Biochemical and Biophysical Research Communications 422 (2012) 121–127

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Potential role of 20S proteasome in maintaining stem cell integrity of human bone marrow stromal cells in prolonged culture expansion

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ARTICLE INFO

Article history:
Received 18 April 2012
Available online 30 April 2012

Keywords:
Proteasome
Replicative senescence
Stemness

ABSTRACT

Human bone marrow stromal cells (hBMSCs) could be used in clinics as precursors of multiple cell lineages following proper induction. Such application is impeded by their characteristically short lifespan, together with the increasing loss of proliferation capability and progressive reduction of differentiation potential after the prolonged culture expansion. In the current study, we addressed the possible role of 20S proteasomes in this process. Consistent with prior reports, long-term in vitro expansion of hBMSCs decreased cell proliferation and increased replicative senescence, accompanied by reduced activity and expression of the catalytic subunits PSMB5 and PSMB1, and the 20S proteasome overall. Application of the proteasome inhibitor MG132 produced a senescence-like phenotype in early passages, whereas treating late-passage cells with 18α-glycyrrhetinic acid (18α-GA), an agonist of 20S proteasomes, delayed the senescence progress, enhancing the proliferation and recovering the capability of differentiation. The data demonstrate that activation of 20S proteasomes assists in counteracting replicative senescence of hBMSCs expanded in vitro.

1. Introduction

Human bone marrow stromal cells (hBMSCs) constitute a multipotent adult stem cell population capable of differentiation into various cell types upon appropriate induction [1,2]. To obtain the sufficient numbers for clinical application, hBMSCs are routinely propagated in vitro following the primary isolation. Nevertheless, the culture-expanded cells are notorious for their short proliferative lifespan, characterized by the morphologic heterogeneity, retarded cell growth, and progressive loss of differentiation potential after limited number of divisions [3].

Replicative senescence accompanies cell mitosis from the first passage onwards, but the underlying mechanisms are unclear. Such senescence may depend on environmental insults and certain genetic predispositions. At the subcellular level, senescence is displayed as DNA damage, oxidative injuries, and accumulation of pathogenic substances [4–6].

Proteasomes are the major components of the mammalian proteolytic system which degrade misfolded, damaged, or excessive numbers of intracellular proteins to preclude their pathologic accumulation [7]. The 20S particle, consisting of 28 subunits to form the quadruple-stacked heptameric rings with a molecular mass of approximately 700 kDa in mammalian cells, is the core element and constitutes the classic 26S proteasome complex together with two 19S regulatory caps [8]. Although the quantity of specified subunits varies in different species, the principle for designated protein removal is evolutionarily conserved [9]. All 20S particles comprise α and β subunits: the former acts as the docking domain or the gate preventing the unplanned entry of substrates, while the later subserves as the essential catalytic site, consisting of subunits β1–7 [8,10]. Previous reports have documented that the proteolytic activities and expression levels of several subunits are substantially decreased in human dermal fibroblasts obtained from aged donors [11]; proteolytic activity and subunit expression also fall in human embryonic fibroblasts with increase in passage number [12]. While partial inhibition of proteasomes in juvenile cells induces a senescence-like phenotype [12], overexpression of subunits β1 and β5 [7,12] restores the impaired activity. Similarly, stimulating the 20S proteasome by 18α-glycyrrhetinic acid (18α-GA) enhances the transcriptional activity of nuclear erythroid factor 2, subsequently delaying the aging process in human fibroblasts [13].

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.119

In this study, we investigated whether the 20S proteasome potentially regulates hBMSC's replicative senescence during culture expansion, by analyzing the responses following drug treatments. Given the significance of the 20S proteasome in retaining cellular homeostasis, identifying its role in hBMSC's aging may permit large-scale production of cells with unchanged vigor.

2. Materials and methods

2.1. Cellular models

Human bone marrow stromal cells (hBMSCs) obtained from ScienCell Research Laboratory (San Diego, CA) were maintained as previously described [14]. Cells were 1/2 subcultivated upon 90% confluence, and studied from passages 2–14.

2.2. Senescence-associated β-galactosidase staining

Activity of intracellular senescence-associated β-galactosidase (SA-β-gal) was estimated with the SA-β-gal Staining Kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Senescent cells expressing SA-β-gal were stained blue.

2.3. Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was measured using the BrdU incorporation method as previously documented [15]. Samples sequentially stained with anti-BrdU (Abtech Biotechnology, South Yorkshire, UK) and Cy3-conjugated anti-mouse antibodies (Invitrogen, Carlsbad, CA) were observed by fluorescent microscopy (Olympus BX51, Tokyo, Japan).

2.4. Western-immunoblotting

Procedure and reagents were as recorded before [16]. The antibodies used for detection were: anti-β actin (Sigma–Aldrich, St. Louis, MO); anti-20S α + β, anti-PSMB5, anti-mouse and anti-rabbit IgG-HRP (Abcam, Cambridge, MA).

2.5. Immunophenotypic analysis

Cells were immunostained as previously mentioned [14], using the following primary antibodies: anti-proteasome 20S α + β (Abcam), anti-human CD34-PE, anti-human CD44-FITC, anti-human CD71-FITC (BD Pharmingen, Temecula, CA), and anti-human CD90-Cy3 (Millipore, Billerica, MA).

2.6. 20S proteasome activity assay

Protein lysates were prepared as mentioned above, and proteasomal activity was determined with the 20S Proteasome Activity Assay Kit (Millipore) following the manufacturer's instruction.

2.7. Cell proliferation assay and growth curve

Cell proliferation was estimated using the MTT Cell Growth Assay Kit (Millipore) following the company's recommendation. Dye absorbances at 570 nm were obtained with the xMark Microplate Spectrophotometer (Bio-Rad, Hercules, CA).

To identify potentially different propagation rates due to senescence, cells of the early and late stages were initially seeded into 24-well plates at a density of 8000 cells per well, and allowed to recover overnight. Thereafter, cells were trypsinized and recounted every 24 h for five consecutive days. The cell quantity at each time point was averaged from three replicate wells and normalized to the initial number recorded at time point zero.

2.8. Real-time PCR

Gene expression levels were quantified as previously described [16]. Primers utilized in the assays are listed in Supplementary Table 1.

2.9. Drug treatment

hBMSCs were incubated with the proteasome blocker MG132 (Beyotime) or equal volumes of DMSO for 2 h, followed by recovery in normal media for 22 h. Such cyclic treatments were repeated for additional 3 times. In contrast, hBMSCs were continuously treated with the proteasome activator 18α-GA (Sigma–Aldrich) for 4 weeks. Upon completion of drug treatments, cells were used for designated assays immediately.

2.10. Neural differentiation assay

The neural differentiation ability was evaluated as previously described [14]. Differentiated cells were identified using antibodies against neural marker Tuj1 (Covance, Berkeley, CA) and glial marker GFAP (Millipore).

2.11. Statistical analysis

The quantitative results are presented as mean ± SEM. Student's *t*-test and one-way ANOVA were applied in comparing 2 or $\geqslant 3$ sets of data, respectively. At least three replicates were used in each testing group, and $P < 0.05$ was considered significant.

3. Results

3.1. Effects of culture expansion on cell proliferation and replicative senescence of hBMSCs

We first examined the changes accompanying in vitro expansion of hBMSCs. Using a commonly-followed method [6], hBMSCs were divided into the early (P2–4), middle (P7–9) and late (P12–14) stages according to their passage numbers. Consistent with previous reports [17,18], culture-expanded hBMSCs initially exhibited the typical spindle-like morphology in the early stage (Fig. 1Aa). Following repeated subculture, they became considerably enlarged, appearing flattened and irregular in shape. Their nuclei concomitantly became more circumscribed by phase-contrast microscopy, with granule-like inclusions and aggregations increasingly produced in the cytosol (Fig. 1Ab). We next evaluated the cell proliferation capability following in vitro expansion.

The propagation rates of hBMSCs in the early and late stages were significantly different from each other. As illustrated in Fig. 1B, unlike cells in the early stage which kept actively growing, those of passages 12–14 propagated much more slowly, whose number was not doubled even 96 h later. Since retarded growth could indicate cell aging, we subsequently checked whether these changes during expansion indeed suggest cell replicative senescence.

The percentage of senescent cells was quantified by the SA-β-gal assay. As expected, in the early stage, very few were positively stained (9 ± 1%; Fig. 1Ca and d), whereas blue cells were significantly increased in the middle (53 ± 2%; Fig. 1Cb and d) and the late stage (87 ± 1%; Fig. 1Cc and d). We again estimated if cell proliferation capability was really compromised utilizing the BrdU incorporation assay. The percentages of BrdU-positive hBMSCs in the

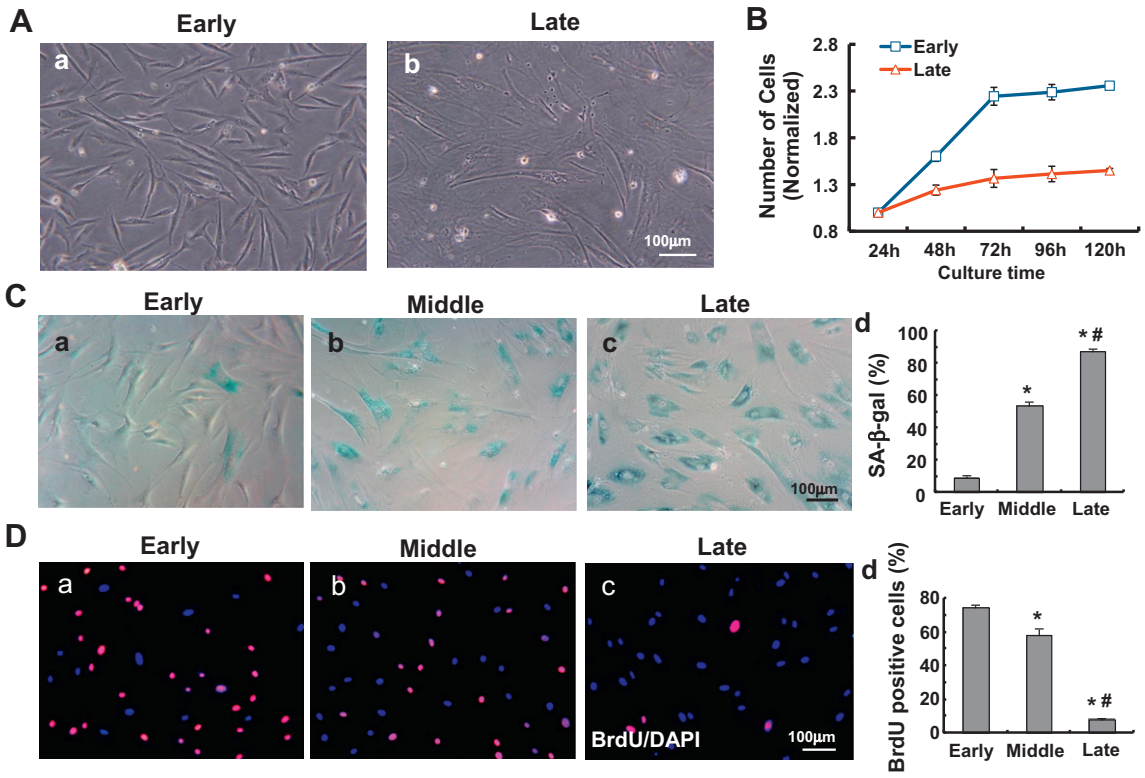

Fig. 1. Replicative Senescence of hBMSCs with Prolonged Culture Expansion. (A) Morphological changes upon replicative senescence. Representative morphologies of hBMSCs in the early (P3; a) and late passage (P13; b). (B) Growth curves of hBMSCs. Cell proliferation was retarded in the late-stage hBMSCs. (C) SA-β-gal activity was determined in hBMSCs at the early (a), middle (b), and late (c) stage. SA-β-gal (%) represents the percentage of positive cells with blue staining. (D) Immunostaining of BrdU-positive hBMSCs (red) in the early (a), middle (b), and late (c) stage. (d) The quantified data. Note that BrdU-incorporated cells were significantly reduced in the late stage. $^{*}P < 0.05$ vs. the early-stage controls, and $^{\#}P < 0.05$ vs. the middle-stage controls. Scale bars: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

early, middle, and late stages were 74 ± 2%, 58 ± 4%, and 8 ± 1%, respectively ($P < 0.05$; Fig. 1D), supporting a passage-dependent reduction in proliferation capability. Altogether, these results demonstrate that even if hBMSCs are stem cells, they develop senescence during prolonged in vitro culture.

3.2. Decreased expression and activity of 20S proteasome during in vitro propagation

As a multicatalytic proteinase complex whose proteolytic core is the 20S particle, the proteasome selectively degrade unwanted proteins to minimize harm to the cell. Dysfunctional proteasomes with impaired digestibility have been documented in aged cells from diverse origins [19]. We tested whether proteasomes are likely involved in the replicative senescence of hBMSCs.

20S proteasomes were mostly distributed in the cytoplasm of hBMSCs at all three stages (Fig. 2A). However, they were absent from some cells in the late passages (Fig. 2Ac). The percentage of 20S proteasome-expressing hBMSCs was significantly decreased in the late stage (32 ± 3%), in contrast to the 100% positive staining in either the early or middle stage (Fig. 2B).

This observation was complemented by semi-quantitative Western-blotting and real-time PCR. In the late stage, the protein expression levels of 20S proteasome and PSMB5, a critical subunit of the former, were significantly reduced by 55% and 53%, respectively, compared with those at the early stage (Fig. 2C). Likewise, the mRNA expression levels of certain β subunits of 20S proteasome were altered in the senescent hBMSCs. PSMB5 and PSMB1 were significantly decreased by 36% and 13%, respectively, in contrast to PSMB6 that remained unaffected (Fig. 2D). As anticipated, the activity of 20S proteasomes in the late phase of cells was substantially decreased to 69 ± 4% ($P < 0.01$ vs. control of the early passages; Fig. 2E). Taken together, these results suggested that 20S proteasome activity and expression, particularly of the PSMB5 and PSMB1 subunits, were lowered with the prolonged culture expansion, which potentially accounts for the replicative senescence of hBMSCs.

3.3. Accelerated senescence-like process of hBMSCs by MG132 application

To pursue the potential link between the proteasome activity reduction and the replicative senescence of hBMSCs, the specific proteasome inhibitor MG132, was applied to treat cells of the early passages. In agreement with other observations [12,20], hBMSCs incubated with MG132 (10 μM) developed the senescence-like phenotype, substantiated by the considerably increased SA-β-gal-positive cells (71 ± 3%; Fig. 3Ab and c), compared with the DMSO control (11 ± 3%; Fig. 3Aa and c). Likewise, the BrdU assay demonstrated that the percentage of cells exhibiting uptake was decreased in a concentration-dependent manner, with a ~50% reduction following MG132 treatment at 20 μM (Fig. 3B and C). The retarded proliferation was also suggested by the MTT assay. The absorbance at 570 nm of samples obtained from the MG132-treated cells was significantly lowered compared with that from the DMSO controls ($P < 0.01$; Fig. 3D). Additionally, the activity of 20S proteasome was reduced by ~60% after MG132 (10 μM) application (Fig. 3E). These data together verified that MG132-triggered inhibition of proteasomes speeds up the senescence and hinders propagation of hBMSCs.

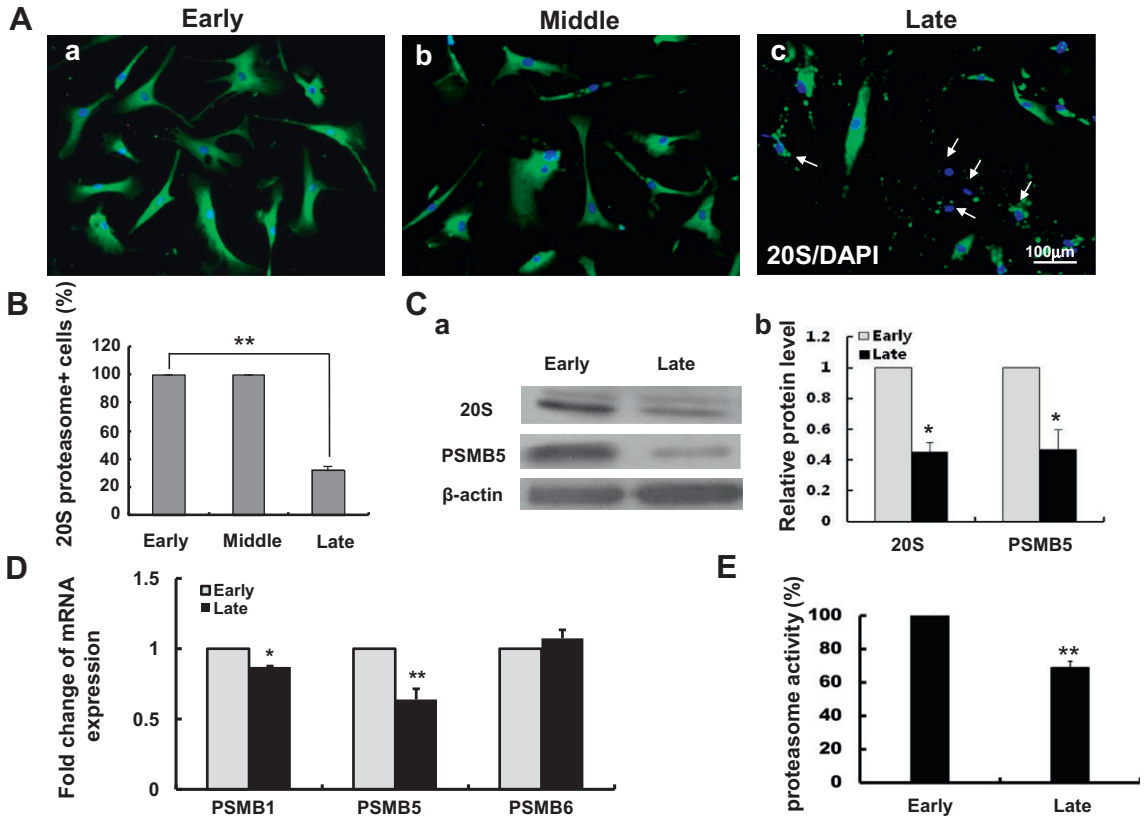

Fig. 2. Decreased expression and activity of the 20S proteasome in the late-stage hBMSCs. (A) Immunofluorescence analysis of the 20S proteasome expression (green) of hBMSCs in the early (a), middle (b), and late (c) stage. Scale bar: 100 μm. (B) The percentage of cells positively stained by the anti-20S proteasome antibody in three stages. All of the cells were stained in the early and middle stage. (C) Semi-quantitative Western-blotting confirmed the reduced protein expression of the 20S proteasome and its subunit PSMB5 in the late-stage hBMSCs (a). (b) The conclusive data of (a). (D) mRNA expression levels of the 20S proteasomal subunits by real-time PCR. (E) The proteolytic activity of 20S proteasomes was reduced in the late-stage hBMSCs. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the early-stage controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Delayed replicative senescence and restored proliferation and differentiation of the aged hBMSCs by 18α-GA treatment

Recent reports suggest that certain pharmacologic drugs, such as 18α-GA, are able to enhance the expression as well as activity of certain proteasome subunits [13]. We next complemented the MG132-mediated inhibition experiments by applying 18α-GA to activate proteasomes in the cells already senescent.

Preincubating the late-passage hBMSCs with 18α-GA (2 μg/ml) promoted 20S proteasome and PSMB5 protein expressions to 159 ± 18% and 230 ± 12%, respectively (Fig. 4A), associated with raised proteasome activity by ~20% (Fig. 4B). Notably, replicative senescence quantified by the SA-β-gal staining was retarded upon proteasome activation, with the percentage of blue cells decreased from 88 ± 3% (DMSO control; Fig. 4Ca and c) to 64 ± 3% (Fig. 4Cb and c). Meanwhile, in the late passages, a significantly higher percentage of BrdU-positive cells were observed after 18α-GA addition (74 ± 3%; Fig. 4Db and c), compared with the control (35 ± 3%; Fig. 4Da and c). We subsequently explored whether 18α-GA had an impact on maintaining differentiation capability of hBMSCs, which are potentially induced into neuronal cells [14].

hBMSCs with prolonged culture expansion in the presence of 18α-GA (2 μg/ml) remained the putative expression of stemness markers, including CD44, CD71, and CD90, but not CD34 (Supplementary Fig. 1A). Induction of hBMSCs at the early stage produced Tuj1-expressing and GFAP-positive cells, accounting for 30 ± 3% (Supplementary Fig. 1Ba and d) and 71 ± 3% (Supplementary Fig. 1Be and h) of the total population, respectively. With the development of senescence, the percentages of both Tuj1-postive cells (Supplementary Fig. 1Bb and d) and GFAP-positive cells (Supplementary Fig. 1Bf and h) were significantly decreased. However, promoting proteasome activity by the 18α-GA treatment counteracted the loss after induction (Supplementary Fig. 1Bc, d, g and h), successfully raising the percentages ($P < 0.01$ vs. DMSO controls). The aforementioned observations suggest that the activation of proteasomes help postpone the replicative aging and retain the stem cell integrity of hBMSCs.

4. Discussion

Introducing mobilized hBMSCs into autologous or allogenic therapies represents a promising strategy in the field of the regenerative medicine [21]. Nonetheless, replicative senescence of hBMSCs following ex vivo expansion has been reported before, which is characterized by morphological changes, proliferation retardation, as well as alterations in global gene expression patterns and miRNA profiles [6].

Consistent with previous studies [17,18], we have also observed the transition of cell shapes from the thin, fibroblast-like spindle shape in the early stage, to the flattened, greatly enlarged, and lost morphological unity after repeated in vitro propagation. Additionally, the nuclei of senescent cells appeared more circumscribed, and the cytosol started to seem granular due to the increasing numbers of inclusions and aggregations. Proliferation capability was also gradually impaired in the course of long-term cultivation, indicated by the reduced BrdU incorporating ratio, and lengthened doubling time. The percentage of SA-β-gal-positive cells was significantly increased, suggesting the development of senescence. Alto-

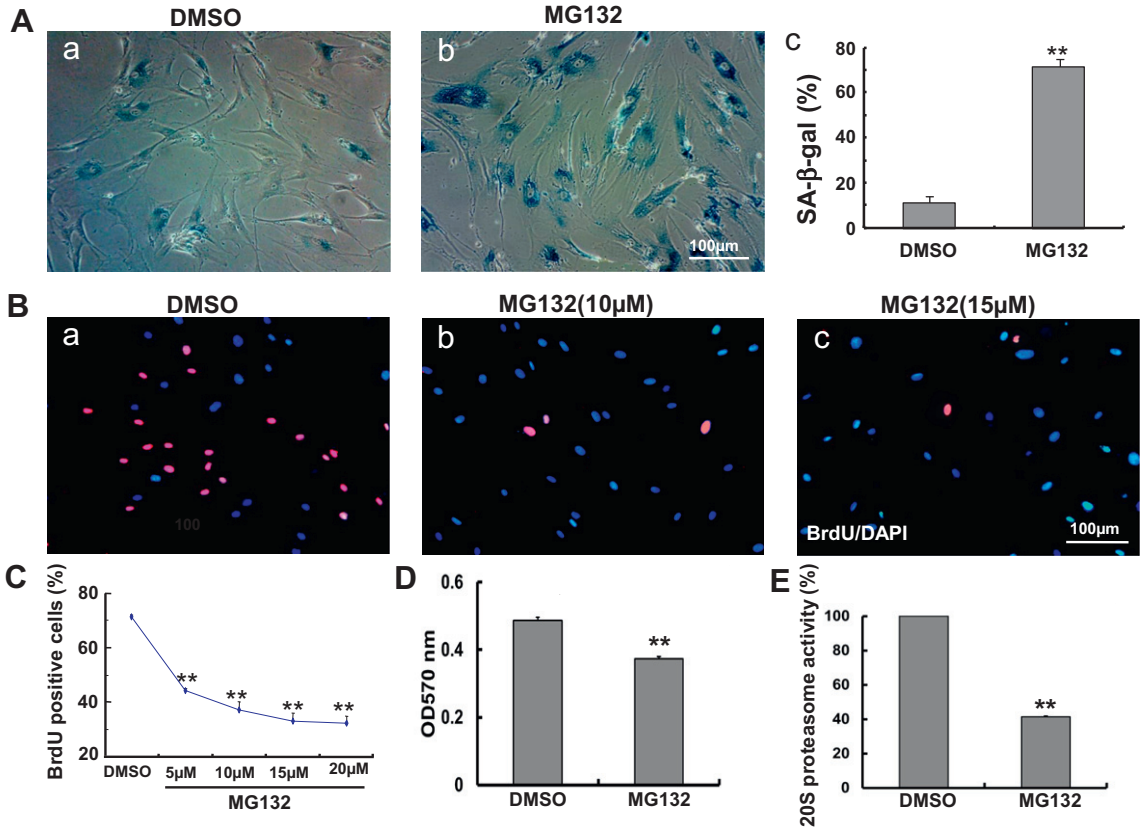

Fig. 3. Induced senescence-like phenotype of the early-stage hBMSCs by the proteasomal inhibitor MG132. (A) SA-β-gal staining of the early-stage hBMSCs following DMSO (a) or MG132 (10 μM for 4 days; b) treatment. A significant increase of senescent cells was observed after inhibiting proteasomal activity (c). (B) The BrdU incorporation assay suggested a delayed proliferation of hBMSCs after the four-day treatment with MG132 at 10 μM (b) or 15 μM (c), compared with the DMSO control (a). (C) The percentage of BrdU-positive cells was lowered after MG132 application in a concentration-dependent manner. (D) The MTT assay suggested proliferation retardation in the early-stage cells following MG132 addition. (E) MG132-triggered proteolytic activity decreased in the early-stage hBMSCs. $^{**}P < 0.01$ vs. DMSO controls. Scale bars: 100 μm.

gether, the data support that replicative senescence is a continuous process during in vitro culture propagation of hBMSCs.

As the essential component of mammalian proteolytic systems in charge of intracellular protein degradation, the proteasomes of tissues obtained from aged donors, as well as of senescent primary cell cultures, have been reported to display diminished proteolytic activity [11,22,23]. In contrast, activation of 20S proteasome by 18α-GA treatment decelerates senescence in human fibroblasts [13], by enhancing the transcriptional activity of nuclear erythroid factor 2. Consequently, the 20S proteasome is hypothesized to be fundamental to replicative senescence, balancing protein production and elimination in order to insure intracellular homeostasis.

The results of our work have confirmed prior observations, showing that the expression of the senescence-related 20S proteasome changed continuously during the in vitro propagation of hBMSCs. First, immunofluorescence analysis identified that ~68% of cells displayed no staining or only punctate stains in the late passages, suggesting a lowered 20S proteasome expression level. This observation was associated with the reduction in protein products of 20S proteasome and its key catalytic subunit PSMB5 by immunoblotting. Likewise, the real-time PCR confirmed the decreased mRNA levels of subunits PSMB1 and PSMB5, but not PSMB6 at the late stage. We subsequently measured the activity of 20S proteasome, and found a significant reduction. To further address the potential link between replicative senescence and 20S proteasome activity, we adapted MG132 as a specific blocker to 20S proteasome catalycity. In accordance with other studies [12], partial inhibition of proteasome activity in juvenile hBMSCs resulted in an induced senescence-like phenotype which has been detected in the late-stage cells, including the inhibited proliferation by the MTT and BrdU assays, and the increased SA-β-gal-positive cells. Collectively, the concurrence of decreased 20S proteasome proteolytic capability and replicative senescence suggests the correlation between the two, while the successful induction of the senescence-like phenotype in the early-stage passages by MG132 indicates that the former should be initiator rather than the follower of the latter.

Loss of stemness due to replicative senescence has been regarded as a barrier preventing hBMSCs from clinical use. Therefore, how to overcome cell aging during their in vitro expansion is drawing increasing attention. Based on others' reports as well as our data mentioned above, enhancing proteasome activity could be a promising way in dealing with this issue. For example, overexpression of the cellular proteasome activator PA28 increased the survival of neuronal model cells of Huntington's Disease [24]. Oleuropein, a small molecule proteasome activator, also extended the lifespan of human embryonic fibroblasts [25]. Unfortunately, given the limited number of agents with the definite and controllable proteasome-agonizing effect, the underlying mechanisms have not been fully studied.

Glycyrrhetinic acid (GA) is a pharmacological chemical widely-used in clinics for treating peptic ulcer [26], viral infection [27] and so forth. As expected from its structural similarity to cortisone, it also displays an anti-inflammatory action [28]. 18α-GA has been suggested to take part in the phosphorylation or changes in the aggregation of connexin subunits to inhibit gap junctional communication [29]. It has also been reported to activate the nuclear erythroid factor 2, subsequently stimulating 20S proteasome to delay

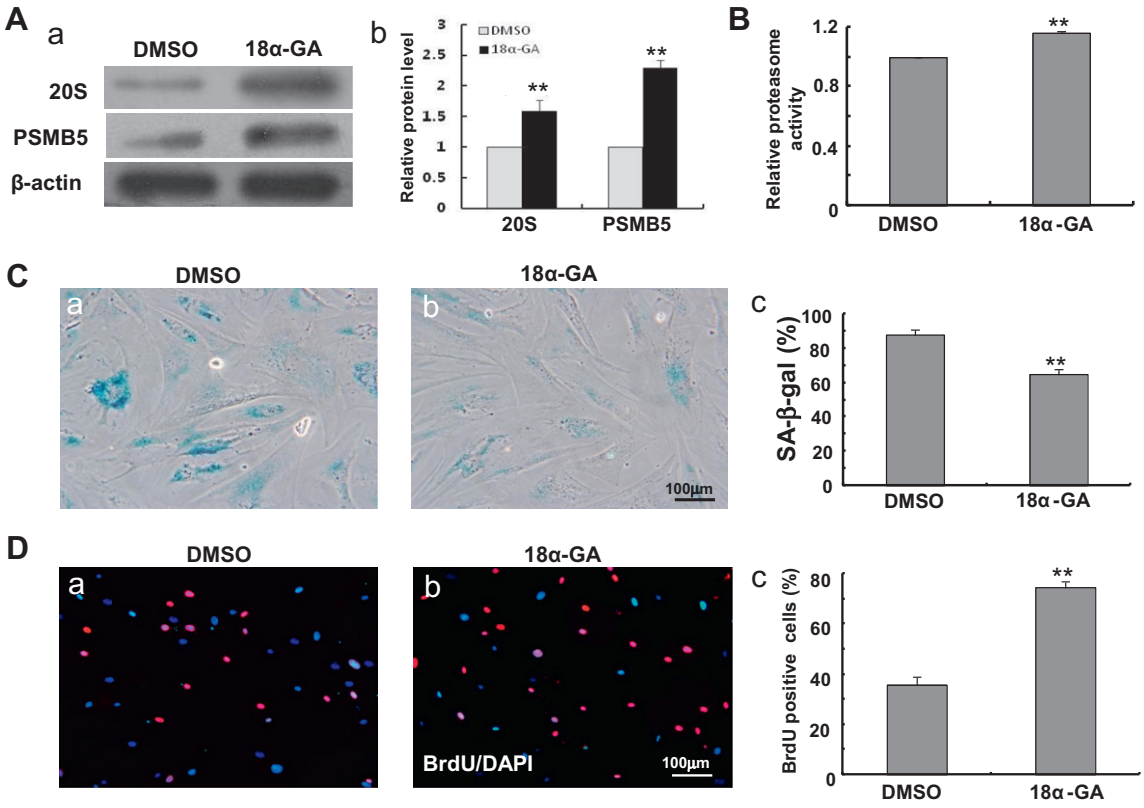

Fig. 4. Delayed replicative senescence of the Late-Stage hBMSCs by 18α-GA-Mediated Proteasome Activation. (A) Representative Western-blotting analyzes of the 20S proteasome and its subunit PSMB5 in hBMSCs treated with either DMSO or 18α-GA (2 μg/ml) for 4 weeks (a). (b) Shows the normalized data. (B) Activity of the 20S proteasome in hBMSCs with or without 18α-GA application. (C) SA-β-gal activity staining of hBMSCs incubated with DMSO (a) or 18α-GA (2 μg/ml for 4 weeks; (b) a significant decrease of positive cells was detected after enhancing proteasomal activity (c). (D) Immunostaining of BrdU-incorporated cells in hBMSCs with (b) or without (a) 18α-GA treatment. Cell proliferation indicated by the percentage of BrdU-positive cells was greatly improved after 20S proteasomal activation. $^{**}P < 0.01$ vs. DMSO controls. Scale bars: 100 μm.

replicative senescence in human fibroblasts [13]. Here, we have shown that treating late-stage hBMSCs with 18α-GA improved the activity and expression of the 20S proteasome, restored the normal capability of proliferation and differentiation of hBMSCs, thereby alleviating their replicative senescence.

In conclusion, hBMSCs become senescent during prolonged in vitro expansion, indicated by morphological abnormalities, proliferation retardation, and reduced differentiation capability, all of which may be possibly related to the decreased activity of 20S proteasome. 18α-GA enhances the proteolytic ability and restores other above-mentioned capabilities, and may thereby maintain stem cell integrity of hBMSCs during prolonged culture expansion.

Acknowledgments

This work was supported by Grants to L. Lu from the National Natural Science Foundation of China (#30973094), the Natural Science Foundation for Young Scientists of Shanxi Province (#2009021045), and the Shanxi Provincial Foundation for Returned Scholars (#2009-50); and to Z.C. Xiao from Talent Program of Yunnan Province, China, and The Professorial Fellowship of Monash University, Australia. We thank Drs. K.F. So and M.M. Civan for helpful discussions, Dr. J. Xie, Y. Dou and Y.Z. Li for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.119.

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