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Long-term Cryopreservation of Mesenchymal Stem Cells Derived from Human Eyelid Adipose and Amniotic Membrane: Maintenance of Stem Cell Characteristics

Yeonhwa Song, Seah Park, Sujin Yun, Hye Jin Yang, A Young Yoon and Haekwon Kim†

Dept. of Biotechnology, Seoul Women's University, Seoul 139-774, Korea

ABSTRACT : Human eyelid adipose-derived stem cells (hEAs) and amniotic mesenchymal stem cells (hAMs) are very valuable sources for the cell therapeutics. Both types of cells have a great proliferating ability *in vitro* and a multipotency to differentiate into adipocytes, osteoblasts and chondrocytes. In the present study, we evaluated their stem cell characteristics after long-time cryopreservation for 6, 12 and 24 months. When frozen-thawed cells were cultivated *in vitro*, their cumulative cell number and doubling time were similar to freshly prepared cells. Also they expressed stem cell-related genes of SCF, *NANOG*, *OCT4*, and TERT, ectoderm-related genes of *NCAM* and *FGF5*, mesoderm/endoderm-related genes of *CK18* and *VIM*, and immune-related genes of *HLA-ABC* and *β2M*. Following differentiation culture in appropriate culture media for 2-3 weeks, both types of cells exhibited well differentiation into adipocyte, osteoblast, and chondrocyte, as revealed by adipogenic, osteogenic or chondrogenic-specific staining and related genes, respectively. In conclusion, even after long-term storage hEAs and hAMs could maintain their stem cell characteristics, suggesting that they might be suitable for clinical application based on stem cell therapy.

Key words : Human eyelid adipose-derived stem cell, Human amniotic mesenchymal stem cell, Cryopreservation, Multipotency

INTRODUCTION

Stem cells are promising as a tool of regenerative therapies for replacing damaged cells or supporting regeneration of damaged tissues. Human embryonic stem cells (ESCs) have been evaluated as therapeutic sources in terms of their multi-lineage differentiation potential including neural cells (Reubinoff et al., 2000), blood cells (Kaufman et al., 2001) and pancreatic β-cells (Assady et al., 2001). However, clinical use of ESCs is faced with the ethical issue and has a potential of teratoma formation. In contrast, adult stem cells do not accompany an ethical issue and there is no report of the teratoma formation after transplantation *in vivo*. Previously, mesenchymal stem cells (MSCs) can proliferate multiple times *in vitro* and differentiate into a variety of cell types of mesodermal lineage (Prockop, 1997). Recently, they have also been shown to differentiate into endodermal lineage such as hepatocytes (Shin et al., 2010), pancreatic β-cells (Kang et al., 2009), and ectodermal lineage including neurons (Woodbury et al., 2000).

MSCs reside in various tissues including bone marrow, adipose tissue, umbilical cord, and amniotic membrane (Pittenger et al., 1999, Minguell et al., 2001). Bone marrow-derived mesenchymal stem cells (BM-MSCs) are the most well-known and characterized MSCs, yet their retrieval from bone marrow accompanies pain in donors during harvest. Thus many studies have been looking alternative sources. Human eyelid adipose-derived stem cells (hEAs), which could be obtained during plastic surgery, show the characteristics of neural crest cells and multi-lineage differentiation potentials. When they were cultivated under the appropriate condition, they could differentiate into insulin-secreting cells which normalized the blood glucose levels of diabetic mice after transplantation. hAMs expressing Oct-4 (Prusa et al., 2003) have also exhibited a potential for osteogenic,

† Corresponding author: Haekwon Kim, Dept. of Biotechnology, College of Natural Sciences, Seoul Women's University, Seoul 139-774, Korea. Tel: +82-2-970-5665, Fax: +82-2-974-2473, E-mail: hwkim@swu.ac.kr

adipogenic, and chondrogenic differentiation (Kim et al., 2007; Alviano et al., 2007). hAMs could be nonsurgically obtained at the time of delivery and overcome the ethical issues. Thus both hEAs and hAMs appear to be useful as cell sources for cell therapeutics.

Cryopreservation of stem cells is important for their late-use in future clinical application. Thus it needs to be verified whether cryopreserved cells could exhibit the same or similar characteristics as the freshly obtained cells. While long-term cryopreservation of hematopoietic stem cells has been well studied, cells such as hEAs and hAMs have not been investigated yet. The purpose of this study was to investigate the characteristics of frozen-thawed hEAs and hAMs after 6, 12, 24 months. The proliferation ability and expression of stem cell-related genes were examined after thawing. The frozen-thawed cells were induced into adipocyte, osteoblast, and chondrocyte, investigated expression of adipogenic, osteogenic, and chondrogenic-related genes by RT-PCR, and stained with oil red O, von Kossa, and alcian blue, respectively.

MATERIALS AND METHODS

1. Isolation and Culture of hEAs

hEAs were obtained from the patients undergoing cosmetic surgery with informed consent. All experiments were approved by Institutional Review Boards of Seoul Women's University. Adipose tissue was treated with 0.075% type I collagenase (Gibco, Grand Island, NY) in Dulbecco's phosphate-buffered saline (DPBS) (Gibco) for 1 hr at 37℃ with gentle stirring. Cell suspensions were cultured in Dulbecco's modified Eagle's medium low glucose (DMEM-LG) (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 1% penicillin/streptomycin solution (Gibco), and 3.7 ㎎/㎖ sodium bicarbonate at 37℃ in 5% CO_2 humidified atmosphere. Medium was changed twice a week. Cumulative population doubling at each subculture was calculated by using the formula, $2^X = N_H/N_I$, where N_I= the inoculum cell number, N_H= cell harvest number at confluence (>80%), and X= population doublings (Cristofalo et al., 1998). The population doubling increase that was calculated was then added to the previous population doubling level to yield the cumulative population doubling level.

2. Isolation and Culture of hAM

Placenta was obtained at cesarean section from the volunteers with the informed consent. Isolation and culture of hAMs were performed as described previously (Kim et al., 2007). Briefly, the amnion was peeled off mechanically from the chorion and washed in DPBS to remove blood and cellular debris. Amnion tissue was minced and washed with DPBS and pelleted by centrifugation, and then supernatant was discarded. The remaining tissue was incubated with 0.25% trypsin (Gibco) three times with continuous stirring for 30 min in 37℃. The remaining amnion tissue pieces were treated with 2 ㎎/㎖ collagenase A (Roche, Rotkreuz, Switzerland) and 0.05 ㎎/㎖ DNase (Roche) for 1 hr to liberate dissociated cells. The dispersed mesenchymal cells were collected by centrifugation. After two times washing with DMEM-LG, the cells were seeded in DMEM-LG supplemented with 1% penicillin/streptomycin solution, 3.7 ㎎/㎖ sodium bicarbonate and 10% FBS and cultured at 37℃ in 5% CO_2 humidified atmosphere. Cell culture media were changed twice a week after cells were attached in flask. When achieving confluence, cells were treated with 0.125% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 2 min. Released cells were collected and subcultured again. Morphologically homogeneous population of fibroblast-like cells, namely hAM, was obtained after 2 or 3 subcultures.

3. Freezing and thawing of Cells

Passage 2-3 (p2-3) hEA and hAM were suspended in DMEM-LG containing 10% dimethylsulfoxide (DMSO) and 25% FBS at concentration of 1×10^6/㎖ and placed in the cryovials (Nunc, Rochester, NY). Cells were frozen in freezing container containing isopropyl alcohol in the base for 24 hr in the −80℃. Each sample was cooled at a rate

of −1℃/minute to -80℃. After 24 hr, they were stored in −196℃ liquid nitrogen tank until use. After 6, 12 or 24 months, cells were thawed in a 37℃ water bath and transferred into 15 ㎖ conical tube and added DMEM- LG supplemented with 10% FBS. After centrifuge at 1,450 rpm for 5 min, supernatant was discarded and cell pellet was washed twice with DMEM-LG. Cells were stained with 0.4% trypan blue solution and plated onto the hematocytometer. Unstained cells were counted as live cells and live cells were seeded by 200 cell/㎠ with DMEM-LG containing 10% FBS and cultivated at 37℃ in 5% CO_2 humidified atmosphere.

4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated using Tri-reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The purity of RNA was assessed by determining the ratio of absorbance at 260 nm to that at 280 nm (>1.8). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a GeneAmp PCR system 2400 (PerkinElmer, Boston). Subsequent PCR reactions were performed using cDNA, primer pairs and PCR mixture (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Human adipose total RNA (hAD) and human osteogenic sarcoma total RNA (hOsteo) were purchased from Ambion (Austin, TX).

5. Multilineage Differentiation

1) Adipogenic Differentiation

To induce adipogenic differentiation, frozen-thawed hEAs at p4 were cultivated in an adipogenic medium consisting of DMEM-LG supplemented with 10% FBS, 1 ㎛M dexamethasone, 0.5 M 3-isobutyl-1-methylxanthine (IBMX), 0.05 ㎍/㎖ human recombinant insulin, 200 ㎛M indomethacin. Frozen-thawed hAMs were cultivated in a commercially-provided, adipogenic differentiation medium (Invitrogen). Medium was changed twice a week. After cultivation for 2 weeks, cells were fixed with 4% paraformaldehyde for 1 hr and stained with oil red O dye for 30 min at room temperature (RT) to visualize intracellular lipid droplets. Counter stain was performed with 10% hematoxylin. Photographs were taken using Zeiss microscope.

2) Osteogenic Differentiation

To induce osteogenic differentiation, frozen-thawed hEAs and hAMs each at p4 were cultivated in a medium consisting of DMEM-LG supplemented with 10% FBS, 0.01 ㎛M dexamethasone, 100 mM β-glycerol phosphate, 200 ㎛M ascorbic acid-2-phosphate, 10 ng/㎖ EGF (Peprotech, USA) and 10 nM vitamin D (Peprotech). Medium was changed twice a week. After cultivation for 2 weeks, cells were fixed with 4% paraformaldehyde for 1 hr and mineralization of accumulated calcium were visualized by 5% silver nitrate treatment for 20 min under the UV followed by 2% sodium thiosulphate for 2 min. Counter staining was done by 1% neutral red or 10% hematoxylin dye.

3) Chondrogenic Differentiation

To induce chondrogenic differentiation, frozen-thawed hEAs and hAMs each at p4 were cultivated in a medium consisting of DMEM-HG supplemented with 1x ITS-premix, 0.1 ㎛M dexamethasone, 10 ng/㎖ TGF-β3 (Peprotech), 50 ㎍/㎖ ascorbate-2-phosphate, 100 ㎍/㎖ pyruvate and 40 ㎍/㎖ L-proline. Medium was changed twice a week. After cultivation for 3 weeks, cells were fixed with 4 % paraformaldehyde for 1 hr and then stained with alcian blue for 30 min at RT. Counter stain was performed with nuclear fast red or 10% hematoxylin.

All reagents and chemicals were purchased from the Sigma (St. Louis, MO, USA) unless otherwise designated.

RESULTS

1. Morphology of Frozen-thawed hEAs and hAMs

When hEAs cryopreserved for 6, 12 and 24 months were thawed and cultivated, cells exhibited neuron-like bipolar shape similar to fresh hEAs (Fig. 1A). Cryopreserved

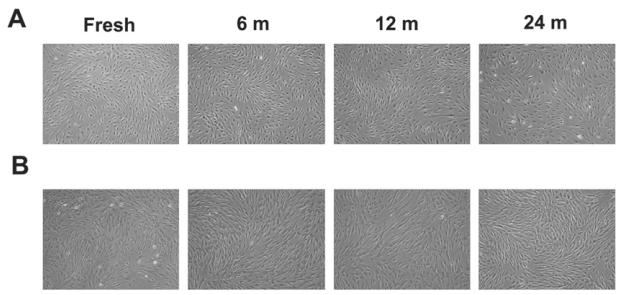

Fig. 1. Morphology of frozen-thawed hEAs and hAMs. hEAs (A) and hAMs (B) which had been cryopreserved for 6, 12 or 24 months (m) exhibited similar morphology to that of fresh cells when they were thawed and cultivated *in vitro* until p4, respectively. Magnification, ×40.

hAMs also showed fibroblast-like morphology similar to fresh hAMs (Fig. 1B).

2. Proliferative Capacity of Frozen-thawed hEAs and hAMs

Throughout the culture, both hEAs and hAMs consistently proliferated until their senescence around p14 regardless of their cryopreservation period (Fig. 2). Frozen-thawed hEAs showed cumulative population doubling number of 40 for 6-month frozen cells, 41 for 12-month frozen cells, and 39 for 24-month frozen cells. Fresh hEAs exhibited 42 population doubling number (Fig. 2A). Population doubling number of fresh hAMs was 53, while frozen-thawed hAMs showed 56 for 6-month frozen cells, 55 for 12-month frozen cells, and 57 for 24-month frozen cells (Fig. 2B).

3. Stem Cell Properties of Frozen-thawed hEAs and hAMs

Following 6, 12, or 24 months of cryopreservation, hEAs and hAMs were thawed and expression of stem cell-related genes were analyzed at p4, 8 and 12. Both hEAs and hAMs expressed consistently stem cell-related genes of *OCT4, SCF*, and *NANOG,* ectoderm-related genes of *FGF5* and *NCAM*, mesoderm/endoderm-related genes of

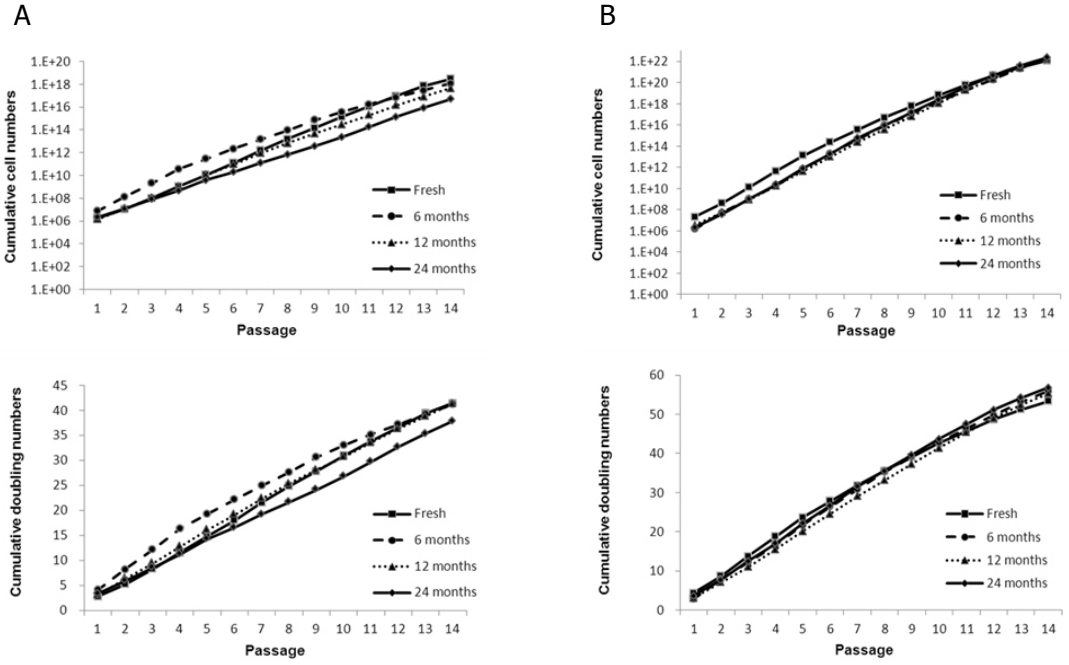

Fig. 2. Cumulative cell numbers and doubling numbers of fresh and frozen-thawed hEAs and hAMs. Cumulative cell and doubling numbers of fresh or frozen-thawed hEAs (A) and hAMs (B) which had been cryopreserved for 6, 12 or 24 months were examined following cultivation *in vitro* until their senescence, respectively.

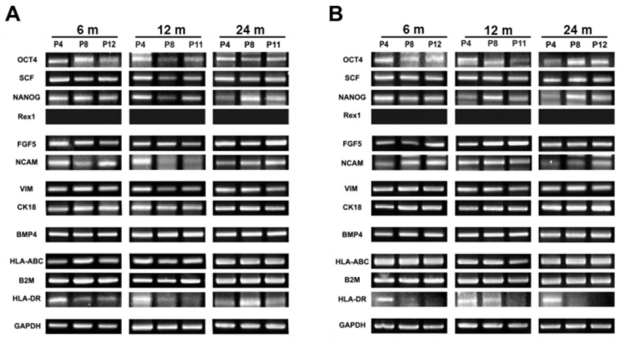

Fig. 3. Gene expression of frozen-thawed hEAs and hAMs following cultivation *in vitro*. During *in vitro* cultivation of hEAs (A) and hAMs (B) which had been cryopreserved for 6, 12 or 24 months (m), their gene expression profile at p4, p8, p11 or p12 was analyzed using RT-PCR, respectively.

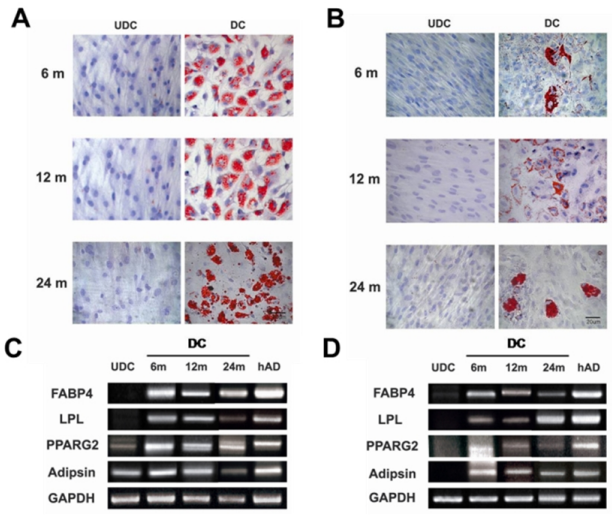

Fig. 4. Adipogenic differentiation of hEAs and hAMs after cryopreservation. After cultivation of cells in adipogenic differentiation media following cryopreservation for 6, 12, or 24 months (m), both hEAs (A) and hAMs (B) exhibited intense staining with oil Red O, respectively. Expression of adipocytes-related genes of hEAs (C) and hAMs (D) was assessed by RT-PCR. Human adipose tissue mRNA (hAD) was used as a positive control. UDC, undifferentiated cells; DC, differentiated cells.

vimentin (VIM) and *CK18*, and endoderm-related genes of *BMP4*. However, both types of cells did not express *REX1* gene (Fig. 3). Frozen-thawed hEAs distinctly expressed *HLA-DR* gene at p4 but the intensity decreased at p8 and p11/p12, the pattern of which was similarly observed regardless of the cryopreservation period. Frozen-thawed hAMs also expressed *HLA-DR* gene at p4 and showed weaker expression at p8. However, hAMs at p11/p12 no longer expressed the gene. The expression pattern was observed throughout the cryopreservation period of 6, 12 or 24 months.

4. Differentiation Potential of Frozen-thawed hEAs and hAMs

Both hEAs and hAMs cryopreserved for 6, 12 or 24 months were cultivated in adipogenic medium for 2 weeks and then were stained with oil red O. All hEAs and hAMs exhibited a lot of lipid droplet accumulation within their cytoplasm (Fig. 4A, B). RT-PCR analysis demonstrated that both types of cells expressed adipocyte-specific genes of *FABP4, LPL, PPARG2* and *Adipsin*, of which expression are typical of human adipose cells (Fig. 4C, D). Moreover, the expression pattern was not severely affected by the cryopreservation period in both types of cells. In contrast, undifferentiated control cells did not express genes of *FABP4* and *LPL* although they expressed *PPARG2* and *Adipsin* genes. These results suggest that adipogenic differential potential of both cell types was not impaired even after 24 months of cryopreservation.

After culture of hEAs and hAMs, which have been cryopreserved for 6, 12 or 24 months, in osteogenic medium for 2 weeks, von Kossa staining exhibited intense accumulation of calcium compounds in the cytoplasm of hEAs and hAMs (Fig. 5A, B). Both types of cells following osteogenic differentiation distinctly expressed osteoblast-specific genes of *Osteocalcin, PTHR, CBFA1, Osteopontin, Col1* and *Osteonectin*, of which expression also seen in human osteogenic sarcoma (Fig. 5C, D). The expression pattern was not affected by cryopreservation period in both types of cells. Among these genes, expression of *CBFA1, Osteopontin, Col1* and *Osteonectin* genes was also observed by undifferentiated cells of both cell types.

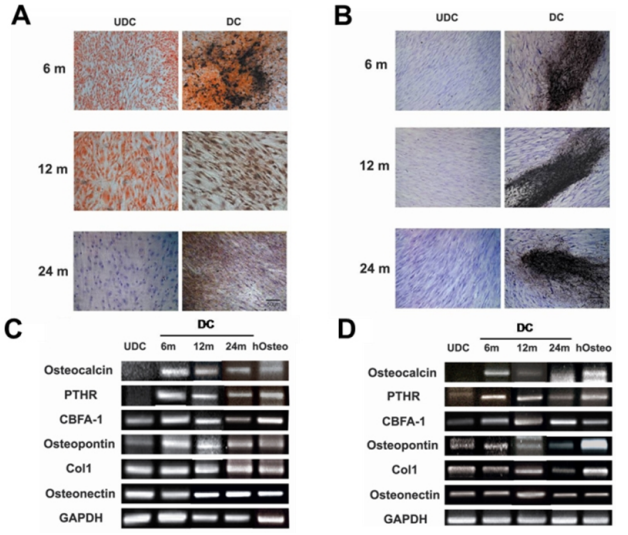

Fig. 5. Osteodgenic differentiation of hEAs and hAMs after cryopreservation. After cultivation of hEAs (A) and hAMs (B) in osteogenic differentiation media following cryopreservation for 6, 12, or 24 months (m), both types of cells were distinctly stained with von Kossa staining solution. Expression of osteoblasts-related genes of hEAs (C) and hAMs (D) was assessed by RT-PCR. Human osteogenic sarcoma mRNA (hOsteo) was used as a positive control. UDC, undifferentiated cells; DC, differentiated cells.

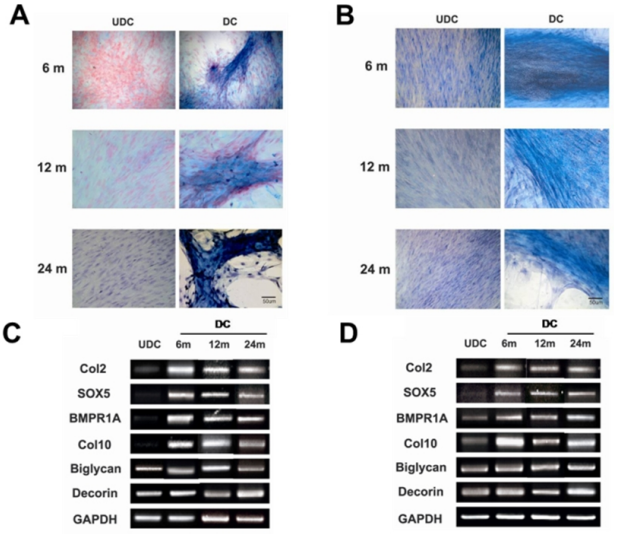

Fig. 6. Chondrogenic differentiation of hEAs and hAMs after cryopreservation. After cultivation of cells in chondrogenic differentiation media following cryopreservation for 6, 12, or 24 months (m), both hEAs (A) and hAMs (B) were deeply stained with alcian blue, respectively. Expression of chondrocytes-related genes of hEAs (C) and hAMs (D) was assessed by RT-PCR. UDC, undifferentiated cells; DC, differentiated cells.

However, these undifferentiated cells did not express genes of *Osteocalcin* and *PTHR*. These results show that the ability of osteogenic differentiation of hEAs and hAMs could be maintained up to 24 months of cryopreservation.

hEAs and hAMs cryopreserved for 6, 12 or 24 months were differentiated into chondrocyte by culture in chondrogenic medium for 3 weeks. After induction, both cells formed multi-layer structures and cytoplasm of both types of cells was deeply stained with alcian blue (Fig. 6A, B). Following chondrogenic differentiation, both hEAs and hAMs expressed chondrocyte-related genes of *Col2, SOX5, BMPR1A, Col10*, *biglycan* and *Decorin*, regardless of cryopreservation period (Fig. 6C, D). In contrast, undifferentiated hEAs showed little expression of *Col2, SOX5, BMPR1A* and *Col10* genes, while hAMs expressed all of these four genes though not as strong as differentiated hAMs. Genes of *biglycan* and *Decorin* were distinctly expressed in both types of cells whether they were differentiated or not. These gene expression patterns were not affected by cryopreservation period. These results demonstrate that frozen-thawed hEAs and hAMs could differentiate into chondrocytes even after 24-month cryopreservation.

DISCUSSION

Cryopreservation is a well-known method of long-term preservation of various tissues and cells. DMSO is a typical cryoprotectant, which protects formation of intracellular ice and disruption of cell membrane under freezing. Slow rate freezing using DMSO has been widely used for cryopreservation of various stem cells including HSCs (Zhao et al., 2008; Yamamoto et al., 2011) as well as a variety of MSCs established from bone marrow (Lee et

al., 2004a; Liu et al., 2008a; Rust et al., 2006), umbilical cord blood (Erices et al., 2000; Romanov et al., 2003; Lee et al., 2004a), dental pulp (Perry et al., 2008; Woods et al., 2009) and adipose tissue (Lee et al.,2004b; Liu et al., 2008b).

Long-term cryopreservation is particularly important for the cells which might be utilized after months to years, depending on the demand for matched tissue (Ayello et al., 1998). Studies about long-term cryopreservation of HSCs have shown that functional HSCs could be recovered with high efficiency after cryopreservation of human cord blood for 15 years (Broxmeyer et al., 2003). Bone marrow-derived progenitor cells have also been successfully cryopreserved for 11 years without loss of progenitor activities (Donnenberg et al., 2002). However, unlike HSCs, little studies have been done regarding to the long- term such as 24-month cryopreservation effect on the MSCs. Short-term freezing effects of human MSCs have been demonstrated that there was little change in the stem cell characteristics as well as viability of MSCs after cryopreservation for 2 weeks to 2 months (Gonda et al., 2008; Liu et al., 2008b). Recent studies reported that cryopreservation of BM-MSCs and hADSCs for 1 year do not impair stem cell characteristics (Zhao et al., 2008; De Rosa et al., 2009). In this study, we showed that cryopreservation of hEAs and hAMs using conventional freezing method did not influence the stem cell characteristics even after 24 months based on the various criteria.

When cryopreserved hEAs and hAMs were cultivated *in vitro*, they displayed the same morphology as their freshly prepared counter parts and could expand over 35 passages similar to unfrozen cells. Both types of cells expressed consistently the stem cell-related genes regardless of their preservation period. Lineage-specific differentiation analyses showed that their differentiation ability into adipocytes, osteoblasts, and chondrocytes was not influenced by cryopreservation as revealed by tissue-specific stainings and gene expressions.

In conclusion, both frozen-thawed hEAs and hAMs could maintain stem cell characteristics as the freshly prepared hEAs and hAMs, in terms of *in vitro* proliferating capability, stem cell-related gene expression and multi-lineage differentiation potential even after 24 months of cryopreservation. Based upon these observations, it is suggested that both hEAs and hAMs can be used as attractive cells for the clinical stem cell therapy.

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