# Age-Related Senescence of Rat Bone Marrow-Derived Mesenchymal Stem Cells

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**Abstract.**To study whether bone marrow-derived mesenchymal stem cells (MSCs) from aged rat present cell senescence. In the present study, cell morphology were observed, and cell proliferation and cell cycle were analyzed. The natural senescence of MSCs from aged rat was testified by investigation of SA-β-gal activity,ROS levels, DNA damage, telomerase activity and senescence-associated factors.Our results demonstrated that MSCs from aged rat showed senescent morphology and cell growth slowed down. SA-β-gal activity was elevated, the intracellularROS was enhanced, cellular DNA was severely damaged, telomerase activity was declined, and the expression of senescence-associated factors $p16^{INK4A}$and $p21^{WAF1/CIP}$ were upregulated. Taken together, our findings reveal that MSCs display biological changesand natural senescence in the process of physiological individual aging.

## Introduction

With the increasing age, almost all the organs and systems inevitably show degenerative changes in physiological functions and organizational structures.This process is actually defined as individual aging or natural aging. Cell senescence is the basis of individual aging[1]. In recent years, amounting studies have shown that stem cells are not immortal, and stem cells are invariably accompanied by degenerative changes with aging[2]. Aging and age-related diseases are attributed to stem cell aging[3]. The function of MSCs obtained from aged individual will bediminished because of the physiological aging, which severely restricts thetherapeutic effects during the autologous stem cell transplantation.It is also the mainreason for the declined organ functions in elderly people. Cell senescence will present the changes of the function and morphology, such as irreversible growth cessation, metabolic abnormalities and fat brown pigment deposition[4]. Thus, the present study aims to investigate the biological characteristicsand cellular senescence of MSCs in the process of physiological individual aging.

## Materials and Method

### Ethics Statement

All experimental protocols used in this study were approved by the Ethics Committee of Jilin University (Permit Number: SYXK 2013-0005).

## Animal and Cell Culture

Male Sprague-Dawley (SD) rats were obtained from the Experimental Animal Center of Jilin University, Changchun, P.R. China.1-2month-oldrats were used as young group, while 15-18 month-oldrats were chosed as agedgroup. Primary MSCs were isolated by using the whole bone marrow adherent method. MSCs at passage 3 were used in subsequent process of the study.

## Cell Growth Assay and Population Doubling Time

$5\times10^3$MSCs each wellwere seeded into 24-well platewith the complete medium. The cell numbers in threewells of each group have been counted chronologically for 7 days after cultivation. For cell doubling time, at a time (t1), we plated $7\times10^5$ cells into a 10cm dish. Thiswas the initial number of cells (Nf).Then at time (t2) wecounted the number of cells (Ni) and determined the DT by using the following formula:PD=In(Nf/Ni)/In(2); Ct=t2-t1; DT=Ct/PD.

## Cell Cycle Analysis and Apoptosis Evaluation

Cell cycle analysis was performed by using a Cell Cycle Detection Kit (KeyGENBioTECH,China) according to the manufacturer'sprotocols.Cell apoptosis was determined by staining cells with PI- AnnexinVApoptosis Detection kit I (BD, USA).

## Senescence-Associated B-Galactosidase Assay

To determine MSCs senescence, senescence-associated-β-galactosidase (SA-β-gal) stainingwas performed byusing a senescence cell histochemicalstaining kit(Beyotime, China). After stainingthe cells were imaged under a bright-field microscope. The percentage of senescent cells was calculated by counting the number of blue cells (β-galactosidase positive cells) at least 200 cells in different microscopic fields.

## Intracellular ROS Measurement

Intracellular accumulation of ROS was measured by Reactive Oxygen Species Assay kit(Beyotime, China) according to the manufacturer's instructions. The fluorescence intensity were examined by flow cytometry.

## Comet Assay

DNA damage was examined byCometAssayKit(Trevigen,USA) following the manufacturer's instructions. $4\times 10^3$cells were harvested and mixed with low-meltingagarose. Then the mixture was spread on a slide evenly. After the mixture evolvingfrom liquid to solid, put them in the lysis buffer for2hours at 4℃. The slideswere then immersed in alkaline unwinding solution for 20 minutes at 4℃. After that step, submerged the slides with the mixture in ice-cold electrophoresis buffer, and then started running electrophoresis at 300 mA for 20minutes.The cells were stained with PI soon afterwards and observed under a fluorescence microscope.

## Telomerase Activity

The telomerase activity was analyzed using a TeloTAGGG Telomerase PCR ELISA PlusKit (Roche, Germany). 10μg of protein extract was used for each assay. Afteramplification by using PCR,the extended products were detected with the method of ELISA.

## Gene Expression Measurement

Total RNA was extracted from MSCs usingTrizol(Takara, China) and reverse transcription was performed with RNA PCR Kit(AMV)Ver.3.0(Takara, China).Gene expression was determined byrealtimePCR with TransStar Top Green qPCRSuperMix(TRANS, China)in 7300 Real-Time PCR System(ABI, USA). Data were normalized relative to those for β-actin expression using the $2^{-\Delta\Delta Ct}$ method.

## Results

### Age-Related Changes In The Morphology

Primary MSCs isolated from either young or aged rats show inapparent difference in the general morphological characteristics. However, cells at passage 3 in aged groupappeared flattened and enlarged, andlost the stereoscopic perception with obvious particles in the cytoplasm as well, whilecells in young group looklikelong fusiform shapeand flocked arrangement.The analytic results revealed that cell areas in MSCs from aged rats increased progressively, but cell aspect ratio decreased gradually.

### Age-Induced Variations In The Cell Proliferation

A statistically significant difference can be observed in the cell growth curves between two groups, indicating that MSCs isolated from aged rats grew much slower. The population doubling time inaged group was markedly extended. In addition, the cell cycle also presents an obvious distinction. MSCs from agedrats displayed longer G1 phase and shorter S period,suggesting that most cells stuck in G1 phase and occured permanent cell cycle arrest. Statistical analysis confirmed thatboth S-phases fraction(SPF)and proliferation index (PI) in the aged group are lower than those in young group.

### Cellular Senescence Increases In Mscs From Old Rats

SA-β-Gal results displayed that the ratio of SA-β-Gal-positive cells was much higher in MSCs obtained from aged rats when compared to those from young rats. Although the apoptosis rate in MSCs from aged rats is higher than that in young group, the difference between the two groups had no statistical significance. To further confirmwhether senescent cells increased in the aged group, we usedother methods comprehensively to judge the cell senescence. The intensity of ROS fluorescence in aged group was much weaker than that in young group,which implied that MSCs in aged group generated excessive ROS compared with the young group. Furthermore, the result in flow cytometry was in accordance with the fluorescent analysis. The comet assay datadisplayed that the length of OTMincreased in the aged group. Then we found that the telomerase activity observably declined in MSCs from aged rats.Moreover, we discovered that both expression of pl6INK4Aand p21WAF1/ CIP1 presented the different degree of elevation in aged group compared with young groupby using RT-qPCR.

## Discussion

In the present study, MSCs derived from both 1-2-month -old rats and 15-18-month-old rats were used to investigate the effect of age on cell biological characteristics and cellular senescence.

We found that there were obvious differences in biologicalcharacteristics between both MSCs. Although primaryMSCs in both groups are similar in cellular morphology, MSCs at passage 3 presented a significant morphological alteration. MSCs from aged group not only became enlarged and flattened, but also lost the stereoscopic perception with obvious particles in the cytoplasm, which had decreasedcell aspectratio and increased cell areas.In addition, flow cytometry analysis proved that accumulating MSCswere arrested in G1 phase and s-phases fraction(SPF) and proliferous index (PI) in the aged group were lower than those in young group.All the evidences manifest that MSCs growthin agedgroup was permanently inhibited and individual agingis accompanied by the alteration of MSCs biological features.

To further confirm the senescence of MSCsfrom aged rats, we carried out a series of assays. The percentage of SA-$\beta$-gal positive cellsin aged group was far higher than that in young group. Nevertheless, no statistical difference in our studyhad been found in apoptosis rate between both MSCs. The increased oxidative stress levelis another feature of senescent cells[5]. Thus, we investigated the level of intracellular ROS by flow cytometry. We found that intracellular ROS in aged group were increased by 3.5folds compared to the young group. Moreover,the degree of DNA damage will also increase with age[6, 7].The comet assay can be applied to estimate extent of DNA breaks, reflect the number and the severity of the damaged cells[8]. In a sense, the longer Olive tail moment (OTM) is, the more serious DNA damage will be. It was confirmed by our findings that the damaged cells in the aged group were much more than those in the young group. In most cells, telomere length is maintained by telomerase activity[9].Whentelomerase activitydeclines to a certain extent, it is insufficient to overcome the progressive telomere shortening caused by replication and oxidative stress, and then senescence occurs[10].Telomerase activity in MSCs from aged rats was only equivalent to half of the young MSCs. Harris deemed that the expression of pl6 $^{INK4A}$ and p21 $^{WAF1 / CIP1}$ can be used as important biological indicators to determine whether AT-MSCs senescence occurs or not[11]. In accordance with the Harris, we discovered that bothexpression of pl6$^{INK4A}$and p21$^{WAF1/ CIP1}$presented the different degree of elevation in aged group compared with young group. Based on the results mentioned above, we finally achieved a conclusion that MSCs isolated from aged rats revealed senescence associated phenotypes,in other words, MSCs senescence naturally appeared during physiological individualaging.

## Conclusion

Taken together, our results indicate that MSCs present biological changes and natural senescence in the process of individual aging. Our findings will provide a research basis to elucidate the mechanisms of stem cell senescence.

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