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Endogenous ROS levels are increased in replicative senescence in human bone marrow mesenchymal stromal cells

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ABSTRACT

Cellular senescence is characterized by functional decline induced by cumulative damage to DNA, proteins, lipids, and carbohydrates. Previous studies have reported that replicative senescence is caused by excessive amounts of reactive oxygen species (ROS) produced as a result of aerobic energy metabolism. In this study, we established human bone marrow mesenchymal stromal cells (hBM-MSCs) in replicative senescence after culture over a long term to investigate the relationship between ROS levels and stem cell potential and to determine whether differentiation potential can be restored by antioxidant treatment. Intracellular ROS levels were increased in hBM-MSCs; this was accompanied by a decrease in the expression of the antioxidant enzymes catalase and superoxide dismutase (SOD)1 and 2 and of phosphorylated forkhead box O1 (p-FOXO1) as well as an increase in the expression of p53 and p16, along with a reduction in differentiation potential. When the antioxidant ascorbic acid was used to eliminate excess ROS, the levels of antioxidant enzymes (catalase, SOD1 and 2, p-FOXO1, and p53) were partly restored. Moreover, differentiation into adipocytes and osteocytes was higher in hBM-MSCs treated with ascorbic acid than in the untreated control cells. These results suggest that the decline in differentiation potential caused by increased endogenous ROS production during in vitro expansion can be reversed by treatment with antioxidants such as ascorbic acid.

1. Introduction

Human bone marrow mesenchymal stromal cells (hBM-MSCs) can differentiate into various cell types [1–3], and also act as trophic mediators by secreting growth factors [4,5] that provide protection against oxidative stress and inflammation. As such, hBM-MSCs have many potential applications in stem cell medicine. However, the efficacy of stem cell therapy differs markedly depending on donor age and health [4,6,7]. To overcome this obstacle, cells must be enhanced prior to use.

Primary cell cultures from human tissues have a limited life span and gradually enter a state of senescence, which is characterized by telomere shortening and a decline in telomerase activity [8,9], which is known as the Hayflick limit [10,11]. Replicative senescence is caused by damage to cellular DNA [12], proteins [13,14], lipids, and carbohydrates induced by an excess of intracellular reactive oxygen species (ROS) that impair DNA repair and cell signaling and thereby accelerate cellular senescence. Progressive senescence in different types of primary cell cultures can be blocked by application of antioxidant agents [15,16], and it has been suggested that removing excess ROS can abolish replicative senescence.

ROS are by-products of aerobic energy metabolism that regulate various cellular processes, including proliferation, apoptosis, differentiation, and cellular senescence [17,18]. Excessive ROS production also suppresses the transcription of genes involved in cellular differentiation and adhesion and mitochondrial function [19,20], and induces the upregulation of the tumor suppressor genes p53, p16, and p21 [21,22]. Thus, ROS are a major cause of functional decline during typical cellular senescence.

Abbreviations: AP, alkaline phosphatase; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; hBM-MSCs, human bone marrow-mesenchymal stromal cells; FOXO1, forkhead box O1; MTT, 3(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SA-β-gal, senescence associated-β-gal; SOD, superoxide dismutase.

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The antioxidant enzymes superoxide dismutase 1 (SOD1/Cu-ZnSOD) and 2 (SOD2/MnSOD) are major scavengers of endogenous ROS [23]. A mutation in the *SOD1* gene is associated with familial amyotrophic lateral sclerosis and impairment of mitochondrial function [24], while SOD1 deficiency leads to age-related pathologies [25]. In *Drosophila*, SOD mutation compromises heart function as a result of ROS accumulation [26]. In stem cells, forkhead box (Fox) O proteins regulate ROS production by modulating SOD and catalase expression [27] in conjunction with mutated in ataxia-telangiectasia (ATM) [28]. This cross-talk with the ATM-p53 pathway converges on p16 to induce stem cell senescence [29]. Thus, cellular senescence is closely associated with antioxidant enzyme deficiency and the intracellular accumulation of ROS. However, this has never been examined in hBM-MSCs. In this study, we investigated the relationship between ROS levels and the differentiation potential of hBM-MSCs and tested whether the latter can be restored by antioxidant treatment.

2. Materials and methods

2.1. hBM-MSC culture

The hBM-MSCs were purchased from Cell Engineering For Origin (Seoul, Korea). Cells were negative for viral infection and mycoplasma contamination, and flow cytometric analysis revealed a CD73+/CD105+/CD31− phenotype (data not shown). Cells ($0.9 \times 10^3/cm^2$) were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone; Logan, Utah, USA), L-glutamine, penicillin, and streptomycin at 37 °C in a humidified incubator of 95% air and 5% CO_2. Cells were subcultured every 5 days, with medium replacement every 3 days.

2.2. Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining was carried out using the Senescence β-Galactosidase Staining kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Cells were seeded in 6-well plates at a density of 1×10^4 cells/well and incubated until confluence was reached. Cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde and 0.2% glutaraldehyde in distilled water for 15 min at room temperature, then washed twice with PBS containing 1 mM $MgCl_2$ (pH 7.2) and stained overnight in β-galactosidase staining solution containing 1 mg/ml X-gal, 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, 2 mM $MgCl_2$, 40 mM citric acid/sodium phosphate (pH 6.0), and 150 mM NaCl in distilled water at 37 °C without CO_2. Staining was visualized by light microscopy (Nikon Eclipse TS100; Tokyo, Japan) and images were acquired with a Canon i-Solution IMTcam3 digital camera (Tokyo, Japan).

2.3. Immunoblot analysis

Total protein was extracted from hBM-MSCs of different passages with 400 μl radioimmunoprecipitation buffer containing phenylmethylsulfonyl fluoride, sodium orthovanadate, and protease inhibitor cocktail (Santa Cruz Biotechnology, Dallas, Texas, USA) for 30 min at 4 °C and centrifuged at 16,000 × *g* for 20 min. Protein samples were then analyzed by immunoblotting with antibodies against catalase (1:500), SOD1 (1:500), SOD2 (1:5000), FOXO1 (1:500), p-FOXO1 (1:1000), p53 (1:500), p16 (1:500), and β-actin (1:5000), followed by the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

2.4. Detection of intracellular ROS

Intracellular ROS levels were measured using the cell permeable substrate 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma–Aldrich, St. Louis, MO, USA), which is converted to the detectable fluorescent product 2′,7′-dichlorodihydrofluorescein. hBM-MSCs were treated with ascorbic acid or left untreated for 2 days at 37 °C, followed by incubation with 20 μM DCFH-DA at 37 °C for 1 h. After washing with PBS, cells were fixed with 4% paraformaldehyde for 10 min and mounted with 4,6-diamidino-2-phenylindole-containing mounting medium (ProLong Gold Antifade Reagent; Molecular Probes, Eugene, OR, USA). Cells were visualized by fluorescence microscopy with an Eclipse 80Ti microscope and images were acquired with a DS-Rl1 digital camera (Nikon).

2.5. 3(4,5-Dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide (MTT) assay

The toxicity of ascorbic acid was evaluated with the MTT assay (Sigma–Aldrich) according to the manufacturer's instructions. Briefly, 8×10^3 cells were seeded in 96-well plates. The following day, cells were incubated with 0–2000 μM ascorbic acid for 2 days and then assayed.

2.6. Osteocyte and adipocyte differentiation

The potential for ascorbic acid-treated passage (P)-17 hBM-MSCs to differentiate into osteocytes and adipocytes was evaluated as previously described [30,31]. For osteogenic differentiation, cells were cultured for 14 days with osteogenic medium consisting of DMEM (Gibco) with 10% FBS, 100 nM dexamethasone (Cayman Chemical, Ann Arbor, Michigan, USA), 100 μM L-ascorbic acid (Sigma–Aldrich), and 10 mM β-glycerophosphate (Sigma–Aldrich). The medium was replaced every 3 days. To assess the efficiency of osteogenic differentiation, cells were fixed in 4% paraformaldehyde for 15 min, and then stained with 1% Alizarin Red S for 20 min at room temperature, with staining solution prepared by dissolving 1 mg Alizarin Red S powder (Sigma–Aldrich) in 1 ml distilled water. For adipogenic differentiation, cells were seeded in 24-well plates and incubated for 14 days with adipogenic medium composed of DMEM, 10% FBS, 500 μM 3-isobutyl-1-methylxanthin (Cayman Chemical), 1 μM dexamethasone, 100 μM indomethacin (Cayman Chemical), and 10 μg/ml insulin (Tocris Bioscience, Bristol, UK). Differentiated cells were identified by staining with Oil Red O (0.7 g Oil Red O powder in 200 ml of 100% isopropanol). Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. After washing twice with distilled water, cells were stained with Oil Red O for 10 min at room temperature, and visualized by microscopy with an Eclipse TS100 microscope. Images were captured with an i-Solution IMTcam3 digital camera.

2.7. Quantitative real-time (qRT)-PCR

hBM-MSCs were treated with 500 μM ascorbic acid or left untreated, and total RNA was extracted using RNAiso reagent (Takara Bio; Otsu, Japan) according to the manufacturer's instructions. The Primescript II 1st Strand cDNA Synthesis kit (Takara Bio) was used to reverse transcribe 3 μg of RNA using 5 μM of oligo(dT) primers, 1 mM each dNTP, and the supplied buffer. First-strand cDNA was amplified using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) with gene-specific primers for human *adiponectin*, *fatty acid binding protein* (*FABP*)*4*, *alkaline phosphatase* (*AP*), *runt-related transcription factor* (*RUNX*)*2*, *matrilin*, *musashi*, *nestin*, or *β-actin*. The cycling parameters were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for

1 min. Primers were synthesized by GenoTech (Daejeon, South Korea) and Integrated DNA Technologies, Coralville, IA, USA); sequences are listed in Supplemental Table S1.

2.8. Statistical analysis

Data are presented as mean ± standard deviation of the mean of three or more independent experiments. Statistical comparisons between groups were made with an independent t test. P values <0.05 were considered statistically significant.

3. Results

3.1. Long-term hBM-MSC cultures undergo replicative senescence

Replicative senescence in hBM-MSCs cultured over a long term was characterized with the SA-β-gal assay. The number of SA-β-gal-positive cells increased with the number of passages, from 29% at P-7 to 81% at P-17 ($P < 0.05$, $n = 3$; Fig. 1A, B). Moreover, the expression of the senescence marker p16 was higher at P-14 and at −17 than at P-7 (Fig. 1C).

3.2. Endogenous ROS levels are increased in long-term hBM-MSC cultures

To evaluate differences between early and late passage hBM-MSCs, endogenous ROS levels were measured by enzyme-linked immunosorbent assay of DCFH-DA-stained cells. ROS levels increased with passage ($P < 0.01$, $n = 6$; Fig. 2A, B). An immunoblot analysis with antibodies against p53, catalase, SOD1, SOD2, and FOXO1, which are associated with ROS modulation, revealed that the level of p53—which functions as a gatekeeper against ROS damage—was increased, whereas the levels of the antioxidant proteins SOD1, SOD2, and catalase were decreased in P-17 hBM-MSCs. The phosphorylation of the ROS-responsive transcription factor FOXO1 was similarly decreased (Fig. 2C), suggesting that excess intracellular ROS accumulation accompanies the reduction in antioxidant enzyme levels in long-term cultures of hBM-MSCs.

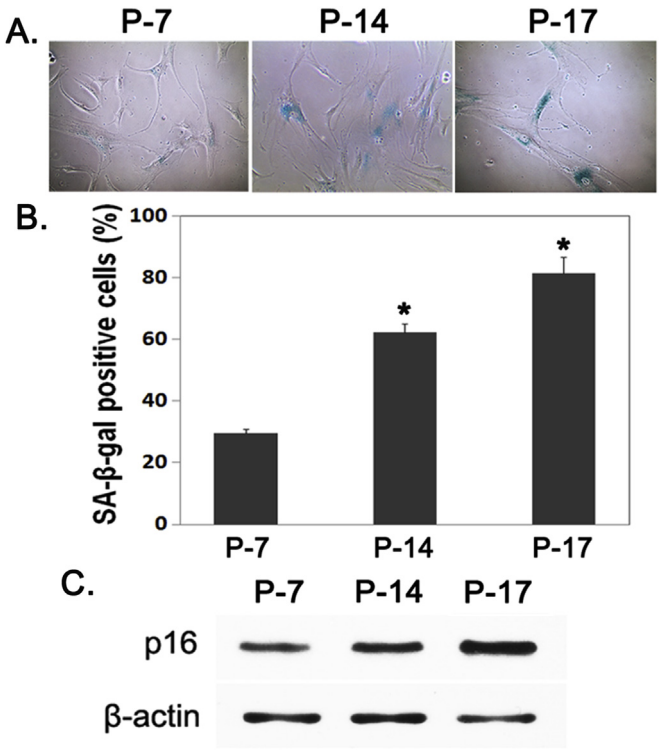

Fig. 1. Replicative senescence in long-term hBM-MSC cultures. (A) The activity of β-galactosidase increased with the number of passages. SA-β-gal-positive cells are visible by a blue color. (B) Quantitative analysis of senescent cells. (C) Expression of the senescence protein p16 from P-7, -14, and −17 hBM-MSCs was detected by immunoblotting, with β-actin used as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Treatment with ascorbic acid restores antioxidant enzyme levels in hBM-MSCs

Excess ROS in P-17 hBM-MSCs were quenched by treatment for 2 days with the antioxidant agent ascorbic acid [27]. ROS levels were measured by DCFH-DA staining and enzyme-linked immunosorbent assay. Ascorbic acid concentrations of 500–2000 μM decreased ROS levels ($P < 0.005$, $n = 7$; Fig. 3A). In the SA-β-gal assay, fewer β-gal-positive cells were observed upon treatment with 500 μM ascorbic acid relative to untreated cells (Fig. 3B, upper panel). Similar results were obtained by immunoblot analysis of p16 expression (Fig. 3B, lower panel). Cellular toxicity was not observed for ascorbic acid concentrations below 500 μM; however, concentrations of 1000–2000 μM were toxic, as determined by the MTT assay ($P < 0.05$, $n = 5$; Fig. 3C).

To examine the effect of depleting ROS from long-term cultures, P-17 hBM-MSCs were treated with 500 μM ascorbic acid and the expression of antioxidant enzymes was measured by immunoblotting. Catalase, SOD1 and 2, and p-FOXO1 levels were reduced in P-17 as compared to P-7 hBM-MSCs, whereas the levels were increased by ascorbic acid treatment (Fig. 3D, P-17/AA). Moreover, the expression of the senescence-related protein p53 was downregulated in P-17 ascorbic acid-treated relative to untreated hBM-MSCs (Fig. 3D). These data indicate that ascorbic acid suppresses excessive ROS generation and partially restores the expression of antioxidant enzymes in long-term cultures of hBM-MSCs.

3.4. Restoration of hBM-MSC differentiation potential by ascorbic acid

hBM-MSCs can differentiate into diverse cell types, including osteocytes and adipocytes [2]. To determine whether antioxidant treatment can restore the differentiation potential of hBM-MSCs cultured over a long term, the expression of tissue-specific progenitor marker genes—including *adiponectin* and *FABP4* (adipocytes); *AP* and *RUNX2* (osteocytes); *matrilin* (chondrocytes); *musashi* and *nestin* (neurons)—in P-17 hBM-MSCs was measured by qRT-PCR. The expression of all marker genes was downregulated in hBM-MSCs at P-17 as compared to P-7; however, levels in ascorbic acid-treated P-17 hBM-MSCs were comparable to those of untreated P-7 hBM-MSCs ($P < 0.05$ or $P < 0.005$, $n = 4$; Fig. 4A). To confirm these findings, untreated and ascorbic acid-treated P-17 hBM-MSCs were stimulated to differentiate into osteocytes and adipocytes. Differentiation potential relative to P-7 cells was reduced in untreated but similar in ascorbic acid-treated hBM-MSCs (Fig. 4B). These results suggest that the decline in differentiation potential caused by excess ROS can be restored by ascorbic acid treatment.

4. Discussion

States of cellular senescence and associated functional decline can undermine the efficacy of stem cell therapy [7,32]. The risks are increased when cells are expanded in long-term cultures that accumulate ROS [10,11]. In the present study, we established a long-term culture of hBM-MSCs in replicative senescence and examined

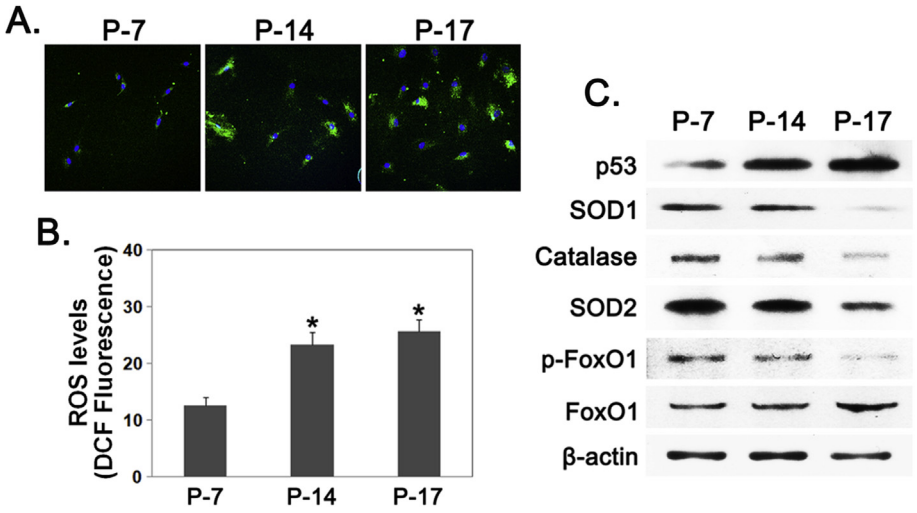

Fig. 2. ROS production is increased in long-term hBM-MSC cultures. (A) Endogenous ROS levels (green) were evaluated in hBM-MSCs stained with DCFH-DA by fluorescence microscopy. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue). (B) Quantitative analysis of ROS level by enzyme-linked immunosorbent assay of DCFH-DA-stained cells. (C) Total protein was extracted from P-7, P-14, and P-17 hBM-MSC, and p53, catalase, SOD1, SOD2, FoxO1, and p-FoxO1 expression was assessed by immunoblotting, with β-actin used as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the relationship between ROS levels and stem cell potential. As expected, the expression of senescence markers increased whereas the potential for differentiation was reduced with the number of passages.

Steady-state, intracellular ROS generation has many beneficial effects, such as enhancing cell proliferation and migration [33]; however, an excess of ROS can lead to the degradation of DNA, proteins, and lipids, which can in turn block gene expression and induce apoptosis [34,35], and can also perturb stem cell differentiation [36,37]. Here, we found that ROS accumulated during hBM-MSC expansion, leading to a decrease in differentiation potential.

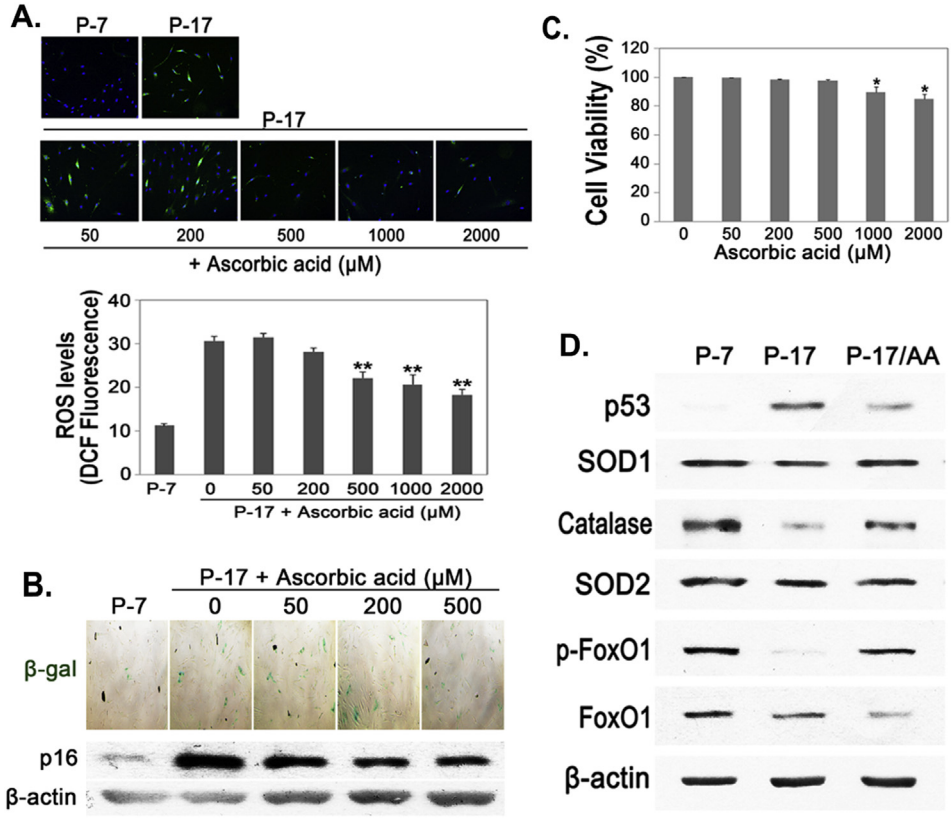

Fig. 3. Modulation of excess ROS and antioxidant enzyme expression by ascorbic acid. (A) Intracellular ROS was detected in P-17 hBM-MSCs treated with indicated concentrations of ascorbic acid (upper panel) and quantified by enzyme-linked immunosorbent assay of DCFH-DA-stained cells (lower panel). (B) SA-β-gal activity was assessed in untreated P-7 and P-17 and ascorbic acid-treated P-17 cells (upper panel); p16 protein expression was detected by immunoblotting (lower panel). (C) Cell viability in ascorbic acid-treated hBM-MSCs was evaluated with the MTT assay. (D) Untreated P-7 and -17 and ascorbic acid (AA)-treated P-17 hBM-MSCS were examined for catalase, SOD1, SOD2, FoxO1, p-FoxO1, and p53 expression by immunoblotting. β-actin was used as a loading control for immunoblotting experiments.

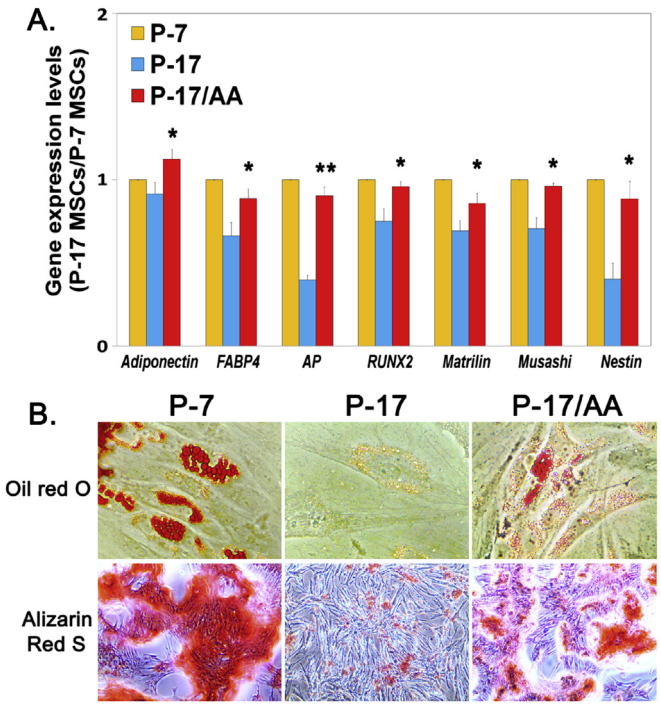

Fig. 4. Differentiation potential of hBM-MSCs is restored by ascorbic acid. (A) The expression of progenitor marker genes in untreated P-7 and -17 and ascorbic acid (AA)-treated P-17 hBM-MSCS was evaluated by qRT-PCR. (B) Differentiation into adipocytes (upper panels) and osteocytes (lower panels) was assessed by staining with Oil Red O and Alizarin Red S, respectively.

The antioxidant enzymes SOD1, SOD2, and catalase are key regulators of ROS level [23], with SOD expression regulated by p53 and FOXO1 [38]. In the present study, SOD1, SOD2, and catalase expression was downregulated at P-14 and -17; this was accompanied by a decrease in FOXO1 phosphorylation. Based on these findings, we suggest that increased levels of ROS in long-term hBM-MSCs cultures alter the expression of antioxidant enzymes, further stimulating the production of intracellular ROS.

We tested whether the increase in ROS levels caused by long-term culture of hBM-MSCs could be reversed by treatment with ascorbic acid, a widely used antioxidant [39,40]. We found that a 2-day treatment with 500 μM ascorbic acid reduced endogenous ROS in P-17 hBM-MSCs and restored the expression of catalase, SOD1, SOD2, p-FOXO1, and p53. Differentiation potential was likewise restored by the treatment, as evidenced by the increase in expression of various progenitor cell-specific markers and enhanced differentiation into adipocytes and osteocytes. These results suggest that scavenging excess intracellular ROS using antioxidants can prevent the functional decline of stem cells resulting from replicative senescence.

In this study, we determined that the increased ROS levels and reduced antioxidant enzyme expression in long-term cultures of hBM-MSCs could be partly reversed by ascorbic acid treatment. This indicates that elevated ROS production likely plays a crucial role in the progression of replicative senescence, and that reducing the levels during long-term expansion may yield stem cells of a higher quality, thereby improving the therapeutic efficacy of stem cell therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.136.

Transparency document

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