell Signaling Technology, Boston, MA, USA) for 30 minutes at 37°C. A Becton Dickinson FACS Scan (Tokyo, Japan) was used for fluorescence-activated cell sorting analysis.

Cells were transfected with lentivirus at a multiplicity of infection (MOI) of 10, 20 or 40 for 72 hours. Then, 1–5 days after reaching 80% confluence, cells were plated onto 96-well plates and incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) for an additional 4 hours and then lysed in dimethyl sulfoxide (Sigma-Aldrich). Optical density was measured at 490 nm with a spectrometer. In the following experiments, all cells were transfected at an optimal MOI.

ADSC transplantation

The mdx mice were originally purchased from the Model Animal Research Center of Nanjing University of China (license No. SYXK(Su)2016-0012). They were subsequently established by in-house breeding at the Laboratory Animal Center of Sun Yat-sen University of China, and mice were housed in a specific-pathogen-free animal facility. All the following animal experiments accorded with the Sun Yat-sen University Guidelines for animal care. Five mdx mice aged 14–16 months were subjected to radiotherapy and ADSC injection in the right gastrocnemius muscle. The left gastrocnemius muscle served as a control. Five age-matched C57BL/c mice (Model Animal Research Center of Nanjing University, Nanjing, Jiangsu Province, China), which were subjected to radiotherapy only, were used as a control group.

All mice were irradiated with 4.5 Gy from a ^{60}Co source. Three days later, 6×10^6 ADSCs were infused into the right gastrocnemius muscle at eight different sites per mouse. Simultaneously, the same amount of saline was injected into the left gastrocnemius muscle of each mouse.

Histology and immunofluorescence analysis

Mice were intraperitoneally anesthetized with 5% chloral hydrate (200 μL/20 g) and euthanized by cervical dislocation 12 weeks after ADSC transplantation. The gastrocnemius muscle was frozen rapidly in isopentane cooled with liquid nitrogen. Specimens were kept at –80°C until sectioning. Serial transverse cryosections of 6 μm thickness were made for hematoxylin-eosin staining, Masson trichrome staining and immunofluorescence analysis. Slides were blocked with 10% normal goat/rabbit serum after air-drying at room temperature with cold acetone for 10 minutes, and were then incubated overnight at 4°C with rabbit anti-dystrophin polyclonal antibody (1:100; Abcam, Cambridge, UK) and vimentin antibody (1:200; Sigma-Aldrich). After three washes with phosphate-buffered saline, slides were incubated with goat anti-rabbit Cy3-conjugated secondary antibodies (1:400; Chemicon, Temecula, CA, USA) for 1 hour at room temperature.

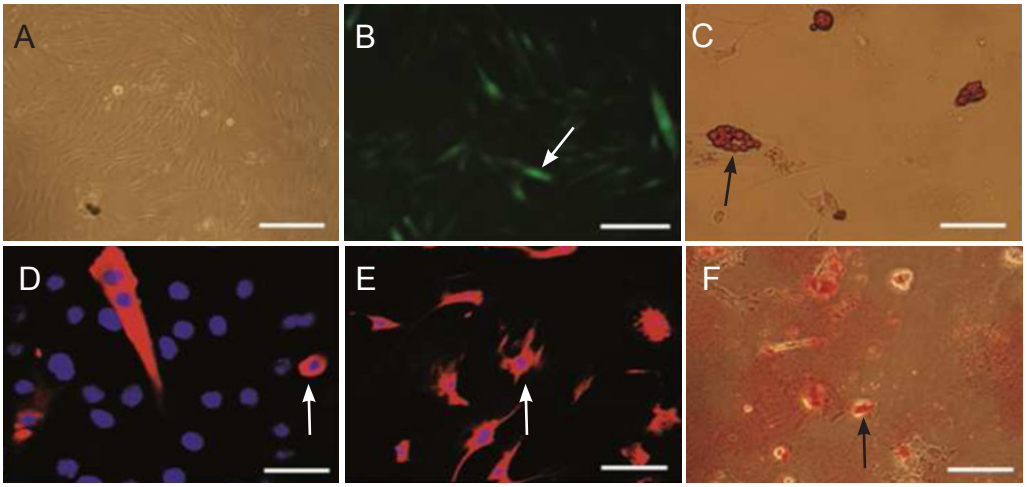

Figure 1 Characterization and identification of rat ADSCs.
(A) Passage five ADSCs displayed a fibroblast-like morphology and were arranged in a spiral or radial pattern. (B) Nearly all ADSCs were green, as they were successfully infected by the lentivirus containing enhanced green fluorescent protein (arrow). (C) The cells were filled with a large number of lipid droplets (arrow), as shown by oil red O staining, when cultured in adipose-specific induction medium, at 16 days. (D) The cells exhibited double nuclei and were positive for major histocompatibility complex (arrow) under myogenic inductive medium at 20 days. (E) The cells exhibited a multipolar neuron-like morphology and were positive for β-III-tubulin (arrow) under neural inductive medium at 14 days. (F) The cells displayed some dark red calcium nodules (arrow) surrounded by alizarin red S staining under osteogenic-specific inductive medium at 14 days. Scale bars: 50 μm in A–C, E, F; 100 μm in D. ADSCs: Adipose-derived stem cells.

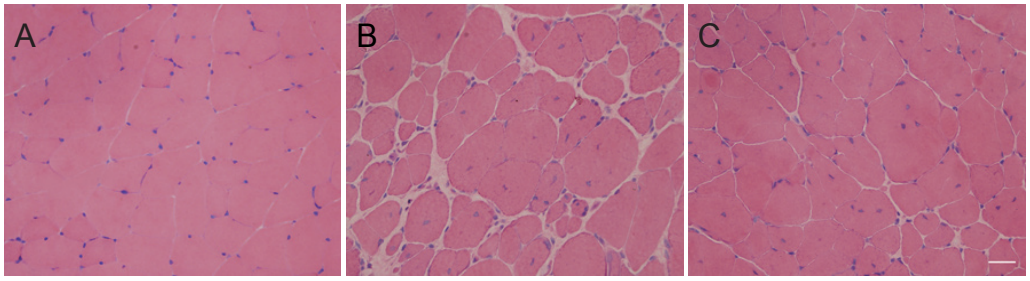

Figure 3 Pathological changes in the gastrocnemius muscle of mdx mice 12 weeks after ADSC transplantation (hematoxylin-eosin staining).
(A) Muscle fibers from C57BL/c mice were polygonal, uniform in size, tightly-arranged, and without central nuclei. (B) Muscle fibers in mdx mice showed a degenerative process involving sarcolemmal damage with muscle cell necrosis, central nucleation, large variation in size and connective tissue infiltration. (C) Myocytes displayed a reduced rate of apoptosis, greater consistency in cell size, and less connective tissue after ADSC transplantation. Scale bar: 100 μm. ADSCs: Adipose-derived stem cells.

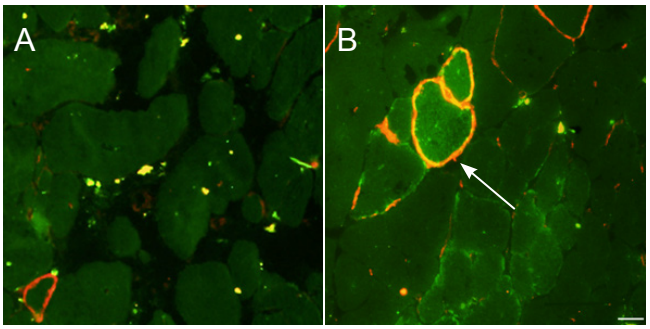

Figure 2 GFP expression in the gastrocnemius muscle of mdx mice 12 weeks after ADSC transplantation.
(A) In the saline-injected left gastrocnemius muscle (control) of *mdx* mice, no myocytes expressed GFP. (B) In the ADSC-injected right gastrocnemius muscle of mdx mice, a few GFP-positive myocytes were found with dystrophin expression (arrow). Scale bar: 51 μm. ADSC: Adipose-derived stem cell; GFP: green fluorescent protein.

Western blot assay

Tissue samples from the gastrocnemius muscle were homogenized in a lysis buffer containing 10% sodium dodecyl sulfate, 70 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol and 10 mM EDTA supplemented with a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). Nuclei and cellular debris were discarded by centrifuging at 10,000 r/min for 10 minutes at 4°C. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, homogenates were separated by 6% and 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% bovine serum albumin, the membranes were probed with the following rabbit antibodies: GAPDH, cleaved-caspase 3, 4E-BP1, phospho-4E-BP1 (Thr37), Akt, phospho-Akt (Ser473), S6 and phospho-S6 (Thr389), mTOR,

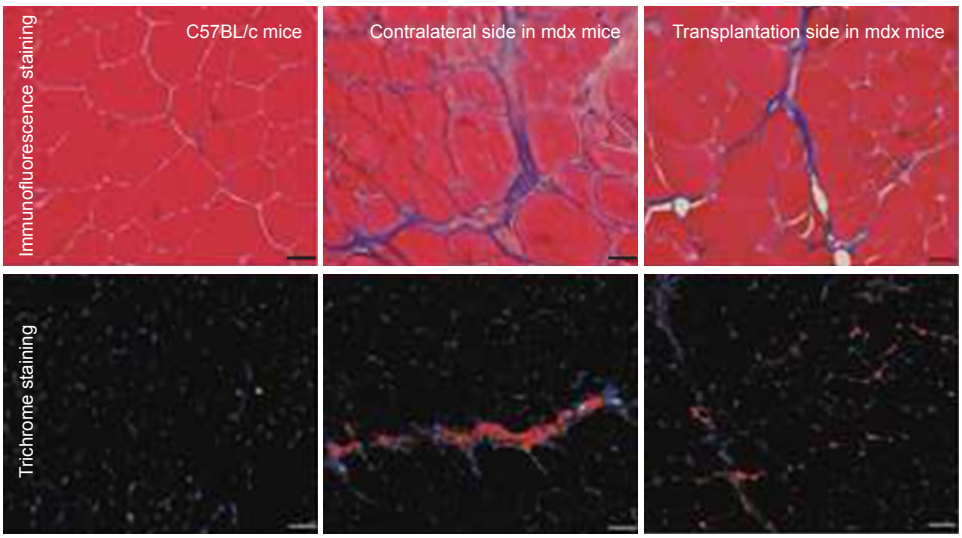

Figure 4 Accumulation of collagen and fibrosis in the gastrocnemius muscle of mdx mice 12 weeks after ADSC transplantation.
Muscle fibers in C57BL/c mice showed little interstitial fibrosis along the muscle fibers. Muscle fibers in mdx mice showed large foci of interstitial fibrosis along the muscle fibers. Muscle fibers in mdx mice given ADSC transplantation displayed a tendency for reduced fibrous tissue content along the muscle fibers. Scale bars: 48 μm. ADSC: Adipose-derived stem cell.

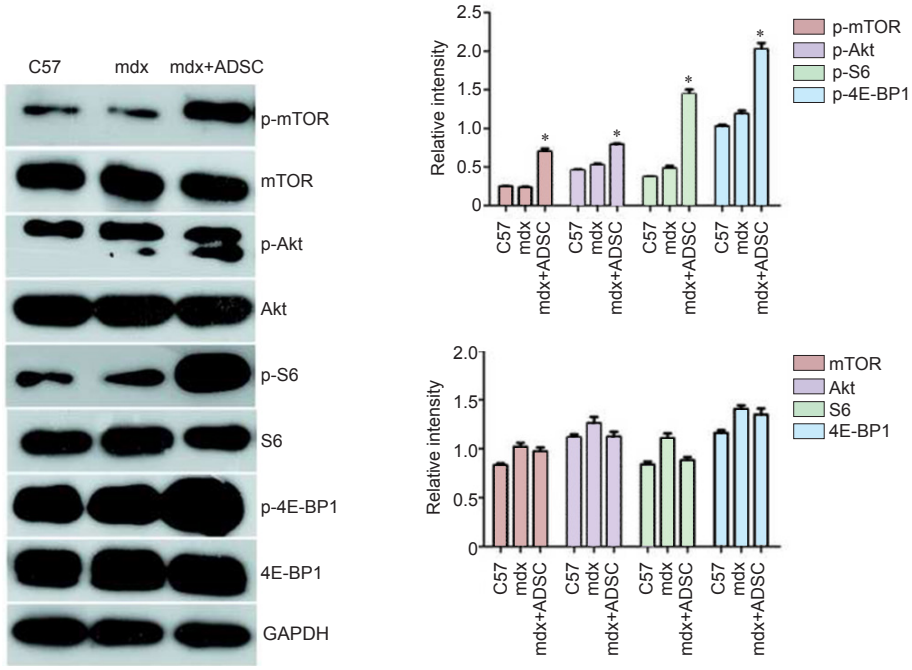

Figure 5 Western blot assay for mTOR pathway proteins in the gastrocnemius muscles 12 weeks after ADSC transplantation.
Target protein levels are presented as the ratio of the optical density to that of GAPDH. Data are expressed as the mean ± SD (n = 5), and were analyzed by one-way analysis of variance. *P < 0.05, *vs.* mdx. C57: C57BL/c mice; mdx: contralateral side in mdx mice; mdx + ADSC: transplantation side in mdx mice. ADSCs: Adipose-derived stem cells; mTOR: mammalian target of rapamycin; S6: S6 kinase 1; 4E-BP1: eIF-4E binding protein 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

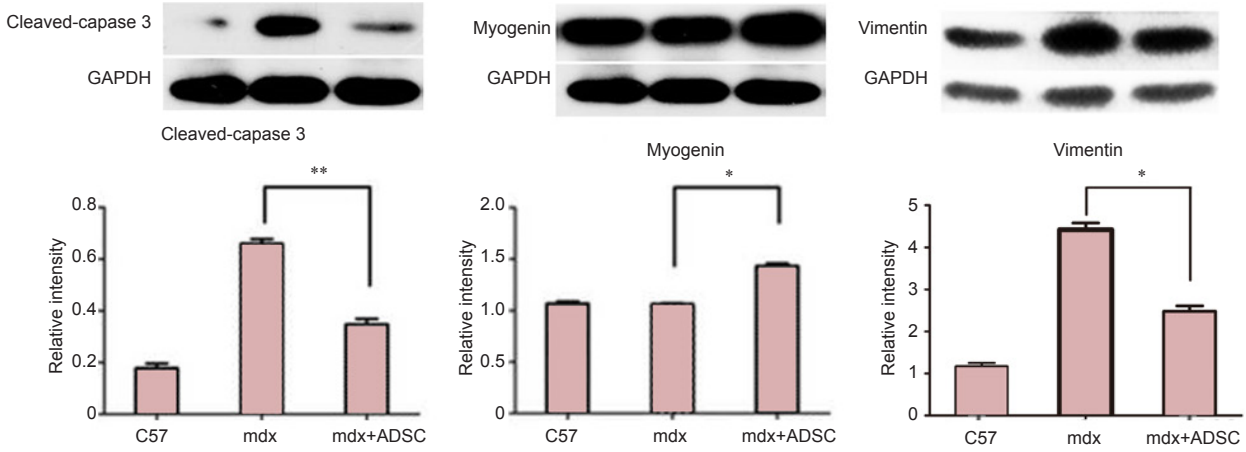

Figure 6 Cleaved caspase 3, myogenin and vimentin in the gastrocnemius muscles 12 weeks after ADSC transplantation (western blot assay).
Target protein levels are presented as the ratio of the optical density to that of GAPDH. Data are expressed as the mean ± SD (n = 5), and were analyzed by one-way analysis of variance. *P < 0.05, **P < 0.01. C57: C57BL/c mice; mdx: contralateral side in mdx mice; mdx + ADSC: transplantation side in mdx mice. ADSCs: Adipose-derived stem cells; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

and phospho-mTOR (all obtained from Cell Signaling Technology). Mouse antibodies to myogenin and vimentin were from Abcam. After immunostaining with the appropriate secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Cell Signaling Technology), bands were detected using the enhanced chemiluminescence method and quantified by optical density using the public domain software Image J for Microscopy.

Statistical analysis

One-way analysis of variance was used for statistical analysis using SPSS 18.0 software (SPSS, Chicago, IL, USA). All data are expressed as the mean ± SD, and values at $P < 0.05$ were considered statistically significant.

Results

Characterization and identification of rat ADSCs

The fifth passage ADSCs grew with a fibroblast-like morphology and were arranged in a spiral or radial pattern (**Figure 1A**). The cells were characterized by high expression of CD29, CD44 and CD105, and by low expression of CD34 and CD45. The optimal MOI was determined to be 10 as assessed by the best cell viability (Geng et al., 2012). Three days after ADSCs were infected with the lentivirus containing GFP, green fluorescence was observed. The vast majority of ADSCs were infected with lentivirus (**Figure 1B**). In adipose-specific inductive medium, the adipocyte-like cells became filled with lipid droplets at 16 days (**Figure 1C**). In myogenic inductive medium, the myogenic cells exhibited two or more nuclei and expressed skeletal muscle-specific major histocompability complex (**Figure 1D**). In neural inductive medium, the cells exhibited a multipolar neuron-like morphology and were positive for β-III-tubulin at 14 days (**Figure 1E**). In osteogenic inductive medium, the cells displayed some dark red calcium nodules surrounded by alizarin red S staining at 14 days (**Figure 1F**).

Changes in the histopathology of the gastrocnemius muscle in mdx mice after ADSC transplantation

Twelve weeks after ADSC transplantation, a few GFP-positive cells expressing dystrophin were found in the right gastrocnemius muscle of mdx mice (**Figure 2A**). No GFP expression was found in the left control muscles of mdx mice (**Figure 2B**). Hematoxylin-eosin staining showed a degenerative process involving sarcolemmal damage with necrotic muscle cells, central nucleation, and a reduction in size of the bilateral gastrocnemius muscles in mature mdx mice. Intramuscular transplantation of ADSCs into the right gastrocnemius muscle resulted in a mild improvement in muscle morphology, compared with the control (left) gastrocnemius muscle, during the twelfth week. Muscle cells showed less necrosis and a greater uniformity in cell size after transplantation (**Figure 3**). In addition, a decrease in the accumulation of collagen and a reduction in fibrosis was revealed by Masson's trichrome staining, and there was a decrease in immunofluorescence staining for the fibrosis marker vimentin in the right gastrocnemius muscle after intramuscular transplantation (**Figure 4**).

Expression of mTOR, cleaved caspase 3, myogenin and vimentin in the gastrocnemius muscle of mdx mice 12 weeks after transplantation

Next, we investigated whether ADSCs alleviate the muscular dystrophy phenotype by promoting muscle cell regeneration and survival and by inhibiting apoptosis and fibrosis. To this end, we examined the cell survival marker mTOR, the apoptotic marker cleaved caspase 3, the muscle satellite cell proliferation and differentiation marker myogenin, and the fibrosis marker vimentin by western blot assay. As shown in **Figure 5**, the levels of phospho-4E-BP1 (Thr37), phospho-Akt (Ser473), phospho-S6 (Thr389) and phospho-mTOR were higher in the right gastrocnemius muscle injected with ADSCs compared with the left control muscle ($P < 0.05$). In comparison, there were no significant differences in the levels of 4E-BP1, Akt, S6 or mTOR between these muscles ($P > 0.05$). As shown in **Figure 6**, myogenin levels were substantially increased, and the levels of cleaved caspase 3 and vimentin were significantly decreased in the ADSC-transplanted gastrocnemius compared with the control non-transplanted gastrocnemius ($P < 0.05$ or $P < 0.01$).

Discussion

In this study, we explored the effects of ADSCs on muscle regeneration and survival, as well as apoptosis and fibrosis in dystrophic mdx mice. Consistent with previous studies (Liu et al., 2007; Vieira et al., 2008; Nitahara-Kasahara et al., 2012), ADSCs were capable of self-renewal and differentiation into multiple lineages *in vitro*. When injected into mdx mice, ADSCs attenuated muscle degeneration.

A few myocytes were from ADSCs after transplantation by GFP observation. As in previous studies, ADSC differentiation into myogenic cells was very limited, especially into dystrophin-positive myocytes (Goudenege et al., 2009; Lee et al., 2015). Our previous study showed that dystrophin-positive fibers accounted for approximately 3% of all muscle fibers after ADSC transplantation (Geng et al., 2012). Muscle fibrosis and apoptosis were significantly decreased as shown by vimentin and cleaved caspase 3 expression in this study, suggesting that a mechanism other than myogenic differentiation is involved in ameliorating muscle dystrophy in mdx mice.

Gehmert et al. (2014) showed that ADSCs secrete multiple paracrine factors, including insulin-like growth factor. Insulin-like growth factor-1 was reported to promote myoblast proliferation and inhibit fibrosis (Xiong et al., 2013; Gehmert et al., 2014). Insulin-like growth factor-II was reported to reduce the elevated programmed cell death in skeletal muscles of mdx mice (Smith et al., 2000). Moreover, Smith et al. (2000) reported elevated levels of programmed cell death in the skeletal muscles of mdx mice, especially muscle satellite and stem cells. In this study, we observed that the levels of phosphoproteins in the mTOR and myogenin pathways were significantly elevated, and that vimentin and cleaved caspase 3 levels were significantly decreased in muscles injected with

ADSCs. These results suggest that ADSCs exert myoprotective effects that alleviate the dystrophic pathology in mdx mice by secreting paracrine factors.

Our findings provide insight into the mechanisms that underlie the protective effects of stem cell transplantation in mdx mice. The upregulation of multiple paracrine factors secreted by stem cells might have therapeutic potential for the treatment of muscular dystrophy in humans as well. However, there are a number of limitations of the present study, including small sample size and selection bias. Further studies are required to clarify the myoprotective actions of ADSCs in muscular dystrophy.

Author contributions: *JQC collected the data, designed and performed the experiment and wrote the paper. YYL, YQL, HLZ and YLZ collected and analyzed the data. JG, LQY, SWF, JY and JK performed the experiment and analyzed the data. CZ designed the study, revised the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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