Tissue Engineering and Regenerative Medicine, Vol. 11, No. 2, pp 137-142 (2014)
DOI 10.1007/s13770-014-0410-3

| Original Article |

Effects of Harvesting Sites and Ages on Adipose Tissue-derived Stem Cells in Rat

Hae-Gyeong Jung[1], Eun-Kyung Ahn[1], Ji-Hyun Lee[2], Young-Ho Kim[1], Sun-Hee Leem[3], Jeonghoon Heo[1,4]*, and Hyun Kim[2]*

[1]*Department of Molecular Biology and Immunology, College of Medicine, Kosin University, 34 Amnam-dong, Seo-gu, Busan 602-703, Korea*
[2]*Department of Anatomy, College of Medicine, Kosin University, 34 Amnam-dong, Seo-gu, Busan 602-703, Korea*
[3]*Department of Biological Science, College of Natural Science, Dong-A University, 840 Hadan2-dong, Saha-gu, Busan 604-714, Korea*
[4]*Institute of Medical Science, College of Medicine, Kosin University, 34 Amnam-dong, Seo-gu, Busan 602-703, Korea*

(Received: September 23th, 2013; Revision: October 14th, 2013; Accepted: December 17th, 2013)

Abstract : Adipose tissue-derived stem cells (ADSCs) are able to self-renew and to differentiate into various types of cells. For clinical application of ADSCs, the quality of ADSCs in terms of growth and differentiation should be considered. This study investigated whether the ability of ADSCs for growth and differentiation is affected by the harvesting sites of tissue or ages of animals. ADSCs were harvested from subcutaneous (Sub), inguinal (Ing), epididymal (Epi) and mesenteric (Mes) fat pads of Sprague Dawley rats. The expression levels of CD29 and CD90 were not different among harvesting sites of fat tissue. The growth of ADSCs were significantly higher in Ing and Sub during early passages (p2-p4) of cultures than in Epi and Mes. The growth of ADSCs decreased during the late passages (p6-p10) of cultures. Cell growth of ADSCs from animals of different ages was higher in 8weeks-old animals than in 4weeks-, 16 weeks- and 32 weeks-old animals. The expression levels of CD29 and CD90 were similar in all aged animals. There was no expression of CD34 and CD45 in ADSCs of all aged animals. In cell aggregation assay in ADSCs from different aged animals, ADSCs from 8 weeks- and 16 weeks-old animals formed larger cell aggregates than ADSCs from 4 weeks- and 32 weeks-old animals. The frequency of cell aggregation was higher in early passage of 8 weeks-old animals. These results showed that the harvesting sites of tissue and age of animals may influence the growth and differentiation potential of ADSCs. Therefore, the source of ADSCs should be considered for efficient clinical application.

Key words: *Adipose tissue-derived stem cells, harvesting site, age*

1. Introduction

The rise of tissue engineering and repair of the rapid clinical development has brought new therapeutic approach. Stem cells have ability to self-renew for unlimited proliferation and to differentiate into various cell types. Therefore, stem cells have been focused on their possible application in tissue engineering. Although embryonic stem cells are the most potent stem cells, there are major problems regarding ethical concerns and tumor formation.[1] Unlike embryonic stem cells are obtained from embryo, stem cells can be obtained from the adult tissue including bone marrow, periosterum and muscle.[2, 3]

Adipose tissue is also another attractive reservoir of stem cells, because it is easily accessible and abundant. The stromal vascular fraction (SVF) from adipose tissue contains an abundant population of multipotent adipose-tissue derived stem cells (ADSCs) that have the capacity to differentiate into adipocytes, chondrocytes, osteoblasts, and myocytes.[4-9] The favorable characteristics of ADSCs have been growing in the application of ADSCs for cell-based therapies.[10]

For successful clinical practice, however, the sufficient ADSCs should be harvested and immediately applied to the patients. The therapeutic use of ADSCs may be limited by the unpredictable variation in the capacity of ADSCs for proliferation and differentiation. This variation of ADSCs may be associated with their niche tissues and biological age of donors.[11, 12] It has been reported that the adipose tissue-harvesting sitesaffected the yield of ADSCs,[13] which suggests that stem cell activation may be affected by extracellular signals from their niche.

*Corresponding authors
Tel: +82-51-990-6413
e-mail: jeonghoonheo@kosin.ac.kr (Jeonghoon Heo)
Tel: +82-51-990-6410
e-mail: drkim@kosin.ac.kr (Hyun Kim)

Previous studies indicate that the function of stem cells is linked to the biological aging of stem cells or the age of the donor, and the oxidative stress is accumulated in cells from aged animals.[14, 15] The quantity of mesenchymal stem cells in bone marrow declined in adult rhesus monkey compared to fetal rhesus monkey.[16] It is also shown that a decline of proliferation capacity in aged ADSCs causes accumulation of non-replicative ADSCs.[17] However, other study claims that that age does not affect the ability of MSC to regenerate the injured tissue.[18] There is also a report that age does not affect the viability of ADSCs and *in vitro* differentiation.[19]

The maintenance of the pluripotency of ADSCs in culture is important for therapeutic application. If pluripotency of stem cells decrease during culture, the therapeutic application will be restricted. It has been reported that the pluripotency of embryonic stem cells is affected by the number of passages for subculturing.[20]

Taken together the functionality of ADSCs in terms of growth and pluripotency is important for clinical application. The growth and pluripotency of ADSCs may be influenced by the harvesting site of adipose tissue and/or the ages of donors. Therefore this study investigated whether the ability of ADSCs for growth and differentiation is affected by the harvesting sites of tissue, ages of animals and the number of passages in culture. The observations from this study indicate that the harvesting sites of adipose tissue and age of animals should be considered to select the sufficient ADSCs for clinical treatments.

2. Materials and Methods

2.1 Cell harvesting and Culture

ADSCs were harvested from 4 weeks-, 8 weeks-, 16 weeks- and 32 weeks-old Sprague Dawley rats. All procedures were performedaccording to Institutional Animal Care and Use Committee at Kosin University College of Medicine. Briefly, animals were anaesthetized with Ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg). Fresh adipose tissue was harvested from subcutaneous (Sub), inguinal (Ing), epididymal (Epi) and mesenteric (Mes) fat pads of each animal, and minced with fine scissors. The adipose tissues were digested with 0.1% collagenase type I (Sigma-Aldrich) for 40 min at 37°C in a shaking bath and then centrifuged at 260 x g for 7 min to obtain a pellet. After incubation of the pellet in culture medium DMEM/F12 (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Invitrogen) overnight at 37°C/5% CO_2. The residual non-adherent red blood cells were removed by washing with the medium. The medium was changed every 2 days. The cells were passaged using 0.25% trypsin-EDTA (Welgene) at a ratio of 1:3.

2.2 RNA extraction and RT-PCR

Total RNA was extracted from ADSCs using TRIzol reagent (Invitrogen) as described in manufacturer's protocol. The RNA was electrophoresed in a 1.3% agarose gel containing formaldehyde and stained with ethidium bromide to determine the integrity of total RNA. Total RNA was reverse-transcribed using SuperScript III Fisrt-Strand Synthesis System (Invitrogen) to synthesize the first strand of cDNA. Briefly, 3 μg of total RNA, 1 μLof 10 mM dNTP, 2 μLof random primer were mixed in RNase-free water to a total volume of 10 μL, incubated at 65°C for 5 min, and cooled on ice. Then, 2 μLof 10X RT buffer, 4 μL of 25 mM $MgCl_2$, 1 μLof RNaseOUT were added and incubated at 42°C for 50 min, and 70°C for 15 min. To identify ADSCs, the expression of CD29, CD90, CD34 and CD45 were determined by standard PCRs using the following primer sets: CD29 5'-gcc agt gtc acc tgg aaa at, 5'-tgt gcc cac tgc tga ctt ag; CD90 5'-agc cag atg cct gaa aga ga, 5'-ggg ctg aga atg acc tgg ta; CD34 5'-act tct gtt gcc tcg gag aa, 5'-tct ctg aga tgg ctg gtg tg; CD45 5'-gaa ggc act cgg act ttc ag, 5'-tcg aag ttt cag gag cag gt; GAPDH 5'-aga tgg tga agg tcg gtg tc, 5'-att gaa ctt gcc gtg ggt ag. Thermal cycling was performed for 32 cycles of 94°C/30sec, 51°C/30 sec, 72°C/30 sec. The PCR products were electrophoreses on 1.2% LE agarose gels containing ethidium bromide.

2.3 Cell Growth Assay

To determine the capacity of cell growth, 1×10^5 cells per well in a 6-well plate were plated and cultured in culture medium DMEM/F12 (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Invitrogen) at 37°C/5% CO_2. On 3 days, cells were digested with 0.25% trypsin-EDTA (Invitrogen) and counted on a hemocytometer after staining with 0.4% Trypan Blue solution. Cells from different harvesting sites or from rats of different ages were sub-cultured every 3 days. Cell counting was performed at each passage stage.

2.4 Cell Aggregation Assay

To evaluate ADSCs from rats of different ages, cell aggregation assay was performed at each passage stages of ADSCs from different aged rats. At 70% confluency, cells were dispersed into a single-cell suspension in culture medium DMEM/F12 (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin (Invitrogen). 30-uL of culture drops containing 500 cells were placed onto the undersurface of culture plate lid and then placed over PBS. After 3days, the drops were transferred to suspension culture

medium in tissue culture plate and further cultured for additional 14 days. The number and size of cell aggregates per plate were observed under the inverted microscopy.

2.5 Statistical Analysis

Student t-test was used to estimate differences between groups. A *P value* less than 0.05 was considered statistically significant.

3. Results

3.1 Effects of Adipose Tissue harvesting Site on ADSCs

To determine the effect of adipose tissue harvesting site on ADSCs, adipose tissue was harvested from subcutaneous (Sub), inguinal (Ing), epididymal (Epi) and mesenteric (Mes) fat pads of Sprague Dawley rat (Fig 1A). The ADSCs were selected by plate adhesion in culture medium. The majority of the adherent cells were fibroblast-like cells, but showed heterogenic morphology including spindle shaped or small round cells (Fig 1B). The cultured ADSCs from Sub exhibited

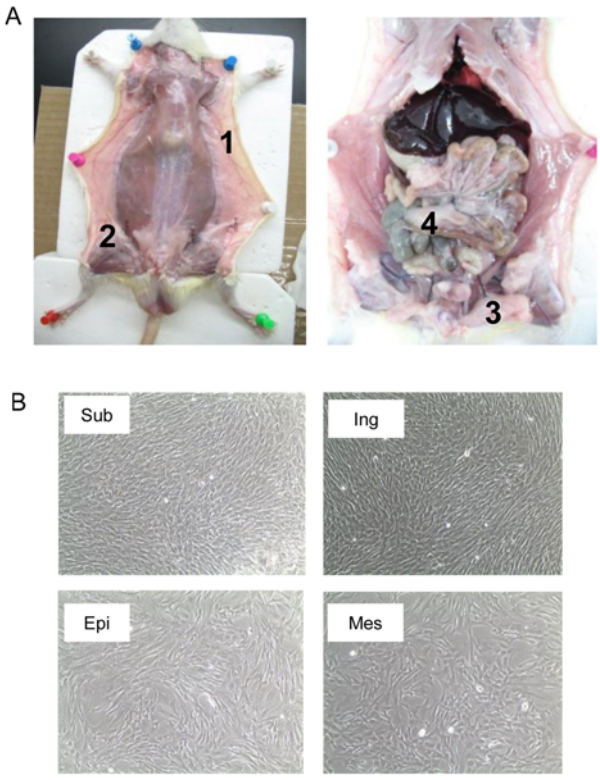


Figure 1. Morphology of ADSCs isolated from different adipose tissue harvesting sites. (A) Harvesting sites of adipose tissues. 1: Subcutaneous fat pad, 2: Inguinal fat pad, 3: Epididymal fat pad, 4: Mesenteric fat pad. (B) Morphology of ADSCs isolated from subcutaneous fat pad (Sub), inguinal fat pad (Ing), epididymal fat pad (Epi) and mesenteric fat pad (Mes) (× 100).

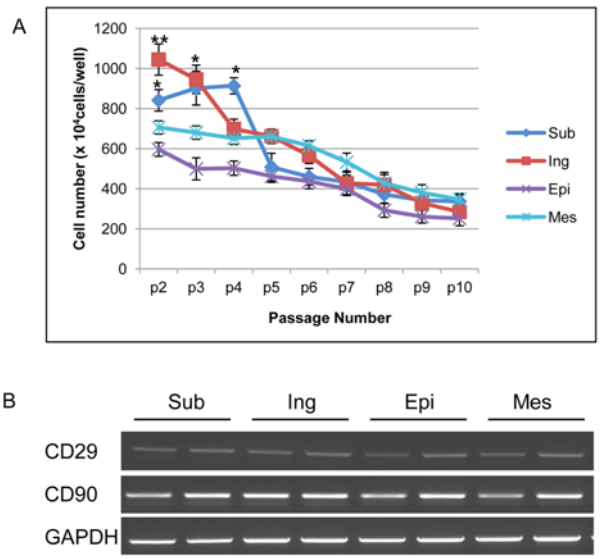


Figure 2. Growth and gene expression profiles of ADSCs isolated from different adipose tissue harvesting sites. (A) Growth curve of ADSCS according to cell passages. *; $p < 0.05$.(B) Expression of ADSCs markers in ADSCs from different harvesting sites.

a similar morphology with ADSCs from Ing, but a little different morphology with ADSCs from Epi and Mes. Whereas the most ADSCs from Epi and Mes were long fibroblast-like cells, the ADSCs from Sub and Ing were small and round cells. To compare the growthof ADSCs from different harvesting sites, the number of cells was counted at each passage during subculturing ADSCs (Fig 2A). The growth of ADSCs was higher in ADSCs from Sub and Ing than from Epi and Mes during early passages. However, there was no difference in the growth of ADSCs from all tissues during late passages. In RT-PCR analysis for the gene expression profile of ADSCs, CD29 and CD90 known as ADSC specific markers were expressed in ADSCs isolated from Sub, Ing, Epi and Mes (Fig 2B). CD34 and CD45 known as hematopoietic markers were not expressed in all samples. There was no difference in the expression levels of CD29 and CD45 among ADSCs from Sub, Ing, Epi and Mes.

3.2 Effects of Age on the Characteristics of ADSCs

To determine the effects of age on the property of ADSCs, ADSCs isolated from 4 weeks-, 8 weeks-, 16 weeks, and 32 weeks-old rates were characterized for morphology, growth and the expression of ADSCs specific markers. The most of cells cultured on plates were fibroblast-like cells, but some of cells exhibited small and round shape (Fig 3). ADSCs from 4 weeks- and 8 weeks- old animals displayed small and round shape during early passage, but ADSCs from 8 weeks-old

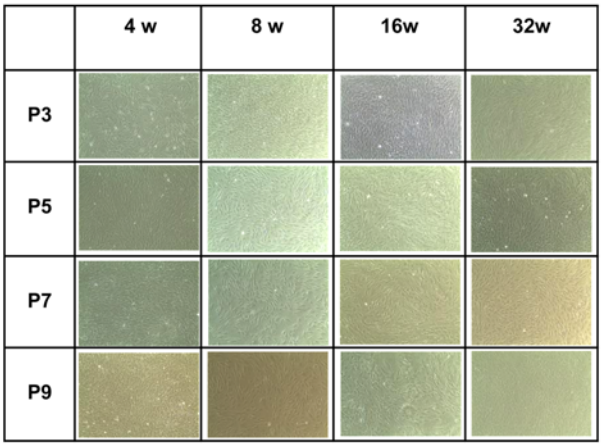


Figure 3. Morphology of ADSCs by the number of passage from different aged animals (× 100).

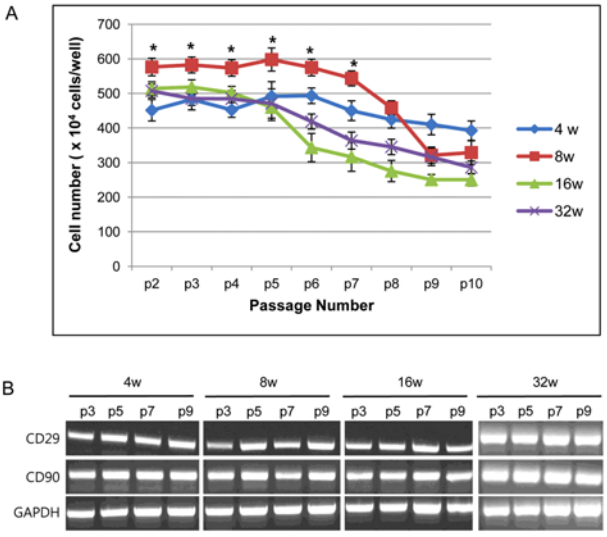
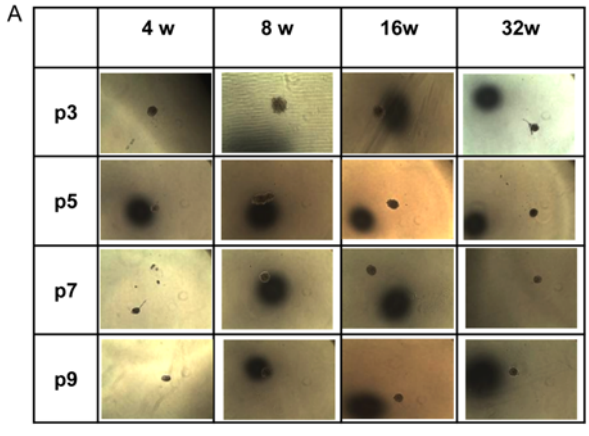


Figure 4. Growth and gene expression profiles of ADSCs isolated from different aged animals. (A) Growth curve of ADSCS according to cell passages. *; $p < 0.05$.(B) Expression of ADSCs markers in ADSCs from different ages.

animals became long and narrow cells like fibroblast cells during late passages. The majority of ADSCs from 16 weeks- and 32 weeks-old animals were small fibroblast-like cells during early passages, but became large cells during late passages.

The growth of ADSCs was compared in ADSCs from different aged animals during subculturing the cells (Fig 4A). The growth of ADSCs from 8 weeks-old animals were higher than from other aged animals. However, the growth of ADSCs from 8 weeks-old animals decreased at late passages and became similar level with ADSCs from other aged animals. All



B

	4 w	8 w	16w	32w
p3	+++	++++	+++	+++
p5	+++	++++	+++	+++
p7	+++	+++	+++	++
p9	+++	+++	+++	++

Figure 5. Cell aggregates formed by ADSCs from different aged animals. (A) Morphology of cell aggregates of ADSCs isolated from different aged animals (× 40) (B) Capacity of ADSCs for formation of cell aggregates. ++++ : high capacity, +++ : medium capacity, ++: low capacity.

ADSCs from different aged animals expressed CD29 and CD90 known as ADSC specific markers, but did not express CD34 and CD45 known as hematopoietic markers (Fig 4B). There was no difference in the expression levels of CD29 and CD45 in all ADSCs isolated from different aged animals.

3.3 Effects of Age on the Formation of Cell Aggregates

To determine the differentiation potential of ADSCs from different aged animals, the capacity of ADSCs for the formation of cell aggregates was measured in ADSCs isolated from different aged animals during subculturing the cells (Fig 5). ADSCs from 8 weeks- and 16 weeks-old animals formed larger cell aggregates than ADSCs from 4 weeks- and 32 weeks-old animals (Fig 5A). The size of cell aggregates was larger in ADSCs at early passages (p3 and p5) than in ADSCs at late passage (p9) (Fig 5B). The frequency of cell aggregation was higher in ADSCs at early passages from 8 weeks-old animals than in other ADSCs, and was the lowest in ADSCs at late passage (p9) from 32 weeks-old animals.

4. Discussion

In the present study, we characterized the capacity of ADSCs

according to harvesting sites of adipose tissue, ages of animals and the number of passages for subculturing to determine the availability of ADSCs for clinical applications. The growth of ADSCs was affected by the harvesting sites of adipose tissue and ages of animals. In addition, the number of passages in subculturing ADSCs also affected the growth and differentiation potential of ADSCs.

Adipose tissue is a highly heterogeneous tissue with various cellular compositions.[21] The metabolic responses of adipose tissue to various hormonal stimuli were dependent on the sites of fat depots.[22] Moreover, it has been reported that the frequency of ADSCs was significantly higher in the SVF of adipose tissue isolated from abdomen region than adipose tissue from the hip/thigh region.[13] Unlike the previous studies, this study has focused on the growth activity of ADSCs isolated from different harvesting sites of adipose tissue, and have showed that the growth activity was significantly higher in ADSCs from adipose tissue of Sub and Ing than in ADSCs from adipose of Epi and Mes. In addition, the growth activity of ADSCs decreased with the number of passages in culture. However, the expression levels of ADSCs specific markers were similar in ADSCs isolated from different harvesting sites of adipose tissue. Therefore, adipose-tissue harvesting sites and the number of passages in culture may influence the growth activity of ADSCs, but not the expression of ADSCs specific markers.

It has been known that aging influences the regeneration capability of an organism. Aging also accumulates oxidative stress in cells,[15] which may cause the senescence of cells. Previous study has demonstrated that the number and differentiation potential of mesenchymal stem cells decreased with age,[23] However, other study has shown that proliferation, attachment and senescence of mesenchymal stem cells was not related with age.[24] Therefore, the effect of age on the function of stem cells is controversial. This study investigated whether the growth activity of ADSCs is influenced by the ages of animals. The morphology of ADSCs in culture from 4 weeks- and 8 weeks-old animals was different from the morphology of ADSCs from 16 weeks- and 32 weeks-old animals. In addition, the growth activity of ADSCs from 8 weeks-old animals was higher than growth activity of ADSCs from other aged animals. However, the growth activity of ADSCs decreased with increase in the number of passage. The expression levels of ADSCs specific markers were not affected by the ages of animals. Therefore, although the growth activity of ADSCs in culture is affected by the age of animals, the effect of age on the growth activity of ADSCs in culture seems to decrease with the number of passages during subculturing.

It has been shown that in murine bone marrow-derived stem cells adipogenic differentiation decreased with donor age and chondrogenic differentiation decreased with increasing passage number,[25] which suggests that the differentiation potential of mesenchymal stem cells may be influenced by donor age and number of passage in cell culture. Conversely, other study has shown that age does not seem to affect in vitro adipogenic differentiation of human adipose tissue-derived stem cells, whereas it does affect osteoblastic differentiation.[19] In this study, the differentiation potential of ADSCs was estimated by the formation of cell aggregates that was used to evaluate in vitro transformation of cells.[26] The size of cell aggregates was larger in ADSCs isolated from 8 weeks- and 16 weeks-old animals than from 4 weeks- and 32 weeks-old animals. The frequency of cell aggregation was high in ADSCs isolated from 8 weeks-old animals. The size of cell aggregates and the frequency of cell aggregation decreased with increase in the number of passage. Taken together, the results of this study suggest that although the differentiation potential of ADSCs is affected by the age of animals as well as the number of passage during subculturing.

In conclusion, the results of this study show that the growth activity of ADSCs may be influenced by the harvesting sites of adipose tissues and age of donors. In addition, the age and the number of passage number in culture may also affect the differentiation capacity of ADSCs. Therefore, the source of ADSCs including harvesting sites of adipose tissue, age of donor, and the number of subculture should be considered to maximize efficient therapeutic application.

Acknowledgements: This study was supported by a grant from Kosin University College of Medicine (2011) and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Korea government (MEST) (2008-0062611).

Disclosure Statement: The authors declare that they have no conflict of interest.

Reference

1. R. Dresser, Ethical issues in embryonic stem cells research, *JAMA*, **285**, 1439 (2001).
2. H Nakahara, VM Goldberg, AI Caplan, Culture-expanded human periosteal-derived cells exhibit osteochondral potential *in vivo*, *J Orthop Res,* **9**, 465 (1991).
3. A Asakura, M Komakim M Rudnicke, Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation, *Differentiation,* **68**, 245 (2001).

4. GR Erickson, M Gimble, DM Franklin *et al*., Chondrogenic potential of adipose tissue-derived stromal cells in vitro and *in vivo*, *Biochem Biophys Res commun,* **290**, 763 (2002).
5. F Guilak, HA Awad, B Fermor *et al*., Adipose-derived adult stem cells for cartilage tissue engineering, *Biorheology* **41**, 389 (2004).
6. YD Halvorsen, D Franklin, AL Bond *et al*., Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells, *Tissu Eng,* **7**, 729 (2001).
7. H Hattori, M Sato, KMasuoka *et al*., Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cells source, *Cells Tissues Organs*, **178**, 2 (2004).
8. V Planat-Benard, JS Silvestre, B Cousin *et al*., Plasticity of human adipose lineage cells toward endothelial cells; physiological and therapeutic perspectives. *Circulation,* **109**, 656 (2004).
9. S Rangappa, C Fen, EH Lee *et al*., Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes, *Ann Thorac Surg*, **75**, 775 (2003).
10. PA Zuk, M Zhu, H Mizuno *et al*., Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng,* **7**, 211 (2001).
11. MJ Oedayrajsingh-Varma, SM Ham, M Knippenberg *et al*., Adipose tissue-derived mesenchymal stem cells yield and growth characteristics are affected by the tissue-harvesting procedure, *Cytotherapy*, **8**, 166 (2006).
12. K Mareschi, I Ferrero, D Rustichelli *et al*., Expansion of mesenchymal stem cells isolated from periatric and adult donor bone marrow, *J Cell Biochem*, **97**, 744 (2006).
13. WJFM Jurgens, MJ Oedayrajsingh-Varma, MN Helder *et al*., Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies, *Cell Tissue Res*, **332**, 415 (2008).
14. A Aguirre, ME Rubio, V Gallo *et al*., Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal, *Nature,* **467**, 323 (2010).
15. G Li, C Luna, PB Liton *et al*., Sustained stress response after oxidative stress in trabecular meshwork cells, *Mol Vis,* **13**, 2282 (2007).
16. CC Lee, F Ye, AF Tarantal, Comparison of growth and differentiation of fetal and adult rhesus mesenchymal stem cells, *Stem Cells Dev*, **15**, 209 (2006).
17. EG Schmuck, JD Mulligan, KW Saupe, Caloric restriction attenuates the age-associated increase of adipose-derived stem cells but further reduces their proliferative capacity, *Age*, **33**, 107 (2011).
18. EE Morrison, RM Costanzo, Regeneration of olfactory sensory neurons and reconnection in the aging hamster central nervous system, *Neurosci Lett*, **198**, 213 (1995).
19. L de Girolamo, S Lopa, E Arrigoni *et al*., Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during *in vitro* osteoblastic differentiation, *Cytotherapy*, **11**, 793 (2009).
20. A Nagy, J Rossant, R Nagy *et al*., Derivation of completely cell culture-derived mice from early-passage embryonic stem cells, *Proc Natl Acad Sci USA*, 90, 8424 (1993).
21. B Prunet-Marcassus, B Cousin, D Caton *et al*., From heterogeneity to plasticity in adipose tissues: site-specific differences, *Exp Cell Res*, **312**, 727 (2005).
22. S Rodriguez-Cuenca, M Monjo, AM Proenza *et al*., Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu, *Am J Physiol Endocrinol Metab*, **288**, E200 (2005).
23. EU Alt, C Senst, SN Murthy *et al*., Aging alters tissue resident mesenchymal stem cells properties, *Stem Cell Res*, **8**, 215 (2012).
24. CG Bellows, W Pei, Y Jia *et al*., Proliferation, differentiation, and self-renewal of osteoprogenitors in vertebral cell populations from aged and young female rats, *Mech Ageing Dev*, **124**, 747 (2003).
25. JD Kretlow, YQ Jin, W Liu *et al*., Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells, *BMC Cell Biol*, **9**, 60 (2008).
26. JS Rhim, Cell aggregation assay: a rapid means of evaluating and selecting *in vitro* transformed cells, *Cancer Detect Prev*, **6**, 381 (1983).