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

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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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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