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Original Article

Effects of donors' age and passage number on the biological characteristics of menstrual blood-derived stem cells

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Abstract: We investigated the effects of donor age and passage number on the biological characteristics of menstrual blood-derived stem cells (MenSCs) by comparing MenSCs derived from donors with three different age ranges and after different passage times. Continuous passage, flat cloning, cell proliferation assays, flow cytometric phenotyping and whole human genome microarray were performed to systematically analyze the relationship between the self-renewal ability of MenSCs as well as their potential to maintain their stem cell characteristics and to resist aging. The results demonstrated that the immunophenotypes and *in vitro* cultural characteristics of MenSCs did not change significantly with the progression of aging. However, some important signal pathways including MAPK, the insulin signaling pathway, pathways involved in carcinogenesis such as PPAR and P53, and cytokines and their receptors, as well as other pathways associated with immune response and aging, changed to various extents under the conditions of aging after a long time *in vitro*. The enriched differentially-expressed genes were mainly involved in transcriptional regulation, stress response, cell proliferation, development and apoptosis. The key differentially-expressed genes associated with age and passage number were identified for use as biomarkers of cell aging.

Keywords: Menstrual blood-derived stem cells, gene expression

Introduction

Human endometrium shows a high capacity for self-renewal during each menstrual cycle, and it is well known that stem cells play an important role in this renewal [1-3]. Endometrial stem cells were initially isolated from endometrial tissues [4, 5]. However, recent studies indicate that there is a special cell population in female menstrual blood which has similar characteristics to those of adult stem cells. Some studies found that MenSCs have telomerase activity, and retain more than 50% of their telomerase activity even at passage 12 when compared to human embryonic stem cells [6]. With a strong multiple-differentiation potential, MenSCs have attracted extensive attention for their potential to treat a variety of diseases including heart failure, neurodegenerative diseases, critical limb ischemia, stroke and endometriosis.

Various studies have shown that the multipotentiality, the ability to express and secrete cytokines, paracrine function, anti-apoptosis and hematopoiesis-supporting ability of MSCs all decrease with aging [7-9]. The aging progression is not only influenced by the number of cell divisions, but also by the "biological age" of the donors. The aging of bone marrow-derived mesenchymal stem cells (BMSCs) has been extensively studied for various reasons, but few studies have investigated the aging mechanisms of MenSCs.

Our study compared the biological characteristics and gene expression profiles of MenSCs which were derived from different age-range donors and through different passage times to identify specifically-expressed genes associated with age and passage number. The effects of age and *in vitro* replicative senescence on MenSCs were investigated in depth. This study provides a theoretical base for MenSC-based clinical cellular therapy.

Materials and methods

Menstrual blood samples

MenSCs were provided by E-vans Biotech (Hangzhou, China). The samples were divided

into three groups according to the age of the donors: 1. Less than 30 years old; 2. 30-40 years old; 3. More than 40 years old. Each group contained 6 samples. The donors were informed of the process and the applications of the donated menstrual blood and signed an informed consent form. The forms were also approved by the Ethics Committee of The First Affiliated Hospital, College of Medicine, Zhejiang University.

Culture and expansion of MenSCs

MenSCs were cultured in an incubator at 37°C, in an atmosphere of 5% CO_2 with saturated humidity. Cells were passaged when they reached 80-90% confluence. The confluent cell layer was digested with 0.25% Trypsin-EDTA (GIBCO, Carlsbad, CA), and cells were harvested and washed. The cell suspension was seeded into flasks at a density of 5,000 cells/cm^2 in menstrual stem cell culture medium (E-vans Biotech). All or half of the medium was replaced every 3 to 4 days depending on the growth state of the cells. Cells were passaged according to the above procedures once they again reached confluence.

Generation of growth curves

The growth curves of cells from the three age groups at the same passage (P5) were created using MTT assays. The cells were seeded into 96-well plates at 5,000 cells per well. Every 24 hours, one measurement point, comprising six parallel measurements, was set. Cells were cultured for 7 days, and then 20 μL MTT solution (Sigma-Aldrich, St. Louis, MO) was added to each pre-tested well. Cells were incubated at 37°C for a further 4 hours then the supernatant was discarded and 150 μL DMSO was added to each well. The plates were then shaken for 10 minutes so that the precipitate could be completely dissolved. The optical absorbance of each well was measured at 490 nm and recorded. The growth curves were plotted with time on the X-axis and absorbance values on the Y-axis.

Assessment of the ability of MenSCs to be continuously passaged

The number of cell passages were recorded in detail and the number of population doublings (PD) by cells of each group were also calculated [10].

Cell colony formation efficiency assay

Cell clone formation assays were performed on P5 (Passage 5) cells during the logarithmic growth phase [10]. Cells were seeded into 6-well plates at densities of 100/well, 200/well and 300/well. The total volume of medium in each well was 3 mL. Cells were cultured in an incubator at 37°C, in an atmosphere of 5% CO_2 with saturated humidity for 2 to 3 weeks and the medium was replaced every 3 days until clones were visible to the naked eye. The supernatant was discarded and each well was washed twice with PBS. Cells were then fixed in 5 mL methanol for 15 min, rinsed, then colonies were stained with Giemsa solution for 10 to 30 min. After staining the stain solution was slowly removed using running water, then the plates were air-dried. The number of colonies consisting of more than 10 cells was counted under a microscope and the rate of colony formation was then calculated.

Flow cytometry Cells at P5 and P10 which had reached 80-90% confluence were harvested and washed. Cell suspensions with a concentration of 1.0×10^5 cells/mL were prepared. Mouse anti-human mABs including anti-CD29, -CD34, -CD45, -CD73, -CD90, -CD105, -HLA-DR, -CD117 and -SSEA-4, as well as isotype controls, were added into different tubes and mixed with cell suspensions by pipetting. The cell suspensions were incubated in the dark at 4°C or on ice for 30 min. After centrifuging, the supernatant was discarded and cell pellets were washed with PBS and centrifuged twice more then resuspended for flow cytometric analysis (FC500MCL, Beckman Coulter, Fullerton, CA).

Analysis and comparison of MenSC gene expression profiles

Microarray analyses were performed according to the instructions provided with the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. The method of collecting cells is shown in **Table 1** and the number of cells used was approximately 1×10^6. RNA was extracted using Trizol and then purified. Double-stranded cDNAs were synthesized from RNA, labeled with biotin then hybridized. The chips were washed and

Table 1. RNA sampling of MenSCs from donors of different ages at different passage numbers

Sample number	Age (y)	Generation (P)
Sample 1	y ≤ 30 years old	P5, P10, P20
Sample 2	30 years old < y ≤ 40 years old	P5, P10, P20
Sample 3	y > 40 years old	P5, P10, P20

scanned, and images were gathered and used for bioinformatic analysis of the whole genome expression profile.

Statistical analysis

The data were analyzed by one-way analysis of variance using SPSS 16.0 software. The data were expressed as mean ± standard deviation. $P < 0.05$ was considered significant.

Results

Effects of donor age on the biological characteristics of MenSCs

Analysis of the proliferative activity of MenSCs from different age donors: The population doubling trends of the three MenSC groups derived from the three age ranges were basically comparable. The population doubling speed was high in P5-P10, and remained relatively stable in P11-P13, then decreased rapidly from P15, especially for the M3 group (**Figure 1A**). The average passage number of the M3 group (the oldest age group) was significantly less than the M1 and M2 groups (relatively younger groups). M3 group cells generally could be passaged as far as P22, while M1 and M2 groups could be passaged consistently to between P24 and P27 (**Figure 1B**). The colony-forming efficiency of a single cell did not significantly differ among the three cell groups and did not change with age. The colony-forming rate of MenSCs was 14-17% (**Figure 1C**), which was similar to the results obtained in previous studies, and higher than that of BMSCs [5, 11, 12].

Analysis of the immunophenotype of MenSCs from donors of different age-groups: There were no significant differences between the immunophenotypes of MenSCs from different age donors. Stromal cell markers including CD29, CD73, CD90 and CD105 were highly expressed in cells of all three age-groups. Other biomarkers, including the hematopoietic stem cell markers CD34 and CD45, the human embryonic stem cell marker-stage-specific embryonic antigen-4 (SSEA-4) and HLA-DR were expressed at negligible levels (**Table 2**). The low level of HLA-DR expression implies low immunogenicity.

Proliferative characteristics of MenSCs at different passage numbers: P5 MenSCs proliferated rapidly; the logarithmic growth phase was reached 2 days after inoculation. Confluence was reached at the 5th day and was followed by a stagnation stage, in which cells ceased proliferation and which continued until the 7th day. Doubling time was around 26 hours. With the progression of passage number, the MenSCs increased in size (**Figure 2C**) and cells exhibited apparent contact inhibition. The proliferative speed of P10 MenSCs was significantly reduced compared to P5 cells. The cell growth trend of P10 cells was similar to that of P5 cells as shown in **Figure 2A** in spite of the obvious low doubling speed of P10 cells. Confluence was reached by the 7th day after inoculation and doubling time was around 31 hours (**Figure 2A**).

Comparison of the genetic stability of MenSCs at different passage numbers: Chromosome karyotyping was performed on M-phase MenSCs at different passage numbers. The results indicated that the karyotype remained normal (46, XX; **Figure 2B**) even when the cells were passaged up to P20. This suggested that the risk of chromosomal aberration of MenSCs did not increase with increasing passage number.

Immunophenotyping of MenSCs at different passage numbers: The expression characteristics of cell surface markers of P10 MenSCs were comparable with those of P5 cells: The cells showed high levels of expression of CD29, CD73, CD90 and CD105. In contrast, the expression level of CD34 and CD45 was below 2%, and HLA-DR and SSEA-4 were barely expressed (**Table 3**). These results indicate that the cell surface markers of mesenchymal stem cells were maintained to a great extent with cell passage even though there were a few differences among cells of different passage numbers. This result is consistent with the study of Domnina *et al*. [13].

Differential expression analysis of MenSCs from different age donors: Three strains of

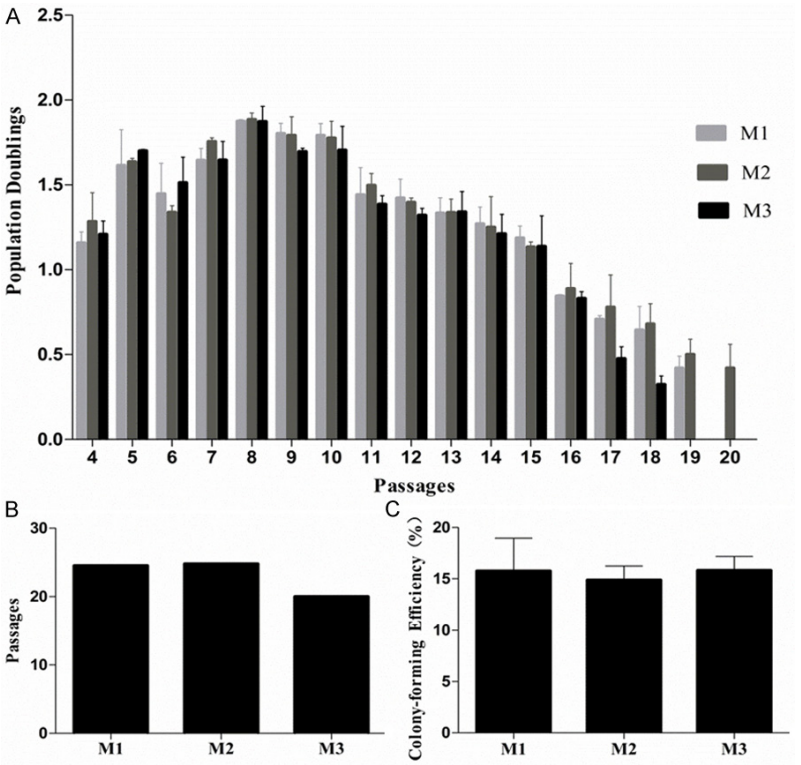

Figure 1. Comparison of the proliferative activities and colony-forming efficiencies of MenSCs from different age donors. The proliferative activity of MenSCs from different age donors were analyzed and compared. M1 represents donors aged between 20 and 30 years, M2, aged between 30 and 40 years and M3, aged over 40 years. A: Population doubling numbers of MenSCs of the three groups (n = 6); B: Comparison of *in vitro* passage times of MenSCs of the three groups; C: Comparison of the colony-forming efficiency of MenSCs from the three groups (n = 6).

Table 2. Comparison of cell surface marker expression by MenSCs (P5) from donors of different age-groups (n = 6)

Cell Surface Marker	Expression level (x ± s, %)		
	M1	M2	M3
CD29	92.33±0.20	91.63±0.12	93.11±0.33
CD34	0.06±0.0.02	1.5±0.40	0.38±0.50
CD45	0.11±0.03	1.10±0.20	1.60±0.70
CD73	99.40±3.80	98.70±4.40	96.80±1.90
CD90	99.5±0.90	98.80±4.70	99.20±7.10
CD105	98.4±0.10	97.80±4.80	98.30±0.80
CD117	6.99±2.30	8.49±5.40	8.20±3.70
HLA-DR	0.19±0.03	0.20±0.12	0.30±0.20
SSEA-4	0.98±0.25	0.96±1.70	1.40±0.60

MenSCs derived from donors of three different age-ranges were chosen to perform microarray analysis (**Table 1**). The experimental design was longitudinal comparison with age as a parameter. Thus MenSCs at the same passage number but from donors of different age-ranges were compared. In this study, we chose MenSCs at two passage numbers (P5 and P10) to perform this analysis. We identified 1,030 genes which were synchronously up-regulated or down-regulated by 1.2-fold with increasing donor age. MenSC genes which were down-regulated with increasing age were mainly involved in the cell cycle, cell proliferation, material metabolism, and cell adhesion, as well as growth factor receptors. Up-regulated genes were mainly related to apoptosis and the immune response (**Figure 3A**). The pathways in which the differentially-expressed genes were enriched were mainly associated with cell aging or with specific functions including MAPK, insulin signaling, oncogenesis, Wnt, and various cytokines (**Table 4**).

Analysis of differentially-expressed genes expressed by MenSCs at different passage numbers: To investigate the effects of passage number on the gene expression of MenSCs, a horizontal comparison experiment with the number of passages as the test parameter was designed and performed. Thus differentially expressed genes were screened by comparing P5 cells with P10 and P20 cells derived from the same donor. The experiment was repeated three times by choosing three different donors. Differentially-expressed genes were identified by picking the intersection of the results of three donors. A total of 237 genes were identified as synchronously up- or down-regulated by at least 1.2-fold with increasing passage number. Down-regulated genes were mainly those involved in cell division and proliferation, material metabolism, stress response, and signal transduction. Up-regulated genes were mainly those associated with apoptosis, cell cycle,

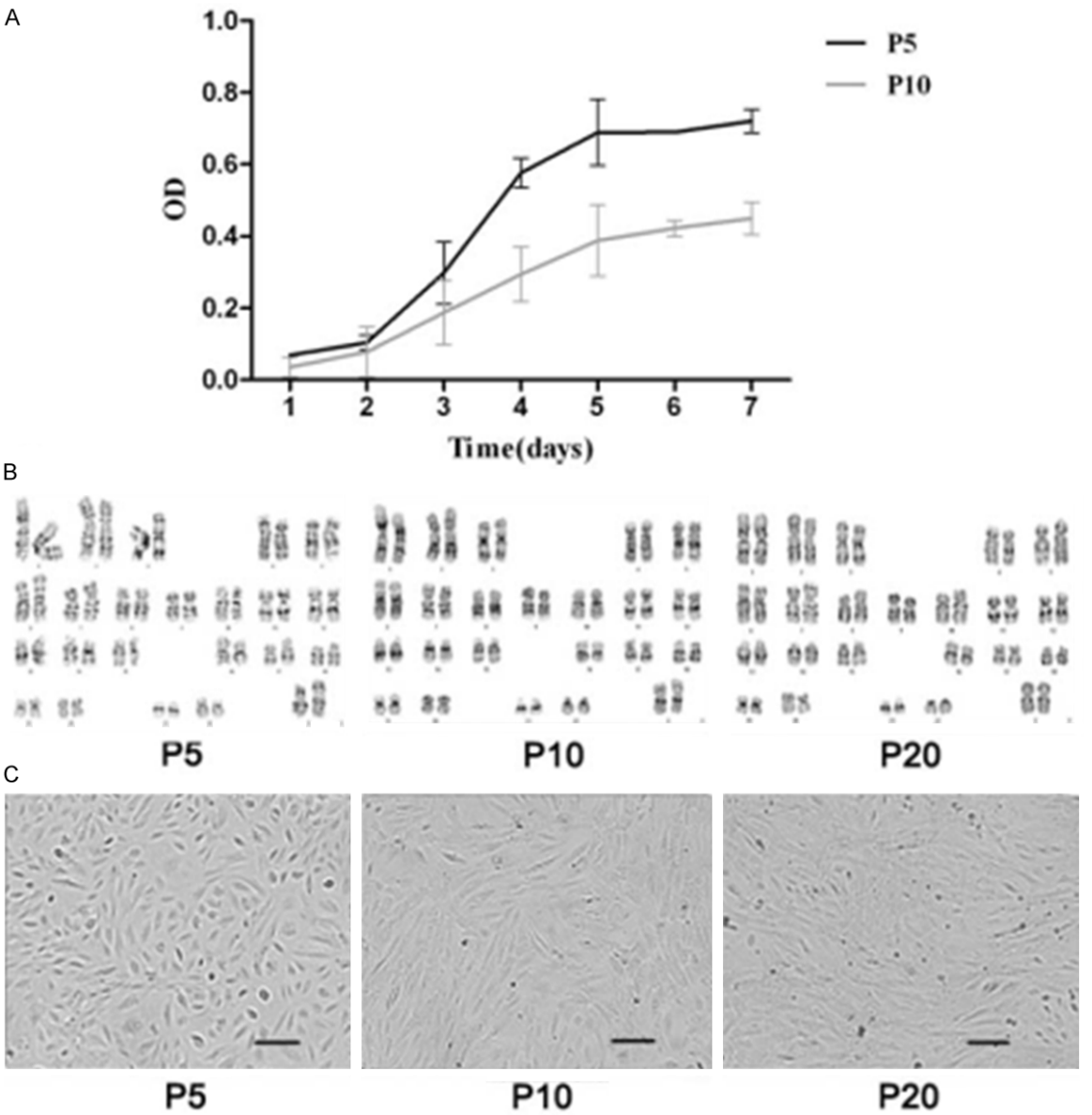

Figure 2. Comparison of proliferative activities and chromosome karyotypes of MenSCs at different passage numbers. MenSCs from donors of the same age range were chosen randomly. The growth curves of P5 and P10 cells were drawn and chromosome karyotypes were analyzed. A: Growth curves of P5 and P10 MenSCs (n = 6); B: Chromosome karyotypes of P5, P10 and P20 MenSCs. C: Morphology of P5, P10 and P20 MenSCs. Scale bar, 100 μm.

transcriptional regulation, and cell adhesion (**Figure 3B**). The pathways in which the differentially-expressed genes were enriched were mainly those associated with cell aging or immune response including MAPK, immune modulation, oncogenesis, PPAR, P53, and cytokines and their receptors (**Table 5**).

Discussion

Most researchers accept that the aging of BMSCs is probably influenced by the age of the donor from whom the BMSCs are derived. Many studies have shown that BMSCs derived from young individuals have higher proliferative activity than those from older individuals [14, 15]. However, to date there have been no studies to elucidate the effects of age on MenSCs. In this study, we compared the biological characteristics of MenSCs derived from donors of different age-groups and found that all donor cells maintained their fibroblast-like morphology well and did not exhibit any aging-related features until P10. Proliferative ability did not

Table 3. Comparison of Cell Surface Markers in MenSCs at Different Passage Numbers (n = 6)

Cell Surface Marker	Expression level (x ± s, %)	
	P5	P10
CD29	91.15±0.32	90.30±0.56
CD34	0.00±0.10	0.09±0.36
CD45	0.03±0.20	0.18±0.07
CD73	99.63±1.24	98.26±0.56
CD90	96.96±2.66	95.54±2.30
CD105	98.78±1.20	96.80±0.51
CD117	6.60±2.30	8.30±2.30
HLA-DR	0.00±0.02	0.08±0.03
SSEA-4	0.83±0.20	1.36±4.56

significantly differ among the different age groups. Colony-forming rates were within the range of previously published values and there were no significant differences among the different age groups. The population doubling trends of different age-groups were comparable. The proliferative ability of P7 to P13 cells was relatively stable and there were no significant differences in terms of population doubling rate. The expression of cell surface markers by MenSCs did not change significantly with aging.

Similar to our results, some studies suggest that the proliferative ability of MSCs is not necessarily influenced by donor age [15-17]. However other researchers presume that there should be a negative relationship between the proliferative ability of MSCs and age [18, 19]. A study by Fickert *et al*. showed that the growth speed of BMSCs (Bone Marrow-derived Mesenchymal Stem Cells) from young and middle-aged donors was much faster than that of BMSCs from elderly people [20]. Meanwhile Choudhery *et al.* found population doubling number of ADSCs (adipose tissue-derived mesenchymal stem cells) derived from donors of different ages was significantly different. The doubling time became gradually prolonged with increasing age. Colony-forming potential also decreased remarkably with increasing age. Additionally, the older the donor, the fewer cells were contained in the clones derived from the donor cells. With regard to cell phenotypes, most studies produced similar results to ours, with the immunophenotype characteristics of cells derived from older individuals being almost the same as those from young individuals [20, 21]. Notably, experimental data of cells in long-term passage culture showed that MenSCs from donors aged over 40 had a weaker potential for long-term passage than those from donors aged below 40. This result is comparable with that reported by Musina *et al*. [22].

These data suggest that age has relatively small effects on the potential of MenSCs in spite of its effects on long-term passage. In this respect MenSCs differ from other MSCs such as BMSCs and this indicates that MenSCs may have potential for wider application.

To obtain enough cells for clinical research, MSCs generally need to be expanded. There have been a few studies on BMSCs and ADSCs which showed that long-term culture can cause malignant transformation of MSCs [23, 24]. In our study, the proliferative speed of MenSCs tended to decrease with increasing passage number. High-passage cells were larger and tended to lose their three-dimensional appearance and become flattened. It became more difficult for cells to converge to form a single layer (**Figure 2C**). However, the immunophenotypes of the cells did not change significantly. Moreover, the cells were able to maintain their normal chromosome karyotypes up to P20. Zemelko *et al.* found that MenSCs underwent aging until the number of population doublings reached 45 [25]. Khademi also observed that MenSCs could be expanded to 68 doublings without losing their normal karyotype or developing tumorigenic potential [26]. Overall, these results indicate that passaging can induce aging of MenSCs. This is consistent with data from other types of stem cells. However MenSCs can maintain their normal chromosome karyotypes and normal immunophenotypes up to P20.

We further investigated the effects of age and passage number on MenSCs by analyzing gene expression level.

To avoid the confounding effects of multiple passages on the analysis of age-related factors, we used MenSCs at two passage numbers (P5 and P10) and from donors of three age-groups and compared their gene expression profiles. The results indicated that with increasing age, down-regulated genes are mainly those involved in the regulation of cell growth and

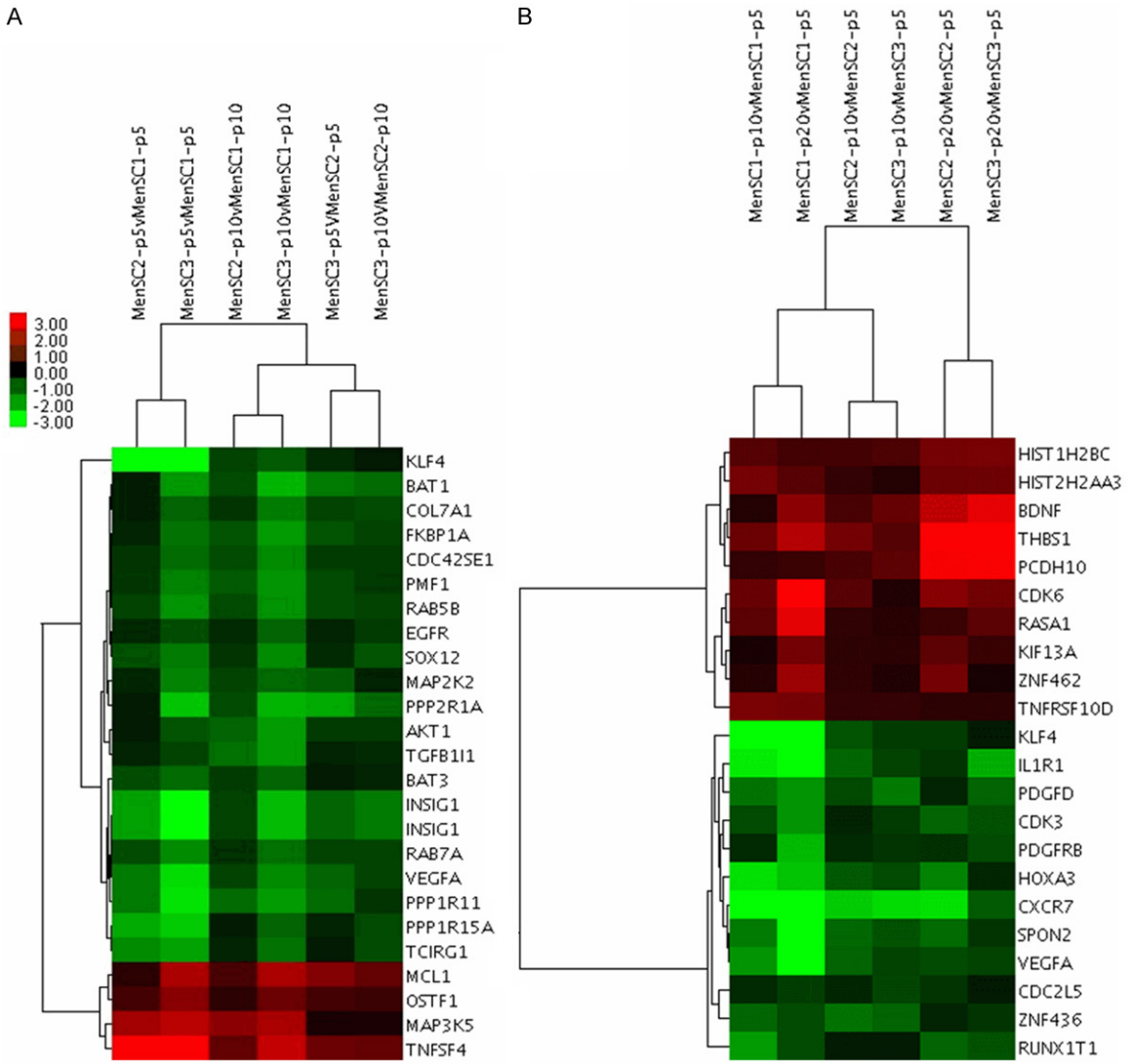

Figure 3. Clustering Heat Map of Differentially-Expressed Genes. Each colored spot represents a signal which was produced by hybridization of a probe with the nucleic acids on the gene chip. Green represents a low signal and red represents a high signal. The brightness of the color represents the intensity of the signal. MenSC1 represents MenSCs from donors aged ≤ 30 years old. MenSC2 represents the MenSCs from donors aged > 30 years old and ≤ 40 years old. MenSC3 represents MenSCs from donors aged > 40 years old. "MenSC2-p5 VS MenSC1-p5" represents passage 5 MenSC2 cells compared with passage 5 MenSC1 cells.

development, including CDC42SE1, which is associated with formation of the cytoskeleton, and EGFR, which functions to promote the proliferation of stem cells as well as contributing to the maintenance of the undifferentiated condition of stem cells. The expression of VEGFA, another gene which can accelerate cell migration and proliferation, was also markedly down-regulated. The expression levels of some genes which are likely associated with cell differentiation decreased continuously, such as INSIG1, which is involved in the metabolism of lipid and cholesterol, and COL7A1, which is associated with cell adhesion and production of extracellular matrix. Another down-regulated gene, TGFB1I1, acts as a hub to coordinate the interactions among a variety of proteins. It is able to transduce intracellular signals to membrane receptors and participates in the regulation of the Wnt and TGF-β signaling pathways, and probably positively regulates cell differentiation. That the above genes are down-regulated with increasing age implies that the multiple differentiation potential of MenSC is influenced

Table 4. Pathway enrichment analysis of differentially-expressed genes of MenSCs from donors of different ages

Pathways	Count	*P*-value	Gene
MAPK signaling pathway	25	2.00E-14	Up: STMN1; RRAS2; CDC42; CDC42P2; MAP3K5;
			Down: MAP2K5; NFATC4; DAXX; TAOK2; RELA; MAP2K7; ECSIT; EGFR; PRKACA; MAP3K12; TP53;
			MAP3K3; MKNK1; AKT1; MAPKAPK2; MKNK2; RAF1; JUN; MAP2K2; FGFR1
Insulin signaling pathway	17	2.30E-12	Up: PRKCI; CALM3; CALM2; CALM1;
			Down: TRIP10; MAP2K2; PRKACA; TSC2; MKNK1; RAPGEF1; AKT1; FASN;
			SREBF1; MKNK2; RAF1; PYGB; MAP2K2; FLOT1
GnRH signaling pathway	15	6.76E-12	Up: CDC42; CDC42P2; CALM3; CALM2; CALM1;
			Down: MAP2K7; MAP2K2; EGFR; PRKACA; MAP3K3;
			RAF1; JUN; MAP2K2; MMP14; MMP14; GNAS; GNAS; MMP2; GNAS; GNAS; GNAS
Focal adhesion	17	1.25E-09	Up: LAMA4; CDC42; CDC42P2; CAV2; XIAP;
			Down: EGFR; VEGFA; ERBB2; RAPGEF1; PXN; AKT1; ITGA11; RAF1; JUN; COL6A2; VEGFA; COL5A1; COL1A2
Prostate cancer	11	1.51E-08	Up:
			Down: RELA; MAP2K2; EGFR; TP53; TCF7L1; ERBB2; AKT1; FGFR1; RAF1; CREB3L1; MAP2K2; FGFR1; CDKN1A;
Ubiquitin mediated proteolysis	13	2.64E-08	Up: CDC23; XIAP; CDC16; UBE2K; UBE2N; SKP1;
			Down: FZR1; PML; LOC652346; CUL7; UBA7; SMURF1; UBE2Z
Pancreatic cancer	10	2.79E-08	Up: CDC42; CDC42P2;
			Down: RELA; EGFR; TP53; ERBB2; AKT1; RAF1; STAT3; VEGFA
Bladder cancer	8	4.83E-08	Up:
			Down: MAP2K2; EGFR; VEGFA; TP53; ERBB2; RAF1; CDKN1A; MMP2
Cholera - Infection	9	5.31E-08	Up: TJP2; ATP6V1D;
			Down: PRKACA; SEC61A1; TCIRG1; KDELR1; ATP6AP1; PDIA4; GNAS
Acute myeloid leukemia	9	5.31E-08	Up:
			Down: PML; LOC652346; STAT5B; RELA; MAP2K2; TCF7L1; AKT1; RAF1; STAT3

The first column is the name of the significant pathway identified by enrichment analysis. The second column displays the number of differentially-expressed genes enriched in this pathway. *P* represents the significance of the enrichment. The smaller the *P*-value, the greater the significance.

Table 5. Pathway enrichment analysis of genes differentially-expressed by MenSCs at different passage numbers

Pathways	Count	*P*-value	Gene
MAPK signaling pathway	13	2.26E-12	Up: RASA1; BDNF; FLNC; RASA1; MAP3K7; MAP2K3;
			Down: PDGFRB; IL1R1; MAP3K12; ECSIT; MAP2K7; AKT2; MAPK7
Systemic lupus erythematosus	7	2.59E-07	Up: HIST1H2BC; HIST1H2BE; HIST1H2BF; HIST1H2BG; HIST1H2BI; HIST2H2AA3; HIST2H2AA4;
			Down:
GnRH signaling pathway	6	8.30E-07	Up: PLCB4; MAP2K3; CAMK2D;
			Down: MAP2K7; MAPK7
Melanoma	5	2.30E-06	Up: MET; CDK6; CDK6; CDK6;
			Down: PDGFRB; PDGFD; AKT2
Focal adhesion	7	2.77E-06	Up: THBS1; MET; FLNC; THBS1;
			Down: PDGFRB; VEGFA; PDGFD; AKT2
Toll-like receptor signaling pathway	5	1.37E-05	Up: MAP3K7; MAP2K3;
			Down: MAP2K7; AKT2
Biosynthesis of unsaturated fatty acids	3	3.70E-05	Up:
			Down: SCD; FADS2; FADS1
Glioma	4	4.25E-05	Up: CDK6; CDK6; CDK6; CAMK2D;
			Down: PDGFRB; AKT2
p53 signaling pathway	4	5.38E-05	Up: THBS1; CDK6;
			Down: MDM4; SFN

The first column is the name of the significant pathway identified by enrichment analysis. The second column shows the number of differentially-expressed genes enriched in this pathway. *P* represents the significance of the enrichment. The smaller the *P*-value, the greater the significance.

by aging. Other down-regulated, differentially-expressed genes are mainly involved in the regulation of transcription and translation including AKT1, FKBP1A, PPP1R15A, BAT1 and BAT3.

Up-regulated osteoclast-stimulating factor-1 (OSTF1) is able to promote the development of osteoclasts and activate the secretion of related cytokines through a cascade of signaling reactions. This suggests that MenSCs from elderly donors may be more likely to differentiate toward osteoclasts. The role of the MAPK pathway is to transduce extracellular signals into cell nuclei to activate cellular biochemical reactions. MAP3K5, an important member of this pathway, is up-regulated. This change may facilitate the transduction of inflammatory and apoptotic signals. TNFSF4, a ligand of inflammatory factors, is up-regulated with the progression of aging. This suggests that the microenvironment can change when cells experience aging to accelerate the expression of internal TNF as an inflammatory response to promote the body's defensive ability.

To investigate the effects of passage number on the gene expression of MenSCs, we set P5 cells as a control to compare the expression of genes of P5 P10, and P20 cells. Three biological repeats of our experiments were set up to screen the differentially-expressed genes which were synchronously up-regulated or down-regulated. The results indicated that with increasing passage number, up-regulated genes included THBS1, RASA1, BDNF, CDK6, PCDH10, and KIF13A. Among them, BDNF is one of the neurotrophins and has the potential to maintain the functions of neurons and to promote the differentiation and growth of new neurons and synapses [27]. For high-passage MenSCs, high expression of BDNF implies that the MenSCs may maintain good neural-induction ability. Upregulation of BDNF is probably related to the increasing purity of MenSCs in the whole cell population following several passages. THBS1 can affect the osteogenic differentiation of MSCs by regulating the activity of latent TGF-β. The osteogenic potential of BMSCs will be significantly decreased when latent TGF-β is activated [28]. CDK6 is a key molecule involved in regulating cell-cycle progression and its expression increases dramatically in certain tumor cells. In our study, expression of CDK6 significantly increased with increasing passage number. Upregulation of CDK6 is probably involved in the regulation of certain pathways associated with development of tumors. Acting as a protocadherin, the expression of PCDH10 can induce the apoptosis of a variety of tumor cells, so PCDH10 is considered to be a tumor suppressor gene [29].

The expression of PCDH10 is up-regulated when MenSCs undergo long-term culture. It is hypothesized that, in order to protect themselves from malignancies, MSCs regulate the interactions of other anti-apoptotic and pro-apoptotic genes by up-regulating the expression of PCDH10 when multiple tumorigenic pathways switch on during the progression of aging [30].

Some genes were down-regulated during passage including PDGFRB, IL1R1, RUNX1T1, CDC2L5, VEGFA, CXCR7, SPON2, PDGFD, KLF4, CDK3, and HOXA3. The two growth factors PDGFD and VEGFA as well as the PDGF receptor are all closely associated with cell migration, proliferation and anti-apoptosis mechanisms. Acting as angiogenesis-related genes, the expression levels of these genes reflect the hematopoiesis-supporting ability of MSCs. Downregulation of these genes therefore im plies a decrease in the hematopoiesis-supporting ability of MenSCs. IL1R1 is an important regulatory gene which is involved in the immune and inflammatory responses induced by cytokines. In combination with cytokines, IL1R1 participates in regulating a variety of cellular functions such as proliferation, differentiation, and apoptosis. Expression of IL1R1 by high-passage MenSCs is also down-regulated. This implies that the immune-regulatory ability of cells tends to weaken with the progression of replicative senescence. MSCs widely express many kinds of chemokines and their receptors. Previous studies found that the expression of chemokines continuously decreased from the early stage of *in vitro* culture. Similarly, CXCR7 showed such a trend in our study. This indicates that the expression of chemokines is greatly affected by aging and is thought to be related to cell autonomous apoptosis [29]. CDK3 and CDC2L5 positively regulate the progression of cell proliferation by promoting cell division. The expression of both genes decreased continuously during passage. KLF4 is a transcription factor whose function is to maintain the undifferentiated status and pluripotency of embryonic stem cells. Its expression was down-regulated to a certain extent during culture and passage. This result suggests that self-renewal ability tends to decrease with replicative senescence.

Our study compared the differentiated expression profiles of MenSCs at different stages of replicative senescence or derived from donors of different ages. It is worthwhile to note that two genes, VEGFA and KLF4, not only were negatively regulated by age but also were down-regulated with continuous passage in culture. These genes are mainly involved in maintaining the integrity of the genome and regulating transcription [31].

Conclusion

In conclusion, *in vitro* data show that both donor age and passage number impose certain effects on MenSCs. This results in decreased capacity of MenSCs from older donors for long-term passage and decreased proliferative efficiency of MenSCs which have undergone multiple passages. Further, the differentiated gene expression profiles elucidate the differences among these cells more sensitively. The differentially-expressed genes screened in our study are mainly associated with aging and with certain biological functions. The differentially expressed genes identified can be used to evaluate the application potential of MenSCs in clinical practice.

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Disclosure of conflict of interest

None.

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References

[1] Prianishnikov VA. On the concept of stem cell and a model of functional-morphological structure of the endometrium. Contraception 1978; 18: 213-23.

[2] Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, King NW Jr, Longcope C, Kaiserman-Abramof IR. The basalis of the primate endometrium: a bifunctional germinal compartment. Biol Reprod 1989; 40: 681-90.

[3] Padykula HA. Regeneration in the primate uterus: the role of stem cells. Ann N Y Acad Sci 1991; 622: 47-56.
[4] Cho NH, Park YK, Kim YT, Yang H, Kim SK. Lifetime expression of stem cell markers in the uterine endometrium. Fertil Steril 2004; 81: 403-7.
[5] Chan RW, Schwab KE and Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. Biol Reprod 2004; 70: 1738-50.
[6] Patel AN, Park E, Kuzman M, Benetti F, Silva FJ, Allickson JG. Multipotent menstrual blood stromal stem cells: isolation, characterization, and differentiation. Cell Transplant 2008; 17: 303-11.
[7] Asumda FZ and Chase PB. Age-related changes in rat bone-marrow mesenchymal stem cell plasticity. BMC Cell Biol 2011; 12: 44.
[8] Alt EU, Senst C, Murthy SN, Slakey DP, Dupin CL, Chaffin AE, Kadowitz PJ, Izadpanah R. Aging alters tissue resident mesenchymal stem cell properties. Stem Cell Res 2012; 8: 215-25.
[9] Yu JM, Wu X, Gimble JM, Guan X, Freitas MA, Bunnell BA. Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. Aging Cell 2011; 10: 66-79.
[10] Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J Cell Biol 1999; 145: 769-82.
[11] Schwab KE and Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Hum Reprod 2007; 22: 2903-11.
[12] Dimitrov R, Timeva T, Kyurkchiev D, Stamenova M, Shterev A, Kostova P, Zlatkov V, Kehayov I, Kyurkchiev S. Characterization of clonogenic stromal cells isolated from human endometrium. Reproduction 2008; 135: 551-8.
[13] Dominina AP, Fridliandskaia II, Zemel'ko VI, Pugovkina NA, Kovaleva ZV, Zenin VV, Grinchuk TM, Nikol'skiĭ NN. [Mesenchymal stem cells of human endometrium do not undergo spontaneous transformation during long-term cultivation]. Tsitologiia 2013; 55: 69-74.
[14] Kassem M, Ankersen L, Eriksen EF, Clark BF, Rattan SI. Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. Osteoporos Int 1997; 7: 514-24.
[15] Stenderup K, Justesen J, Eriksen EF, Rattan SI, Kassem M. Number and proliferative capacity of osteogenic stem cells are maintained during aging and in patients with osteoporosis. J Bone Miner Res 2001; 16: 1120-9.
[16] Scharstuhl A, Schewe B, Benz K, Gaissmaier C, Bühring HJ, Stoop R. Chondrogenic potential of human adult mesenchymal stem cells is independent of age or osteoarthritis etiology. Stem Cells 2007; 25: 3244-51.
[17] Suva D, Garavaglia G, Menetrey J, Chapuis B, Hoffmeyer P, Bernheim L, Kindler V. Non-hematopoietic human bone marrow contains long-lasting, pluripotential mesenchymal stem cells. J Cell Physiol 2004; 198: 110-8.
[18] Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 2004; 22: 675-82.
[19] Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M, Madon E, Fagioli F. Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 2006; 97: 744-54.
[20] Fickert S, Schröter-Bobsin U, Gross AF, Hempel U, Wojciechowski C, Rentsch C, Corbeil D, Günther KP. Human mesenchymal stem cell proliferation and osteogenic differentiation during long-term ex vivo cultivation is not age dependent. J Bone Miner Metab 2011; 29: 224-35.
[21] Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. J Transl Med 2014; 12: 8.
[22] Musina RA, Belyavski AV, Tarusova OV, Solovyova EV, Sukhikh GT. Endometrial mesenchymal stem cells isolated from the menstrual blood. Bull Exp Biol Med 2008; 145: 539-43.
[23] Buyanovskaya OA, Kuleshov NP, Nikitina VA, Voronina ES, Katosova LD, Bochkov NP. Spontaneous aneuploidy and clone formation in adipose tissue stem cells during different periods of culturing. Bull Exp Biol Med 2009; 148: 109-12.
[24] Tarte K, Gaillard J, Lataillade JJ, Fouillard L, Becker M, Mossafa H, Tchirkov A, Rouard H, Henry C, Splingard M, Dulong J, Monnier D, Gourmelon P, Gorin NC, Sensebé L; Société Française de Greffe de Moelle et Thérapie Cellulaire. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. Blood 2010; 115: 1549-53.
[25] Zemel'ko VI, Grinchuk TM, Domnina AP, Artsybasheva IV, Zenin VV, Kirsanov AA, Bichevaia NK, Korsak VS, Nikol'skiĭ NN. [Multipotent mesenchymal stem cells of desquamated endometrium: isolation, characterization and use as feeder layer for maintenance of human embryonic stem cell lines]. Tsitologiia 2011; 53: 919-29.
[26] Meng X, Ichim TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW,

Thébaud B, Riordan NH. Endometrial regenerative cells: a novel stem cell population. J Transl Med 2007; 5: 57.

[27] Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM. A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 1995; 374: 450-3.

[28] Bailey Dubose K, Zayzafoon M and Murphy-Ullrich JE. Thrombospondin-1 inhibits osteogenic differentiation of human mesenchymal stem cells through latent TGF-beta activation. Biochem Biophys Res Commun 2012; 422: 488-93.

[29] Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW, Putti T, Murray P, Chan AT, Tao Q. Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Oncogene 2006; 25: 1070-80.

[30] Schenk S, Mal N, Finan A, Zhang M, Kiedrowski M, Popovic Z, McCarthy PM, Penn MS. Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. Stem Cells 2007; 25: 245-51.

[31] Ryu E, Hong S, Kang J, Woo J, Park J, Lee J, Seo JS. Identification of senescence-associated genes in human bone marrow mesenchymal stem cells. Biochem Biophys Res Commun 2008; 371: 431-6.