



## Original Article

# Enhancement of individual differences in proliferation and differentiation potentials of aged human adipose-derived stem cells



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## ABSTRACT

**Background:** Adipose-derived stem cells (ASCs) are a robust, multipotent cell source. They are easily obtained and hold promise in many regenerative applications. It is generally considered that the function of somatic stem cells declines with age. Although several studies have examined the effects of donor age on proliferation potential and pluripotency of ASCs, the results of these studies were not consistent.

**Objective:** This study tested whether the donor age affects the yield of ASCs from adipose tissue, as well as the proliferation and differentiation potentials of ASCs.

**Methods:** This study used ASCs obtained from adipose tissues of 260 donors (ages 5–97 years). ASCs were examined for individual differences in proliferation, and adipogenic, osteogenic and chondrogenic differentiation potentials *in vitro*. Characteristics of ASCs from each donor were evaluated by the principal component analysis (PCA) using their potential parameters.

**Results:** Analyses on ASCs demonstrated that adipogenic potentials declined with age, but proliferation, osteogenic and chondrogenic potentials were not correlated with age. Interestingly, in all ASC potentials, including adipogenesis, individual differences were observed. Principal component analysis (PCA) revealed that individual differences became evident in the elderly, and those variations were more prominent in females than in males.

**Conclusions:** This study demonstrated age-related changes in the potentials of ASCs and revealed that the individual differences of ASCs become significant in people over 60 years of age (for females over 60, and for males over 80). We believe that it is important to carefully observe ASC potentials in order to achieve effective regenerative medicine treatments using ASCs.

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**Abbreviations:**  $\alpha$ MEM,  $\alpha$  minimum essential medium; CFU-F, colony-forming unit-fibroblast; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; GAGs, glycosaminoglycans; PCA, principal component analysis; PDL, population doubling level.

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## 1. Introduction

Mesenchymal stem cells (MSCs) are adult stem cells capable of differentiating into mesenchymal cells such as osteoblasts, adipocytes, chondrocytes and muscle cells, and are thought to hold great promise in the field of regenerative medicine. MSCs can be obtained from several tissues such as bone marrow, adipose, muscle, skin and umbilical cord, and MSCs from any of these tissues have similar

morphological characteristics and immunophenotypes [1–3]. Among these stem cells, bone marrow-derived stem cells (BMSCs) have been studied over many years; a large number of studies including clinical data have been published [4–11]. However, BMSCs are not always easy to obtain because of the donor's health condition. Furthermore, the percentage of BMSCs contained in bone marrow is only 0.001–0.01% [12] of all nucleated cells.

On the other hand, as many as 600,000 to 800,000 stromal vascular fraction (SVF) cells can be isolated per gram of adipose tissue, and a great number of adipose-derived stem cells (ASCs) are contained in the SVF cells [13]. More specifically, approximately 500-times more MSCs are available from adipose tissue than from the same amount of bone marrow, thus, adipose tissue makes it easier to obtain MSCs from all donors with a minor risk. As ASCs are characterized by excellent proliferation potential, and can be cultured in large quantities and cryopreserved [14], ASCs have gained attention in recent years as a source of cells for application in cellular treatments [15,16].

It is generally considered that the function of somatic stem cells declines with age. Some studies have suggested that proliferation and osteogenic potentials of BMSCs also decline with age [17–19]. Although several studies have demonstrated the effects of donor age on proliferation potential and pluripotency of ASCs, the findings were not consistent across these studies [20–30]. In our previous study, we examined age-related changes using mouse ASCs and found that the number of ASCs obtained, as well as their adipogenic and osteogenic potentials decline with age [31]. In this study, we evaluated age-related changes in the numbers and potentials of human ASCs obtained from 260 donors (ages 5–97 years, mean 61.8).

## 2. Materials and methods

### 2.1. Collection and processing of adipose tissue-derived mesenchymal stem cells

This study was conducted with an ethical approval from the Research and Ethics Committee of Fujita Health University (approval No. 15-235). After obtaining informed consent, subcutaneous adipose tissue was obtained from excess normal skin of total 260 patients aged 5 to 97 with median age of 68 (male:  $n = 115$ , 5–97 years old, median age: 66; female:  $n = 145$ , 5–96 years old, median age: 69) who underwent a dermatological surgery. Subcutaneous adipose tissue (0.1–11 g, median 0.64 g) from a single part of body of each patient was used to prepare a specimen. The tissues were collected from various body parts (abdomen: 29.2%, groin: 15.8%, lower limb: 14.2%, back: 10.4%, buttock: 7.3%). The subcutaneous adipose tissue was shredded and incubated for 1 h at 37 °C in 0.5% collagenase L (Nitta Gelatin Inc., Osaka, Japan), followed by filtration using 100  $\mu\text{m}$  nylon cell strainer (Falcon<sup>®</sup>, Coning, NY, USA). After centrifugation, cell precipitates were suspended by tris-buffered ammonium chloride (ACTB) to eliminate red blood cells and washed by PBS. The cells separated in this way are defined as SVF cells, and among SVF cells, only cells which were able to adhere and multiply are referred to as ASCs in this study. The separated SVF cells and ASCs were cultured in complete medium (D/ $\alpha$  medium) consisting of 50% Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA)/50%  $\alpha$  minimum essential medium ( $\alpha$ MEM, Invitrogen) supplemented with 1% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1  $\times$  ITS-X (Invitrogen), 10 ng/mL basic FGF (PeproTech, Rocky Hill, NJ, USA), and 0.4  $\mu\text{g}/\text{mL}$  hydrocortisone and 1% Antibiotic-Antimycotic (Gibco BRL, Rockville, MD) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> until passage 5. Population doubling level (PDL) and doubling time were determined by counting the number of viable cells in

each passage using a hemocytometer. Seeding density was 1–2  $\times 10^4/\text{cm}^2$ . Culture media were replaced every 2–3 days.

### 2.2. CFU-F assay

To measure a colony-forming unit-fibroblast (CFU-F), ASCs (passage 0) were seeded at 1  $\times 10^2$  cells/well in triplicate in 6-well plates (Falcon<sup>®</sup>) and cultured at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> in D/ $\alpha$  medium. After 14-day culture, cells were fixed with methanol and stained with a solution of 0.5% (w/v) crystal violet (Sigma-Aldrich). After the individual colonies were counted, the frequency of CFU-F was expressed as a percentage. The culture medium was replaced once after 6 days.

### 2.3. Measurement of adipogenesis

ASCs (passage 5) were seeded in triplicate in a 24-well plate (Falcon<sup>®</sup>) at a density of 4  $\times 10^4$  in D/ $\alpha$  medium. After 4 days, differentiation induction was carried out using adipogenic induction medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 33  $\mu\text{M}$  Biotin (Sigma-Aldrich), 10  $\mu\text{g}/\text{mL}$  Insulin (Sigma-Aldrich), 1  $\mu\text{M}$  dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), and 1% Antibiotic-Antimycotic (Gibco) for 6 days. Additionally, ASCs were cultured using adipogenic differentiation medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 3  $\mu\text{M}$  Biotin (Sigma-Aldrich), 10  $\mu\text{g}/\text{mL}$  Insulin (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco) for 4 days. The medium was replaced every 2–3 days. After adipogenic differentiation was induced, optical densities were determined using Cell Counting Kit-8 (cck8; DOJINDO Laboratories, Kumamoto, Japan) solution mixed with D/ $\alpha$  medium. The adipogenic differentiation medium was replaced with this solution, and then the differentiated ASCs were incubated for 1 h at 37 °C under 5% CO<sub>2</sub>. Next, the supernatants (100  $\mu\text{l}$ ) were transferred into a 96-well plate, and the absorbance values were measured at 450 nm. All analyses were carried out in triplicate.

Adipogenesis was assayed by lipid accumulation in differentiated cells with Oil Red O staining. After staining, Oil Red O was extracted with 100% isopropanol and the optical density (OD) of the solution was measured at 520 nm as well as cck8 [31]. Optical densities of Oil Red O were adjusted by the result of cck8. For comparison of Oil Red O/cck8 values among donors, ASCs were cultured with standard cells (UE7T-13; immortalized bone marrow-derived mesenchymal stem cell) for differentiation induction. Oil Red O/cck8 values of ASCs was adjusted relative to those of UE7T-13, and the adjusted values were used for evaluation of adipogenic potential.

### 2.4. Measurement of osteogenesis

ASCs (passage 5) were seeded in triplicate in a 24-well plate (Falcon<sup>®</sup>) at a density of 4  $\times 10^4$  in D/ $\alpha$  medium. After 4 days, differentiation was carried out using osteogenic differentiation medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 50  $\mu\text{M}$  ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01  $\mu\text{M}$  Dexamethasone (Sigma-Aldrich) and 1% Antibiotic-Antimycotic (Gibco) for 21 days. Culture medium was replaced every 2–3 days. After induction was completed, the cell densities were determined using cck8.

Osteogenesis was assayed by calcium content in differentiated cells and matrix mineralization with Alizarin Red S staining. The total calcium content was determined by a colorimetric assay using Calcium E-Test Wako (Wako Pure Chemical Industries, Osaka,

Japan) after eluted by 10% (w/v) Formic acid (Wako) [31]. By the same method as for adipogenesis, chondrogenic potential was evaluated by the values adjusted by standard cells.

### 2.5. Measurement of chondrogenesis

ASCs (passage 5) were seeded in triplicate in a 15 mL polypropylene conical tube (Falcon®) at a density of  $1 \times 10^5$ . After centrifuged, cells were suspended by chondrogenic differentiation medium consisting of DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 50  $\mu$ M ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01  $\mu$ M Deamethasone (Sigma-Aldrich), 0.5  $\times$  ITS-X (Invitrogen), 10 ng/mL BMP-2 (PeproTech Inc.), 10 ng/mL TGF- $\mu$ 1 (PeproTech Inc.) and 1% Antibiotic-Antimycotic (Gibco). Cells were further centrifuged for 2 min at 500 g. After the 14-day differentiation culture, cells were washed in PBS. The differentiation medium was replaced every 2–3 days.

Chondrogenesis was assayed by the sulfated glycosaminoglycans (GAGs) content [32] and normalized with DNA content [33]. By the same method as for adipogenesis, osteogenic potential was evaluated by the values adjusted to standard cells.

### 2.6. Gene expression analysis

We examined the expression levels of CD44, CD73, CD90, CD105 and CD271, which have been reported as MSC surface markers [34–37], and that of NANOG, an undifferentiated cell marker by qPCR. Total RNA of ASCs passaged five times was extracted with RNAiso Plus (Takara Bio Inc., Shiga, Japan), and 1  $\mu$ g of RNA was used for reverse transcription with PrimeScript™ RT Master Mix (Takara) to get cDNA. Real-time semi-quantitative RT-PCR was performed with the THUNDERBIRD® SYBR® qPCR Mix (TOYOBO Co., LTD, Osaka, Japan) using the StepOnePlus Real-time RT-PCR system (Life Technologies Japan, Tokyo, Japan). The primer sequences are shown in Table 1. Amplification was normalized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Differences between samples were quantified based on the  $\Delta\Delta C_t$  method.

### 2.7. Statistical analysis

The relationship between each ASC potential (i.e. proliferation, adipogenesis, osteogenesis and chondrogenesis) and donor age was determined by Spearman's rank correlation coefficient, and a test for no correlation was performed. To see if the expression levels are significantly different, t-tests were conducted.

Characteristics of ASCs from each donor were evaluated by principal component analysis (PCA) using the following parameters: doubling time, and adipogenic, osteogenic and chondrogenic potentials. Each measured value was normalized to use for PCA. Distribution charts of each donor were made with reference to an X axis as the first principal component (PC1) and a Y axis as the second principal component (PC2). The distance between the mean values of each age group was determined. Variations of each group

were evaluated by non-parametric Shirley–Williams' multiple comparison test. A p value of <0.05 was considered statistically significant.

All statistical analyses were performed by R program (version 3.1.1, R Development Core Team 2012).

## 3. Results

### 3.1. Effects of donor age on ASC growth

SVF cells, including ASCs, were successfully separated from adipose tissue of all donors (n = 260, ages 5–97 years). The number of SVF cells per gram of tissue was determined by counting cells using a hemocytometer. From one gram of adipose tissue, 750–1,198,590 SVF cells (median 9923 cells) were separated. The numbers of separated cells were not correlated with donor age (r = 0.103, Fig. 1A). Subsequently, colony formation assay was performed for 75 randomly chosen samples. ASCs were seeded at a concentration of 100 cells/well in a 6-well plate and cultured for 2 weeks to count colonies. For each age group, 1–50 colonies (median 11 colonies, mean  $14 \pm$  SEM 10.58 colonies) were found. There were varied numbers of colonies regardless of donor age (r = -0.037, Fig. 1B). Therefore, the number of cells separated from adipose tissue and the number of cells capable of adhesive proliferation were not correlated with donor age, thereby yielding variations among the samples.

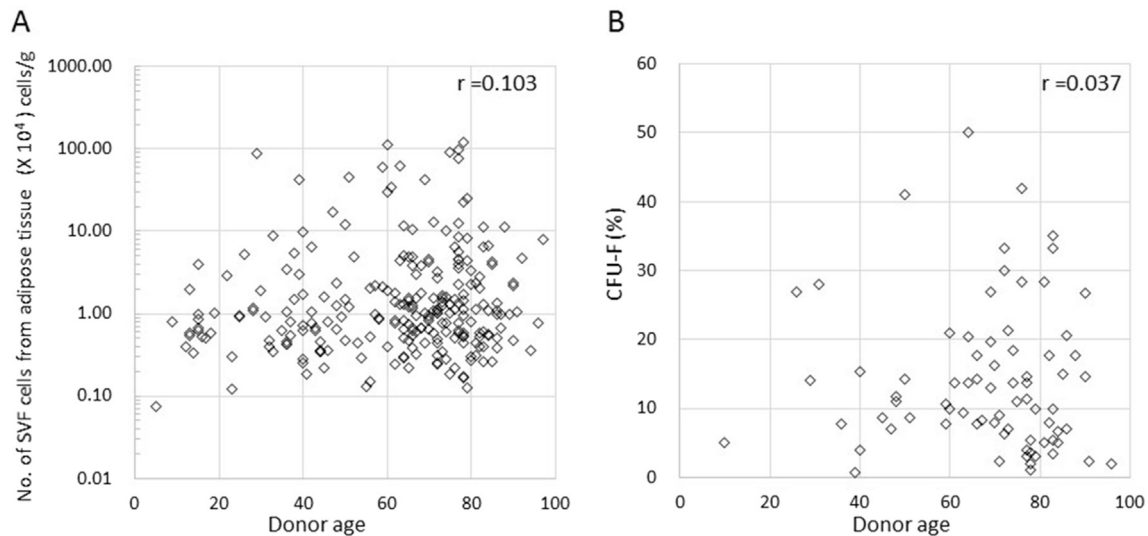
All ASCs were able to be cultured and passaged, and the population doubling levels (PDL) varied as follows; median 4.51 h (range 1.42–8.10) at passage 1 (P1), median 7.58 h (range 4.73–19.60) at P2, median 10.32 h (range 7.56–22.61) at P3, median 12.74 h (range 8.83–24.20) at P4 and median 14.96 h (range 9.94–26.31) at P5. Doubling time changed through passages as follows; median 29.18 h (range 17.76–68.34) at P1, median 30.96 h (range 19.61–105.98) at P2, median 36.06 h (range: 26.26–166.60) at P3 and median 45.34 h (range 27.23–205.70) at P4 (date not shown). By analyzing the doubling time of ASCs (passage 4) of 254 samples from the donors aged 5 to 97, the doubling time became longer in an age-dependent manner, although there was not a significant correlation between proliferation potential and donor age (the correlation coefficient, r = 0.099; Fig. 2A). In the other analyses, correlations between proliferation potential and donor age were not observed either. However, individual differences were observed in all age groups.

All ASCs exhibited a fibroblast-like morphology, and morphological characteristics of ASCs varied depending on their proliferation potential. ASCs with a good proliferation potential were relatively small and exhibited a sharp shape, whereas ASCs with a reduced proliferation potential were larger, appeared swollen and did not exhibit a spindle shape (Fig. 2B).

In both the young donor group (<40 years of age) and the elderly group ( $\geq$ 60 years of age), ASCs from 9 donors each were randomly chosen from both ASC groups demonstrating a high proliferation potential and those with a low proliferation potential. Then, the expression levels of MSC surface markers, CD44, CD73, CD90, CD105 and CD271, and that of the undifferentiated cell marker, NANOG, in these ASC samples were examined by qPCR (Fig. 2C–H). ASCs of the elderly group exhibited a significantly higher expression of CD73 (p < 0.01) compared with those of the young group. When the gene expressions were compared between the ASCs of high and low proliferation potential, in the elderly group, there was no significant difference. On the other hand, in the young group, ASCs of high proliferation potential had a significantly lower expression of CD105 (p < 0.05) than those in the low potential group. Other than the differences described above, there were no

**Table 1**  
Primers used for real-time quantitative PCR.

Gene	Forward sequence	Reverse sequence
GADPH	TGCACCACCAACTGCTTAGC	TCCTCTGGGTGGCAGTGATG
CD44	CGTGATGGCACCCGCTAT	GGGTAGATGTCTTCAGGATTCGT
CD73	ACTGGGACATTCGGGTTTTG	GACGTCCACACCCCTCACTT
CD90	CCGCTCCCAACCAACT	GGCGGATAAGTAGAGACCTTCA
CD105	ATCACCCACAGCGAAAAAGG	GGTAGAGGCCAGCTGGAA
CD271	CATCTGGCTGCTGTGTT	TGCAGCTGTCCACCTCTTG
NANOG	CCTTCCTCCATGGATCTGCTT	AAGTGGGTGTTTGCTTGG



**Fig. 1. Effect of donor age on the number and CFU-F of SVF cells.** (A) The number of SVF cells per gram separated from adipose tissue was not correlated with donor age ( $n = 251$ ). SVF cells were obtained from adipose tissue of all donors. There was no correlation between the numbers of SVF cells and donor age ( $r = 0.103$ ). (B) CFU assay was performed on fresh SVF cells ( $n = 75$ , 10–96 years old). There was no correlation between the number of colonies and donor age ( $r = 0.037$ ). The correlation coefficient was determined by Spearman's rank correlation coefficient, and the significance was tested by a test for no correlation.

differences in the comparisons of young vs. elderly, nor in high vs. low potential.

### 3.2. Effects of donor age on adipogenic potential of ASCs

Oil Red O staining was performed to assess adipogenic potential of ASCs (Fig. 3B). Although the amount of lipid droplets stained with Oil Red O varied from donor to donor (Fig. 3B; d, high; e, moderate; f, low), all ASCs had adipogenic potential. The effects of donor age on adipogenic potential of ASCs were examined (Fig. 3A). The result of non-parametric analysis by Spearman's rank correlation coefficient demonstrated that the adipogenic potential of ASCs significantly declined with donor age ( $r = -0.283$ ,  $p < 0.01$ ). Individual difference in adipogenic potential was great, and observed in all age groups including elderly donors.

From both the young and elderly groups, 10 samples each from ASCs exhibiting high or low adipogenic potential were randomly chosen, and qPCR was performed to examine the expression levels of MSC surface markers, CD44, CD73, CD90, CD105 and CD271, and that of an undifferentiated cell marker, NANOG (Fig. 3C–H). Compared with ASCs from the young donor group, ASCs of the elderly group had significantly higher expression of CD73 and CD105 ( $p < 0.01$  and  $p < 0.05$ , respectively). When ASCs were compared between the high and low adipogenic potential group, in the elderly group, expression of CD73 was significantly higher in the ASCs with low adipogenic potential ( $p < 0.05$ ), although no difference was observed in the young group. There were no other significant differences in the gene expressions between the young and elderly groups, as well as between the high and low groups.

### 3.3. Effects of donor age on osteogenic potential of ASCs

After osteogenic differentiation, alizarin red staining and evaluation of Ca accumulation were performed to assess osteogenic potential of ASCs. Most ASCs had an osteogenic potential, though the amount of Ca accumulation differed among ASCs (Fig. 4B). The result of non-parametric analysis by Spearman's rank correlation coefficient demonstrated that osteogenic potential of ASCs was not correlated with donor age ( $r = -0.005$ ), but individual differences

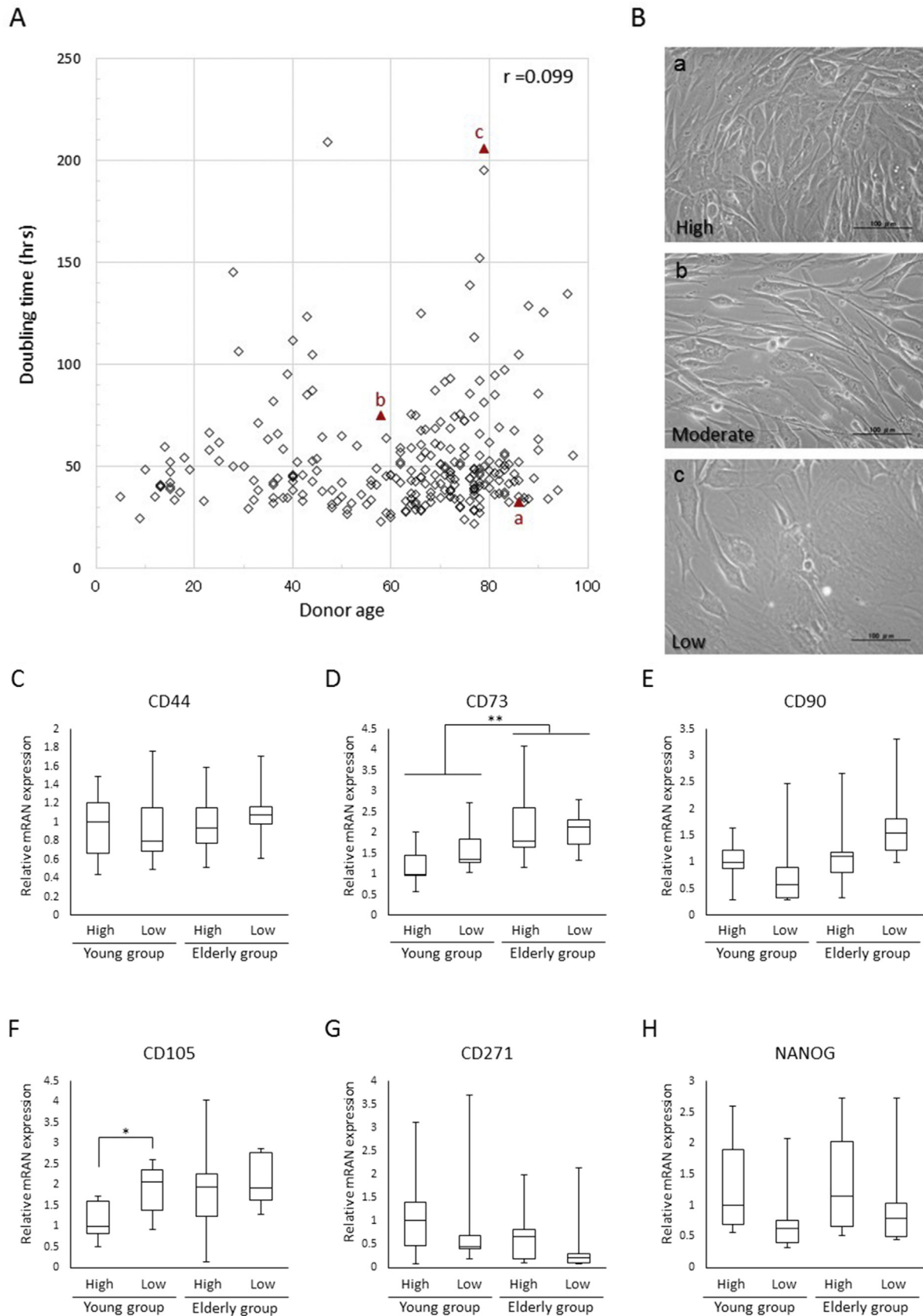
were noted in all age groups (Fig. 4A). Interestingly, individual differences in osteogenic potential were incrementally increased in proportion to the donor age.

In order to see characteristics of ASCs with different osteogenic potentials from different age groups, we again examined the expression levels of MSC surface markers (CD44, CD73, CD90, CD105 and CD271) and that of the undifferentiated cell marker (NANOG) by qPCR, using 10 samples each from the high or low osteogenic potential groups of both the young and elderly groups (Fig. 4C–H). When compared with the young group, the elderly group of ASCs exhibited a significantly higher expression of CD73 and CD105 ( $p < 0.01$ ,  $p < 0.05$ , respectively). In the comparison between the high and low group, ASCs with a low osteogenic potential had a significantly reduced expression of CD44 ( $p < 0.01$ ) in only the elderly group. Other than these differences, no significant differences were observed in young vs. elderly or high vs. low.

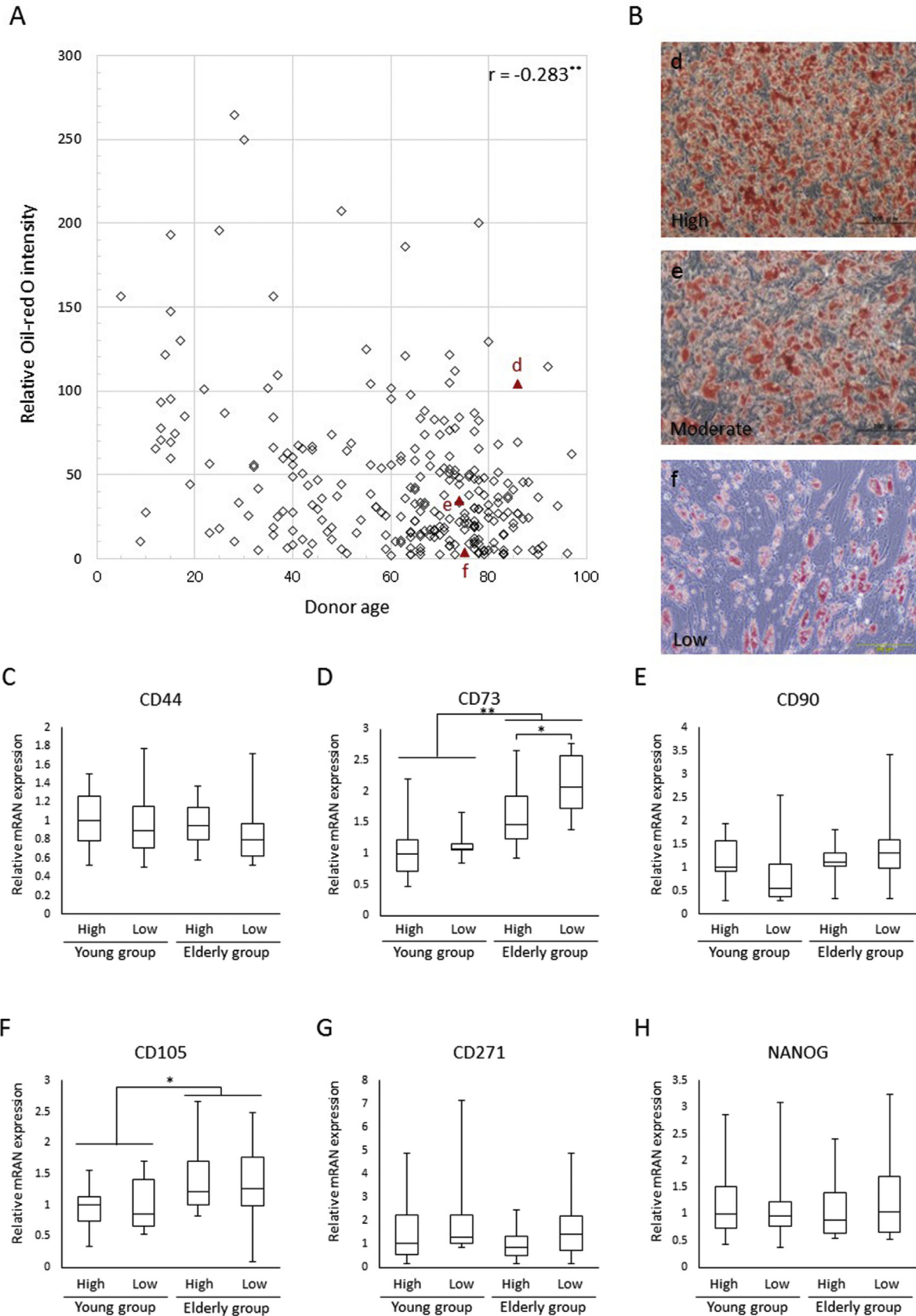
### 3.4. Effects of donor age on ASC chondrogenic potential

All ASCs had chondrogenic potential, and chondrocyte aggregates looked similar regardless of their differences in differentiation potential (Fig. 5B). Chondrogenic potential of ASCs was assessed using GAGs as an indicator. The result of non-parametric analysis by Spearman's rank correlation coefficient demonstrated that chondrogenic potential of ASCs was not correlated with donor age ( $r = 0.059$ ), but individual differences were observed in all age groups (Fig. 5A).

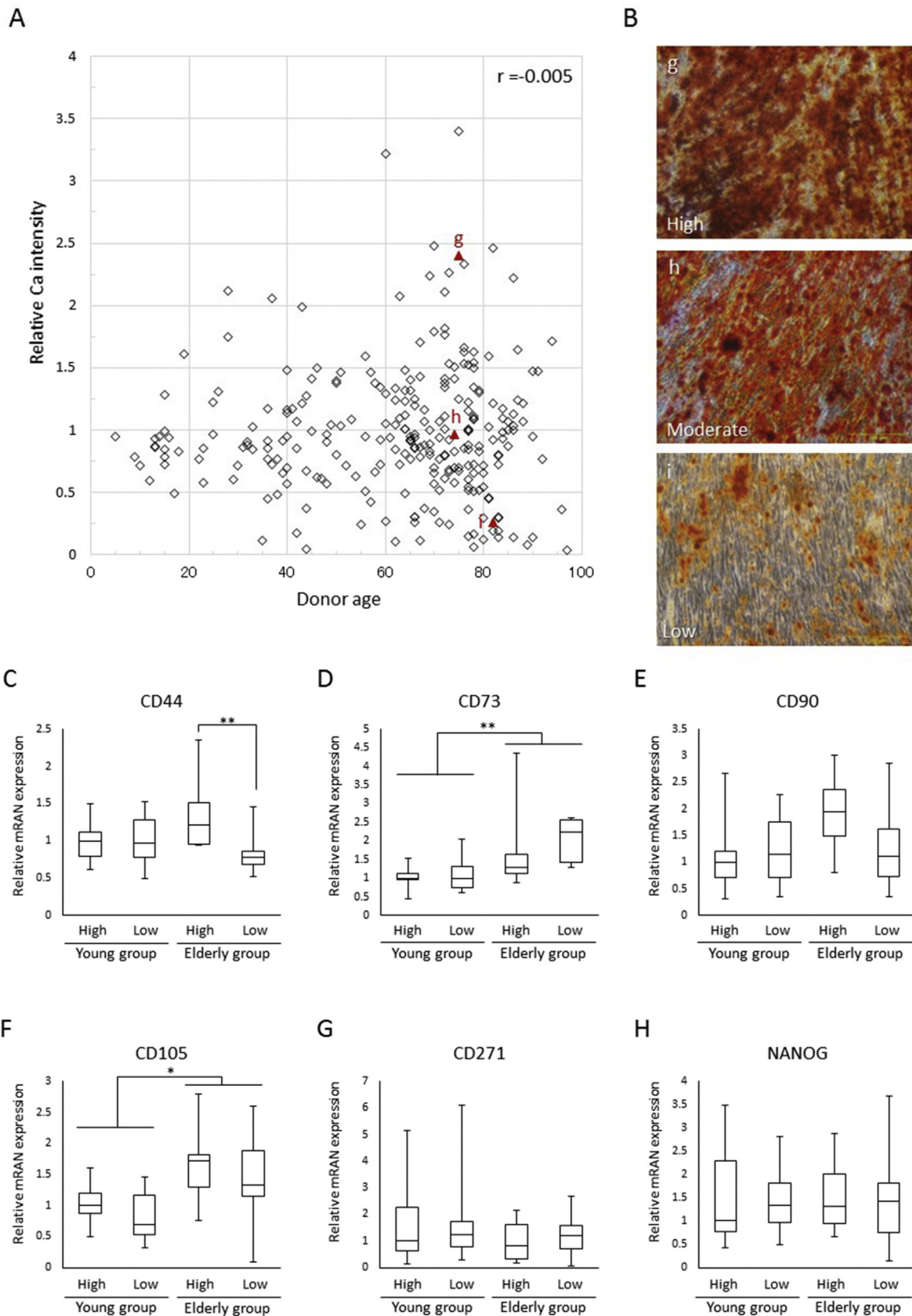
To assess chondrogenicity, we again examined the expression levels of MSC surface markers (CD44, CD73, CD90, CD105 and CD271) and that of the undifferentiated cell marker (NANOG) by qPCR, using 10 samples each for ASCs with high or low potential from both the young and elderly group (Fig. 5C–H). When compared with the young group, the elderly group of ASCs exhibited a significantly higher expression of CD73 and CD105 ( $p < 0.01$ ,  $p < 0.05$ , respectively), and a lower expression of CD271 ( $p < 0.05$ ). In other respects, no significant differences were observed between young vs. elderly and high vs. low.



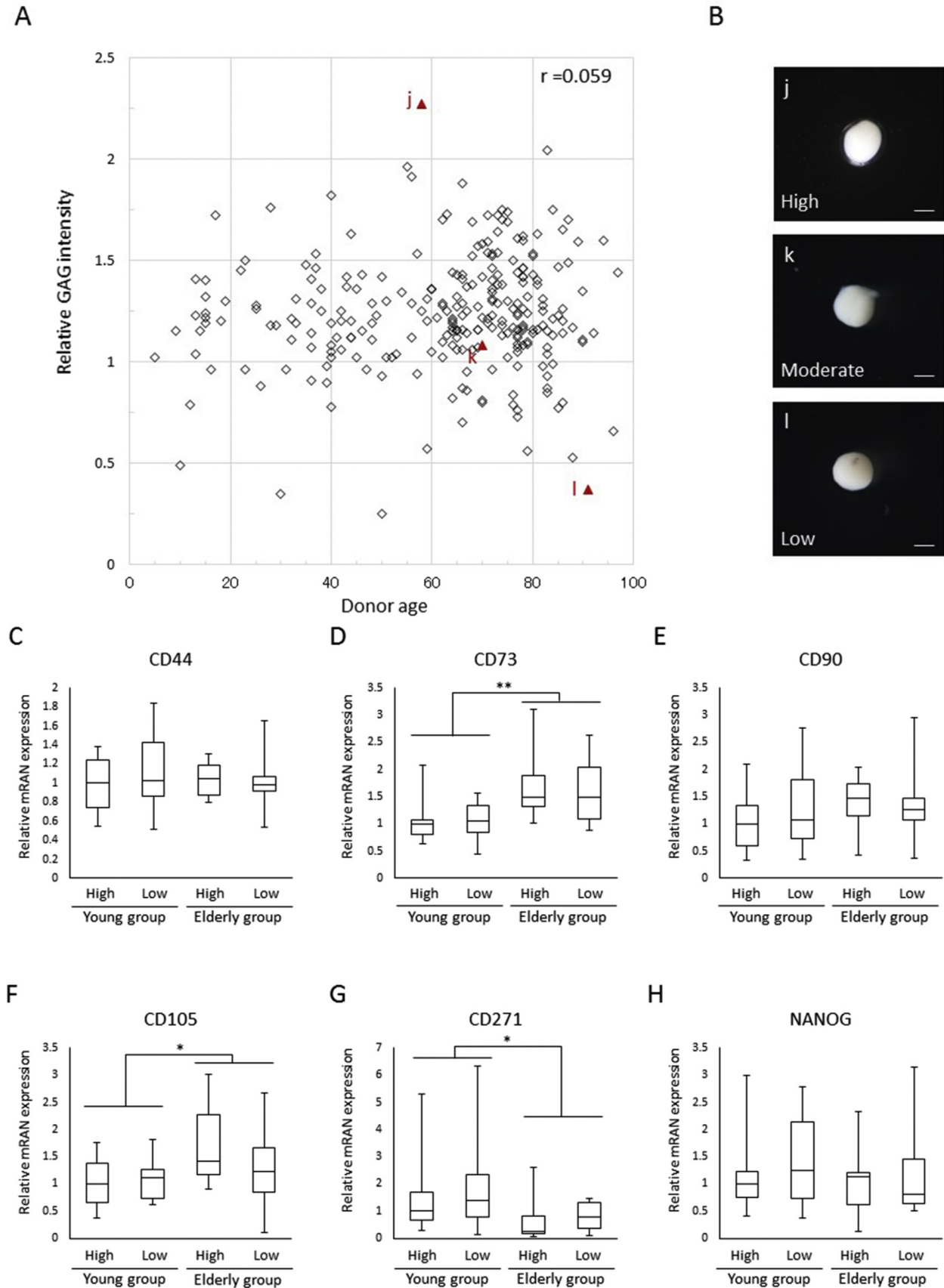
**Fig. 2.** Effect of donor age on the reciprocal of doubling time in cultured ASCs. (A) Each open diamond indicates an ASC sample from a single donor. Cell population doubling time (hours) at passage 4 of ASCs obtained from 254 subjects was shown with donor age. The correlation coefficient by Spearman's rank correlation coefficient was  $r = 0.099$  indicating no correlation between the proliferation potentials and donor age. (B) Observation of cellular morphology for ASCs under phase contrast microscopy at passage 4 ( $\times 100$ ). a–c show representative ASC samples having a different degree of proliferation potential, indicated by red triangles in A. (C–H) Examination of expression of MSC surface markers such as CD44, CD73, CD90, CD105, CD271 and stemness marker (NANOG) by using qPCR.  $n = 9$  each for high and low group (young group:  $< 40$  years of age, elderly group:  $\geq 60$  years of age). \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Fig. 3. Effect of donor age on adipogenic potential of ASCs.** Adipogenic differentiation was analyzed by extracted Oil Red O concentration. (A) Normalized concentration of eluted Oil Red O from ASCs of each donor ( $n = 260$ ; 5–97 years old) showed a significant correlation with age,  $r = -0.283$ ;  $**p < 0.01$ . The correlation was calculated by Spearman's rank correlation coefficient test. (B) d–f show Oil Red O-stained lipid inclusions of ASCs from representative donors having a different degree of adipogenic potential, indicated by red triangles in A. (C–H) Examination of expression of MSC surface markers, CD44, CD73, CD90, CD105, CD271 and stemness marker (NANOG) by using qPCR.  $n = 10$  each for high and low group (young group:  $<40$  years of age, elderly group:  $\geq 60$  years of age).  $*p < 0.05$ ;  $**p < 0.01$ .



**Fig. 4.** Effect of donor age on osteogenic potential of ASCs. Osteogenic differentiation was analyzed by calcium (Ca) deposition. (A) Normalized quantification of Ca deposition from ASCs of each donor ( $n = 260$ ; 5–97 years old) did not show a correlation with age ( $r = -0.005$ ), but large variations were noted. The correlation was calculated by Spearman's rank correlation coefficient test. (B) g–i show Alizarin red-stained ASCs of representative donors having a different degree of osteogenic potential, indicated by red triangles in A. (C–H) Examination of expression of MSC surface markers, CD44, CD73, CD90, CD105, CD271 and stemness marker (NANOG) by using qPCR.  $n = 10$  each for high and low group (young group: <40 years of age, elderly group:  $\geq 60$  years of age). \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Fig. 5.** Effect of donor age on chondrogenic potential of ASCs. Chondrogenic differentiation was analyzed by GAG synthesis. (A) Normalized quantification of GAG synthesis from ASCs of each donor ( $n = 260$ ; 5–97 years old) did not show a correlation with age ( $r = 0.059$ ), but a large variation was noted. The correlation was calculated by Spearman's rank correlation coefficient test. (B) j–l show cellular deposition of ASCs of representative donors having different degrees of chondrogenic potentials, indicated by red triangles in A. (C–H) Examination of expression of MSC surface markers, CD44, CD73, CD90, CD105, CD271 and stemness marker (NANOG) by using qPCR.  $n = 10$  each for high and low group (young group: <40 years of age, elderly group:  $\geq 60$  years of age). \* $p < 0.05$ ; \*\* $p < 0.01$ . Bars = 1 mm.



### 3.5. Age-related individual differences in proliferation and multi-differentiation

Proliferation and differentiation potentials (adipogenesis, osteogenesis and chondrogenesis) of ASCs were assessed. The results demonstrated that there were individual differences in pluripotency of ASCs. Potentials of ASCs varied in each individual; for example, some ASCs had all around excellent potentials, and others had excellent proliferation and adipogenic potential, but neither osteogenic nor chondrogenic potential. Therefore, we performed PCA by measuring proliferation and differentiation potentials (adipogenesis, osteogenesis and chondrogenesis) to examine the variations in individual ASC differences. Fig. 6A–C indicates distributions of potentials of ASCs classified by each age group and 95% prediction areas (95% prediction interval ellipse). In addition, the distances between the center of the prediction ellipse and each distribution point were measured (Fig. 6D–F). The result of analysis by non-parametric Shirley–Williams' multiple comparison test (control group:  $\leq 20$ 's) revealed that potentials of ASCs generate significant individual differences (variations) over 60 years of age. Furthermore, to address the sex difference, we separated the samples by sex and conducted the analysis independently. We found that for ASCs from female donors, individual differences (variations) significantly increased over 60, when the 20's age group was used to as a control. In contrast, ASCs from male donors exhibited a significant increase in individual differences (variations) over 80, when compared with the combined age group of 20's and 30's.

## 4. Discussion

In this study, ASCs were obtained from subcutaneous tissue from 260 donors over a broad age range (5–97 years). Although the numbers of SVF cells, including ASCs, per gram of adipose tissue markedly varied (750–1,198,600 cells, median 9,923 cells), ASCs were successfully obtained from all donors. We previously examined age-related changes in ASCs of mice and found that the number of ASCs per gram of adipose tissue declines with age [31]. However, in this study, there was no correlation between the number of SVF cells per gram of adipose tissue and donor age (Fig. 1A). In addition, in the colony tests to determine the percentage of ASCs, the cells which were able to adhere and proliferate, among the separated SVF cells, a correlation between the numbers of ASC cells and donor age was not observed. Moreover, it was found that individual differences in proliferation potential were prominent (Fig. 1B).

We observed morphological characteristics related to proliferation potential; ASCs with reduced proliferation potential were large and exhibited a spread-out morphology (Fig. 2B). As it is reported that senescent cells generally become hypertrophied and flattened, and eventually lose their ability to divide [38], ASCs may be susceptible to cellular senescence.

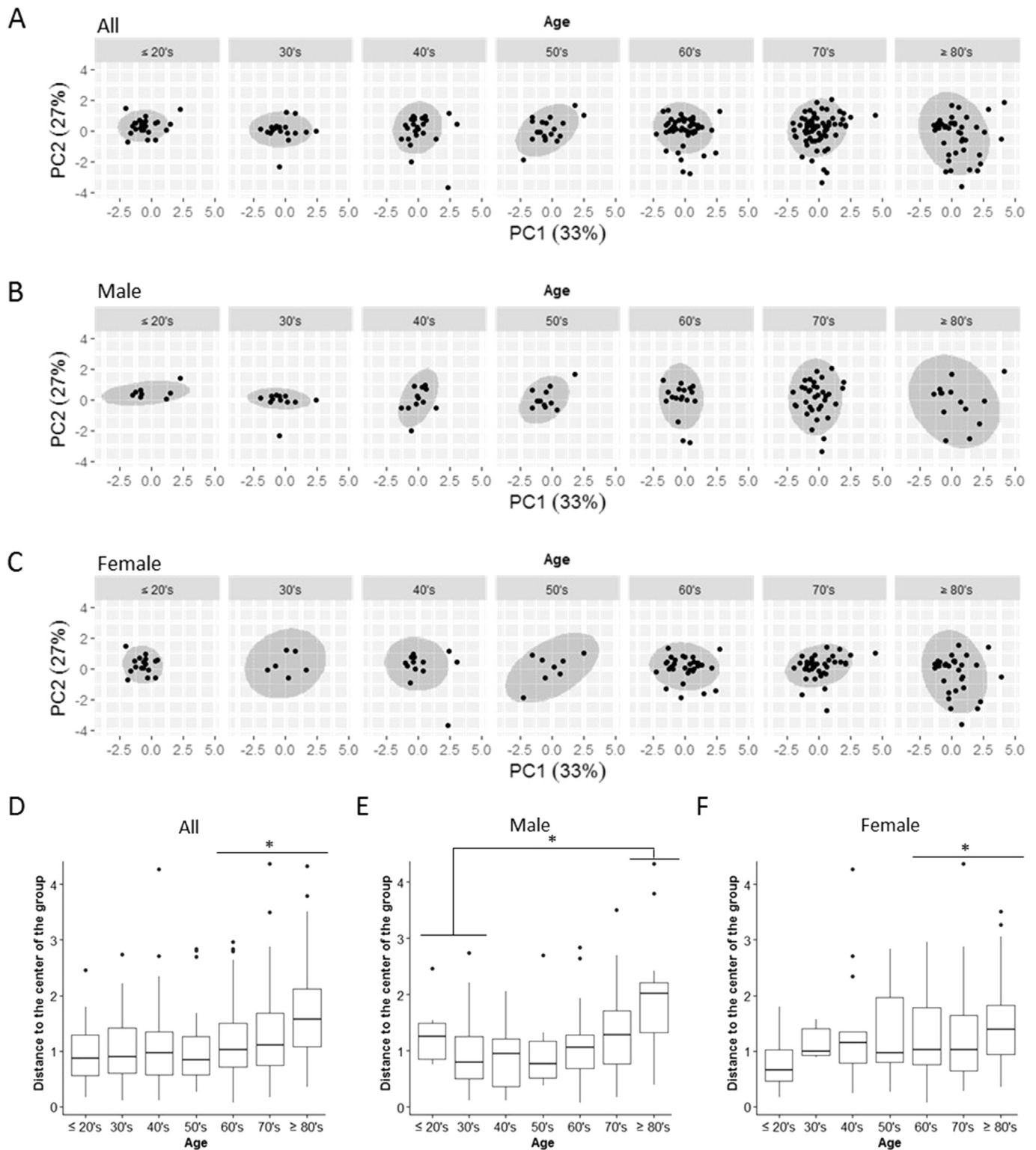
Next, we assessed the proliferation potential (doubling time) and pluripotency (adipogenesis, osteogenesis and chondrogenesis) using ASCs from all donors. All of the ASCs had subculture potential, and differentiated into adipocytes, osteoblasts or chondrocytes, although there were variations (individual differences) in the proliferation rate and the frequency of differentiation. Correlation between the donor age and the potentials (proliferation, adipogenic, osteogenic and chondrogenic potentials) of ASCs was analyzed by non-parametric Spearman's rank correlation coefficient test to examine if aging was associated with the variations observed (individual differences). The results demonstrated that adipogenic potentials decline with age (Fig. 3A) and that proliferation, osteogenic and chondrogenic potentials are not correlated

with donor age (Figs. 2A, 4A and 5A). Other than donor age, we also analyzed the effects of sex and difference in tissue collection sites (data not shown). Jurgens et al. reported that no differences were detected in differentiation capacity between ASCs harvested from the abdomen and hip/thigh region [39], and we did not observe significant differences in ASC potentials from different sites, consistent with the previous report. The sex of donors did not significantly affect ASC potentials either.

Recently, it has been reported that ASCs from obese donors have a greater proliferation potential and a less accumulation of oil droplets compared with those from donors of standard weights [40], and that obesity decreases the osteogenic potential [41]. The correlation between BMI of donors and the numbers of SVF cells or ASCs obtained from adipose tissue has also been reported [26,28,42]. In this study, no correlation was observed between BMI and the yield of SVF cells from adipose tissue, or each ASC potential we tested (data not shown). The BMI of donors in our study ranged from 15.7 to 43.1 (median 22.7), and the majority (64%) was of standard body weight ( $18.5 \leq \text{BMI} < 25$ ), which may explain the discrepancy.

Moreover, we identified several patterns of ASC potentials. Characteristics of ASCs of each donor were evaluated by PCA and distributed in the chart which represents their potentials. The results demonstrated that individual differences in ASC potentials changed with aging, and the individual differences became significant after the age of 60 (Fig. 6A and D). When the analysis was conducted separately by sex, ASCs from male donors exhibited a significant increase in individual differences after the age of 80, compared with the donor group aged less than 40 (Fig. 6B and E). For female donors, the increase of individual differences in ASC potentials appeared significantly after the age of 60 (Fig. 6C and F), and these results indicate that females have their ASC potentials affected earlier than males. Recently, the effects of menopause and female hormones on stem cell potentials have been studied. Bodle et al. reported that ASCs from postmenopausal donors demonstrated a relatively high proclivity for osteogenic differentiation and a relatively lowered proclivity for adipogenic differentiation as compared with ASCs from pre- and perimenopausal donors [43]. On the other hand, Niada et al. demonstrated that 17 $\beta$ -estradiol improved adipogenic differentiation of ASCs and negatively affected osteogenic potential of ASCs [44]. Although these results were inconsistent, their view that ASC potentials are affected by menopause and female hormones is intriguing. Our data demonstrated that the significant increase in individual differences of ASC potentials were observed (when compared to the young age groups) at an earlier age group in females than in males, which may be partly due to the change in female hormones caused by menopause. Additionally, it is widely accepted that such age-related changes in the individual differences may occur because of lifestyle or environmental factors causing epigenetic changes. For example, twins have different life spans and appearances due to different lifestyles and environments, although they carry identical genes [45,46]. It is reported that the degree of differentiation potential of adult stem cells, including ASCs, is controlled by methylation [47–49]. It has been demonstrated that ASCs from healthy donors and ischemic heart disease patients varied in proliferation rate [50], and that ASCs from obese or type 2 diabetic patients have an altered cellular metabolism [51]. It is possible that DNA methylation in ASCs varies among donors under the influence of their lifestyles, environmental factors or diseases.

In this study, the expression levels of MSC surface markers (CD44, CD73, CD90, CD105 and CD271) and the undifferentiated cell marker (NANOG) were examined in ASCs with high and low potential for each of the four potentials, from both the young and elderly donor groups (C–H in Figs. 2–5). In the young group, the



**Fig. 6.** Principal component analysis of ASC potentials (proliferation and differentiation of adipogenesis, osteogenesis and chondrogenesis). ASCs of each age group are plotted in the first and the second principal components which account for 33% and 27% of variance, respectively. Gray areas indicate probable ellipse (95%) areas. Both sexes (A), males (B), females (C). (D–F) Box plots indicate spread data of the distances between the center of prediction ellipse and each distribution point. In total, individual differences of ASCs were significantly increased after the age of 60's. \* $p < 0.05$ . (E) For males, individual differences significantly increased over 80 years old, when the combined age group of 20's and 30's was referred to as a control. (F) ASCs from females showed a significant increase over 60, when compared with the age group of 20.

expression level of CD105 was different depending on the proliferation potential level. In the elderly group of ASCs, there were differences in the expression of CD73 and CD44, depending on their adipogenic and osteogenic potential, respectively. In the

comparison between the young and elderly groups, the expression of CD73 and CD105 was generally increased in the elderly group. There were no other differences related to ASC potentials and aging in NANOG expression levels or the other MSC surface markers. This

suggests that the differences in ASC potentials observed in this study did not come from the level of stemness. Interestingly, changes in the expression levels of CD44, CD73 and CD105 were observed for some ASC potentials and aging, and these changes may be involved in the enhancement of individual differences by age. In addition to further examination on the involvement of these marker genes in individual differences, we will discover factors contributing to individual differences of ASCs that significantly arise with old age by analyzing associations with the constitution of donors and DNA methylated region.

This study demonstrated the age-related change in the potentials of ASCs and revealed that the individual differences of ASCs become significant over the age of 60 years old, and the increase in individual differences with age is more prominent in females than in males. We believe that it is important to carefully observe ASC potentials in order to achieve effective treatments in regenerative medicine using ASCs. Considering that ASC potentials may be influenced by lifestyle factors, such as diet, exercise and psychological stress in daily life, further investigation on lifestyle, environmental factors and epigenetic analysis will lead to the identification of factors that regulate ASC potentials.

## 5. Conclusion

ASCs were successfully obtained from donors of all ages and cultured. In our examination on proliferation, and adipogenic, osteogenic and chondrogenic differentiation potentials of ASCs *in vitro*, individual differences were observed for all ASC potentials. PCA revealed that the individual differences of ASCs become significant in people over 60 years old, particularly in females. Thus, this study indicates that an age-based prediction of proliferation and differentiation potentials of ASCs may not be valid. We believe that it is important to carefully observe ASC potentials in order to achieve effective regenerative medicine treatments using ASCs.

## Conflict of interest

All authors declare no conflict of interest.

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