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Isolation of Mesenchymal Stem Cells From Shoulder Rotator Cuff: A Potential Source for Muscle and Tendon Repair

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The self-healing potential of each tissue belongs to endogenous stem cells residing in the tissue; however, there are currently no reports mentioned for the isolation of human rotator cuff-derived mesenchymal stem cells (RC-MSCs) since. To isolate RC-MSCs, minced rotator cuff samples were first digested with enzymes and the single cell suspensions were seeded in plastic culture dishes. Twenty-four hours later, nonadherent cells were removed and the adherent cells were further cultured. The RC-MSCs had fibroblast-like morphology and were positive for the putative surface markers of MSCs, such as CD44, CD73, CD90, CD105, and CD166, and negative for the putative markers of hematopoietic cells, such as CD34, CD45, and CD133. Similar to BM-MSCs, RC-MSCs were demonstrated to have the potential to undergo osteogenic, adipogenic, and chondrogenic differentiation. Upon induction in the defined media, RC-MSCs also expressed lineage-specific genes, such as Runx 2 and osteocalcin in osteogenic induction, PPAR-γ and LPL in adipogenic differentiation, and aggrecan and Col2a1 in chondrogenic differentiation. The multipotent feature of RC-MSCs in the myogenic injury model was further strengthened by the increase in myogenic potential both in vitro and in vivo when compared with BM-MSCs. These results demonstrate the successful isolation of MSCs from human rotator cuffs and encourage the application of RC-MSCs in myogenic regeneration.

Key words: Mesenchymal stem cells (MSCs); Rotator cuff (RC); Muscle repair

INTRODUCTION

The rotator cuff (RC), composed of four tendons of supraspinatus, infraspinatus, subscapularis, and teres minor muscles, is critical for the normal function of the shoulder joint. When the RC is disrupted (referred to as an RC tear), patients experience pain, limited motion, and weakness. An RC tear is a common injury of the shoulder joint; the prevalence of RC tears among the elderly is 22% (10). This injury is affected by both intrinsic and extrinsic factors. Intrinsic factors include poor blood supply over the critical zone and ischemic change of the tendon (32), while extrinsic factors include trauma, shape of the acromion, and overuse (29). The treatment for an RC tear usually consists of conservative treatment followed by surgery (28,30). The gold standard surgery of RC repair includes acromioplasty. For a large RC tear, muscle transfer or allograft with mesh can help restore function. When patients suffer a more severe tear, it is difficult to repair the injury and the tear will most likely become unrepairable. The shoulder joint will then degenerate, turning into severe osteoarthritis. It remains a challenge for surgeons to treat patients in this severe situation.

Cell-based therapies and tissue engineering might overcome the limitation of muscle transfer and the shortage of allografts in treating large or massive RC tears. Recent experiments with the use of fibroblasts seeded in chitosan-based hyaluronan hybrid scaffold or tenocytes seeded in porcine-derived bioscaffold have shown superior graft quality and osseointegration for RC reconstruction in a rabbit model (3,12). In particular, multipotent mesenchymal stem cells (MSCs) have initiated new therapeutic procedures to regenerate damaged or diseased skeletal tissues with long-term engrafting (2,7). MSCs were originally isolated from bone marrow (25)

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but have now be isolated from a variety of tissues such as the umbilical cord, placenta, amniotic fluid, liposuction waste, synovial tissues, and cruciate ligaments (5,11,16,19). MSCs can self-renew and differentiate into mesenchymal tissues such as bone, fat, and cartilage (25). Moreover, MSCs could also be induced to differentiate into skeletal muscle lineage cells and heal the damaged tendon in a rabbit model (9,24).

The self-healing potential of each tissue belongs to endogenous stem cells residing in the tissue; therefore, it is important to identify and isolate MSCs from RC tissues. Fatty degeneration (22), calcification (15), as well as chondroid metaplasia (15) have been reported in RC lesions. Therefore, we hypothesized that MSCs, which can be differentiated into fat, bone, and cartilage, could be isolated from the human RC. In this paper, we first isolated RC tissue-derived MSCs (RC-MSCs). We further characterized the surface marker profile of RC-MSCs and determined their potential to differentiate along osteogenic, adipogenic, and chondrogenic lineages. Furthermore, we compared the myogenic differentiation potential between bone marrow-derived MSCs (BM-MSCs) and RC-MSCs.

MATERIALS AND METHODS

Cell Isolation and Culture

Bone marrow samples were collected from five patients (28-, 33-, 42-, and 77-year-old males and 27-year-old female), while RC samples were collected from five patients (34-, 40-, and 60-year-old males and 45- and 56-year-old females). All of these patients received surgeries after the approval of protocol and informed consent form by the institutional review broad. To isolate BM-MSCs, bone marrow mononuclear cells isolated by density gradient centrifugation (Ficoll Paque, specific gravity = 1.077; GE Healthcare, Munich, Germany) were suspended in complete culture medium [CCM: α-MEM (α-minimal essential medium; Gibco-BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, USA)] and seeded at a density of $10^6/cm^2$. For isolation of RC-MSCs, RC samples were minced with scissors and digested with 3 mg/ml type I collagenase (Sigma-Aldrich, Milwaukee, WI, USA) for 6 h at 37°C. The cell suspension was filtered through a 40-μm cell strainer (BD Falcon; BD Biosciences, Bedford, MA, USA), and the nucleated cells were plated in six-well plastic dishes at clonal density and cultured in CCM. At 14 days after seeding, these cells were reseeded in 10-cm plastic dishes at an initial density of 4×10^3 cells/cm^2 and the culture medium was changed twice per week. After reaching 80% of confluence, they were replated at a ratio of 1:5.

Differentiation Protocol

For in vitro differentiation into osteoblasts, adipocytes, chondrocytes, and muscle cell, cells were induced in osteogenic induction medium [OIM: CCM supplemented with 50 μg/ml ascorbate-2 phosphate, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate (all from Sigma-Aldrich)], adipogenic induction medium [AIM: CCM supplemented with 50 μg/ml ascorbate-2 phosphate, 10^{-7} M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-1-methyl-xanthine and 10 μg/ml insulin (all from Sigma-Aldrich)], chondrogenic induction medium {CIM: cell pellets in serum-free α-MEM supplemented with, ITS+ [insulin, transferrin, selenous acid, bovine serum albumin (BSA) and linoleic acid; GIBCO] and 10 ng/ml transforming growth factor-β1 (TGF-β1; Preprotech, Rocky Hill, NJ, USA)}, and myogenic induction medium [MIM: CCM supplemented with 5% horse serum, 0.1 mM dexamethasone and 50 mM hydrocortisone (all from Sigma-Aldrich)], respectively. After the appearance of morphological features of differentiation, cells treated in OIM and AIM were stained for Alizarin Red S (ARS; Sigma-Aldrich) and Oil Red O (Sigma-Aldrich), respectively. Cells induced in CIM were prepared for Alcian blue (ScyTek Laboratories, West Logan, UT, USA) staining and type II collagen immunohistochemistry. Cells induced in MIM were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 10 min and then prepared for MyoD and myogenin immunohistochemistry (see later). For quantification of histochemistry or immunohistochemistry, the images were saved in a TIFF format for analysis with Image-Pro Plus 4.5 software (Media Cybernetics; Silver Spring, MD, USA) using histogram-based quantification (18).

Flow Cytometric Analysis

Specific surface antigens of isolated RC-MSCs were characterized by flow cytometry. Cells were harvested from culture dishes by treatment with 5 mM EDTA in PBS. Cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies with optimal concentration in 100-ml blocking buffer (PBS, 2% FBS) on ice for 30 min. Tested markers included cluster of differentiation 34 (CD34, BD Pharmingen, San Diego, CA, USA), CD44 (BD Pharmingen), CD45 (BD Pharmingen), CD73 (BD Pharmingen), CD90 (BD Pharmingen), CD105 (Ancell, Bayport, MN, USA), CD133 (Miltenyi Biotec, Auburn, CA, USA), and CD166 (Ancell). After incubation, cells were washed twice with PBS and fixed in 1% paraformaldehyde in PBS. Cells were analyzed using a fluorescence-activated cell sorter (FACS Vantage SE; Becton Dickinson, Mountain View, CA, USA) using a 525-nm bandpass filter for green

FITC fluorescence and a 575-nm bandpass filter for red PE fluorescence.

RT-PCR and Real-Time PCR

Total RNA was extracted using a TRIzol kit (Invitrogen). RNA was reverse transcribed in 20 μL using 0.5 μg of oligo dT and 200 U Superscript III RT (Invitrogen) for 30 min at 50°C, followed by 2 min at 94°C to inactivate the reverse transcriptase. PCR amplification of the resulting cDNAs was performed under the following conditions: 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The reaction products were resolved by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. β-Actin was used for endogenous reference in each sample.

For real-time PCR, the amplification was carried out in a total volume of 10 μl containing 0.5 μM of each primer, 5 μl of SYBR Green PCR Master Mix (Applied Biosystems, Framingham, MA, USA) and 4 μl of 1:20 diluted cDNA. PCR reactions were prepared in duplicate and heated to 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 15 s. Standard curves (cycle threshold values vs. template concentration) were prepared for each target gene and for the endogenous reference [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] in each sample. The quantification of the unknown samples was performed by the StepOne™ software version 2.0 (Applied Biosystems). The sequences of primers were listed in Table 1.

Western Blotting

Cell extracts were prepared with M-PER (Pierce, Rockford, IL, USA) plus phosphatase inhibitor (Halt™; Pierce) and protease inhibitor cocktail (Halt™; Pierce). Protein concentrations were determined using the BCA assay (Pierce). Aliquots of protein lysates were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide (JT Baker, Phillipsburg, NJ, USA) gels and transferred to polyvinylidene difluoride (PVDF) membrane filters (PerkinElmer, Boston, MA, USA), which were blocked with 5% blotting grade milk (Bio-Rad, Hercules, CA, USA) in TBST [20 mM Tris-HCl (pH 7.6, MDBio, Taipei, Taiwan), 137 mM NaCl (Merck), 1% Tween 20 (Sigma-Aldrich)]. Membranes were then probed with the indicated primary antibodies, reacted with corresponding secondary antibodies, and detected using a chemiluminescence assay (Millipore, Billerica, MA, USA). Membranes were exposed to X-ray film to visualize the bands (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primary antibodies were anti-MyoD (5.8A, sc-32758), anti-myogenin (F5D, sc-12732), and anti-β-actin (I-19, sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse antibodies (Santa Cruz Biotechnology).

In Vivo Muscle Regeneration Model

The animal research protocol was reviewed and approved by the animal center committee of National

Table 1. Primer Sets for PCR

Name	Primer Sequence	Size	NCBI Reference Sequence
β-Actin	F: GGACTTCGAGCAAGAGATGG	234	NM_001101.3
	R: AGCACTGTGTTGGCGTACAG		
Runx2	F: GTTTGTTCTCTGACCGCCTC	317	NM_001024630.3
	R: CCAGTTCTGAGGCACCTGAAA		
OC	F: CATGAGAGCCCTCACA	310	NM_199173.3
	R: AGAGCGACACCCTAGAC		
PPAR-γ	F: AGCCTCATGAAGAGCCTTCCA	120	NM_015869.4
	R: TCCGGAAGAAACCCTTGCA		
LPL	F: AATTTTTCCGTCTGCCCTTT	224	NM_000237.2
	R: AGCTTTCCCTTGAGGAGGAG		
Col2a1	F: TTCAGCTATGGAGATGACAATC	473	NM_033150.2
	R: CAGAGTCCTAGAGTGACTGAG		
Aggrecan	F: AAACCACCTCTGCATTCCAC	211	NM_001135.3
	R: TCTCCGCTGATTTCAGTCCT		
GAPDH	F: GTTCCAATATGATTCCACCC	400	M33197.1
	R: TGAGTCCTTCCACGATACC		
MyoD	F: GGGGCTAGGTTCAGCTTTCT	128	NM_002478.4
	R: CTACATTTGGGACCGGAGTG		
Myogenin	F: CCCTTTCCAGGGAGGTAAAG	100	NM_002479.4
	R: GAGGCCGCGTTATGATAAAA		

Ying-Ming University. BM-MSCs or RC-MSCs were infected with green fluorescent protein (GFP) lentivirus (RNAi core; Academic Sinica, Taipei, Taiwan) in the presence of 8 μg/ml polybrene (Sigma-Aldrich). At 24 h postinfection, medium was replaced with fresh growth medium containing puromycin (3 μg/ml) for 48 h to select infected cells. Male nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice were obtained from the Taiwan University Animal Center (Taipei, Taiwan). Care and use of the animals were in accordance with protocols approved by the Taipei Veterans General Hospital Institutional Animal Care and Use Committee. For muscle regeneration experiments, we injected 100 μg in 50 μl doxorubicin (Sigma-Aldrich) into the left side tibialis anterior muscle of NOD-SCID mice with age at 6–8 weeks. After 48 h, 5×10^5 BM-MSCs or RC-MSCs were injected into the same side of tibialis anterior muscle. The contralateral tibialis anterior muscle served as a control. Mice were sacrificed at 4 weeks for purposes of the experiment.

Histology and Immunohistochemistry

Specimens from in vitro chondrogenesis and in vivo muscle regeneration experiment were fixed in 4% paraformaldehyde in PBS before routine processing and paraffin embedding. Embedding specimens were performed on 4-μm tissue sections. Sections were stained with H&E (Medical Chemical Corporation, Torrance, CA, USA), Masson's trichrome (Sigma-Aldrich) and immunohistochemistry stain for histological examination. For immunohistochemistry, the sections from chondrogenesis were first reacted with primary antibodies against human type II collagen (Chemicon, Temecula, CA, USA) followed by incubation with biotinylated secondary antibodies (Biogenex, San Ramon, CA, USA). Detection was accomplished using streptavidin-peroxidase conjugate and diaminobenzidine (DAB) as a substrate (LAB Vision, Fremont, CA, USA). Counterstaining was carried out with hematoxylin. Sections from in vivo muscle regeneration experiments were used for double immunohistochemistry. Sections were stained first with primary antibody against MyoD (5.8A, sc-32758) or myogenin (F5D, sc-12732) (Santa Cruz Biotechnology) as described above. After incubation with DAB substrate, they were washed and blocked with 5% FBS. Afterward, slides were incubated with anti-green fluorescent protein (GFP) antibody (Genetex, Irvine, CA, USA), which was visualized with NBT substrate (Sigma-Aldrich). Finally, the slides were mounted and analyzed using an optical microscope.

Statistical Analysis

All values are expressed as mean ± SD. Comparisons between two groups were analyzed by the Student's *t* test. For comparisons of myogenic differentiation potential between BM- and RC-MSCs, nonparametric analysis using Mann–Whitney *U* test or Wilcoxon signed ranks test were applied. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Isolation of RC-Derived MSCs by Plastic Adherence

For isolation of MSCs from the human RC, cell suspension was seeded in plastic dishes after treatment of minced RC samples with collagenase, and cells were harvested by discarding the nonadherent cells during subsequent changes of the medium at 24 h. Three RC samples from patients who received RC reconstruction were used to isolate the cells. Cells were cultured in 10% FBS-supplemented αMEM, which was usually used to culture BM-MSCs. Like BM-MSCs, these cells adopted a fibroblastic-like morphology (Fig. 1A). Cells were recovered from the dishes when they reached 80% confluence and replated at a ratio of 1:5. To characterize whether cells isolated by plastic adherence from RC had the same profile of surface markers as BM-MSCs, flow cytometric analysis for several surface markers was performed. Cells had the same profile of surface markers with BM-MSCs; they were negative for the hematopoietic markers CD34, CD45, and CD133 and positive for CD44, CD73, CD90, CD105, and CD166, the putative markers of MSCs (Fig. 1B), indicating these cells had the same surface protein profile with BM-MSCs. We therefore refer to these cells as RC-MSCs.

Differentiation Potential of RC-Derived MSCs In Vitro

In order to examine whether RC-MSCs have a similar differentiation potential as BM-MSCs, cells from each sample were examined using the differentiation protocols that are routinely used for MSCs from a variety of sources. Commitments of various lineage-specific differentiations were identified by typical morphological changes or histo- and immunohistochemistry. In the case of osteogenic differentiation, cells were demonstrated to have matrix stained positive for Alizarin Red S staining. After 3 weeks of osteogenic differentiation, all samples were obviously positive by Alizarin Red S staining while cells cultured under control conditions were not stained by Alizarin Red S (Fig. 2A). Adipogenic differentiation was clearly demonstrated by the accumulation of Oil Red O-stained lipid vesicles. Although different in the intensity of Oil Red O staining, all samples induced to undergo adipogenic differentiation were stained by Oil Red O, while cells cultured under control conditions were not (Fig. 2B). Regarding chondrogenic differentiation, Alcian blue staining and type II collagen immunostaining were used to reveal deposition of glycosaminoglycan and type II collagen by pellet cultures, respectively. Similar to BM-MSCs, the RC-derived MSCs of all samples were found to undergo

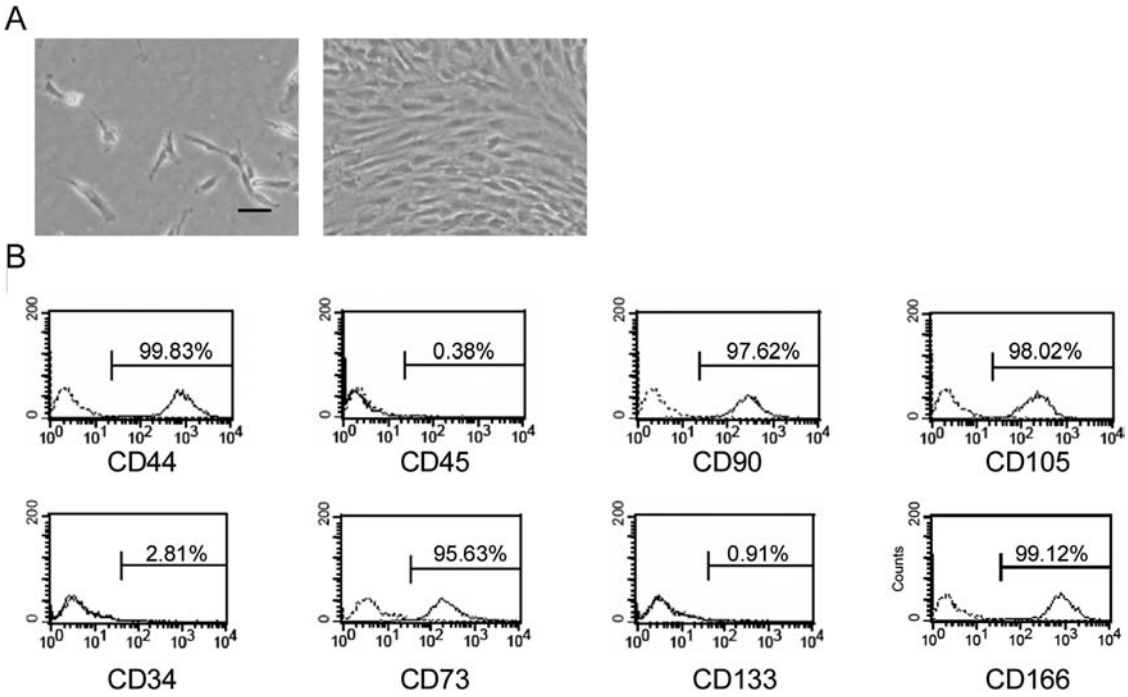

Figure 1. Morphology of rotator cuff-derived mesenchymal stem cells (RC-MSCs) at (A) initial seeding (left) and after confluence (right). (B) Characterization of the surface protein profile of RC-MSCs by flow cytometry. Scale bar: 50 μm.

differentiation to chondrocytes, with the expression of proteoglycan and type II collagen (Fig. 2C).

In order to confirm successful differentiation into various lineages, RT-PCR analysis of gene expression for markers of osteogenic, adipogenic, and chondrogenic differentiation was performed. RC-MSCs, shown to be tripotential by histo- and immunohistochemistry, also expressed runt-related transcription factor 2 (Runx2) and osteocalcin (OC) when induced in osteogenic differentiation medium, expressed peroxisome-proliferator activated receptor (PPAR)-γ and lipoprotein lipase (LPL) in adipogenic differentiation medium, and expressed aggrecan and Collagen, type II, α1 (Col2a1) upon undergoing chondrogenic differentiation while the control cells did not express these differentiated genes (Fig. 2D). These data suggest that RC-MSCs have the same differentiation potential as MSCs derived from other sources.

Myogenic Potential of RC-Derived MSCs In Vitro

Previous studies have shown that MSCs could be induced to differentiate into skeletal muscle lineage cells (9). Therefore, it is logical to assume RC-MSCs, which were isolated from muscle tendon, have better myogenic differentiation potential than MSCs derived from other origins. To demonstrate this, an in vitro myogenic differentiation protocol has been applied to compare myogenic potential between BM-MSCs and RC-MSCs (13). After 1 week of myogenic differentiation, BM-MSCs began to adopt flat and enlarged morphology, while RC-MSCs maintained the fibroblastic-like morphology and began to arrange in lines (Fig. 3A). MyoD and myogenin have been reported to be activated during myogenic differentiation (26). RT-PCR analysis (Fig. 3B) and real-time quantitative RT-PCR (Fig. 3C) revealed RC-MSCs increased in the expression of MyoD and myogenin after differentiation compared to BM-MSCs. After statistical analyses, we found the expression of MyoD and myogenin was significantly induced in RC-MSCs after induction of differentiation (Fig. 3D), while there were no differences in the expression of these genes between RC-MSCs and BM-MSCs without induction of differentiation and between BM-MSCs with and without induction of differentiation. Western blot analysis further confirmed that the protein levels of MyoD and myogenin were increased in RC-MSCs when compared to BM-MSCs (Fig. 4A). Further, immunocytochemistry demonstrated that the expression of MyoD and myogenin was observed in the nuclei after 1 week of differentiation (Fig. 4B). These data suggest that RC-MSCs have better myogenic differentiation potential than BM-MSCs.

Myogenic Potential of RC-Derived MSCs In Vivo

To compare the in vivo myogenic differentiation between BM-MSCs and RC-MSCs, a muscle regeneration model induced by doxorubicin (Dox) toxicity was applied (20,23). Injection of tibialis anterior muscle with 100 μg

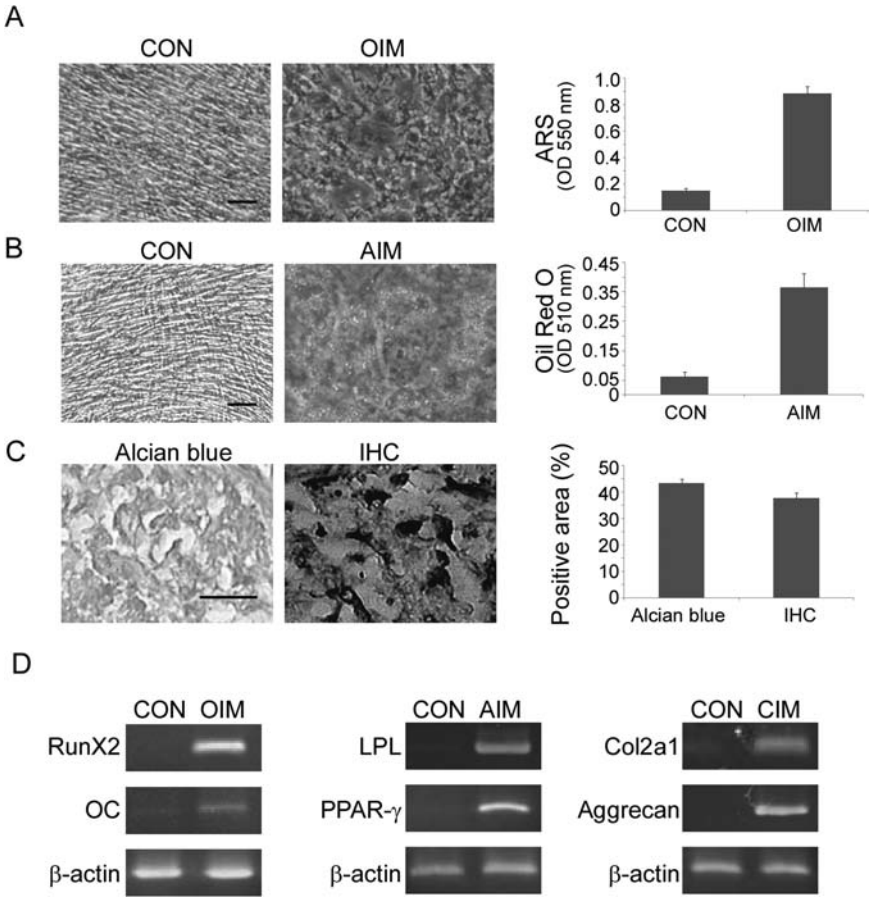

Figure 2. Differentiation potential of RC-MSCs. (A) Alizarin Red S (ARS) staining for RC-MSCs in control medium (CON) and osteogenic induction medium (OIM) for 3 weeks. Quantification of staining was by O.D. measurement of extracted dye. (B) Oil Red O staining for RC-MSCs in control medium (CON) and adipogenic induction medium (AIM) for 3 weeks. Quantification of staining was by O.D. measurement of extracted dye. (C) Alcian blue staining and immunohistochemical staining for type II collagen (IHC) for RC-MSCs in chondrogenic induction medium (CIM) for 3 weeks. Quantification of staining was by Image-Pro Plus. (D) RT-PCR detection of mRNAs of lineage-specific markers in RC-MSCs in control medium or in OIM for 3 days, AIM and CIM for 7 days. Scale bar: 50 μm. Runx2, runt-related transcription factor 2; OC, osteocalcin; LPL, lipoprotein lipase; PPAR-γ, peroxisome proliferator-activated receptor-γ; Col2a1, Collagen, type II, α1.

of Dox-induced muscle shrinkage at 4 weeks, while that injected with vehicle alone did not induce any change in size (Fig. 5A). Muscle samples that were injected with Dox and also received BM-MSCs or RC-MSCs 2 days later had increased in muscle size compared to those injected with Dox but only additionally receiving PBS (Fig. 5A). Histological examination also revealed muscle samples that were injected with Dox had decreased muscle fiber number and size compared to vehicle control, while those which also received BM-MSC or RC-MSC transplantation restored muscle number and size (Fig. 5B). Moreover, muscle number and size were greater in muscle samples treated with RC-MSCs compared to that treated with BM-MSCs (Fig. 5B). Masson's trichrome stain also showed that injection with Dox increased muscle fibrosis. If treated with BM-MSCs or RC-MSCs, there was decreased muscle fibrosis, with a greater effect with RC-MSCs (Fig. 5C). To track cell fate of transplanted cells, BM-MSCs and RC-MSCs were genetically engineered to express GFP constitutively followed by transplantation into Dox-injected muscle. Since some skeletal muscle fibers such as oxidative muscle fibers have bright green autofluorescence that may induce potential for stem cell engraftment artifacts (17), we used immunohistochemistry to track transplanted cells. Immunohistochemistry revealed RC-MSCs rather than BM-MSCs expressed MyoD and myogenin following transplantation (Fig. 5D). These data together suggest that RC-MSCs, compared to BM-MSCs, have increased muscle differentiation potential and the ability to regenerate Dox-induced muscle injury following transplantation in vivo.

DISCUSSION

The RC-MSCs isolated here had the same morphology and surface protein profile of BM-MSCs and were

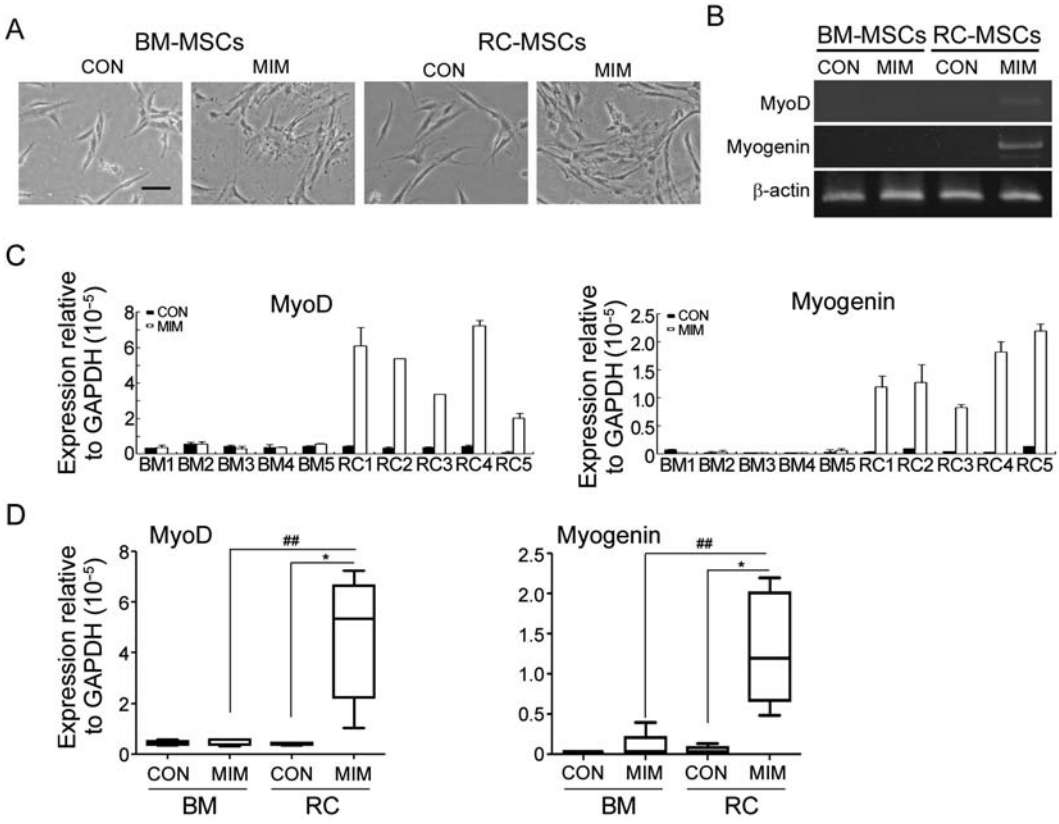

Figure 3. Myogenic differentiation potential of bone marrow (BM)-MSCs and RC-MSC in vitro. (A) Representative morphology, (B) representative RT-PCR, and (C) quantitative (real-time) RT-PCR analysis for myogenic markers of BM-MSCs and RC-MSCs in control medium (CON) and myogenic induction medium (MIM) for 1 week. The numbers listed after BM or RC indicate cells derived from different individuals. Moreover, BM and RC are not derived from the same individual. (D) Values are the relative mRNA levels of myogenic differentiation (MyoD) and myogenin before and after induction of differentiation counted from (C). $^{*}p<0.05$ (Wilcoxon signed ranks test); $^{\#\#}p<0.01$ (Mann–Whitney *U* test). Scale bar: 50 μm.

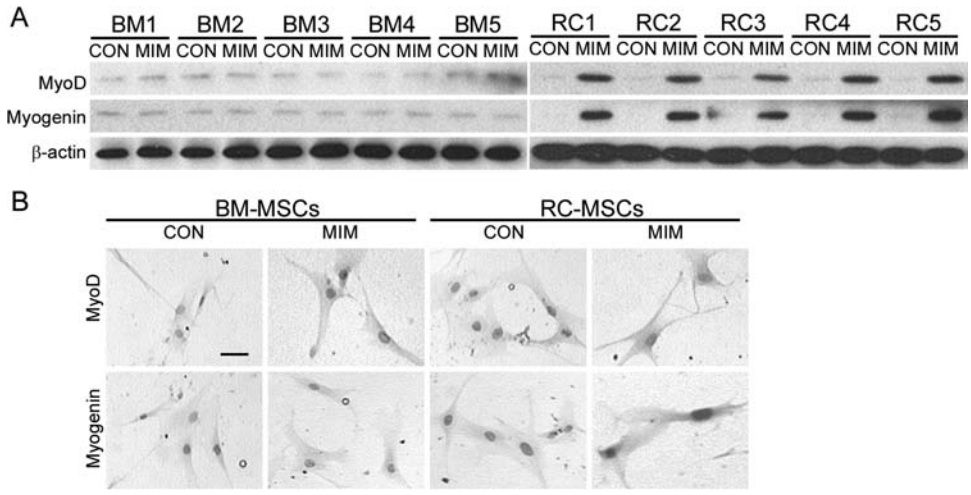

Figure 4. Expression of myogenic markers in BM-MSCs and RC-MSCs. (A) Western blotting and (B) representative immunohistochemistry analysis for myogenic markers of BM-MSCs and RC-MSCs in control medium (CON) and myogenic induction medium (MIM) for 1 week. The numbers listed after BM or RC indicate cells derived from different individuals. Moreover, BM and RC are not derived from the same individual. Scale bar: 50 μm.

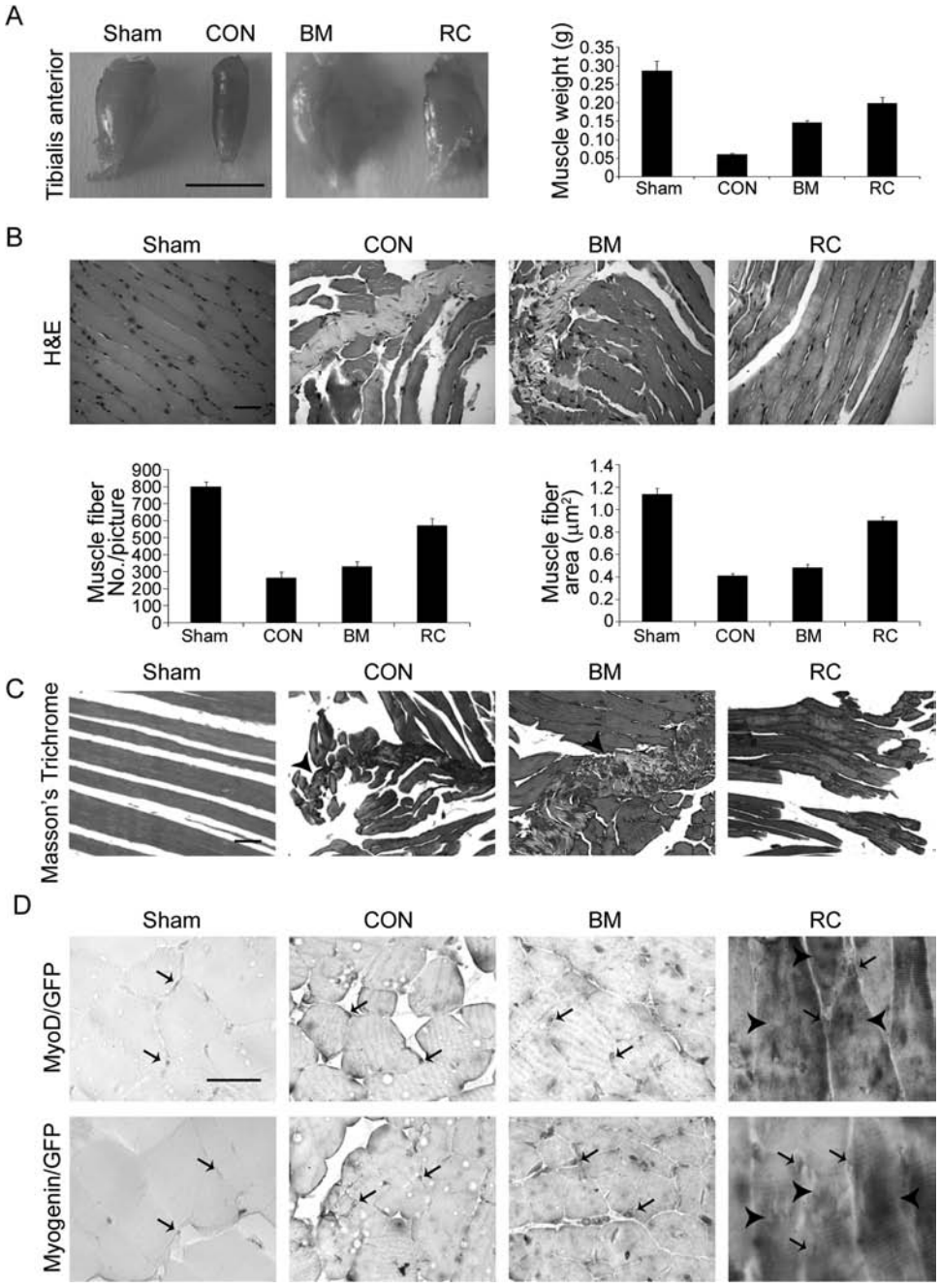

Figure 5. Muscle regeneration potential of BM-MSCs and RC-MSCs in vivo. (A, left) Gross morphology and (A, right) muscle weight (at 14 days) of tibialis anterior muscles without (Sham) or with 100 μg doxorubicin injection followed by injection with PBS (CON), 5×10^5 BM-MSCs (BM), or 5×10^5 RC-MSCs (RC) 2 days later. Scale bar: 5 mm. (B) H&E staining of tibialis anterior muscle from (A). Muscle fiber numbers and per muscle fiber size calculated from three to five randomized micropictures (200×). Scale bar: 50 μm. (C) Masson's trichrome staining of tibialis anterior muscle from (A). Scale bar: 50 μm. (D) Double immunohistochemical staining of tibialis anterior muscle from (A). Arrows indicate MyoD- or myogenin-positive areas, while arrow heads indicate GFP-positive areas. Scale bar: 50 μm.

demonstrated to have the potential to undergo osteogenic, adipogenic, and chondrogenic differentiation. In addition, RC-MSCs also expressed lineage-specific genes upon differentiation into mesenchymal lineages such as bone, fat, and cartilage. To our knowledge, there are no reports regarding the isolation of MSCs from RC tissues; therefore, this is the first paper to demonstrate the isolation of RC-MSCs. These data further support the hypothesis that MSCs, originally derived from bone marrow, reside in a variety of tissues (31) and are responsible for regeneration when tissues are injured or degenerated.

The differentiation potential of RC-MSCs was further enhanced by their increase in myogenic potential both in vitro and in vivo in a myogenic injury model. The myogenic differentiation potential of MSCs was first demonstrated in BM-MSCs, which were induced for myogenic differentiation in a defined medium that contained horse serum, dexamethasone, and hydrocortisone (13). Other sources of MSCs, such as umbilical cord blood (UCB-MSCs), can also be induced into myogenic differentiation and express MyoD and myogenin (13). RC-MSCs, which were isolated from muscle tendon, should have better myogenic differentiation potential compared to MSCs derived from other origins. Our RT-PCR and Western blot results showed that RC-MSCs expressed higher MyoD and myogenin than BM-MSCs after a week of myogenic differentiation, indicating the better myogenic potential of RC-MSCs. The osteogenic potential of BM-MSCs is lower than dental tissue-derived MSCs (14), while higher than adipose tissue-derived MSCs (27). We have also demonstrated that MSCs from ligaments have better potential than BM-MSCs to differentiate into ligament tissues (4). Moreover, MSCs from different sources have varied myogenic potential and skeletal muscle regeneration in vivo (8). These data together support the hypothesis that MSCs from different origins have unique profile of differentiation potential, albeit share the same morphology and profile of surface markers.

According to these data, RC-MSCs might become a suitable candidate for future treatment of muscular injury or degeneration. When muscle injury or chronic degenerative myopathy occurs, myogenic precursor cells, which are converted from satellite cells in the muscle fibers, divide and fuse to initiate the muscle repair mechanism (1). Because the percentage of satellite cells in postnatal mature muscle fiber is less than 5%, it is hard for these mature muscle fibers to regenerate once damages occur. Our in vivo data demonstrated that RC-MSCs could regenerate new muscle fibers just as synovial membrane-derived MSCs did in a previous study (6). Compared with BM-MSCs, RC-MSCs increase in muscular repair ability, indicating that RC-MSC is a potential candidate for cell sources of muscle regeneration.

For myogenic differentiation, Gang et al. demonstrate that UCB-MSCs expressed MyoD and myogenin after a week of differentiation but quickly lost the expression of these markers at 2 weeks (13). Moreover, the expression of other muscle-specific markers, such as fast-twitch myosin and myosin heavy chain, was detected after 3–6 weeks of myogenic differentiation. Another study also showed that myogenic precursor cells also expressed MyoD1 and myosin heavy chain during premyogenic differentiation status and fused together to form long and multinucleated myotubes after 3–6 weeks of myogenic differentiation (21). Due to the better myogenic differentiation potential of RC-MSCs, the expression of these later markers and cell fusion event might occur earlier in RC-MSCs than BM-MSCs and need to be further elucidated in future studies.

The capacity of stem cells for cell-based therapy is not only dependent on their multipotent differentiation ability but also on their ease for isolation. Here, we demonstrate that RC-MSCs can differentiate into several lineage-specific cells and have better myogenic potential than BM-MSCs. Moreover, the RC-MSCs in the current study were isolated from the torn RC sample, which were always discarded after surgery. In the future, we can isolate RC-MSCs from patient tissue samples and deliver these cells in a suitable scaffold to create engineered RC tissues for treatment in large and massive RC tears. In conclusion, our data suggest that human RC-MSCs could be another cell sources for cell therapies in patients with tendon or muscle injury or with dystrophic muscle disorders.

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