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Effects of matrix metalloproteinase-1 on the myogenic differentiation of bone marrow-derived mesenchymal stem cells *in vitro*

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ABSTRACT

Matrix metalloproteinase-1 (MMP-1) is a member of the family of zinc-dependent endopeptidases that are capable of degrading extracellular matrix (ECM) and certain non-matrix proteins. It has been shown that MMP-1 can enhance muscle regeneration by improving the differentiation and migration of myoblasts. However, it is still not known whether MMP-1 can promote the myogenesis of bone marrow-derived mesenchymal stem cells (BMSCs). To address this question, we isolated BMSCs from C57BL/6J mice and investigated the effects of MMP-1 on their proliferation and myogenic differentiation. Our results showed that MMP-1 treatment, which had no cytotoxic effects on BMSCs, increased the mRNA and protein levels of MyoD and desmin in a dose-dependent manner, indicating that MMP-1 promoted myogenic differentiation of BMSCs *in vitro*. These results suggest that BMSCs may have a therapeutic potential for treating muscular disorders.

1. Introduction

The discovery of adult stem cells has proven indisputably that there are regenerative populations of cells within developed organs and tissues and has led to a growing interest in using these cells as therapeutics. Unlike the adult stem cells obtained from other sources, bone marrow-derived mesenchymal stem cells (BMSCs) are abundant and easy to isolate, and they have an unlimited potential for expansion in culture. Importantly, BMSCs have low immunogenicity [1].

An emerging concept is that preconditioning BMSCs with lineage-specific agents *in vitro* could enhance their efficacy in therapy, for example, inducing BMSCs with myogenic differentiation media before transplanting them into Duchenne muscular dystrophy (DMD) patients. The chemical 5-azacytidine (5-Aza) is commonly used to induce myogenic differentiation *in vitro*. However, this strong demethylating agent can cause extensive cell death [2,3]. Therefore, it is necessary to search for new agents that are less toxic and more potent. Wang et al. reported that matrix metalloproteinase-1 (MMP-1) could enhance muscle regeneration by improving the differentiation and migration of myoblasts [4]. Their study also showed that the efficacy of myoblast transplantation into the dystrophic skeletal muscles of *mdx* mice *in vivo* was significantly improved by MMP-1 treatment [4]. In this study, we investigated whether the myogenesis of BMSCs could be enhanced by MMP-1 because BMSCs are easy to obtain and can differentiate into muscle cells.

2. Materials and methods

2.1. Materials

Six- to eight-week-old male C57BL/6J mice were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Sun Yat-sen University.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum (HS) were purchased from Gibco (USA). CCK-8 was purchased from Dojindo (Japan). RNAiso Plus, the PrimeScript RT reagent Kit and SYBR Premix Ex Taq were

Abbreviations: 5-Aza, 5-azacytidine; ARS, Alizarin red S; bHLH, basic helix-loop-helix; BMSCs, bone marrow-derived mesenchymal stem cells; CCK-8, cell counting kit-8; DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; HRP, horse radish peroxidase; mdx, x-linked muscular dystrophy; MMPs, matrix metalloproteinases; MRFs, myogenic regulator factors; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Sca-1, stem cell antigen-1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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purchased from Takara (Japan). Recombinant human MMP-1 and 5-Aza were purchased from Sigma–Aldrich (USA). PE-conjugated anti-CD29, anti-CD34, anti-CD45 and anti-stem cell antigen 1 (Sca-1) monoclonal antibodies were purchased from Biolegend (USA). Anti-MyoD polyclonal antibody was purchased from Abcam (UK). Anti-desmin polyclonal antibody was purchased from Bioworld (USA). Anti-GAPDH monoclonal antibody was purchased from Cell Signaling Technology (USA). Horse radish peroxidase (HRP)-conjugated sheep anti-rabbit IgG was purchased from MultiSciences (China). All other chemicals were purchased from Sigma–Aldrich (USA).

2.2. Methods

2.2.1. Isolation and culture of BMSCs [5]

Bone marrow was flushed from the tibias and femurs with DMEM. The marrow cells were pooled and dissociated by passage through a 25-gauge needle (BD, USA). The single-cell suspension was centrifuged at 1000 rpm for 5 min, re-suspended in complete medium (DMEM, 10% FBS and 100 IU/ml antibiotics), and plated in 25 cm^2 flasks at a density of 1×10^7 cells/ml. After 24 h of incubation in a humidified incubator (37 °C and 5% CO_2), the medium containing the non-adherent cells was removed, and the adherent cells were gently washed 2 times with PBS to reduce the degree of hematopoietic lineage cell contamination. The cells were cultured in DMEM for 3–4 weeks, until they reached 70–80% confluence. Fresh medium was added every 3 days. The cells were expanded by replating at a density of 1×10^6 cells/ml or used directly for the experiments.

2.2.2. Flow cytometry

The cells were trypsinized and washed, then incubated for 30 min at 4 °C with phycoerythrin (PE)-conjugated antibodies against murine CD29, CD34, CD45 and Sca-1. The labeled cells were analyzed using a FACScalibur Cytometer (BD, USA) and CellQuest Software (BD, USA).

2.2.3. Differentiation of BMSCs[5]

2.2.3.1. Osteogenesis. Each well of a 6-well plate was seeded in duplicate with 1×10^6 nucleated cells that were treated with osteogenic induction medium (DMEM supplemented with 10% FBS, 50 mg/l ascorbic acid, 0.1 μM dexamethasone and 0.5 mM β-sodium glycerophosphate). The cells were fixed with 4% paraformaldehyde and stained with an Alizarin red S (ARS) solution on day 14 or a 5% silver nitrate solution (von Kossa stain) on day 21.

2.2.3.2. Adipogenesis. Each well of a 6-well plate was seeded in triplicate with 2×10^6 nucleated cells that were treated with adipogenic induction medium (DMEM supplemented with 10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 10 μg/ml insulin and 0.5 mM IBMX) for 3 days and then switched to maintenance medium (DMEM supplemented with 10% FBS and 10 μg/ml insulin). Oil red O stain was performed when lipid droplets were observed under the microscope.

2.2.4. Cytotoxicity assays

An aliquot of 100 μl of the cell suspension (5000 cells/well) was dispensed into each well of a 96-well plate and pre-incubated with complete medium (DMEM, 10% FBS and 100 IU/ml antibiotics) for 24 h. The cells were then switched to complete medium supplemented with 10 ng/ml, 1 ng/ml, 0.1 ng/ml, or 0 ng/ml of MMP-1 and cultured for 2-, 4-, 6-, or 8 days before they were harvested. Ten microliters of CCK-8 solution was added to each well and incubated for 4 h before measuring the absorbance at a wavelength of 450 nm.

2.2.5. Effect of MMP-1 on myogenesis

Each of 4 25-cm^2 flasks was plated with 1×10^5 nucleated cells. When the cells reached 70–80% confluence, they were induced with myogenic induction medium (DMEM supplemented with 5% FBS, 5% horse serum and 10^{-5} M 5-Aza) for 24 h. The induction medium was removed and replaced with complete medium containing MMP-1 (10 ng/ml, 1 ng/ml, 0.1 ng/ml or 0 ng/ml). The medium was changed every 3 days.

2.2.5.1. Real-time RT-PCR analysis. Total RNA was extracted using the RNAiso plus reagent. Reverse transcription was performed using the PrimeScript RT reagent kit using 500 ng of total RNA. The expression levels of the MyoD and desmin genes were determined using SYBR Premix Ex Taq (Perfect Real Time). *β-actin* was used as an internal control. The sequences of the primers used in PCR are listed in Table 1. All procedures were performed strictly according to the manufacturer's instructions.

2.2.5.2. Western blot analysis. For protein analysis, the cells were treated with MMP-1 for 7 days. Equal amounts of the protein extracts were separated on SDS–PAGE gels and transferred onto PVDF membranes (Millipore, 0.45 μm), then immunolabeled with anti-MyoD (1:200) or anti-desmin polyclonal antibodies (1:1000) at 4 °C for 12 h and incubated with HRP-conjugated anti-rabbit secondary antibody (1:5000) at room temperature for 1 h. GAPDH (1:2000) was used as a control for the equal loading of the lanes. The blots were developed using a chemiluminescent HRP substrate (Millipore). The results were quantified using Quantity One 4.6.2 Software.

2.3. Statistical analysis

All results were expressed as the mean ± SD. The differences between groups were analyzed by one-way ANOVA (SPSS 18.0 Software package). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Isolation and culture of BMSCs

Bone marrow mesenchymal stem cells are known to have the ability to adhere to plastic plates and to proliferate. Utilizing these properties, we isolated BMSCs from the hematopoietic lineage cells by several rounds of subculturing. After several passages, the hematopoietic lineage cells had died. The spindle-like BMSCs that were the dominant cell population (Fig. 1A) were positive for the surface makers CD29 and Sca-1 and negative for CD34 and CD45, as assessed by flow cytometry (Fig. 1B).

Table 1
Real-time RT-PCR primers for amplification of mouse mRNA.

Genes	Forward	Reverse	Size (bp)
MyoD	ATTCCAACCCACAGAACCTTTGTC	TCAACCCAAGCCGTGAGAGTC	85
Desmin	CGACGCTGTGAACCAGGAGT	TAGTTGGCGAAGCGGTCATT	84
β-Actin	CTGGCACCACACCTTCTACA	GGTACGACCAGAGGCATACA	190

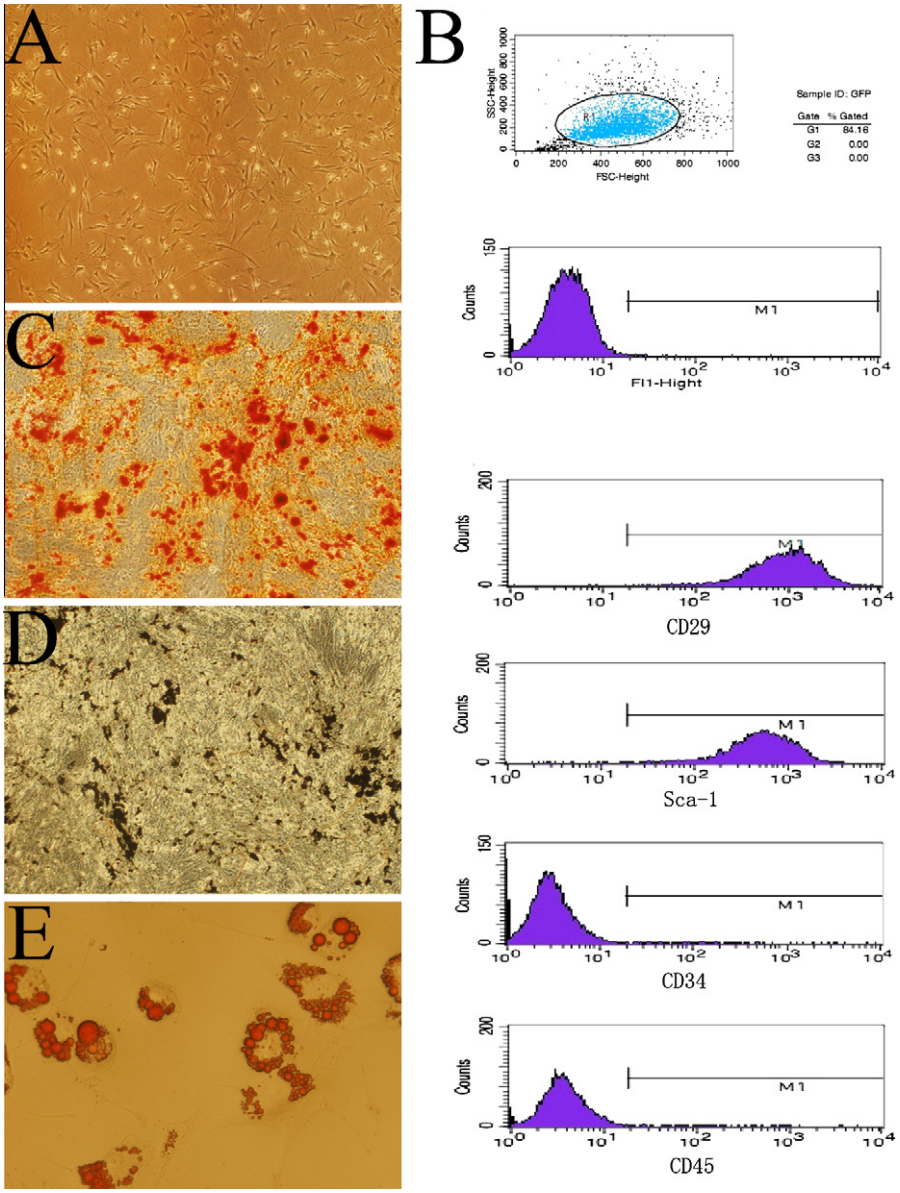

Fig. 1. Culture and identification of BMSCs. (A) Morphology of BMSCs isolated using the differential adhesion technique. (B) Flow cytometry results showing the percentages of cells immunolabeled for surface markers (99.96% or 99.74% positive for CD29 or Sca-1, and 99.28% or 98.86% negative for CD34 or CD45). (C) Mineralized bone nodules stained with ARS (red) after 2 weeks of induction. (D) von Kossa stain showing mineralized bone nodules (black) after 3 weeks of induction. (E) Oil red O stain showing the accumulated lipid droplets inside adipocytes. Original magnification for A, C and D: 100×; original magnification for E: 200×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. BMSC differentiation assays

3.2.1. Osteogenesis

To assess the multipotentiality of the isolated BMSCs, we performed osteogenic differentiation assays. The cells were induced with the osteogenic induction medium, and stained with ARS on day 14 (Fig. 1C) or with von Kossa stain on day 21 (Fig. 1D, black) to visualize the formation of mineralized bone nodules.

3.2.2. Adipogenesis

To assess the adipogenic ability, we subjected BMSCs to the standard adipogenic induction program. After 2 weeks of induction, a substantial number of BMSCs had differentiated into adipocytes, as demonstrated by oil red O stain of the accumulated intracellular lipid droplets (Fig. 1E).

3.3. Effect of MMP-1 on BMSC cytotoxicity

To assess whether MMP-1 is toxic to BMSCs, we performed time-course (2–8 days) and dose–response (0.1–10 ng/ml of MMP-1) experiments. The results showed that MMP-1 treatment had no significant toxic effects on BMSCs, even when the highest concentration of MMP-1 was present over the longest period of treatment (10 ng/ml for 8 days) (Fig. 2).

3.4. Effect of MMP-1 on myogenesis of BMSCs

3.4.1. Effect of MMP-1 on the expression levels of the MyoD and desmin genes

To determine the effect of MMP-1 on the expression levels of the MyoD and desmin genes, we treated BMSCs with MMP-1 for

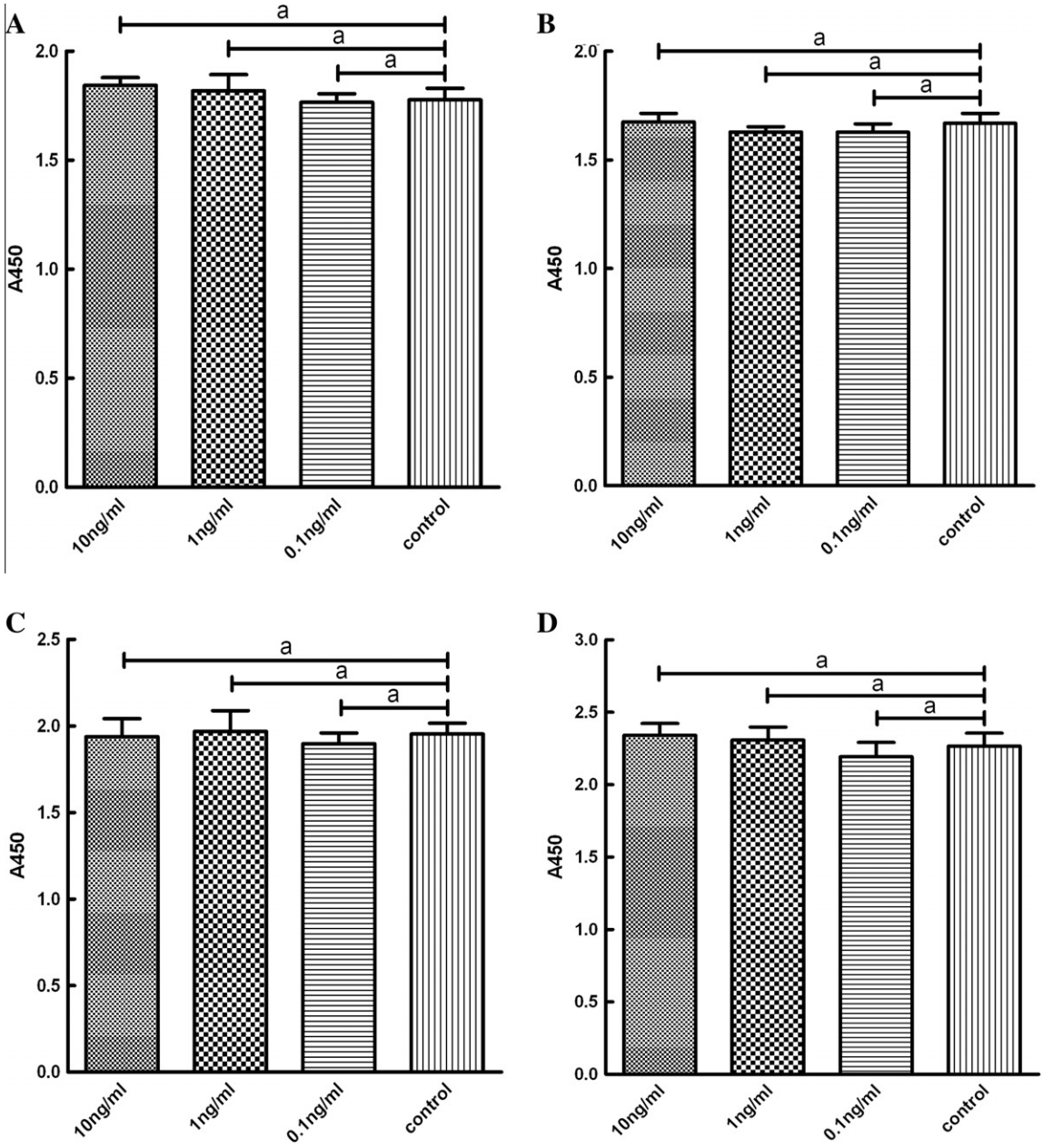

Fig. 2. Effect of MMP-1 on BMSC cytotoxicity. BMSCs were treated with 10, 1, or 0.1 ng/ml of MMP-1 and harvested for cytotoxicity assays using a CCK-8 kit after 2 (A), 4 (B), 6 (C) and 8 (D) days. The results are presented as relative values of absorbance at 450 nm (A_{450}). [a]$P > 0.05$.

2 days and then performed real-time quantitative RT-PCR analysis. Our results showed that MMP-1 increased the mRNA levels of MyoD and desmin, two key factors regulating myogenic differentiation, in a dose-dependent manner. The mRNA levels of MyoD increased 68-, 4- and 2-fold in the cells exposed to the high (10 ng/ml), intermediate (1 ng/ml) or low (0.1 ng/ml) concentrations, respectively, compared to the control group ($P < 0.05$) (Fig. 3A); and the mRNA levels of desmin increased 14-, 3- and 2-fold in the cells exposed to the high, intermediate or low concentrations, respectively, compared to the control group ($P < 0.05$) (Fig. 3B).

3.4.2. Effects of MMP-1 on MyoD and desmin protein expression

To determine the effects of MMP-1 on the expression of MyoD and desmin protein, we performed Western blot analysis using whole cell lysates harvested from BMSCs that had been treated with MMP-1 for 7 days. The results of our Western analysis showed that MMP-1 also upregulated the levels of MyoD and desmin protein in a dose-dependent manner, similar to the patterns of mRNA expression. The expression of MyoD increased 2-, 1.7- and 1.3-fold ($P < 0.05$) (Fig. 3C) and the expression of desmin increased 1.7-, 1.6- and 1.2-fold in the cells treated with the high, intermediate or low concentrations, respectively, compared to the control groups ($P < 0.05$) (Fig. 3D).

4. Discussion

BMSCs represent a heterogeneous cell population present in the bone marrow; similar cell populations can also be isolated from many different tissues throughout the body [1,6]. These cells have the potential to differentiate into many cell types, including adipocytes, chondrocytes, neurons, myocytes and osteocytes [7,8]. BMSCs express a spectrum of cell surface markers, but none is

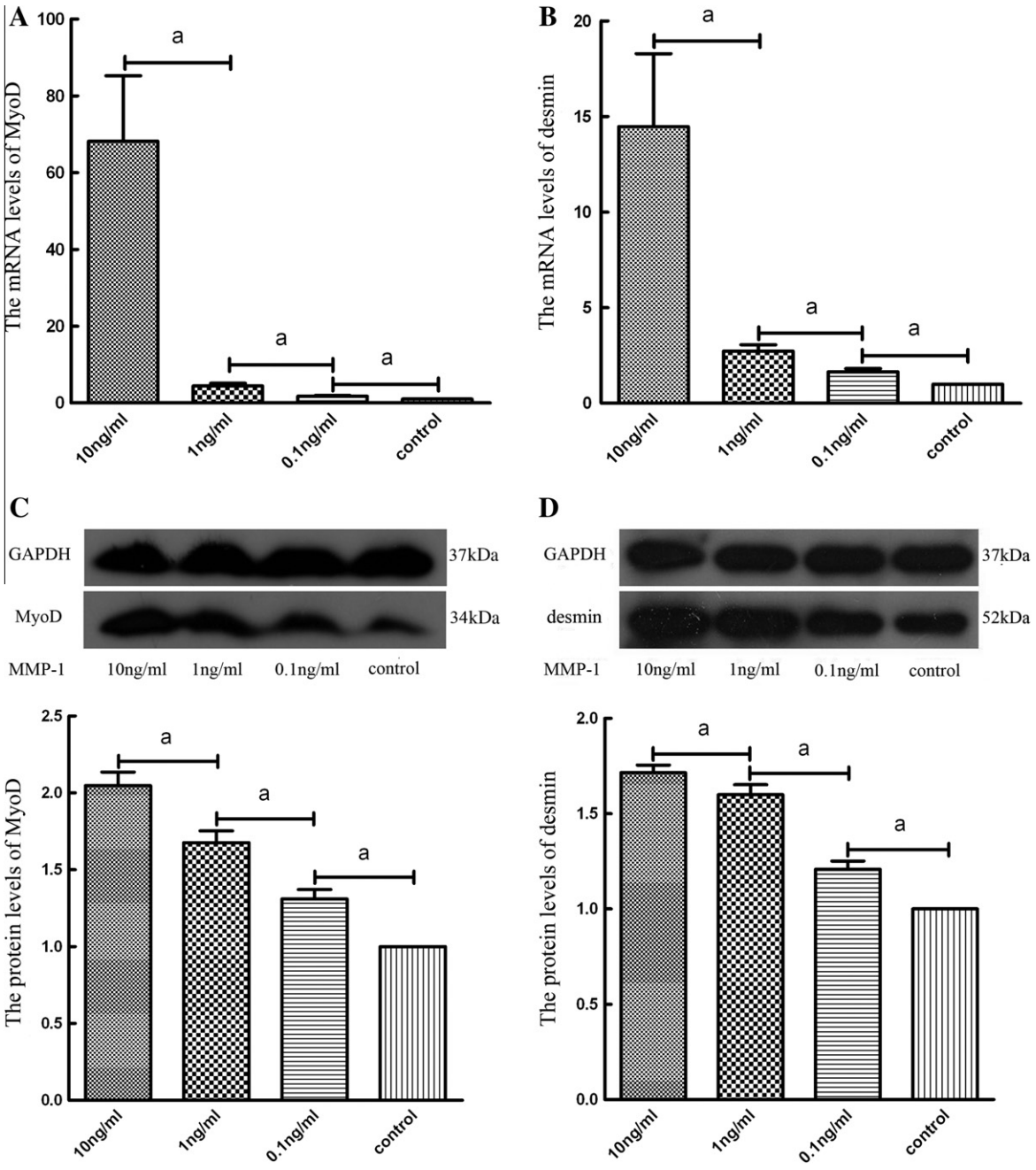

Fig. 3. Effect of MMP-1 on myogenesis of BMSCs. Real-time RT-PCR analysis showing the levels of MyoD (A) and desmin (B) mRNA in BMSCs treated with the indicated concentrations of MMP-1 for 2 days. $^{a}P < 0.05$. Western blot analyses showing the protein levels of MyoD (C) and desmin (D) in BMSCs treated with the indicated concentrations of MMP-1 for 7 days. $^{a}P < 0.05$.

unique to them. Nonetheless, BMSCs are usually identified as positive for CD105, CD73, CD90, CD166, CD44 and CD29 but negative for CD14, CD31, CD34 and CD45 [9,10].

Myogenic regulatory factors (MRFs) including MyoD, Myf5, myogenin and MRF4 are basic helix-loop-helix (bHLH) transcription factors [11]. These factors are critical for the determination and terminal differentiation of skeletal muscle cells. MyoD and Myf5 are required for the commitment of progenitor cells to the myogenic lineage and myogenin plays an important role in the maintenance of the muscle phenotype established by MyoD and Myf5. MRF4 plays roles in both the commitment and maintenance of myocyte [12]. MyoD is a master regulator of myogenesis; it sequentially activates myogenin, myosin and myoglobin. Overexpression of MyoD can induce myogenesis in a variety of cells [13]. Desmin is a muscle-specific intermediate filament protein and one of the best-known early myogenic markers for skeletal muscle [14]. The desmin genes found throughout the vertebrate phylum are highly homologous [15]. For these reasons, we chose to use MyoD and desmin as the myogenic markers in this study.

The chemical 5-Aza is a strong DNA demethylating agent that is utilized extensively to induce stem cells to differentiate into muscle cells. Currently, the molecular mechanisms by which 5-Aza induces myogenesis are unclear. Previous studies showed that 5-Aza inhibited methyltransferase and prevented the methylation of DNA at the C5 atom of the cytosine residue, leading to the reactivation of muscle-specific genes that had been silenced, MyoD in particular [16]. However, 5-Aza can cause extensive cell death and has low potency [17].

Matrix metalloproteinases (MMPs) are a family of highly homologous zinc-dependent endopeptidases that digest components of the extracellular matrix (ECM) as well as non-matrix proteins [18]. Twenty-eight members of the MMPs have been identified. Each MMP interacts specifically with certain elements of the ECM [19]. MMPs play important roles in regulating a spectrum of cell functions including myocyte migration and regeneration as well as the differentiation of muscle precursors [19]. It has been demonstrated that MMP-1, MMP-2, MMP-9 and MT1-MMP also play roles in the migration and differentiation of muscle satellite cells [4,20–22]. Wang et al. reported that MMP-1 enhances myoblast migration and differentiation in an *in vitro* wound-healing model. When myoblasts were induced with differentiation medium in the presence of MMP-1, their differentiation capacity increased in a dose-dependent manner. Moreover, when *mdx* mice were treated with MMP-1 (within their dystrophic skeletal muscles), the efficiency of myoblast transplantation was significantly improved [4].

In this study, we treated BMSCs with different concentrations of MMP-1 and examined the mRNA and protein expression of MyoD and desmin. Our results showed that both the mRNA and protein levels of MyoD and desmin were upregulated by MMP-1 in a dose-dependent manner. MMP-1 participates in the degradation of collagens type I, II and III. MMP-1 also cleaves non-matrix components and activates growth factors, kinases, cytokines, chemokines, membrane receptors, proteoglycans and perlecans [23]. MMP-1 regulates the fate determination of BMSCs and promotes their differentiation into muscle cells by degrading ECM and non-matrix components, thus altering cell morphologies as well as the stiffness of the ECM, and by up-regulating the expression of tension-induced protein-1 (TIP-1) and activating signaling pathways including the RhoA–Rho kinase pathway [24].

In conclusion, we found that MMP-1 significantly enhanced the myogenic differentiation of BMSCs *in vitro*. This finding sheds new light on BMSC transplantation as a therapy for muscular disorders.

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References

[1] H.K. Salem, C. Thiemermann, Mesenchymal stromal cells: current understanding and clinical status, Stem Cells 28 (2010) 585–596.
[2] J. Chan, K. O'Donoghue, M. Gavina, et al., Galectin-1 induces skeletal muscle differentiation in human fetal mesenchymal stem cells and increases muscle regeneration, Stem Cells 24 (2006) 1879–1891.
[3] E.J. Gang, D. Bosnakovski, T. Simsek, et al., Pax3 activation promotes the differentiation of mesenchymal stem cells toward the myogenic lineage, Exp. Cell Res. 314 (2008) 1721–1733.
[4] W. Wang, H. Pan, K. Murray, et al., Matrix metalloproteinase-1 promotes muscle cell migration and differentiation, Am. J. Pathol. 174 (2009) 541–549.
[5] W. Zhang, G. Ou, M. Hamrick, et al., Age-related changes in the osteogenic differentiation potential of mouse bone marrow stromal cells, J. Bone Miner. Res. 23 (2008) 1118–1128.
[6] R.S. Wong, Mesenchymal stem cells: angels or demons?, J Biomed. Biotechnol. 2011 (2011) 459–510.
[7] M.F. Pittenger, A.M. Mackay, S.C. Beck, et al., Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[8] A.S. Rowlands, P.A. George, J.J. Cooper-White, Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation, Am. J. Physiol. Cell Physiol. 295 (2008) C1037–C1044.
[9] M.E. Bernardo, A.M. Cometa, D. Pagliara, et al., Ex vivo expansion of mesenchymal stromal cells, Best Pract. Res. Clin. Haematol. 24 (2011) 73–81.
[10] G. Chamberlain, J. Fox, B. Ashton, et al., Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing, Stem Cells 25 (2007) 2739–2749.
[11] R.L. Perry, M.A. Rudnick, Molecular mechanisms regulating myogenic determination and differentiation, Front. Biosci. 5 (2000) D750–D767.
[12] C.A. Berkes, S.J. Tapscott, MyoD and the transcriptional control of myogenesis, Semin. Cell Dev. Biol. 16 (2005) 585–595.
[13] S. Etzion, I.M. Barbash, M.S. Feinberg, et al., Cellular cardiomyoplasty of cardiac fibroblasts by adenoviral delivery of MyoD ex vivo: an unlimited source of cells for myocardial repair, Circulation 106 (2002) I125–I130.
[14] Y. Capetanaki, D.J. Milner, G. Weitzer, Desmin in muscle formation and maintenance: knockouts and consequences, Cell Struct. Funct. 22 (1997) 103–116.
[15] M.L. Costa, R. Escaleira, A. Cataldo, et al., Desmin: molecular interactions and putative functions of the muscle intermediate filament protein, Braz. J. Med. Biol. Res. 37 (2004) 1819–1830.
[16] B. Balana, C. Nicoletti, I. Zahanich, et al., 5-Azacytidine induces changes in electrophysiological properties of human mesenchymal stem cells, Cell Res. 16 (2006) 949–960.
[17] A.C. Drost, S. Weng, G. Feil, et al., In vitro myogenic differentiation of human bone marrow-derived mesenchymal stem cells as a potential treatment for urethral sphincter muscle repair, Ann. N. Y. Acad. Sci. 1176 (2009) 135–143.
[18] S. Swarnakar, S. Paul, L.P. Singh, et al., Matrix metalloproteinases in health and disease: regulation by melatonin, J. Pineal Res. 50 (2011) 8–20.
[19] X. Chen, Y. Li, Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, regeneration and fibrosis, Cell Adh. Migr. 3 (2009) 337–341.
[20] M.P. Lewis, H.L. Tippett, A.C. Sinanan, et al., Gelatinase-B (matrix metalloproteinase-9; MMP-9) secretion is involved in the migratory phase of human and murine muscle cell cultures, J. Muscle Res. Cell Motil. 21 (2000) 223–233.
[21] F.E. El, Y. Torrente, N.J. Caron, et al., In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity, Exp. Cell Res. 258 (2000) 279–287.
[22] N.J. Caron, I. Asselin, G. Morel, et al., Increased myogenic potential and fusion of matrilysin-expressing myoblasts transplanted in mice, Cell Transplant. 8 (1999) 465–476.
[23] S. Kar, S. Subbaram, P.M. Carrico, et al., Redox-control of matrix metalloproteinase-1: a critical link between free radicals, matrix remodeling and degenerative disease, Respir. Physiol. Neurobiol. 174 (2010) 299–306.
[24] F. Guilak, D.M. Cohen, B.T. Estes, et al., Control of stem cell fate by physical interactions with the extracellular matrix, Cell Stem Cell 5 (2009) 17–26.