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BMP4 inhibits myogenic differentiation of bone marrow–derived mesenchymal stromal cells in mdx mice

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Abstract

Background aims. Bone marrow–derived mesenchymal stromal cells (BMSCs) are a promising therapeutic option for treating Duchenne muscular dystrophy (DMD). Myogenic differentiation occurs in the skeletal muscle of the mdx mouse (a mouse model of DMD) after BMSC transplantation. The transcription factor bone morphogenic protein 4 (BMP4) plays a crucial role in growth regulation, differentiation and survival of many cell types, including BMSCs. We treated BMSCs with BMP4 or the BMP antagonist noggin to examine the effects of BMP signaling on the myogenic potential of BMSCs in mdx mice. *Methods.* We added BMP4 or noggin to cultured BMSCs under myogenic differentiation conditions. We then injected BMP4- or noggin-treated BMSCs into the muscles of mdx mice to determine their myogenic potential. *Results.* We found that the expression levels of desmin and myosin heavy chain decreased after treating BMSCs with BMP4, whereas the expression levels of phosphorylated Smad, a downstream target of BMP4, were higher in these BMSCs than in the controls. Mdx mouse muscles injected with BMSCs pretreated with BMP4 showed decreased dystrophin expression and increased phosphorylated Smad levels compared with muscles injected with non-treated BMSCs. The opposite effects were seen after pretreatment with noggin, as expected. *Conclusions.* Our results identified BMP/Smad signaling as an essential negative regulator of promyogenic BMSC activity; inhibition of this pathway improved the efficiency of BMSC myogenic differentiation, which suggests that this pathway might serve as a target to regulate BMSC function for better myogenic differentiation during treatment of DMD and degenerative skeletal muscle diseases.

Key Words: *BMPs, BMSCs, Duchenne muscular dystrophy, dystrophin, mdx mice, myogenic differentiation, Smad*

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked inherited muscular disorder characterized by the absence of dystrophin [1]. Without dystrophin, the muscles undergo repetitive degeneration and regeneration cycles leading to muscle fibrosis and weakness [2]. Various attempts have been made to overcome DMD, including steroid treatment [3,4], gene therapy [5,6] and stem cell therapy [7]. Stem cell–based therapy provides a promising treatment option for DMD because healthy stem cells can restore dystrophin expression *in vitro* and *in vivo*. Many studies have demonstrated that mesenchymal stromal cells induce dystrophin protein production in mdx mice [8], dogs [9] and humans [10]. On the basis of previous studies, no high-yield myogenic cells were obtained in mdx mouse skeletal muscle after bone marrow–derived mesenchymal stromal cell (BMSC) transplantation [11]. However, because defective muscle regeneration cannot counteract repetitive degeneration, the myogenic differentiation capacity of BMSCs has unique therapeutic potential.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF–β) superfamily, and they regulate cell commitment and differentiation through intracellular proteins called Smads [12,13]. Initially, BMP4 was shown to play critical roles in osteoblast differentiation [14]. More recently, it was revealed that BMP4 also modulates myogenic differentiation in BMSCs. Grajales *et al.*

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[15] reported that BMP4 enhanced cardiac myogenic development and suppressed skeletal myogenesis, leading to loss of "stemness" in BMSCs. Another study showed that retinoic acid enhanced skeletal myogenesis in stem cells by inhibition of BMP signaling [16]. Noggin, a BMP antagonist, is crucial for proper differentiation of muscle progenitor cells, and it acts through inhibition of local BMP signaling during embryonic muscle differentiation [12,17]. Thus, we hypothesized that inhibition of BMP/Smad signaling may promote the myogenic differentiation of BMSCs *in vitro* and increase restored dystrophin in muscles of mdx mice. Therefore, the objective of the present study was to determine the effects of BMP4 on the myogenic differentiation potential of BMSCs *in vivo* and *in vitro* and on the molecular mechanism of BMP4 function in this context.

Methods

Bone marrow isolation and culture

Bone marrow was obtained from 6- to 8-week-old C57 mice. Mice were euthanized by means of cervical dislocation, and bone marrow was slowly flushed out the tibia and femur into lymphocyte separation medium, in which it was proportionally diluted with phosphate-buffered saline (PBS). After density gradient centrifugation, mononuclear cells were collected and centrifuged for 4 min at 1000*g* twice, after dilution in PBS. The cells were then cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM, Gibco, Invitrogen) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen) and 1% penicillin/streptomycin and were subsequently incubated at 37°C in 5% CO_2. After 3 days, the medium was changed, and the non-adherent cells were discarded. The adherent cells were trypsinized with 0.25% trypsin–ethylene diamine tetra-acetic acid (Gibco, Invitrogen) when well-developed colonies reached near-confluency, and they were seeded into fresh plates for further expansion.

Characterization of BMSCs

To determine the purity of BMSCs, flow cytometric analysis of BMSC cell surface marker expression was performed. Cells that were 80% to 90% confluent were washed in PBS and centrifuged at 1000*g* for 4 min after trypsinization. Cells were then re-suspended in equal volumes of ice-cold PBS and 100% ethanol and incubated on ice for 30 min. Cells were incubated in PBS containing CD29, CD34, CD44, CD45 and CD105 primary antibodies (Cell Signaling Technology) for 30 min at 37°C. After incubation, cells were washed with PBS containing 1% bovine serum albumin (BSA) 3 times and then were centrifuged at 12,000*g* for 5 min at 4°C between each wash. A Becton Dickinson FACS Scan was used for fluorescence-activated cell-sorting (FACS) analysis. Cells were then tested for the ability to differentiate into adipogenic and osteogenic lineages through the use of oil red O staining and alizarin red staining, as described previously [18].

Induction of myogenic differentiation

BMSCs that reached sub-confluence were treated with 5-azacytidine (5-Aza, Sigma) for 24 h; cells were then assigned to three treatments groups, and their media was changed as follows: group 1 (control), DMEM supplemented with 2% horse serum (HS, Gibco, Invitrogen); group 2, DMEM supplemented with 10 ng/mL recombinant human BMP4 (Invitrogen) and 2% HS; group 3, DMEM supplemented with 100 ng/mL recombinant human noggin (Invitrogen) and 2% HS. The culture media was changed every 2 days. The expression of target proteins was analyzed after 14 days and 21 days of culture.

Animal injection

Twenty-two mdx mice (6–8 weeks old) were divided into four groups (with four mice in the control group, and six mice each in the other three treatment groups): group 1 (control), radiotherapy only; group 2, transplantation with BMSCs pre-treated with 5-Aza; group 3, transplantation with BMSCs pre-treated with 5-Aza + BMP4 (3 days); group 4, transplantation with BMSCs pretreated with 5-Aza + noggin (3 days). All mice were irradiated (total body with 6 Gy) from a 60 Co source. Three days after irradiation, 3×10^6 BMSCs were infused through the tail vein per mouse. Muscle tissue was harvested 12 weeks after injection. The mice were housed in a specific pathogen-free animal facility at the Laboratory Animal Center of Sun-Yet University (Guang Zhou, China), which was purchased from The Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All animal experiments were performed in accordance with the Sun-Yet University Guidelines for Animal Care.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde and blocked with 5% BSA; they were then incubated with the following primary antibodies: rabbit polyclonal anti-desmin (1:200; Abcam), rabbit polyclonal anti-myosin heavy chain (MHC) (1:50; Santa Cruz) and rabbit polyclonal anti-Smad or anti–phosphor-Smad

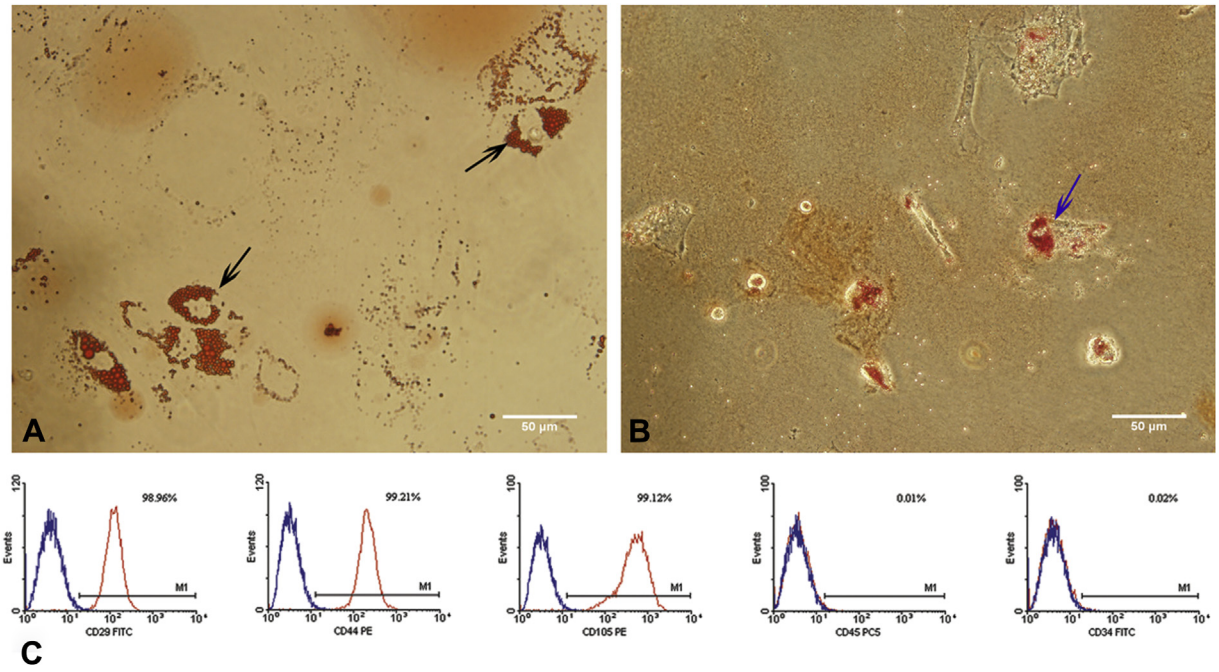

Figure 1. Characterization of cultured BMSCs. (A) Oil red O staining (blue arrow) shows the formation of lipid vacuoles, which indicates adipogenic differentiation of BMSCs. (B) Alizarin red staining (black arrow) shows the formation of calcium deposits, which indicates osteogenic differentiation of BMSCs. (C) Flow cytometric analysis of BMSCs stained with antibodies against myogenic surface antigens. Y-axis: number of events; X-axis: fluorescence intensity; red lines: specific antibodies; blue lines: control antibodies.

(p-Smad) (1:100; Cell Signaling Technology) for 60 min at room temperature. Cells were then washed 3 times with PBS. Cy3-conjugated goat anti-rabbit secondary antibody (1:400, Millipore) was used to detect the localization of anti-desmin and anti-MHC antibodies. Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (Sigma). Immunofluorescence analysis was performed 12 weeks after BMSC transplantation on transverse, serial cryosections (6 μm in thickness) after fixation with cold acetone. Slides were incubated overnight at 4°C with an anti-dystrophin rabbit polyclonal primary antibody (1:400, Abcam) followed by 1-h incubation with Cy3-conjugated goat anti-rabbit secondary antibody (1:400, Millipore) in 1% BSA. The other steps were performed as described above.

Western blotting

At predetermined experimental time points, cells were harvested and lysed in Radio Immunoprecipitation Assay (RIPA) buffer (Millipore) (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 1 mg/mL aprotinin and 1 mg/mL leupeptin) after ice-cold PBS (×3). Protein concentration was determined with the use of a BCA assay kit (bicinchoninic acid; Millipore). Cell lysates were loaded and separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel. Proteins were transferred to polyvinylidene fluoride membranes (Millipore) that were blocked with 5% BSA for 1 h. The membranes were probed with rabbit anti-desmin (1:1000; Abcam), anti-MHC (1:200; Santa Cruz), anti-phosphorylated Smad (p-Smad) (1:1000, Cell Signaling Technology), anti-Smad (1:1000; Cell Signaling Technology) and anti–glyceraldehyde-3-phosphate dehydrogenase (1:1000; Cell Signaling Technology) monoclonal antibodies at 4°C, overnight. The membranes were incubated with a horseradish-polypeptidase–conjugated secondary antibody (1:1000; Cell Signaling Technology) for 1 h at room temperature. Protein bands were visualized by means of enhanced chemiluminescence, and band intensity was quantified with the use of ImageJ software (National Institutes of Health). Tissue samples from the gastrocnemius muscle were homogenized in a lysis buffer containing 10% SDS, 70 mmol/L Tris-HCl, pH 6.8, 5% β-mercaptoethanol and 10 mmol/L ethylene diamine tetra-acetic acid supplemented with a cocktail of protease and phosphatase inhibitors (Sigma). Nuclei and cellular debris were discarded by centrifuging at 10,000*g* for 10 min at 4°C. Homogenates were separated by use of 6% SDS-PAGE. The membranes were probed using the following antibodies: anti-dystrophin, anti-Smad and anti–p-Smad (described above). The remaining steps were carried out as described above.

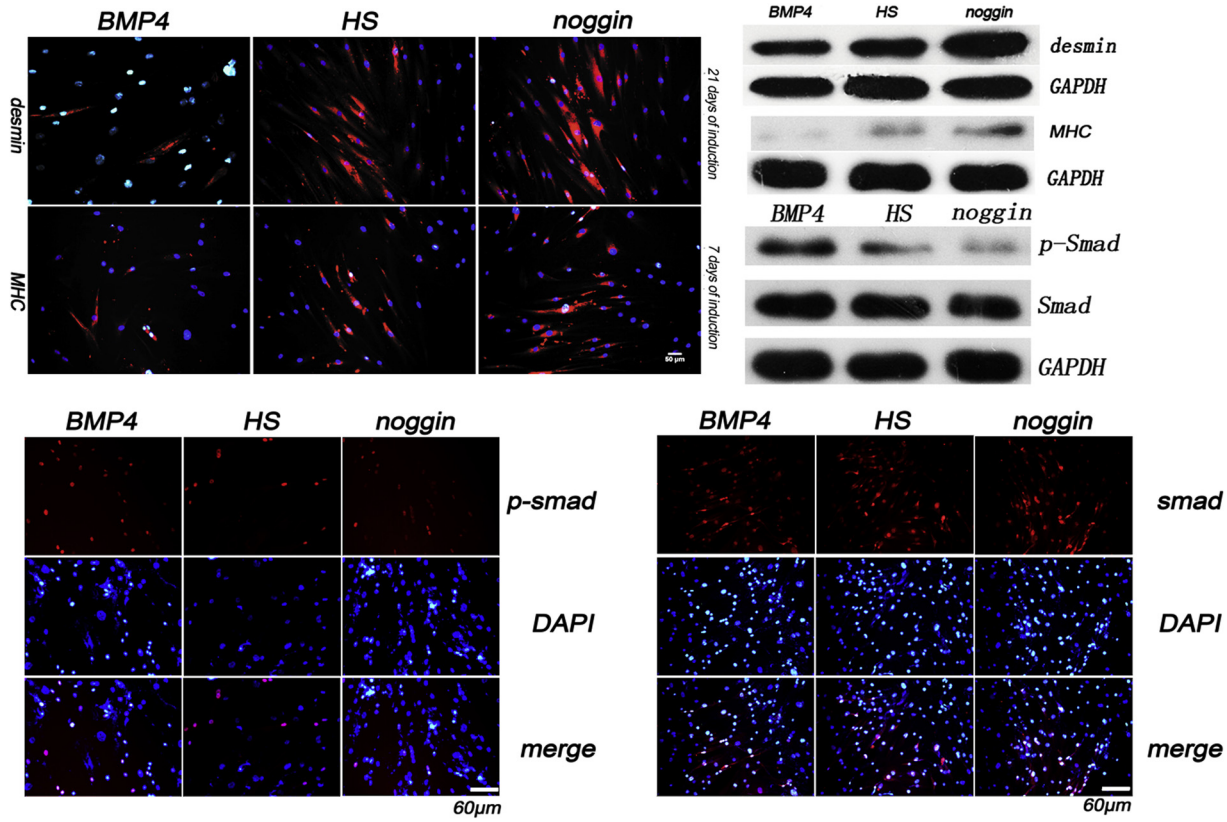

Figure 2. Expression of desmin/MHC and Smad/p-Smad in BMSCs exposed to BMP4/noggin after induction with 5-Aza. Immunofluorescent staining and Western blot analysis of desmin and MHC expression indicated baseline expression in BMSCs after induction with 5-Aza and 2% HS for 13 days and 20 days, respectively. On exposure to BMP4, expression of desmin and MHC was decreased, and, on exposure to noggin, expression was enhanced. Immunofluorescent staining and Western blot analysis of Smad and p-Smad showed that p-Smad levels were clearly upregulated after BMSC exposure to BMP4 and clearly downregulated after BMSC exposure to noggin after induction with 5-Aza. Levels of Smad showed no significant difference after exposure to BMP4 or noggin when compared with controls.

Results

Characterization of mouse BMSCs

BMSCs were defined by cell surface antigen expression and *in vitro* differentiation potential. We found that BMSCs in our study were able to adopt adipogenic (Figure 1A) and osteogenic (Figure 1B) phenotypes. Flow cytometric analysis showed that BMSCs were positive for the MSC markers CD29, CD44 and CD105 and negative for the hematopoietic markers CD45 and CD34 (Figure 1C).

Expression of desmin/MHC and Smad/p-Smad in BMSCs exposed to BMP4/noggin after induction with 5-Aza

As shown in Figures 2 and 4, expression of desmin and expression of MHC were detected by immunofluorescent staining and Western blot analysis. Desmin expression and MHC expression were detected after 5-Aza treatment. However, after exposure to BMP4 (10 ng/mL), the expression of desmin and MHC were repressed. Conversely, desmin expression and MHC expression were enhanced after cells were stimulated with noggin (100 ng/mL). Because BMP4 activates the members of the Smad family, initiating an intracellular cascade, we further investigated the effects of BMP4/noggin on the myogenic differentiation of BMSCs after induction with 5-Aza by analysis of Smad activation. We performed immunofluorescent staining and Western blot analysis to examine protein Smad and p-Smad expression levels. As shown in Figures 2 and 4, Smad levels determined after treatment with 5-Aza alone did not change after exposure to BMP4 or noggin. However, p-Smad levels were clearly upregulated or downregulated when BMSCs treated with 5-Aza were exposed to BMP4 or noggin, respectively.

Expression of dystrophin and p-Smad in muscles of mdx mice injected with BMSCs pre-treated with BMP4/noggin

To test whether BMP4/noggin treatment changed the myogenic differentiation potential of BMSCs in muscles of mdx mice, the expression of dystrophin

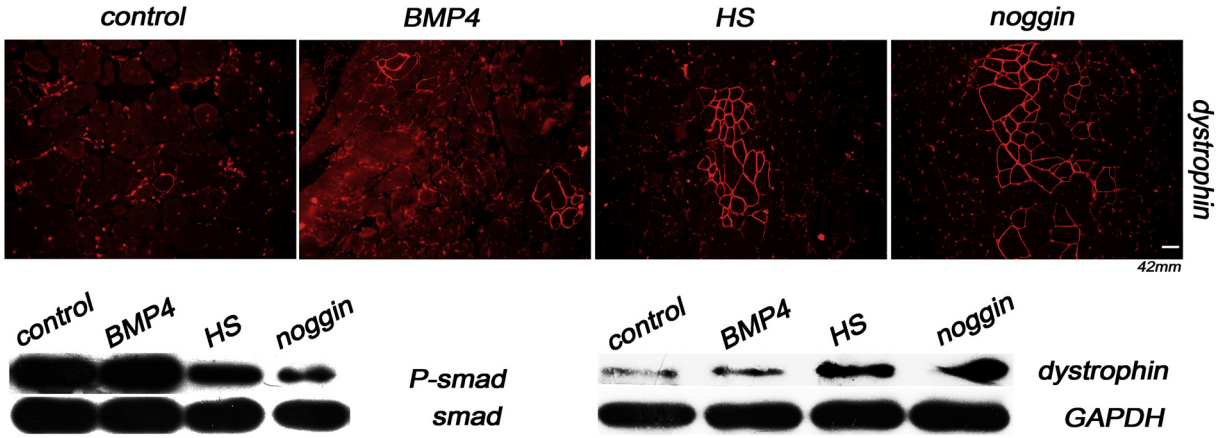

Figure 3. Expression of dystrophin and p-Smad in the muscles of mdx mice injected with BMSCs pre-treated with BMP4/noggin after induction with 5-Aza. Immunofluorescent staining and Western blot analysis of dystrophin and p-Smad was performed in muscle tissue from mdx mice injected with BMSCs. Low expression of dystrophin was seen on the cell membrane of mdx mice without BMSC transplantation, and dystrophin expression was increased in cell membranes after BMSC transplantation. However, expression of dystrophin was clearly decreased in BMSCs pretreated with BMP4 compared with BMSCs induced with 5-Aza alone. Conversely, dystrophin was clearly increased in groups of BMSCs pretreated with noggin after 5-Aza induction, compared with groups of BMSCs induced with 5-Aza alone. Expression of p-Smad was clearly upregulated in BMSCs pretreated with BMP4, and it was clearly downregulated in groups of BMSCs pretreated with noggin after 5-Aza induction, compared with BMSCs induced with 5-Aza alone.

and p-Smad were analyzed. As shown in Figures 3 and 5, in comparison to muscles of mdx mice injected with BMSCs (controls), dystrophin expression was clearly decreased in muscles injected with BMSCs that had been pretreated with BMP4, whereas levels were evidently increased in muscles pretreated with noggin. We also found that p-Smad expression was clearly increased in muscles of mdx mice injected with BMP4-treated BMSCs and visibly decreased in muscles of injected with noggin-treated BMSCs.

Discussion

In the present study, we cultured BMSCs with 5-Aza, a compound that promotes DNA hypomethylation, which, in turn, leads to gene activation. This compound activates myogenic gene expression in mesenchymal stromal cells, inducing myogenic differentiation. To investigate the effects of BMP4 on the myogenic differentiation potential of BMSCs, cell cultures were supplemented with BMP4 or noggin. The addition of BMP4 to BMSC cultures inhibited myogenesis *in vitro*. Conversely, noggin induced a myocyte phenotype when added to 5-Aza–treated BMSC cultures, as determined by analysis of myogenic protein expression. This suggested that BMP4 has an inhibitory role in the myogenic differentiation of BMSCs after 5-Aza treatment. This was consistent with previous reports by Grajales *et al.* [15] and Kennedy *et al.* [16], who reported that BMP4 suppressed skeletal myogenic differentiation. The BMP4 intracellular signaling pathway involves a specific set of receptor-mediated Smad proteins, and the induction of Smads was shown to negatively regulate myogenic differentiation of C2C12 myoblasts, embryonic stem cells and mesenchymal stromal cells [12]. We further investigated changes in Smad phosphorylation in BMSCs exposed to BMP4/noggin after induction with 5-Aza. As expected, BMP4 increased p-Smad expression, whereas exposure to noggin had the opposite effect. These results demonstrated that BMP4 inhibited the myogenic differentiation of BMSCs through the Smad signaling pathway.

Previous studies showed that expression of TGF-β superfamily members (including BMP4) increased in the muscles of mdx mice [12,19]. These studies implicated intracellular Smad signaling as a novel player in DMD pathology, suggesting that the BMP/Smad signaling pathway may be directly involved in regulating myogenic differentiation of transplanted BMSCs. On the basis of our *in vitro* results, we injected BMP4-treated or noggin-treated BMSCs into the muscles of mdx mice to determine their myogenic potential. Our results showed that BMSCs pre-treated with BMP4 reduced dystrophin expression in the muscles of mdx mice by upregulating the Smad signaling pathway, whereas BMSCs pre-treated with noggin showed increased dystrophin expression in the muscles of mdx mice. This may have been caused by downregulation of the Smad signaling pathway before transplantation.

It is well known that the main obstacle preventing the application of BMSC transplantation in the treatment of muscle disorders is ineffective myogenic differentiation *in vivo*. For example, Gussoni *et al.* [10] reported that on analysis of muscle biopsies from

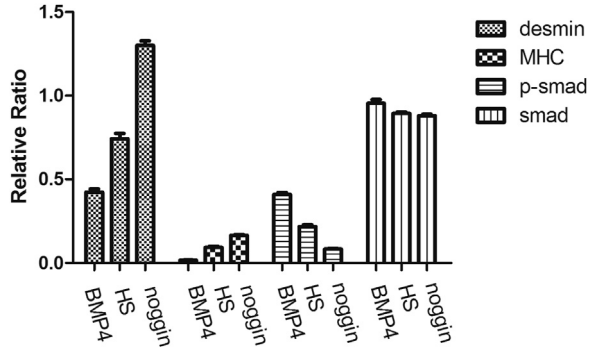

Figure 4. Quantitative analysis of expression of desmin, MHC, p-smad, and smad in BMSCs exposed to BMP4/noggin detected by Western blot.

a DMD patient who received bone marrow transplantation, the presence of donor nuclei was only 0.5% to 0.9%. Nair *et al.* [20] did not detect dystrophin in a 9-year-old boy with Diamond-Blackfan anemia and Duchenne muscular dystrophy who underwent successful allogeneic hematopoietic stem cell transplantation. Our results showed that suppression of BMP/Smad signaling promoted the myogenic differentiation of BMSCs and restored dystrophin expression in the muscle tissue of mdx mice. This suggests that the inhibition of BMP/Smad signaling in BMSCs undergoing myogenic differentiation before transplantation can efficiently increase the number of myogenic cells and the expression of dystrophin in skeletal muscles of mdx mice. However, myogenic differentiation of BMSCs varies with the concentration of BMP4 and is affected by several unknown factors. Thus, further studies are required to investigate the effect of the BMP/Smad signaling on the myogenic differentiation potential of BMSCs. This may be achieved by transfecting the cells with a lentivirus containing BMP4 and assessing the effects on skeletal muscle in mdx mice.

In summary, our results indicate that BMP/Smad signaling is an essential negative regulator of myogenic differentiation in BMSCs. The identification of BMP/Smad signaling as an essential negative regulator of promyogenic BMSC activity suggests that this pathway might serve as a pharmacological target to regulate BMSC function during treatment of DMD and other degenerative skeletal muscle diseases and might improve the efficiency of BMSC therapy.

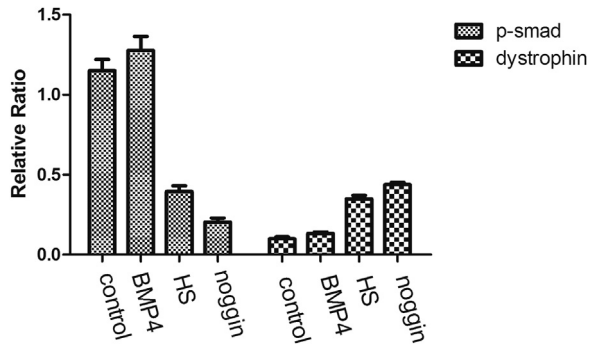

Figure 5. Quantitative analysis of expression of dystrophin and p-smad in muscles of mdx mice injected with BMSCs pre-treated with BMP4/noggin detected by Western blot.

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