Aging alters tissue resident mesenchymal stem cell properties

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Abstract

Tissue resident mesenchymal stem cells (MSCs) are known to participate in tissue regeneration that follows cell turnover, apoptosis, or necrosis. It has been long known that aging impedes an organism's repair/regeneration capabilities. In order to study the age associated changes, the molecular characteristics of adipose tissue derived MSCs (ASCs) from three age groups of healthy volunteers, i.e., young, middle aged, and aged were investigated. The number and multilineage differentiation potential of ASCs declined with age. Aging reduces the proliferative capacity along with increases in cellular senescence. A significant increase in quiescence of G2 and S phase was observed in ASCs from aged donors. The expression of genes related to senescence such as CHEK1 and cyclin-dependent kinase inhibitor p16ink4a was increased with age, however genes of apoptosis were downregulated. Further, an age-dependent abnormality in the expression of DNA break repair genes was observed. Global microRNA analysis revealed an abnormal expression of mir-27b, mir-106a, mir-199a, and let-7. In ubiquitously distributed adipose tissue (and ASCs), aging brings about important alterations, which might be critical for tissue regeneration and homeostasis. Our findings therefore provide a better understanding of the mechanism(s) involved in stem cell aging and regenerative potential, and this in turn may affect tissue repair that declines with aging.

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Abbreviations: MSCs, Mesenchymal stem cells; ASCs, adipose tissue MSCs; miRNA, microRNAs; 3′UTR, 3′ un-translated region; CFU, colony forming unit; DT, doubling time; FACS, fluorescent-activated cell sorter; ALP, alkaline phosphatase activity.

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Introduction

Aging is an intricate process characterized by a variety of disorders associated with generalized decline and inability to maintain tissue homeostasis. Throughout life, stem cells persist in numerous tissues replacing mature cells lost during physical activity or injury. However, the function of stem cells and other progenitors declines with age (Maslov et al., 2004), subsequently affecting tissue structures and increased repair capacity and increased preponderance for degenerative disorders (Campisi, 2005). To date, the role of stem cells in aging, more specifically, mechanisms responsible for age-related decline in function of stem/progenitor cells is hotly debated. Tissue resident stem cells or mesenchymal stem cells (MSCs) are ubiquitously distributed and have specifically been characterized in tissues including bone marrow, adipose, skeletal muscle, dermis, and umbilical cord (Tuan et al., 2003). Morphologically and immunophenotypically, no significant differences have been observed among the MSCs regardless of tissue origin (Docheva et al., 2007; Izadpanah et al., 2006). Age-related decline in the quantity of MSCs in bone marrow has been reported in rodents, monkeys, and humans (Lee et al., 2006a; Mareschi et al., 2006; Yue et al., 2005). These studies, however, focused on the effect of aging on reduction of differentiation properties of bone marrow derived MSCs (Karagiannides et al., 2001; Tokalov et al., 2007; Zheng et al., 2007). We and others have reported the impact of prolonged in vitro expansion of two types of adult MSCs, bone marrow and adipose tissue derived MSCs (ASCs), and have provided some insight into the probable age-related decline in self-renewal and differentiation potential of MSCs (Izadpanah et al., 2008). It has recently been shown that proliferation potential decreased in aged ASCs compared to young ASCs (Efimenko et al., 2011), which ultimately causes accumulation of non-replicative ASCs and have been associated with caloric restriction (Schmuck et al., 2011). It is also shown that aging alters the availability of adipose tissue-derived CD45-/CD34+/CD133+ cells and their angiogenic function (Madonna et al., 2011). However, there are claims that age does not seem to affect ASC viability and in vitro adipogenic differentiation significantly, whereas it does affect osteogenic differentiation (de Girolamo et al., 2009). Furthermore, as to what mechanisms for self-renewal and differentiation underlie the process of aging, and the effects on the biology of cell growth, cell cycle, and promote cell senescence and apoptosis in MSCs are not well understood.

The genetic and epigenetic alterations responsible for aging of stem cell are reported, and include changes that directly affect cell cycle, genetic stability, and DNA damage and repair (Marasa et al., 2010). Considerable effort has been made toward identifying the role of microRNAs (miRNA) in regulating the aging process. miRNAs are 19–25 nucleotides in length, which bind to a 3′-untranslated region (3′UTR) of an RNA transcript in a complimentary and sequence-specific manner resulting in antagonizing the translational efficiency of the transcript (Bartel, 2009). Evidence suggests that miRNAs are important regulators of a variety of cellular processes including cell survival, proliferation, differentiation, and senescence (Bartel, 2009; Ambros, 2004). Further, with model organisms, change in the expression patterns of miRNA during a cell’s lifespan and existence of variations in their expression that may directly affect the aging process are reported (Bates et al., 2010; Li et al., 2009).

In the present report, we characterized several age-related alterations in ASCs. Using ASCs from healthy young, middle aged, and aged volunteers (Groups 1, 2, and 3, respectively) we demonstrate a significant decrease in the cell number concomitant with the volunteer age paralleled by increased population doubling time and cell senescence. We also profiled miRNA expression in both young and aged individuals and identified miRNAs that are differentially expressed in aged volunteers. The observations from our studies indicate that various molecular, genetic and epigenetic alterations take place in ASCs during the progression of age. These changes impact on proliferation and differentiation potential of tissue resident stem cells, and ultimately affect the tissue homeostasis and repair capabilities.

Results

Characterization of ASC properties in different age groups

ASCs isolated from the age Groups 1, 2, and 3 exhibit identical characteristics such as expression of CD44, CD90, CD105, and CD146 and absence of surface markers CD3, CD4, CD11b, CD34, and CD45, respectively. In order to assess the number of growing adherent cells/g of adipose tissue and evaluate their ability to form colony, equal number of cells were plated after processing the tissue and the number of individual cells and colonies were recorded on days 5 and 8. There was a decrease in the number of single cells from day 5 to day 8 in all three groups with group 1 showing the highest number of single adherent cells in culture on day 5 (421/cm² of culture area). This number decreased greatly concurrent with increased number of colonies by day 8 (24/cm²) indicating that these cells were able to form colonies. Group 2 cells also showed a similar decrease in the number of growing cells by day 8, but the absolute decrease in count was lower than Group 1 (358/cm², day 5 to 135/cm², day 8). Compared to Groups 1 and 2, ASCs from Group 3 showed a lower cell (single) count on day 5 (89/cm²), which decreased by day 8 to 21/cm², the decrease however was not as seen in Group 1 or 2 (Fig. 1). The number of colonies increased from day 5 to day 8 in all three groups. The number of colonies was comparable between Groups 1 and 2, however, these cells formed larger colonies which merged rapidly while cells of Group 3 could only form smaller size colonies with limited number of cells.

The cumulative population doublings of ASCs were quantified in all three groups. Compared to Group 1, Group 3 exhibited a significant increase (about 38%) in population time (Fig. 2A). Simultaneously, colony forming unit (CFU) potential of ASCs declined by about 30% in Group 3 compared to Group 1 (P < 0.01) indicating that as age advances the number of ASCs that are capable of replicating and form colonies decrease (Fig. 2B).

For all three groups, the cell cycle distribution of ASCs was determined by FACS analysis following propidium iodide staining of cellular DNA. An assessment of the percentage of cells in G1, S, and G2-M phases among the ASCs of Groups 1 and 3 indicated a slight decrease in the number of cells in the quiescent G1 phase of the cell cycle (Fig. 3A). However,
there was a significant increase in the G2 phase and decrease in the S phase (n=15; P<0.05) (Figs. 3B and C). The increased G2 phase arrest was coupled with decreased G1 and S phase entry in ASCs of Group 3. Using real time RT-PCR, a transcriptome analysis of ASCs from all three groups was performed to obtain an overview of genes associated with regulation of cell cycle. A significant up-regulation of genes that promote a G2 phase arrest such as CHEK1 (4.4 folds) was observed and most significantly a 22.9 fold increase of p16INK4a (related to S phase entry prevention) was detected. The expression of ATM, E2F transcription factor 4 (E2F4), retinoblastoma 1 (RB1), BRCA1, and HMGA2 — that play a crucial role in the control of a cell cycle were marginally upregulated in Group 3 compared to Group 1 (Fig. 3D).

Analysis of genes involved in apoptosis and DNA repair

Real time RT-PCR analysis revealed a significant change in the transcripts encoding apoptotic regulators such as ATR (2.6±0.9 folds), TNFα (5±1.4 folds), and NFκB (4±0.4 folds). However, the expression of hTERT transcript, p53, and caspase family genes including Casp3, Casp8, and Casp9 decreased in Group 3. In addition, the expression of anti-apoptotic genes BCL2 and BAX was upregulated whereas the expression of BIRC5 (survivin) was downregulated in Group 3 (Fig. 4A).

A dramatic reduction of telomerase activity in Group 3 ASCs was also observed (Fig. 4B).

Since it is known that during the aging process there is an accumulation of DNA replication errors and mutations, the DNA repair among three groups was analyzed. The RT-PCR analysis for the expression of genes involved in the process of cellular DNA repair revealed an up-regulation of XRCC4 (3±1.1 folds) and XRCC6 (4.8±0.4 folds) in ASCs of Group 3 compared to Group 1. However, there was a marginal down-regulation of APEX1 (1.15±0.4 folds; n=5) in ASCs from Group 3 compared to Group 1 (Fig. 4C). The comet assay was then used to evaluate the DNA damage; cells from Group 3 showed a small increase in comet tail (Fig. 4D).

To examine the spontaneous tumorigenic potential of ASCs from different age groups, ASCs from all three groups were subcutaneously injected into the flank of immune deficient mice (n =9 per group). No tumors were found in any of the SC populations even after 120 days. In line with this by PCR assay human Alu sequence was not detected in liver, lung, and kidney of these mice.

Age-related decline of multi-lineage differentiation potential in SC

In order to study multilineage differentiation capacity of ASCs from all three groups, cells were differentiated toward the adipogenic, osteogenic, and chondrogenic lineages using

Figure 2  Age-associated decline in ASC’s replication capacity. ASC replication in Groups 1 and 3. A) Cumulative population doublings increased with progression of age. A known number of ASCs from each group were cultured. The total number of cells was determined at different time points to obtain the doubling time. B) Counted colonies of ASCs from the three groups were plated at a density of 25 cells=cm² for 10 days in culture, stained with 1% crystal violet (n=8 volunteers/group; *P<0.01).
lineage-specific induction factors. The percentage of cells capable of undergoing adipogenic differentiation declined from about 33% in Group 1 to about 10% in Group 3 (Fig. 5A). Real time RT-PCR analysis on lineage specific transcriptomes indicated a down-regulation of LPL (7.9±1.2 folds) and CEBPA (4.53±2.7 folds) in Group 3 compared to Group 1 (Fig. 5B).

Osteogenic differentiation potential was found to decline significantly with progression of age. In addition to differences in staining for calcium deposits in differentiated cultures, ALP concentration, representative of the level of osteogenic differentiation activity in Group 3 was markedly reduced when compared to Group 1 (from about 50% in Group 1 to about 22% in Group 3; n=5/group) (Fig. 5C). Accordingly, it was inferred that the chondrogenic potential as well decreased with aging. Analysis of the expression of lineage specific markers revealed a down-regulation of BMP6 (2.8±0.8 folds), COL2A (3±0.4 folds), and Col10A (2.9±1.2 folds; \(P<0.05\)) genes in ASCs from Group 3 compared to Group 1 (Fig. 5D).

microRNA analysis

To determine the extent to which microRNAs (miRNA) contribute to age-related changes in gene expression of ASCs, the miRNA of ASCs from Groups 1 and 3 were profiled using a miRNA microarray platform that probes for 911 mature human miRNAs and 148 viral miRNAs. Microarray results identified miRNAs with change in expression in Group 3 by more than 1 SD compared to the mean expression of each miRNA in Group 1. The results of this microarray analysis suggest that there was a subnormal expression of several miRNAs regulating cell cycle, apoptosis and multilineage potential of stem cells in Group 3. There was substantial downregulation of miRNA implying progression of apoptosis and inhibition of differentiation potential of stem cells including mir-27B and let-7G (Fig. 6A). We further examined the expression of several miRNAs among the three groups with quantitative real-time RT-PCR. The miRNAs were selected on the basis of microarray results. These results demonstrated an identical pattern of downregulation for Let7g (3.95±0.2 folds), mir-27B (3.43±0.7 folds), mir-106a (0.94±0.3 folds), and mir-199a (4.68±0.6 folds; \(P<0.05\), n=5/group) in Group 3 compared to Group 1 (Fig. 6B).

Discussion

The results of our study clearly demonstrate a correlation between age-related decrease in the quantity of tissue resident stem cells, and their impairment in self-renewal and...
differentiation capabilities. While equal amounts of tissue samples were analyzed, there was a substantial reduction in the quantity of available resident stem cells in Group 3. In addition, compared to Groups 1 and 2, only a limited number of ASCs from Group 3 were capable of forming colonies. A weaker CFU ability and increased population doubling time were observed in Groups 2 and 3 compared to Group 1. Some studies on bone marrow derived MSCs have shown a link between aging and declined self-renewal capacity of cells (Kasper et al., 2009; Wagner et al., 2010a). Interestingly, it is reported that these changes in stem cells are accompanied by an abnormal cell cycle profile. Our data show an increase in G2 phase and S phase profiles in ASCs from Groups 2 and 3. Additionally, pathways that regulate cell cycle comprising of p16INK4a and cell cycle checkpoint kinase genes were perturbed in Group 3. Previous studies have shown that p16INK4a gene expression increases with age in various tissues (Zindy et al., 1997; Krishnamurthy et al., 2006). Although induction of p16INK4a expression can cause senescence in a variety of cell types in vitro and in vivo (Sharpless et al., 2004; Michaloglou et al., 2005), some progenitor cells are reported to be unaffected by increase in p16INK4a expression or p16INK4a deletion (Molofsky et al., 2005). The novelty of our findings is the demonstration of a significant increase in expression of p16INK4a in ASCs from Group 3. Although, there was an increase in the expression of CHEK1, other cell cycle regulators such as ATM, E2F4, HMGA2, RB1, and BRCA1 displayed only a marginal increase in Group 3 (Fig. 3D). Our data also show a downregulation of genes associated with apoptosis such as p53, caspase 3, caspase 8, and caspase 9 in Group 3. The report of an age-associated decline in the expression of p53 in bone marrow derived SCs (Wilson et al., 2010) support our findings. Simultaneously the expression of anti apoptotic gene BCL2 was significantly upregulated in ASCs from Group 3. Danial and Korsmeyer have reported that overexpression of BCL2 is associated with enhancement of the survival of many cell types (Danial and Korsmeyer, 2004). Furthermore, in ASCs from Group 3, there was a significant upregulation of NFκB and the genes for their corresponding receptors. There are reports suggesting that NFκB can be activated by TNFα (Krapfmann and Scheiderer, 2005) and engage in the survival pathway by upregulating BCL2 (Li et al., 2002).

An analysis of the expression of apoptosis factors such as p53 and senescence markers including p16INK4a indicates that age-associated decline in ASC proliferation might mainly
be controlled by senescence pathways. In the absence of abnormal environmental factors, the importance of p16INK4a and p53 in tumor suppression indicates that these genes are utilized frequently throughout life and the cancer incidence is indicative of their failure (Ruzankina and Brown, 2007). Although p16INK4a and p53 play an anti-oncogenic role, constant activation of these genes can result in accumulation of senescent/apoptotic cells which, in turn, influence tissue homeostasis and slow down tissue renewal capacity and thus promote aging. ASCs from different age groups were not able to form any detectable tumor up to 120 days even in our studies.

Further, the expression of hTERT and telomerase activity decreased significantly in ASCs from aged volunteers. These findings are in agreement with existing data indicating that telomere dysfunction contributes to aging in human stem cells (Rudolph et al., 1999). In the absence of adequate telomerase activity, telomere shortening occurs. Animal models with abnormal telomere function have been shown to develop features of premature aging related to senescence and apoptotic mechanisms (Rudolph et al., 1999; Allsopp et al., 2003). Bazarov et al. have shown the suppressive effects of p16INK4a on hTERT (Bazarov et al., 2010). Recent studies on hematopoietic stem cells, neuronal stem cells, and pancreatic islet cells have shown that increasing levels of p16INK4a were not only associated with aging but partly contribute to age-induced replicative failure (Krishnamurthy et al., 2006; Janzen et al., 2006; Molofsky et al., 2006).

In addition to abnormal expression of cell cycle genes, an irregular expression of genes involved in DNA break repair such as APEX, XRCC4 and XRCC6 was observed with the progression of age. Age associated downregulation of APEX has been shown in male germ line cells (Allen et al., 2008). Similar to this, we observed a decrease in the expression of APEX in ASCs from Group 3. The irregular expression of DNA repair genes and higher comet tail indicate a higher DNA damage and possible repair activity.

Our data signify that aging processes have drastic effects on the differentiation potential of ASCs. We found a considerable decline in differentiation capacity of ASCs from Group 3. Compared to Group 1, the Group 3 cells displayed a significantly lower osteogenic, adipogenic, and chondrogenic differentiation potential. The decline in differentiation potential could be considered as one of the consequences of abnormal self-renewal in ASCs.

miRNA profiling among cells from Groups 1 and 3 revealed significant downregulation of a limited number of miRNAs in ASCs from Group 3 including mir-27b, mir-106a, mir-199a, and let-7, while the majority remained unchanged in aged ASCs. Downregulation of mir-27b has been shown to have

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**Figure 5** Effect of aging on the differentiation potential of ASCs. A) Percentage of positively stained adipogenic differentiated cells of the three group ASC cultures. Cells were incubated in adipogenic induction medium for 12 days and stained with Oil Red O, and absolute cell numbers were counted (n = 5; P < 0.05). B) Real-time RT PCR analysis of genes in adipogenic differentiated cells. All values were normalized to adipogenic differentiated ASCs from Group 1 (x-axis). C) Levels of alkaline phosphatase in ASCs from the three groups following incubating of cells in the osteogenesis induction medium for 12 days (n = 5). Real-time RT PCR analysis of genes in chondrogenic differentiated ASCs from Groups 2 and 3 compared to Group 1 (n = 5; P < 0.05).
levels of some established target genes, particularly CDK inhibitor p21/CDKN1A in replicative cell aging models (Hackl et al., 2010). However, our data with ASCs did not show any significant downregulation of mir-19b, mir-20a, and mir-17 in Group 3. There is compelling evidence indicating direct involvement of miR-199a in chondrogenic and osteogenic differentiation (Lin et al., 2009). miR-199a has also been reported to directly target SIRT1 (Rane et al., 2009). SIRT1, a mammalian homologue of silent information regulator 2 has been shown to promote longevity in yeast and mammalian cells (Kaeberlein et al., 1999). SIRT1 deacetylates a large number of substrates, including p53, XRCC6, NFκB, and forkhead proteins, and has been reported to affect various physiological processes (Chen et al., 2010). These findings demonstrate that with advancement of age, miRNAs could possibly be playing a role in the aging of ASCs. The loss of miRNA function during the aging process may be due to transcriptional repression, deletion, mutation, epigenetic silencing, or aberrant miRNA processing (Liang et al., 2009). The age-associated decrease in miRNA abundance and its probable role in the normal functioning of tissue resident stem cells as presently reported is an important and novel finding that warrants additional investigations.

We and others have shown the effect of prolonged in vitro replication (in vitro aging) potential of ASCs (Izadpanah et al., 2008; Wagner et al., 2010b). One of the most significant outcomes of in vitro aging in ASCs was the increase in cell senescence and apoptosis. This was also accompanied by the increased expression of DNA repair genes, indicative of more DNA break probabilities. In addition, ASCs from Group 3 showed subnormal cell cycle and miRNA expression. Most importantly, despite all the reported changes in the cells no tumorigenicity was observed in immunodeficient mice.

Considering abundant and ubiquitous distribution of adipose tissue in the body, resident ASCs may play a significant role in normal growth and homeostasis and can be considered as vital components of tissue repair. Recently, in ASCs derived from diabetic donors we reported the existence of similar characteristics such as self-renewal and differentiation potential. Interestingly, in the present study, we observed some cellular events in ASCs from volunteers of aged group that were similar to those seen in ASCs from diabetic donors (Cramer et al., 2010). Importantly, in the present investigation, ASCs were obtained only from non-diabetic healthy volunteers. We are therefore tempted to suggest that both aging and diabetes result in impairment of tissue repair mechanisms as tissue repair is a stem cell mediated process. It is also plausible to speculate that the impairments of tissue repair are accelerated when there is an association of aging and diabetes.

**Conclusions**

In summary, for the first time using ASCs from young, middle age, and aged healthy volunteers, the effect of aging on the self-renewal and differentiation potential of ASCs is presented. Further, ASCs, the residing stem/progenitor cells (within the abundantly distributed adipose tissue) are likely play a significant role in tissue repair and homeostasis. This would then suggest that an impairment of these cells due to the process of aging could be responsible for the underlying mechanisms of
aging and related disorders. In conclusion, our findings may help in the useful application of ASCs as therapeutic options, especially for tissue repair in the elderly.

Materials and methods

Isolation and expansion of ASCs

The volunteers or their legal guardians were consented based on the approved protocol by the Institutional Review Board (IRB) of the Tulane University Health Sciences Center. Healthy volunteers between 15 and 71 years of age were grouped into three groups comprising of Group 1 (<20 years; n=15, mean age 16.75 ± 1.4), Group 2 (30–40; n=17; mean age 34.4 ± 1.6), and Group 3 (> 50 yrs; n=8; mean age 61.33 ± 7.4). The body mass index (BMI) of volunteers (male and female) varied from 17 to 40; n = 17, mean BMI 24.8 ± 1.4). The ASCs were isolated from abdominal adipose tissue specimens from volunteers using a previously described method (Izadpanah et al., 2006). Briefly, 50 g of tissue was minced and digested with collagenase Type I (Invitrogen Corp., Carlsbad, CA, USA) for 60 min at 37 °C. Following this, they were subject to RBC lysis buffer (BioWhittaker, Walkersville, MD, USA) for 60 min at 37 °C. After this, the cells were plated at a fixed density in alpha-MEM medium, supplemented with 20% fetal bovine serum (Atlanta Biologicals, USA). The cells were cultured for ten (10) days before they were fixed and stained with toluidine blue. Multi-lineage potential was also evaluated by real time PCR analyses for expression of lineage-specific genes.

Quantitative real time PCR analysis

Using an RNA isolation kit total cellular RNA was isolated from cell cultures of all three groups and the multi-lineage differentiated cells (GE lifesciences, Piscataway, NJ, USA). From here the cDNA was generated using High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Assay for colony forming unit (CFU) and population doubling time (DT)

ASCs from the three groups were plated at densities of 1000, 500, 250, 100, 50 and 25 cells/cm² in 12-well dishes. Cells were cultured for ten (10) days before they were fixed and stained with 1% crystal violet in methanol. Colonies with diameters larger than 3 mm were considered for counting.

For doubling time experiments, 20,000 ASCs from the three groups were plated in a 75 cm² flask and counted at 48, 72 and 96 hour time points. The population doubling time for each time point was calculated using the formula: DT = n x log₂(2n/m)/log₂ t; where, n = number of cells at the beginning of the time period, m = number of cells counted at the end and t = time period. Thus the doubling time was ascertainment from at least 3 time points and the mean was calculated accordingly.

Flow cytometry and cell cycle analysis

Flow cytometry was performed on trypsin-dissociated ASC cultures stained for CD105, CD90, CD44, CD146, CD34, CD45, CD4, and CD11b (Invitrogen Corp., Carlsbad, CA, USA) on a Beckman-Coulter Epics FC500 flow cytometer.

For analysis of cellular DNA content, cultured cells at the confluence of ~70–75% were fixed in 70% ethanol, rehydrated in PBS, treated for 30 min with RNase A (1 mg/mL), and stained with 1 μg/mL of propidium iodide (PI) for 5 min. The fluorescence intensity was determined using a fluorescent-activated cell sorter (FACS) and the percentage of cells in different phases of cell cycle was assessed.

Multi-lineage differentiation

Adipogenic differentiation was determined in cultures of ASCs from all three groups using previously described methods (Izadpanah et al., 2006). Adipogenic potentials were evaluated by oil red O staining and real time PCR analyses of lineage-specific genes (Diagnostic BioSystems, Pleasanton, CA).

Osteogenic differentiation was induced as previously described (Izadpanah et al., 2005). Differentiated cells were either fixed and stained with Alizarin Red (Diagnostic BioSystems) or quantified for alkaline phosphatase activity (ALP) using the Sensolyte™ pNPP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA, USA). All analyses were carried out in triplicate.

Chondrogenic differentiation was accomplished by using the Stempro® chondrogenesis differentiation kit (Invitrogen Corp., Carlsbad, CA, USA). About 1 × 10⁵ cells were spun in a 15 ml conical tube and grown in chondrogenic media for 21 days. Micro masses were fixed and stained with toluidine blue.

Multi-lineage potential was also evaluated by real time PCR analyses for expression of lineage-specific genes.

Quantitative real time PCR analysis

Total cellular RNA was isolated from ASCs of different age groups using an RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA was obtained by using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

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CAAAAC-3'; BCL2 5'-AGATGGGACACTGGTAGG-3' and 5'-CTTCTCCTTAAAGATGCAA-3'; Leptin 5'-GAAGTGAACACACTCC TGCAA-3' and 5'-TTGATGTTCAAGCATAAG-3'; CEBPA 5'-CT TGGAACAGGGCAGAGCAG-3' and 5'-ATGTTGTTTAGCAGA GACG-3'; BMP-6 5'-AACCTGCTGGAGATGAC-3' and 5'- CGGGTGCACCTAAGGAAATG-3' and 5'-ATCGTTACACTGC CCTGAA-3' and 5'-TGCAACGGATTGGTTGTGTG-3'; COL10A1 5'-CTGGAACCTGCCCTGTGATTG-3' and 5'-TCCAGCTCCTTGGT GATAA-3'; (Realtimeprimers.com, Elkins Park, PA). All reac tions were run at 58 °C using a Bio-Rad iCycler (Bio-Rad Labo ratories, Hercules, CA).

Real-time PCR assays

Real-time PCR assay was performed with 200 ng of target DNA (extracted from homogenized frozen tissues) and Alu specific primers. Values for the amount of target DNA in each sample were corrected by assays for the single-copy mouse albumin gene (Lee et al., 2006b).

Telomerase assay

Telomerase activity was measured using a TRAPEz® ELISA Telomerase Detection Kit (Millipore, MA, USA) according to the standard telomeric repeat amplification protocol (TRAP) based on a previously published method (Kim and Wu, 1997).

Comet assay

The comet assay was performed on ASCs from different age groups according to manufacturer's protocols (Cell Biolabs Inc. San Diego, CA, USA). Briefly, electrophoresis was carried out on 1 × 10⁵ cells layered on comet slides for 30 min at 25 V and 300 mA. Then the slides were stained with 100 μL of PBS obtained using RT2 CDNA synthesis kit according to the manufacturer's protocol (Exiqon, Vedbaek, Denmark). The miRNA-qRT-PCR was then used to analyze selected miRNAs using SYBRgreen MasterMix (Exiqon, Vedbaek, Denmark). Relative expression levels were calculated based on the expression of three constitutive (ub, 5s, snord44) miRNA references. Expression levels of miRNAs were calculated after subtracting the CT-values of the endogenous references, and fold change of gene expression was subsequently calculated using ΔΔCT-method. The primers used for microRNA PCR were mir-let 7g: UGAGGAGUAGUUGUGUACAGU, mi-27b: UUCACA GUUGCUAAGUUCUGC, mi-106a: AAAAGUGCUUACAGUCG AGU, and mi-199a: CCCAGUGUUCAGACUACCUGUUC.

In vivo tumorigenicity

Immune-deficient NIHIII 6-week-old mice were inoculated subcutaneously with 1 × 10⁶ cells (200 μL of PBS) obtained from all three groups in (9 mice per group; n = 3 volunteers/ group). 200 μL of plain PBS was injected in one group of animal as negative control. One group of animals was injected with 1 × 10⁶ MDA-MB231 cells, which is a transformed breast carcinoma cell line as a positive control. All animals were maintained in specific pathogen-free conditions, and all experiments conformed to the requirements of the Animal Welfare Act and the Institutional Animal Care and Use Committee.

Statistical methodology

All data relating to study specific were summarized using descriptive statistics such as mean, standard deviation. The variance method was used to compare the mean differences. Where meaningful, the results were presented graphically. The study hypotheses were tested at 5% level of significance throughout the analysis. Estimates of means and their 95% confidence intervals were calculated. R-computing software was used to plot the graphs.

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