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Research article

Changes in stemness properties of human adenoid-derived mesenchymal stem cells during *in vitro* aging

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Abstract

Mesenchymal stem cells (MSCs) have significant therapeutic potential in gene therapy. *In vitro* replicative senescence causes a decrease in the proliferation capacity of MSCs and changes in stem cell properties. In this study, adenoid tissue was focused as a new MSC source. The stem cell properties and the proliferation potential of adenoid-derived MSCs after the long-term *in vitro* replicative senescence were investigated. Adenoid-derived MSCs (A-MSCs) were cultured up to passage 20 and were analysed for cell morphology, proliferative capacity, differentiation potential, and surface marker expression. In addition, the expression profile of cell cycle, apoptosis, and senescence-related genes were evaluated. After *in vitro* replicative senescence, A-MSCs did not show any significant morphological differences. The proliferation potential of A-MSCs was rapid up to passage 16, and a reduction in the proliferation potential of senescent cells *in vitro* was observed depending on the passage number. The differentiation potential of late-passage A-MSCs was also reduced compared to early-passage cells. A-MSCs also provided significant closure at the 8th hour in early passages in terms of closure of the scratch area, while late passage A-MSCs exhibited a similar closure profile at the 24th hour. At the transcriptional level, the upregulation of the *BAX* gene and the downregulation of the *p21* and *p53* genes suggest that late-passage A-MSCs may not exhibit a senescence profile. In conclusion, A-MSCs have significant potential for clinical use due to the sustainability of MSC properties and their ability to proliferate and migrate with long-term culture.

Keywords: *Adenoid; cell proliferation; characterization; mesenchymal stem cells; senescence*

1. Introduction

Mesenchymal stem cells (MSCs) are adult multipotent cells that can self-renew and differentiate into non-mesodermal origins such as mesodermal and neuronal cells, cardiomyocytes, hepatocytes, or epithelial cells, particularly in fat, bone, and cartilage tissue (Noronha-Matos and Correia-de-Sá, 2016; Hmadcha et al., 2020). After the first identification in the bone marrow (Friedenstein et al., 1976), mesenchymal stem cells derived from many tissues such as adipose tissue (Mazini et al., 2019), muscle (Camernik et al., 2019), peripheral blood (Wu et al., 2015), hair follicles (Wang et al., 2020), teeth (Ledesma-Martinez et al., 2016), placenta (Deng et al., 2024), umbilical cord (Rajput et al., 2024), cord blood (Chang et al., 2009), lung (Hoffman et al., 2011), heart (Hoogduijn et al., 2007), endometrium (Bagheri-Mohammadi et al., 2019) are shown to be promising sources in terms of proliferation and differentiation potential into different cell types.

MSCs exhibit significant potential for tissue repair and gene therapy. Their ability to divide symmetrically and asymmetrically gives these cells self-renewal and versatile differentiation capability, thus making them promising

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candidates for regenerative medicine. (Bonab et al., 2006). Furthermore, their immunomodulatory properties make them advantageous for autologous applications in addition to allogeneic applications (Choudhery et al., 2014). To date, bone marrow MSCs (BM-MSCs) are the best-characterized stem cells widely used in experimental and clinical studies. However, the collection of BMs requires a challenging and invasive process. However, the low number of obtained cells and decreased proliferation ability and differentiation potential due to age led the researchers to search the alternative sources (Kern et al., 2006; Choudhery et al., 2014).

There is an imperative to culture and propagate MSCs *in vitro* before their therapeutic usage in the clinic. Therefore, it is important to determine the effect of *in vitro* aging on cellular properties before applications of MSC-based therapeutic strategies (Donega et al., 2014). The decrease in the proliferative abilities of MSCs, the change in their morphology, differentiation potential, and aging-related gene expression with long-term *in vitro* culture of MSCs have been demonstrated to cause many problems before the clinical use of these cells (Gu et al., 2016). Determination of alternative MSC sources is very important for the development of future cell therapies and stem cell banking due to the difficulty of sample collection, the low number of stem cells, and the decrease in proliferation and differentiation potentials depending on donor age (Feng et al., 2020). Nowadays, preserving distinct stem cell types and making them ready for treatment at the right time have indisputable importance. This seems possible with stem cell biobanks, but there are still significant shortcomings in providing adequate tissue and stem cells. Umbilical cord blood comes to the fore in stem cell banking because it is an easily accessible waste tissue. In addition, new born tissues such as umbilical cord tissue, placenta tissue, amniotic fluid, and amniotic membrane have become popular in the last few years for stem cell sources and banking. However, waste tissues such as adenoid tissue, tonsil tissue, and foreskin also have important potential as stem cell sources for biobanks (Li et al., 2023; Zhu et al., 2024). The importance of the stromal cells involved in secondary lymphoid organs (SLOs), which is an important part of the immune system in defence against invading pathogens, has been highly investigated (Mueller and Germain, 2009; Genovese and Brendolan, 2016). Tonsil-derived MSCs (T-MSCs) obtained from tonsil, one of the SLOs, have recently attracted intense interest as a source of MSCs due to their easy availability as waste tissue after tonsillectomy, their relatively high proliferation rate, and low allogenicity (Ryu et al., 2012; Shin et al., 2018; Cho et al., 2019; Oh et al., 2019). However, insufficient data are available on whether adenoid tissue can be used as a source to isolate multipotent MSCs or not. Adenoid tissue, which is commonly found in childhood and can be easily accessed as waste tissue, has the potential to be an important MSC source. In addition to their capacity to differentiate into adipogenic, osteogenic, and chondrogenic lineages, recent studies have revealed that they can differentiate into immature olfactory sensory neurons *in vitro* (Guo et al., 2023). It is important to investigate what extent they preserve their *in vitro* replicative potential and stem cell properties considering the regenerative potential of MSCs and their importance in cellular therapy. Therefore, in this study, we examined changes in cell morphology, proliferation rate, cell surface antigen expressions, migration ability, differentiation ability and many cellular process-related gene expressions of A-MSCs after replicative senescence *in vitro*.

2. Materials and methods

2.1. Adenoid-derived mesenchymal stem cell isolation and culture

A-MSCs were isolated from adenoid tissue taken during an adenoidectomy operation in the ear and nose and throat diseases department of Samsun Training and Research Hospital under the patient's consent. This study was approved by Ondokuz Mayis University Clinic Research Ethics Committee (Decision number: 2021/352). The adenoid tissues were washed twice with phosphate-buffered saline (PBS) and then incubated in 10 ml PBS supplemented with 10% antibiotic/antimycotic for 15-20 min at room temperature (RT). A-MSC isolation was performed according to the procedure described in the previous publication by Yuce and Albayrak, (2022). Briefly, tissues were divided into small pieces of 1-3 mm^3 in size. They were then enzymatically digested with Dulbecco's modified Eagle's medium (DMEM) supplemented with collagenase type-I at 37°C for 30 min and then filtered through a 100 µm cell strainer. Cells were incubated with DMEM (Sigma) containing 10% FBS, and 1% antibiotic/antimycotic (Sigma Aldrich).

2.2. Immunophenotyping on adenoid-derived mesenchymal stem cells

To evaluate the mesenchymal cell characteristics of the newly isolated adenoid-derived cells with replicative senescence, we performed the immunophenotyping assay. The cells at different passage numbers (p:4, p:12, and p:20) were collected from the culture flask by using 0.25% trypsin. Then, the cells were labelled with fluorescein Isothiocyanate (FITC)-conjugated anti-human CD90, allophycocyanin (APC)-conjugated anti-human CD73, FITC-conjugated anti-human CD44, phycoerythrin (PE)-conjugated anti-human CD34, allophycocyanin (APC)/Cyanine7-conjugated anti-human CD45 monoclonal antibodies at 40°C for 30 min. After the incubation, the labelled cells were analysed by flow cytometry (n=3) (Cytoflex S, Beckman Coulter).

2.3. Determination of multi-lineage potential of A-MSCs

Adipogenic and osteogenic differentiation protocols were performed for A-MSCs at passages 4 and 20. The protocol was performed according to Yuce and Albayrak (2022). A-MSCs at a density of $1x10^5$ were seeded in six-well culture dishes exposed to a differentiation medium for three weeks and renewed every 2 days. At the end of the 3 weeks when cell differentiation was observed, cells were washed with PBS. They were then fixed with 4% paraformaldehyde (PFA) for 30 minutes. After fixation, cells were stained with 2% Oil red O solution (Sigma Aldrich) for 1 hour at room temperature for adipogenic differentiation and 2% Alizarin Red S solution (Sigma Aldrich) for 15 minutes for osteogenic differentiation. After washing with PBS, the cells were observed under inverted microscopy. The experiments were performed in triplicate.

2.4. Determination of growth rate and doubling time

To indicate the effect of replicative senescence on the growth rate and doubling time of A-MSCs, we determined the growth rate and calculated the doubling time. The cells in passages 4, 8, 12, 16, and 20 were used for the determination of

the growth curve graph. The cells at passages 5 and 50 were used for the calculation of doubling time. For this, A-MSCs were seeded in a 12-well plate at a density of $3x10^4$ cells and incubated for 7 days (n=3). The cells were trypsinized and counted following trypan blue (Sigma Aldrich) staining every day post-seeding for determination of growth rate. Besides, population doubling time (PDT) and the number of cell population doubling (NCPD) were calculated with the formulas given below (Yang et al., 2018).

$$NCPD=3.33\times\log(Nt/Ni)$$
$$PDT=(t-ti)\times\log\{2\times[\log(Nt/Ni)]-1\}$$

2.5. Cell proliferation assay

We performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to exhibit the changes in the proliferation ability of A-MSCs with replicative senescence. We seeded the A-MSCs passage 5 and 20 onto 96 well-plate at a density of $2x10^3$ cells (n=3). The proliferation was measured on days 1, 3, 5, and 7. For measurement, MTT reagent was added to the cells at ten percent volume of the culture medium and the cells were incubated at 37°C for 4 hours. After the incubation, the dissolving solution (10% Sodium Dodecyl Sulphate (SDS) in 0.01 M Hydrochloric acid (HCL)) was added into the wells and incubated for 16 hours. The wells were measured by a microplate reader at 570 nm wavelength (Thermo Scientific™Multiskan™GOMicroplate Spectrophotometer) (Alcayaga-Miranda et al., 2015).

2.6. In vitro scratch assay

The migration abilities of A-MSCs post *in vitro* aging were indicated with *in vitro* scratch assay. $5x10^4$ cells at passages 7 and 20 were seeded onto 6 well-plate and the wound was created as a line with a 10 µl pipette tip onto the cells post reaching the confluence (n=3). The cells were washed with PBS to avoid the residues and were incubated in 2% FBS-DMEM at 37°C for 24 hours. The migration on the wound region was observed by phase-contrast microscope in time intervals (at 0, 8, 16, and 24 hours). The migration ability was quantified through the counting of the cells in the region by ImageJ analysis software (Alcayaga-Miranda et al., 2015).

2.7. Gene expression analysis post replicative senescence

The expression profiles of the aging-related genes on *in vitro* aged-adenoid-derived MSCs were analyzed by real-time polymerase chain reaction (RT-PCR). For that, total RNAs were isolated from the $5x10^4$ cells at passages 7 and 20 by NucleoZOL reagent (Macherey-Nagel). After RNA isolation, 5 µg RNA was converted to cDNA by cDNA synthesis kit (ProtoScript® II First Strand cDNA Synthesis Kit). Then, RT-PCR was performed with the interested primers listed in Table 1 by Maxima SYBR Green qPCR Master mix kit (Thermo Scientific). The gene expressions were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene and the Ct values were analysed by the 2-ΔΔCt method. The experiments were performed in triplicate.

2.8. Statistical analysis

The results are expressed as mean ± standard deviation. The statistical analysis was performed using GraphPad Prism software. The experiments were performed in triplicate and analysis of variance was used to analyse the variance among between groups. "2-tailed Student's t-test" was used to determine the significance level. The results were considered statistically significant if the values were $p < 0.05$.

3. Results

3.1. Morphological comparison of in vitro aged A-MSCs

The isolated cells from human adenoid tissue exhibited the fibroblast-like spindle-shaped morphology. We have observed that A-MSCs retained their characteristic spindle-shaped morp-

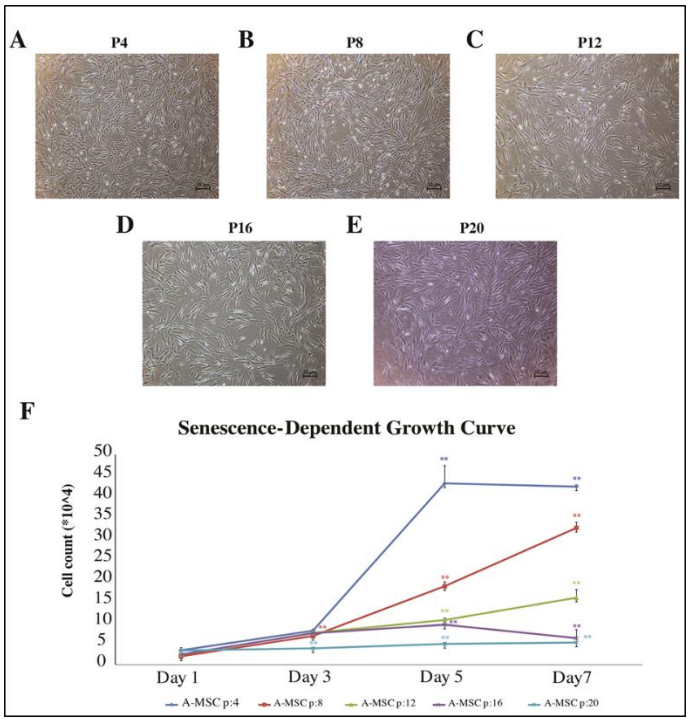

Fig. 1. Morphology of adenoid-mesenchymal stem cells depending on different passage number. The bright field microscopy views are presented for (A) passage 4, (B) passage 8, (C) passage 12, (D) passage 16, (E) passage 20 respectively (F) Senescence depending growth curve. The images are taken under 4X magnification. Scale bar: 10µm.

Table 1
The sequence information of the primers used for RT-PCR.

Gene	Forward primer	Revers primer
h*GAPDH*	5′-GTCTCCTCTGACTTCAACAGCG-3′	5′-ACCACCCTGTTGCTGTAGCCAA-3′
h*CCND1*	5′-TCTACACCGACAACTCCATCCG-3′	5′-TCTGGCATTTTGGAGAGGAAGTG-3′
h*CDKN1A*	5′-AGGTGGACCTGGAGACTCTCAG-3′	5′-TCCTCTTGGAGAAGATCAGCCG-3′
h*BAX*	5′-TCAGGATGCGTCCACCAAGAAG-3′	5′-TGTGTCCACGGCGGCAATCATC-3′
h*P53*	5′-CCTCAGCATCTTATCCGAGTGG-3′	5′-TGGATGGTGGTACAGTCAGAGC-3′
h*C-MYC*	5′-CCTGGTGCTCCATGAGGAGAC-3′	5′-CAGACTCTGACCTTTTGCCAGG-3′

hology until the 12th passage. A-MSCs at higher passage (p16, 20) exhibited a slightly more irregular morphology, although no major changes were observed (Fig. 1A-E). Therefore, we investigated the effect of *in vitro* aging on the growth of A-MSCs and generated growth curves for passages 4, 8, 12, 16, and 20. The decrease observed in the growth curves depending on the number of passages was greater in passages 16 and 20, defined as late passages. Because of the rapid increase in early passage 4 cells until day 5, 90-100% confluency was reached, so there was not significant change between the 5th and 7th day. Besides, we determined that the growth rate of A-MSCs at early passages (passages 4 and 8) was higher than the cells at late passage during *in vitro* aging (Fig. 1F).

3.2. The expressions of A-MSC surface antigens were maintained during in vitro aging.

The cell surface antigens are commonly used for identification of mesenchymal stem cells. The negative markers CD45, CD34, and positive markers CD73, CD90 are identified for mesenchymal stem cell characterization. In addition to them, CD44 expression is specifically important for the identification of A-MSCs. We analysed the expressions of these markers to investigate the effect of *in vitro* aging on A-MSCs. The plots of flow cytometry analysis are representatively shown in Fig. 2A. We compared passages 4, 12 and 20, and we found no significant changes in the ratio of CD34-, CD45-, CD73+, and CD90+ post-replicative senescence. However, although statistically significant change in CD44 ratio due to *in vitro* aging was observed, passages 4, 12, and 20 also showed over 97% positive expression (Fig. 2B). These findings reveal that the expressions of A-MSC surface antigens were maintained during *in vitro* aging.

3.3. The multi-lineage potential of A-MSCs during in vitro aging

Adipogenic and osteogenic differentiation capacities of A-MSCs have been investigated during *in vitro* aging. The findings obtained from the cells at passage 20 (late passage) were compared to the results obtained from the cells at passage 4 (early passage). To determine the adipogenic and osteogenic differentiation capacities of A-MSCs at passages 4 and 20, the cells were stained with Oil red O and Alizarin red, respectively following induction to differentiation with the special medium for 21 days. After the induction, we found that the lipid vesicles, which are the main indicator for adipogenic differentiation, were considerably decreased for passage 20 cells compared to passage 4 cells during *in vitro* replicative senescence (Fig. 3A). Similarly, the osteogenic indicator calcium deposits were decreased for passage 20 cells compared to passage 4 cells (Fig. 3B). These findings reveal that the *in vitro* aging suppressed the multi-lineage potential of A-MSCs.

3.4. In vitro aging caused the reduction of A-MSCs growth rate

MSC usage in clinic applications requires abundant cell numbers for efficiency. Thus *in vitro*, cell proliferation and population doubling time are very important parameters as much as cell sources and cell passage for cellular therapies. Therefore, we calculated the population doubling time and number of cell population doubling parameters. We found that the number of cell population doubling was significantly decreased around 2-fold in passage 20 A-MSCs compared to P:5 A-MSCs. In parallel with this result, the population doubling time was extended from 3.39 to 5.25 (Fig. 4A). These results indicated that the *in vitro* aging negatively regulated the doubling and growth rate of the A-MSCs.

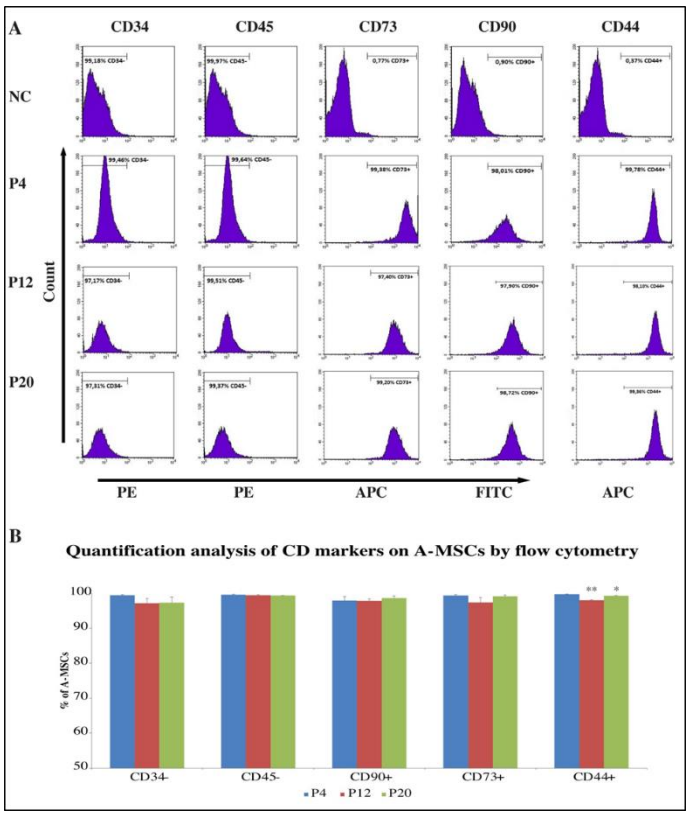

Fig. 2. Cell surface CD marker expression profile on A-MSCs at different passage number. The histogram plots (A) and quantification results (B) which belong to flow cytometry analysis of CD markers are presented. n=3, n=3, **p*<0,05, ***p*<0,01.

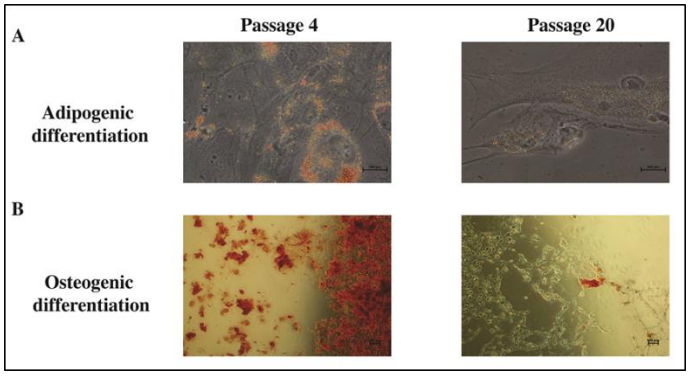

Fig. 3. Comparison of multilineage differentiation ability of adenoid-derived mesenchymal stem cells in different passages. (A) Adipogenic differentiation and (B) osteogenic differentiation abilities of adenoid-derived MSCs were evaluated by detection of oil vesicles and calcium deposition following Oil-Red-O and alizarin red staining on the cells at passage 4 and 20, respectively. Scale bar: 100µm.

In addition, we confirmed the proliferation capacity of A-MSCs with MTT assay during *in vitro* aging. We measured the proliferation in time intervals (day 1, 3, 5, and 7). We found that the absorbance values in passage 20 cells were significantly lower compared to passage five cells in the first 24 hours of culture. This reduction may be due to the low number of seeded cells. However, the proliferative potential of p20 cells was

relatively lower than that of p5 cells. We did not find a significant difference between day 3 and day 5, but a statistically significant decrease was observed on day 7 (Fig. 4B).

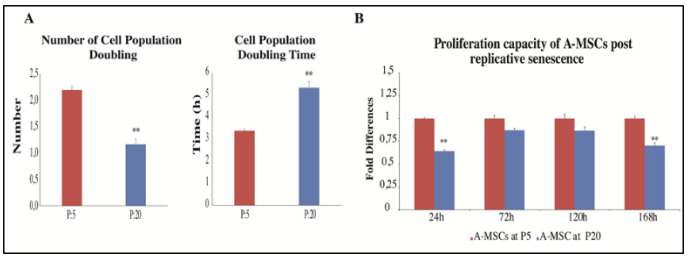

Fig. 4. Growth and proliferation rate of A-MSCs post replicative senescence. A) The number of cell population doubling (left) and the doubling time of the cell population (right) graphs are shown for A-MSCs post replicative senescence. B) The proliferation capacity of late passage A-MSCs is quantified in B compared to A-MSCs at passage 5. n=3, *p<0,05, **p<0,01. A-MSCs: adenoid-mesenchymal stem cells, P: passage number, h: hour.

3.5. In vitro aged A-MSCs maintained the migration ability

Wound healing requires the proliferation and migration of many cells including MSCs. Therefore, wound healing as migration ability is an important parameter for MSCs. We compared the wound healing potential of A-MSCs during replicative senescence. For that, we used passage 7 and passage 20 cells and performed the *in vitro* scratch assay on the cells. We observed the migration process under phase-contrast microscopy post 8, 16, and 24 hours of formation of the scratch. The microscopic views were recorded at 0. hours after formation of the scratch and then, the ratio of wound closure at 8, 16, and 24 hours was compared with the result at 0. hours. We observed the significant migration through the decrease in cell-free wound area in passage 7 A-MSCs in the first 8 hours. Besides, the cells at passage 20 did not show high migration potential at 8 hours as well as early passage cells. However, we recorded the close migration potential for the cells at passage 20 to early passage (passage 7) A-MSCs at 24. h. The wound healing ability of A-MSCs during *in vitro* aging was shown as microscopic views (Fig. 5A-H) and quantification graphs (Fig. 5I). These findings show that *in vitro* aged A-MSCs maintained the migration ability after 24h of scratch formation.

3.6. In vitro aging differentially regulated the cycling, apoptosis, and senescence-related genes on A-MSCs

We evaluated the different gene expressions to confirm the effect of *in vitro* aging on cellular processes on A-MSCs. *CCND1* (*Cyclin D1*) and *CDKN1A* (*P21*) genes from the cycling-related genes, the apoptotic gene *BAX* and also, and senescence-related genes *c-Myc* and *p53* were analysed for evaluating of its effect on MSC proliferation, cell death, and senescence during *in vitro* replicative senescence. We found that the expression of *CCND1*, which regulates G1/S transition during the cell cycle, was upregulated 2-fold on A-MSCs at passage 20 compared to A-MSCs at passage 7. Besides, there was not any significant change in the expression of *CDKN1A*, known as a cell cycle inhibitor (Fig. 6). The transcriptional changes on cell cycle genes with *in vitro* aging confirmed the stable proliferation capacity of A-MSCs instead of *in vitro* replicative senescence.

Interestingly, we also found the downregulation of the *p53* gene, which regulates the cell death, nearly 2-fold on A-MSCs at passage 20. The absence of the correlation between *BAX* and *p53* gene expressions shows that the function of the *BAX* apoptotic gene may occur by a p53-independent pathway. Besides, the upregulation *BAX* gene and downregulation of *CDKN1A* and *p53* genes reveal that the A-MSCs at passage 20 may not exhibit the senescence profile in contrast to other sources of MSCs.

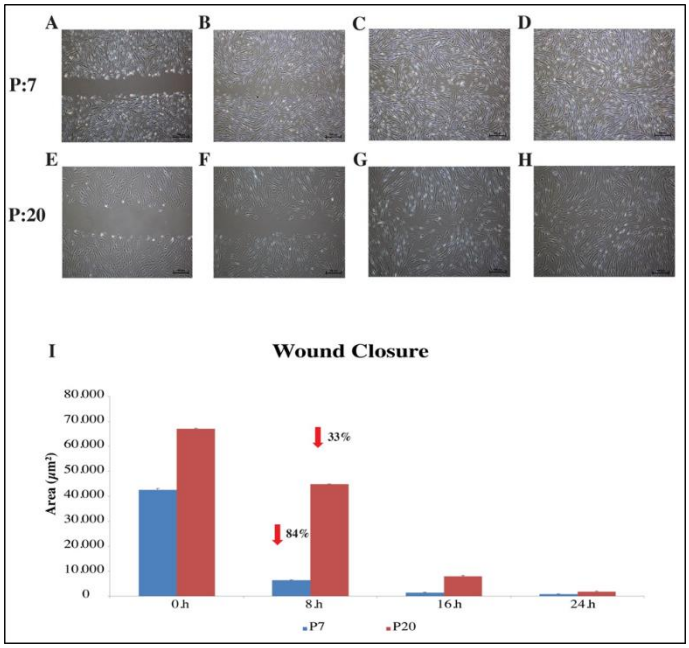

Fig. 5. *In vitro* scratch assay. The bright field microscopy views of A-MSCs post *in vitro* scratch was shown for the observation of A-E) 0.h, B-F) 8.h, C-G) 16.h and D-H) 24.h. I) The quantification of the wound closure is calculated by ImageJ analysis software and also, represented compared to passage 7 cells. Magnification: 100x and scale bar: 100μm H: hour.

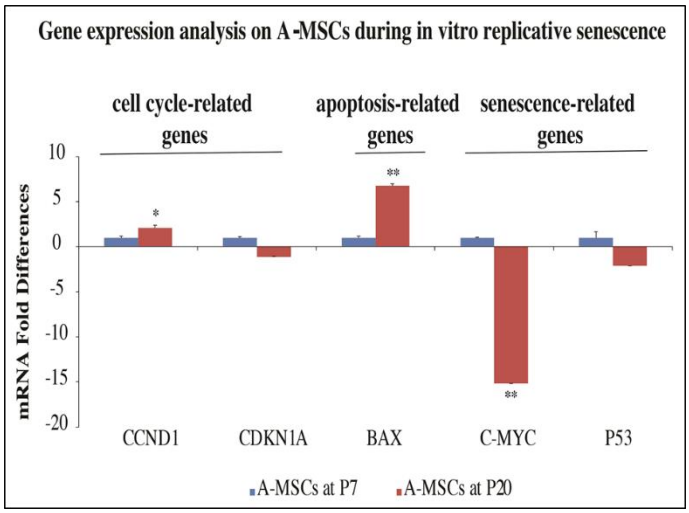

Fig. 6. Gene expression analysis on A-MSCs at early and late passage. The expression level of cell cycle, apoptosis and senescence related genes for A-MSCs at p20 were compared to A-MSCs at p7. The gene expressions were also normalized to *GAPDH* housekeeping gene according to 2-ΔΔCt method. n=2, *p<0,05, **p<0,01.

We found that the expression of *c-Myc* declined 15-fold with the *in vitro* aging process compared to early passage cells. This reduction in *c-Myc* expression was correlated with the decreased growth rate found in our result.

4. Discussion

The immunomodulator and multipotent stem cell properties of mesenchymal stem cells make them important for their therapeutical and clinical use (Gopalarethinam et al., 2023). *In vitro* and *in vivo* studies about the investigation of MSC aging have been carried out until today. The source tissues commonly used for human MSCs are primarily bone marrow and adipose tissue until recent years. The fact that bone marrow is renewable and adipose tissue is easily accessible as waste tissue makes these tissues important. Besides, the different studies showed decreased self-renewal and differentiation potential after *in vivo* and *in vitro* aging of bone marrow and adipose-derived MSCs (Baxter et al., 2004; Stolzing et al., 2008; Zhou et al., 2008; Alt et al., 2012; Wang et al., 2024).

MSCs are a rare cell population compared to the total number of cells in the tissue from which they are isolated. Because of their low numbers, it is important to obtain more MSCs that retain their regenerative ability for their various clinical uses, including regenerative medicine. This is based on the strong proliferative abilities of MSCs *in vitro* (Yang, 2018). Aging occurs with the replicative process and potentially results in reduced stem cell properties and regeneration ability of MSCs. In this study, we investigate the changes in cell morphology, proliferation rate, cell surface antigen expressions, migration and differentiation abilities, and various gene expressions on human A-MSCs that were exposed to senescence *in vitro*. Therefore, MSCs isolated from human adenoid tissue were cultured up to passage 20, and the changes in their morphological features, PDT, the expressions of surface antigens, differentiation potentials, migration abilities, and the cell cycle, apoptosis, and aging-related gene expression profiles were determined due to the increased passages number.

In vitro replicative aged MSCs are reported to have greater morphology and lower spreading potential than young cells (Gu et al., 2016; Gresham et al., 2024). A study reported that after the long-term culture of tonsil-derived MSCs, the cells in passage 15 and passage 2 did not show any significant morphological difference (Choi et al., 2015). In our study, the spindle-shaped cell morphology of A-MSCs with a similar origin to tonsil-derived cells was preserved in the first passages and up to passage 16. As replicative aging progressed, the cells in passage 20 that retained the spindle-shaped morphology were mostly observed, while some had a slightly more irregular morphology. The cell population doubling time in culture varies considerably for *in vitro* senescence. The population doubling time of BM-MSCs has been reported to be significantly prolonged in the later passages, while it is very short in the first passages. In the study, PDT increased to 15.8 days in passage 5 while it was 1.3 days in the first passage (Banfi et al., 2000). According to the results of the study which analyzed the growth characteristics of human umbilical cord-derived MSCs (UC-MSC) after long-term *in vitro* culture, the proliferation rate of cells cultured until passage 17 was significantly reduced to early passage cells. The cells in passage 11, defined as the mid-stage passage, showed a small decrease in their proliferation rate compared to early passage cells (Gu et al., 2016). Similarly, the replicative aging caused a decrease in growth rate in our study. As passage progressed from 7 to 20, the PDT increased from 3.39 days to 5.25 days. The reduction in the growth rate of A-MSCs after *in vitro* replicative aging was similar to T-MSCs (Choi et al., 2015).

The expressions of cell surface antigens are one of the important parameters for the identification of mesenchymal stem cells. It has been reported that (Choi et al., 2015) T-MSCs are highly positive for the expression of CD90, CD44 and CD73 mesenchymal stem cell surface antigens and negative for the hematopoietic stem cell antigens CD34 and CD45. It was not observed that there are no significant differences in the expression of positive and negative surface markers on MSCs after prolonged passages and cryopreservation. According to the results of this study, A-MSCs showed positive expression for MSC-specific cell surface antigens at a high rate similar to T-MSCs until late passages and also showed almost no expression in terms of hematopoietic stem cell antigens. Although there was a statistically significant decrease in CD44 expression, it showed positive expression over 97%. These findings reveal that A-MSCs display a stable profile of MSC marker expressions with *in vitro* aging.

It has been observed that aged BM-MSCs retain significantly the ability to differentiate into adipogenic lineages, while their osteogenic potential is significantly decreased with aging (Yang et al., 2018). The microscopic analysis post Oil Red O and Alizarin Red staining following *in vitro* adipogenic and osteogenic differentiation assays, which were performed to determine the change in differentiation potential of UC-MSCs after *in vitro* aging, showed that adipogenic and osteogenic differentiation capacity was decreased in p11 and p17 compared to p4 cells (Gu et al., 2016). Similarly, in our study, we evaluated the multi-lineage potential of A-MSCs and we found that the lipid vesicles, which are the main indicator for adipogenic differentiation and the osteogenic indicator calcium deposits, were decreased for passage 20 cells compared to passage 4 cells. These findings suggest that the *in vitro* aging suppressed the multi-lineage potential of A-MSCs.

Mesenchymal stem cells are important for their immunomodulatory function through migration to damaged sites. The migration ability varies depending on MSC sources. Recent studies showed that adipose- and bone marrow-derived MSCs had lost their migration ability with cellular aging (Bustos et al., 2014; Liu et al., 2017), while umbilical cord-derived MSCs did not (Naaldijk et al., 2015). Our study observed 85% wound closure after 8 h for early passage cells (passage 7) compared to *in vitro* aged A-MSCs (passage 20). At the end of 24 hours, late passage cells achieved closure of the entire scratch area, similar to early passage, and it was observed that they largely preserved their migration ability at 24 hours.

Finally, we evaluated the different gene expressions to confirm the effect of *in vitro* aging on cellular processes on A-MSCs. The stable proliferation capacity of aged A-MSCs may be explained by the upregulation and slight downregulation of cell cycle regulators *Cyclin-D1* and *CDKN1A*, respectively. In addition, recent studies reported that senescent cells are known to be resistant to apoptosis induced by this genotoxic stress (Rochette and Brash, 2008). In contrast to this information, we found a significant upregulation of the *BAX* gene with *in vitro* aging. Besides, we also found the downregulation of the *p53* gene involved in the regulation of cell death (Amaral et al., 2010) and is the marker for senescence with its function on the regulation of *BAX* and *p21* genes (Zhou et al., 2008) with *in vitro* replicative senescence. The high upregulation *BAX* gene and downregulation of *p21* and *p53* genes reveal that the A-MSCs at passage 20 may not exhibit the senescence profile in contrast to other sources of MSCs. Besides, the absence of the correlation between *BAX* and *p53* gene expressions in our study shows that the function of the *BAX* apoptotic gene may occur by a p53-

independent pathway. In addition, we found that the expression of oncogene *c-Myc* declined 15-fold with *in vitro* aging process compared to early passage cells. It was indicated that overexpression of *c-Myc* induced MSC proliferation and suppressed the multi-lineage differentiation capacity (Melnik et al., 2019). This reduction in *c-Myc* expression was correlated with the slightly decreased growth rate found in our result.

In light of our findings, we indicated that *in vitro* aging is slowly processed on A-MSCs rather than other sources of MSC. Therefore, A-MSCs have more potential for clinic usage depending on their sustainability of MSC characteristics and proliferation and migration abilities with prolonged culture (until passage 20) compared to other sources of MSC. These clinical advantages of A-MSCs make the cells more promising for cellular therapy.

In conclusion, before the clinical use of MSCs, which have an important therapeutic potential in regenerative medicine, it is important to obtain more cells that retain their regenerative ability. It is also known that MSCs obtained from different sources are affected differently by *in vitro* replicative aging. The data from our study showed that A-MSC largely retained the expression of cell surface antigens during long-term *in vitro* culture. However, there is a significant decrease in differentiation potential at passage 20. While no significant difference was observed in the doubling time of cells due to *in vitro* aging, the proliferation ability was significantly preserved. In addition, migration potential was preserved in the 24 hours, although not at the same rate as in the early passage. Therefore, A-MSC will likely be highly promising for future clinical applications with its long-term maintained stemness properties. However, more data and detailed investigations showing the safety and efficiency of A-MSCS are needed for its use in clinics.

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