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Original Article

Age-related methylation of genomic DNA in human adipose-derived stem cells

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Abstract: Objective: Human adipose-derived stem cells (ADSCs) are multipotent stromal cells, andthe cellular functionsof ADSCs are regulated by genomic DNA methylation. The objective of this study was to research the relationship between age and ADSC genomic DNA methylation. Materials and methods: We examined the age-related gene expression and methylation of ADSCs from young (<25 years) and elderly (>55 years) patients. Real-time quantitative PCR was used to analyze OCT-4, NANOG and SOX-2 expression levels. Bisulfite sequencing was performed to determine the density of DNA methylation on target gene promoters. Results: After ADSCs from elderly patients (oldADSCs) were treated with the DNA-demethylating drug 5-aza-2'-deoxycytidine (5-Aza-dC), the OCT-4 and NANOG expression levels were significantly lower in oldADSCs than those in ADSCs from young patients (youngADSCs) (P=0.011 and P=0.030, respectively). Conversely, SOX-2 expression was significantly increased in oldADSCs (P=0.029). OCT-4 and NANOG promoter methylation was extremely dense in oldADSCs, but these promoters were hypomethylated in youngADSCs (P=0.031 and P=0.048, respectively). Moreover, significant associationswere found between methylation and the expression of OCT-4 and NANOG (R=-0.693, P=0.026 and R=-0.839, P=0.002, respectively). However, significant differencesin SOX-2 methylation were not observed between the two groups (P=0.179). Conclusion: ADSCs treated with 5-Aza-dC exhibited significant increases inOCT-4, NANOG, and SOX-2 expression. Our results suggest that DNA methylation plays an important role in ADSC aging and that DNA methylation density increases with patient age. More importantly, demethylation drugs may restore OCT-4 and NANOG expression inoldADSCs and could have implications regarding the potential for autologous stem cell therapies in elderly patients.

Keywords: Aging, adipose-derived stem cells, methylation, genomic DNA

Introduction

Human adipose-derived stem cells (ADSCs) are mesenchymal stem cells (MSCs) that can be collected from adipose tissue [1-3]. ADSCs have the capacity for self-renewal and the potential to differentiate into multiple lineages, including adipocytes, osteocytes, myocytes and chondrocytes [4-8]. Due to their broad differentiation potential and convenient accessibility, ADSCs are an attractive source of adult MSCs for many clinical applications, including orthopedic and reconstructive surgery, as well as cell developmental plasticity research [9-11]. Unfortunately, the physiological function and differentiation potential of ADSCs diminishes with advanced age, which limits the application of ADSCs in elderly patients [12-14].

Epigenetic modifications (DNA methylation, genomic imprinting, maternal effects and gene silencing) can regulate ADSC pluripotency and differentiation. Moreover, the epigenetic regulation of the genome is considered a crucial pathway affecting stem cell aging [15-18]. Recently, the effects of CpG methylation on proliferation, stemness, and differentiation have been carefully studied, particularly for important pluripotency genes, such as OCT-4, NANOG, and SOX-2 [19-23]. DNA methylation is a powerful mechanism regulating gene expression; hypermethylation of promoter regions has been shown to silence gene expression, whereas promoterhypomethylation can activate transcription [24].

However, few reports have examined OCT-4, NANOG and SOX-2 expression and promoter methylation across different ages. This study researched donor-age-related changes in the expression and methylation patterns of these three genes. Furthermore, we assessed wheth-

Table 1. Sequence of primers

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature
OCT-4	TGAAGCTGGAGAAGGAGAAGCTG	TCTTTCTGCAGAGCTTTGATGTCCT	64.0°C
NANOG	AACTGGCCGAAGAATAGCAA	ACTGGATGTTCTGGGTCTGG	69.6°C
SOX-2	CATGCACCGCTACGACG	CGGACTTGACCACCGAAC	64.5°C
GAPDH	GATGGGATTTCCATTGATGACA	CCACCCATGGCAAATTCC	60.0°C
OCT-4-BSP	TGGATTTAATTTAATGGGGTT	CAACAAACACAACAATAAAACC	57.2°C
NANOG-BSP	GGATTATAGGGGTGGGTTAT	CTACATAATAACATAAAACAACCAACTCAA	60.5°C
SOX-2-BSP	TGGTAGGTTGGTTTTGGGAG	TAAAACTCAAACTTCTCTCCCTT	59.8°C

er these age-related changes are reversible after DNA demethylation.

Materials and methods

Cultivation and isolation of ADSCs

The study was approved by the Institutional Ethics Committee of the Affiliated Shanghai Eighth People's Hospital of Jiangsu University.

Human abdominal fat tissue was obtained from young (aged<25 years, n=5) and elderly (aged>55 years, n=5) donors who underwent abdominoplasty. Informed consent was received from all patients for the use of their tissues.

The subcutaneous fat was washed three times with phosphate-buffered saline (HyClone, Logan, UT, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) and gently shaken to removeblood. Then, the samples were cut into small pieces (1 mm^3) using scissors. Adipose tissues were then treated with 0.075% collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 40 minutes at 37°C in a vibrating, constant-temperature water bath. Then, Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin was added to stop the digestion.

The digested fat was centrifuged at 300×g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in complete medium (DMEM/F12 containing 10% FBS) and filtered through a 100 μm nylon mesh filter (Millipore, Billerica, MA, USA). The filtered cell fraction was incubated overnight, and the adherent cells were collected, maintained at 37°C in a humidified atmosphere containing 5% CO_2, and cultured to passage 3 for experiments.

Immunophenotyping

To characterize the ADSCs, we performed a flow cytometric analysis for six MSC markers, CD44, CD73, CD90 and CD105, and two negative markers, CD34 and CD45. Fluorescence-conjugated antibodies were purchased from eBioscience (San Diego, CA, USA). The surface antigens were analyzed witha FACSVerse system (BD Bioscience, San Jose, CA, USA) using FlowJo software (Treestar, Inc., San Carlos, CA, USA).

Adipogenic differentiation of ADSCs

ADSCs from healthy donors (passage 3) were seeded at 2×10^4 cells/cm^2 in 6-well cell culture plates. Cells were allowed to grow to post-confluence in complete medium. Adipogenic differentiation of ADSCs was achieved using an adipogenic kit (Cyagen Biosciences, Guangzhou, China) and was confirmed by Oil Red O (Cyagen) staining of lipid droplets after 16 days in culture.

Osteogenic differentiation of ADSCs

ADSCs (passage 3) were seeded at 2×10^4 cells/cm^2 in 6-well cell culture plates precoated with 0.1% gelatin solution (Cyagen). Cells were grown to 80-90% confluence in complete medium, which was then replaced with osteoinduction medium (Cyagen). Osteoinduction was stopped at day 18, and the cells were stained with alizarin red (Cyagen) for microscopic visualization.

5-Aza-2'-deoxycytidine (5-Aza-dC) treatment of ADSCs

For demethylation studies, ADSCs were seeded at an initial density of 1×10^5 cells/cm^2. After

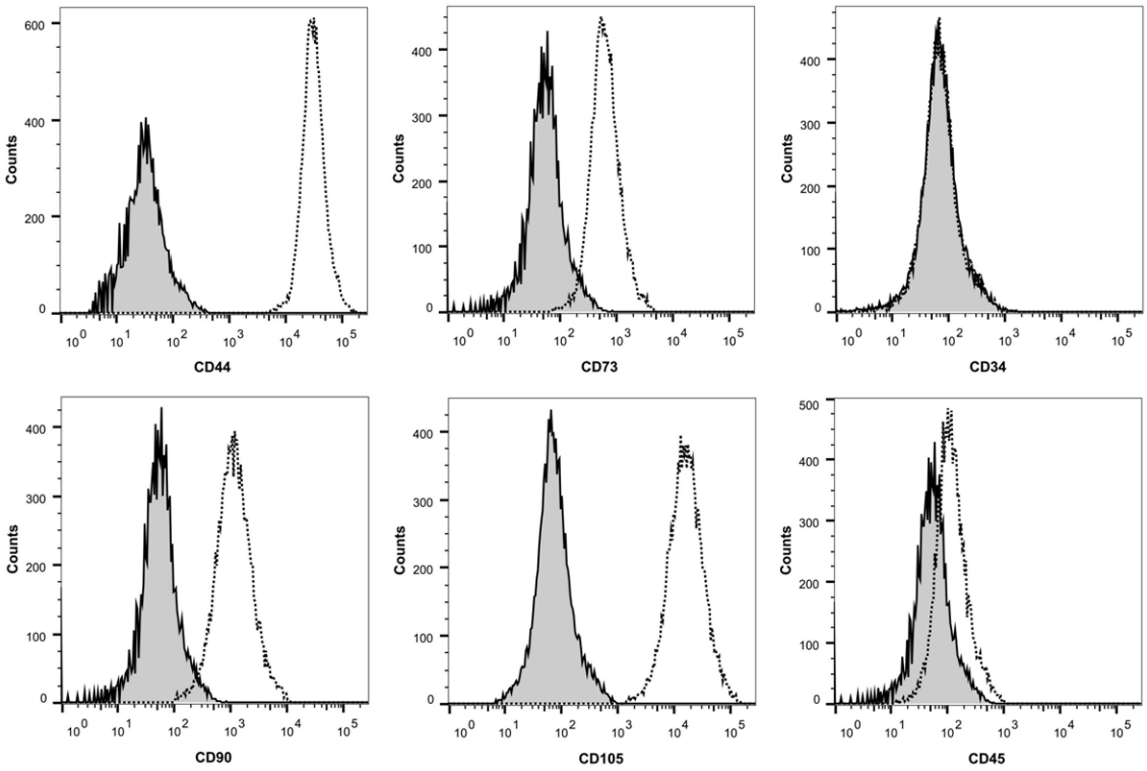

Figure 1. Flow cytometric analysis of cell surface marker expression in adipose-derived stem cells (ADSCs). Isolated ADSCs at passage 3 were prepared for flow cytometric analysis. The dashed lines indicate stained cells, and the straight lines represent the isotype-matched monoclonal antibody control.

attachment, the cells were incubated with culture medium containing 5-Aza-dC (Sigma-Aldrich, Steinheim, USA) (final concentrations of 0 μM, 1 μM, or10 μM) for 72 hours. All the cells were cultured until harvested for RNA and DNA extraction.

RNA isolation, reverse transcription and real-time quantitative PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA. Reverse transcription reactions (40 μL) contained 10 mM dNTPs, 10 μM random hexamers, 10 mM 5× buffer, 80 U RNasin, and 200 U MMLV reverse transcriptase (MBI Fermentas, Hanover, USA). Reverse transcription was performed using an iCycler Thermal Cycler (Eppendorf, Hamburg, Germany). The reaction mixtures were incubated for 10 minutes at 25°C, 60 minutes at 42°C, and then stored at -20°C. qRT-PCR was performed on a 7500 Thermo cycler (Applied Biosystems, CA, USA). The primer sequences used to assess OCT-4, NANOG, and SOX-2 expression are listed in **Table 1**. The PCR reactions contained 20 ng of cDNA, 10 μM SYBR Premix Ex Taq II (Takara, Japan), 0.4 μM 50× ROX (Takara, Japan) and 0.8 μM primers. The RT-PCR reaction conditions were 95°C for 30 s; 40 cycles of 95°C for 5 s, annealing temperature for 30 s (listed in **Table 1**), 72°C for 30 s, and 80°C for 30 s to collect fluorescence, followed by 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. GAPDH was used as the endogenous control. All assays included positive and negative controls.

DNA isolation and chemical modification

A genomic DNA purification kit (Gentra, Minneapolis, MN, USA) was used to isolate DNA. Genomic DNA was modified by the Cp Genome DNA Modification Kit (Chemicon, Ternecula, Canada) according to the manufacturer's instructions.

Bisulfite sequencing (BSP)

BSP was performed to analyze the DNA methylation density on the three gene promoters. Bisulfite-treated DNA was amplified with the sequencing primers listed in **Table 1**. The reaction was performed on an iCycler Thermal

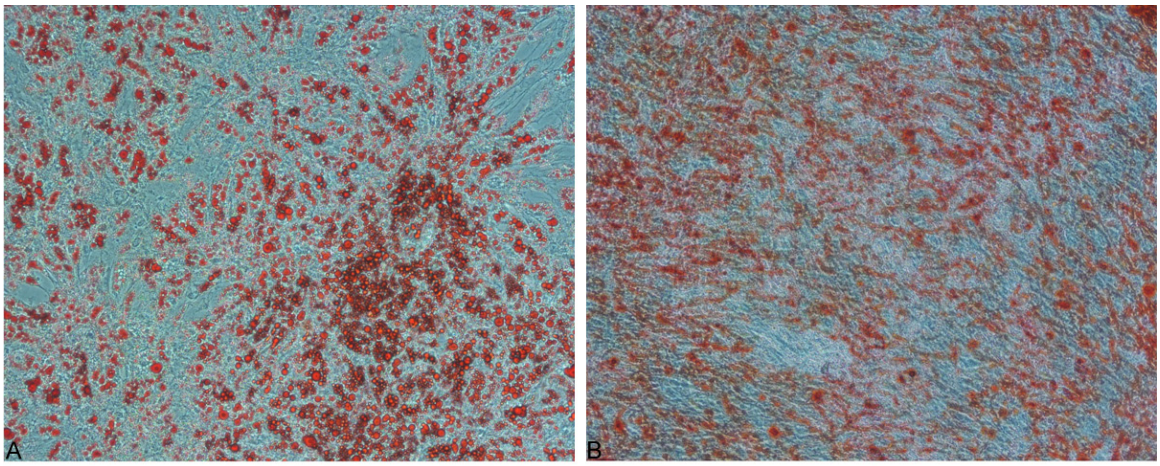

Figure 2. Characterization of isolated ADSCs by adipogenic and osteogenic differentiation. A: ADSCs were cultured in adipogenic media for 16 days and showed positive Oil Red O staining results. The lipid droplets appeared red under the fluorescence microscope (×100). B: ADSCs were cultured in osteogenic media for 18 days and showed positive alizarin red staining results. Red staining marked mineral deposition in the newly formed extracellular matrix (×100).

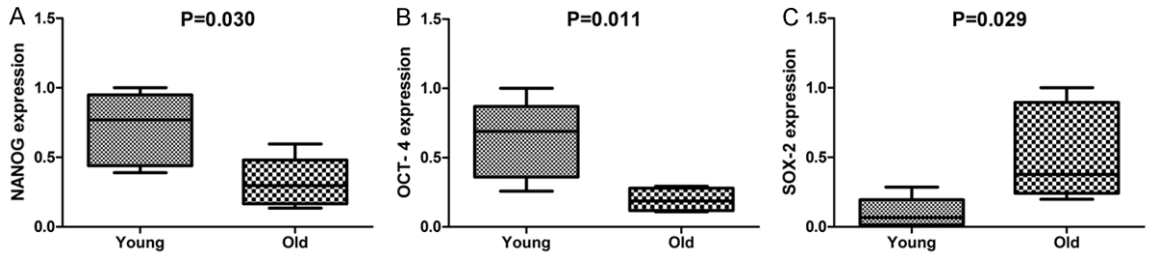

Figure 3. The expression levels of genes in the two groups. A: NANOG; B: OCT-4; and C: SOX-2.

Cycler (Eppendorf, Hamburg, Germany) in 25-μL reaction mixtures containing 10×PCR buffer (0.25 mM KCl), 6.25 μM dNTP mixture, 0.5 μM primers, 0.75 U Hot Dtart DNA polymerase (Takara, Tokyo, Japan), and 20 ng of modified DNA. PCR conditions were 98°C for 10 s; 40 cycles of 10 s at 98°C, 30 s at annealing temperature (listed in **Table 1**), and 30 s at 72°C; followed by a final 7-minute extension step at 72°C. The PCR products were purified using the AxyPrep DNA Gel recovery kit (Axygen, Suzhou, China) and cloned into a pMD®19-T Vector (Takara, Dalian, China); five clones for each sample were sequenced at BGI (Shanghai, China).

Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Science (SPSS) software 20.0 package (SPSS, Chicago, IL, USA). Allresults are expressed as the mean ± SEM of at least 3 independent experiments and were compared with two-tailed Student's t-tests. *P* values<0.05 were considered statistically significant.

Results

Phenotypic characterization of ADSCs

Using our isolation and culture methods, ADSCs were easily expanded *in vitro* and exhibited a fibroblast-like morphology. Cell surface marker expressionwas analyzed using flow cytometry (**Figure 1**), which revealed the expression of the MSC markers CD44, CD73, CD90 and CD105 in the ADSCs. The hematopoietic lineage markers CD34 and CD45 were not expressed in the derived ADSCs. Therefore, our results suggest that ADSCs isolated from fat tissue resemble MSCs rather than hematopoietic stem cells.

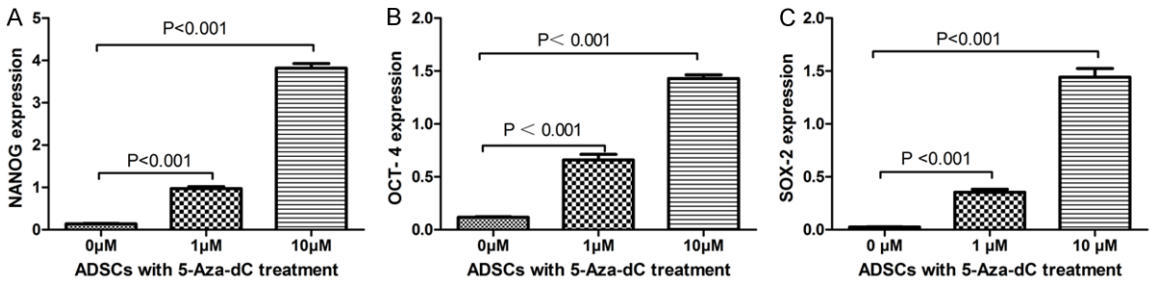

Figure 4. Gene expression levels after 5-Aza-dC treatment. A: NANOG; B: OCT-4; and C: SOX-2.

Induction of multilineage differentiation

Adipogenesis: Adipogenic differentiation of ADSCs was induced with an adipogenic kit. Preadipocytes with lipid droplets appeared after approximately one week of treatment. The lipid vacuoles became larger after 16 days of treatment and were clearly visible without staining under a phase microscope. After Oil Red O staining, the lipid nature of the red vacuoles was evident (**Figure 2A**). The ability of these cells to differentiate into adipocytes was observed in cells derived from both young and elderly donors.

Osteogenesis: After treatment with an osteogenic kit for 18 days, the cell monolayer was covered with a layer of visible deposits indicative of cell differentiation. Positive staining with alizarin red revealed that these deposits were calcified extracellular matrix (**Figure 2B**). The induction of osteogenesis in ADSCs derived from both young and elderlydonorswas widespread, and these results indicated that ADSCs retained their capacity for osteogenic differentiation.

Gene expression levels in ADSCs from patients of different ages

The ADSCs were divided into two groups according to their age (<25 years and >55 years). The relative OCT-4, NANOG and SOX-2 expression levels were evaluated with qRT-PCR, and GAPDH was used as an internal control. NANOG and OCT-4 expression levels were significantly lower in ADSCs from elderly patients than in those from young patients (P=0.011 and P=0.030, respectively) (**Figure 3A**, **3B**). Conversely, SOX-2 expression was significantly elevated in ADSCs from elderly patients (P=0.029) compared with ADSCs from young patients (**Figure 3C**).

Gene expression levels after 5-Aza-dC treatment

The cell lines with low OCT-4, NANOG and SOX-2 expression were selected for 5-Aza-dC treatment at different concentrations (0 μM, 1 μM or 10 μM).

Accordingly, OCT-4, NANOG and SOX-2 expression was all significantly increased after 5-Aza-dC treatment (**Figure 4**).

DNA methylation status of OCT-4, NANOG and SOX-2

To investigate the methylation density of the OCT-4, NANOG and SOX-2 promoters, BSP was performed on cells from five young patients and five elderly patients. The methylation density of OCT-4 and NANOG was extremely high in elderly patients, whereas OCT-4 and NANOG were hypomethylated in young patients (P=0.031 and P=0.048, respectively) (**Figure 5A**, **5B**). However, no significant differences in SOX-2 methylation were observed between the two groups (P=0.179) (**Figure 5C**).

The cell lines were treated with different concentrations of 5-Aza-dC, andthe BSP results showed that OCT-4, NANOG and SOX-2 promoter methylation was decreased and that OCT-4, NANOG and SOX-2 expression levels were significantly increased after 5-Aza-dC treatment (**Figure 6**).

The association between OCT-4, NANOG and SOX-2 methylation and expression

The expression levels of OCT-4, NANOG and SOX-2 in ten patients ranged from 0.109 to 1.000, 0.197 to 1.000 and 0.026 to 1.000, respectively. A significant correlation was observed between OCT-4 and NANOG expres-

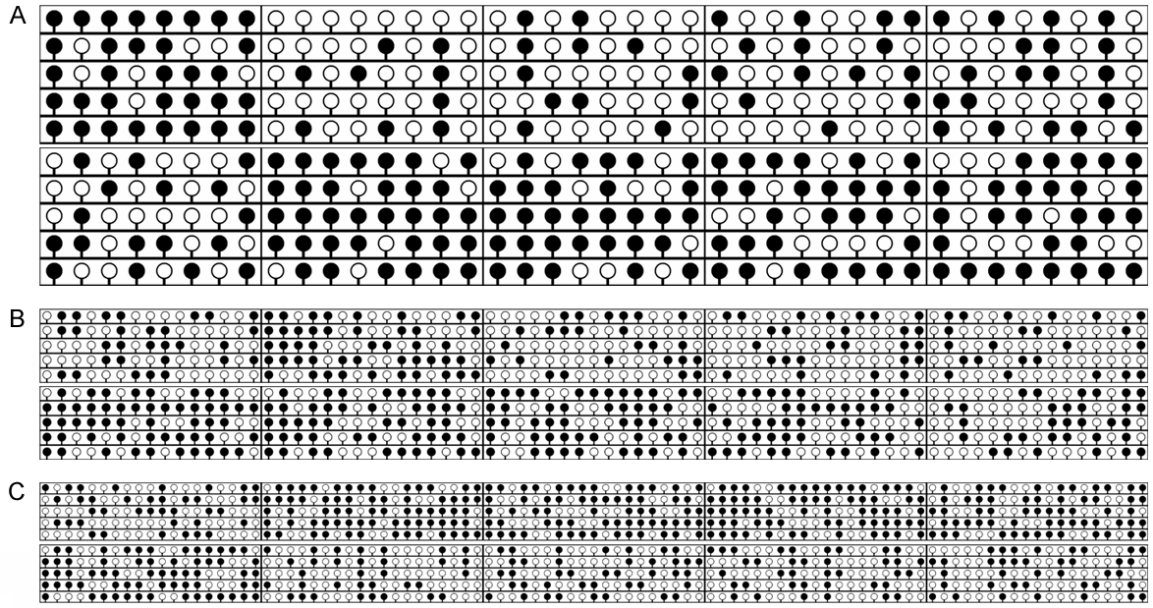

Figure 5. DNA methylation status of genes in the two groups. A: NANOG; B: OCT-4; and C: SOX-2. Upper: DNA methylation density of genes in five young patients. Lower: DNA methylation density of genes in five elderly patients.

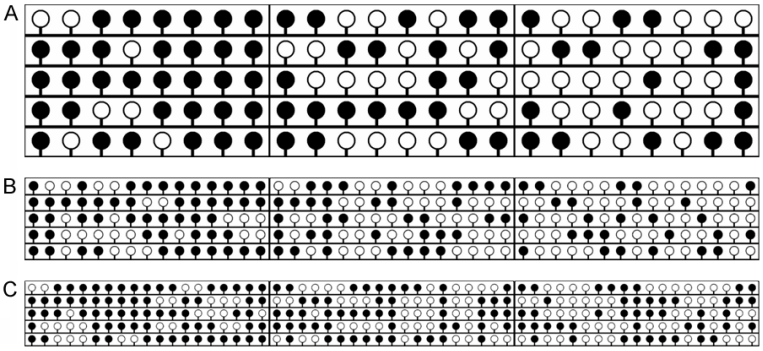

Figure 6. Cell lines were treated with different concentrations of 5-Aza-dC (0 μM, 1 μM, or 10 μM). A: NANOG (0 μM, mean density: 82.5%; 1 μM, mean density: 55%, P=0.009; 10 μM: mean density: 42.5%, P=0.001); B: OCT-4 (0 μM, mean density: 69.33%; 1 μM, mean density: 48%, P=0.013; 10 μM: mean density: 34.67%, P=0.001); and C: SOX-2 (0 μM, mean density: 71.58%; 1 μM, mean density: 55.79%, P=0.014; 10 μM: mean density: 43.16%, P<0.001).

sion and promoter methylation (R=-0.693, P=0.026 and R=-0.839, P=0.002). However, a significant correlation between SOX-2 expression and promoter methylation was not observed (R=-0.315, P=0.375).

Discussion

The study of ADSCs has become popular because ADSCs have the potential to treat various diseases. Owing to their multilineage differentiation potential, ADSCs have been applied in regenerative medicine [21, 25, 26]. In addition, theycan be used totreat immune diseases [27, 28]. Moreover, ADSCs have been shown top romote vasculogenesis, which can enhance engraftment success [29]. However, these therapeutic properties gradually weaken with ADSC senescence, which restricts their application sin elderly patients [12-14].

Some studies have indicated that cellular senescence negatively impacts MSC functions through various molecular mechanisms, such as genomic in stability, telomere attrition, altered intercellular communication, mitochondrial dysfunction, proteostasis loss and deregulated nutrients [30-33]. The increasing age of MSCs and their hosts has been associated with decreased capacities for proliferation, differentiation and mobilization [34]. Campisi J posited that the presence of senescent cells, whether stem cells themselves or surrounding cells in stem cell niches, could disrupt the stem cell microenvironment andthat this senescent microenvironment could then alter the functional capacity of the resident stem cells [35].

Due to their broad multipotent potential and convenient accessibility, ADSCs have become the focus of recent research. Several studies have focused on the mechanisms that affect ADSC functions, especiallythe methylation of certain genes [36-39]. CpG methylation of the CD31 and CD144 promoter regions in ADSCs was demonstrated to limit ADSC differentiation potential [40]. Berdasco M et al. reported that DNA methylation was necessary toregulate ADSC differentiation and proliferation [41]. Furthermore, the use of 5-Aza-dC may promote ADSC differentiation [20].

However, the status of gene expression and promoter methylation at different ages has not been previously described. This study examined donor-age-related changes in the pattern of OCT-4, NANOG and SOX-2 expression and methylation. Furthermore, we assessed whether these age-related changes could be reversed after treatment with a demethylation agent.

In this study, we unexpectedly discovered that OCT-4 and NANOG expression were significantly lower in oldADSCs than in youngADSCs. Additionally, the density of OCT-4 and NANOG methylation was extremely high in oldADSCs, but OCT-4 and NANOG were hypomethylated in youngADSCs. Furthermore, we also observed a significant association between the methylation and expression of OCT-4 and NANOG, suggesting that the age-related methylation of OCT-4 and NANOG along their promoters regulates their expression. Moreover, in ADSCs treated with 5-Aza-dC, both OCT-4 and NANOG expression levels were significantly increased, further confirming that both OCT-4 and NANOG expression can be restored by treatment with a demethylating drug, thereby providing a method to study and treat aging. Zhou GS et al. reported that pretreating MSCs with 5-Aza-dC significantly accelerated their osteogenic differentiation, resulting in DNA hypomethylation and the increased expression of osteogenic genes [42]. Yan X et al. revealed that 5-Aza-dC improved the osteogenic differentiation potential of aged ADSCs by DNA demethylation [20]. Unfortunately, although SOX-2 expression was significantly increased in oldADSCs, no significant differencesin SOX-2 methylation were observed between the two groups. Accordingly, more prospective studies are needed to confirm and elucidate the role of age-related DNA methylation in ADSCs.

In summary, our investigation discovered that DNA methylation plays an important role in ADSC aging and that DNA methylation density increases with donor age. Additionally, demethylating agents can restore OCT-4 and NANOG expression in ADSCs from elderly patients.

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Disclosure of conflict of interest

None.

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