Successful vitrification of human amnion-derived mesenchymal stem cells

Jeong Hee Moon, Jung Ryeol Lee, Byung Chul Jee, Chang Suk Suh, Seok Hyun Kim, Hyun Jung Lim and Hae Kwon Kim

BACKGROUND: A cryopreservation protocol for human amnion-derived mesenchymal stem cells (HAMs) is required because these cells cannot survive for long periods in culture. The aim of this study was to determine whether vitrification is a useful freezing method for storage of HAMs. METHODS: HAMs were cryopreserved using vitrification method. The morphology and viability of thawed HAMs was evaluated by Trypan Blue staining. The expression of several embryonic stem cell (ESC) markers was evaluated using flow cytometry, RT–PCR and immunocytochemistry. Von Kossa, Oil Red O and Alcian Blue staining were used to assess the differentiation potential of thawed HAMs. RESULTS: The post-thawing viability of HAMs was 84.3 ± 3.2% (Mean ± SD, n = 10). The thawed HAMs showed morphological characteristics indistinguishable from the non-vitrified fresh HAMs. The expression of surface antigens (strong positive for CD44, CD49d, CD59, CD90, CD105 and HLA-ABC; weak positive for HLA-G; negative for CD31, CD34, CD45, CD106, CD117 and HLA-DR) and the expression of ESC markers [CK18, fibroblast growth factor-5, GATA-4, neural cell adhesion molecule, Nestin, Oct-4, stem cell factor, Octopodin, B-trypsin, B-2, TRA-1-60, stage-specific embryonic antigen (SSEA-3, SSEA-4)] were maintained in the vitrified-thawed HAMs. The thawed HAMs retained ability to differentiate into osteoblasts, adipocytes and chondrocytes under appropriate culture conditions. CONCLUSIONS: Our results suggest that vitrification is a reliable and effective method for cryopreservation of HAMs.

Keywords: vitrification; amnion; mesenchymal stem cells; cryopreservation; stem cell markers
Formulating a cryopreservation protocol for the HAMs is required because these cells cannot survive for long periods under in vitro culture conditions. Slow rate cooling methods using dimethylsulfoxide (DMSO) as a cryoprotectant have been used for a wide variety of MSC lines established from bone marrow (Lee et al., 2004a,b; Kotobuki et al., 2005), umbilical cord blood (Erices et al., 2000; Romanov et al., 2003; Lee et al., 2004a,b) and hematopoietic progenitor cells (Zhao et al., 2001). Slow freezing has been developed to reduce ice crystal formation and to eliminate toxic and osmotic damage to cells through exposure to low concentration of cryoprotectants while slowly decreasing the temperatures (Vajta et al., 2006). However, it is difficult to eliminate injuries by intracellular ice formation completely. Alternately, vitrification, a rapid cooling method using a high concentration of cryoprotectant, could also be used. Vitrification can completely eliminate the damage caused by ice crystal formation in the cytoplasm of cells during freezing (Kasai et al., 2004). It is also advantageous because the procedure takes a relatively short time and a programmable temperature-decreasing container is not required (Karlsson, 2002). Vitrification has been successfully used as a method of preservation for human oocytes and embryos (Rall and Fahy, 1985; Kuleshova et al., 1999; Cho et al., 2002; Dobrinsky, 2002) and for hematopoietic progenitor cells retrieved from human cord blood (Kurata et al., 1994). Reubinoff et al. (2001) tested vitrification of the human embryonic stem cells (hESCs) with an open-pulled straw; although hESCs could be recovered after thawing, the survival rate was quite low. In addition, it has been shown that the vitrification is an efficient and reliable method for hESCs compared with conventional slow freezing (Fujioka et al., 2004). Li et al. (2008) also reported that vitrification of hESCs with open-pulled straw resulted in a high survival rate of up to 94.3% compared with that (38.6%) of slow freezing in a cryovial. However, as a long-term preservation method for HAMs, the usefullness of vitrification remains to be elucidated. The aim of this study was to investigate whether vitrification is a feasible method for long-term cryopreservation of HAMs.

Materials and Methods

Isolation and culture of hams

Human placentas (n = 10) were obtained during Cesarean section after receiving informed consent from patients. This study was approved by the Institutional Review Board at Seoul National University Bundang Hospital. The peeled amniotic membranes were treated with 0.25% trypsin (Gibco BRL, Grand Island, NY, USA) for 20 min before mechanical mincing. Then, the pieces of amniotic membranes (∼5 × 5 mm²) were incubated at 37°C in Dulbecco’s phosphate-buffered saline (DPBS; Gibco) containing 2 mg/ml collagenase (Roche Diagnostics, Rotkreuz, Switzerland) and 0.05 mg/ml DNase (Roche Diagnostics) for 2 h. The dispersed cells were harvested and then incubated in 25 cm² culture flask (Nunc, Rochester, MN, USA) using low-glucose Dulbecco’s Modified Eagle’s Medium (DMEM-LG; Gibco) containing 100 IU penicillin (Gibco), 100 μg streptomycin (Gibco), 3.7 ml/1 sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA) and 10% fetal bovine serum (FBS; Gibco) under 5% CO₂ at 37°C. Seven days later, non-adherent cells were removed and the culture was continued, replacing medium twice a week. The confluent cells were treated with 0.05% trypsin and then re-plated on 75 cm² culture flask (Nunc) at a density of 10⁶ cells/ml for subculture. Under the same conditions, the retrieved mesenchymal cells continued to be cultured.

Vitrification of hams

The HAMs were cryopreserved by vitrification method using a two-step exposure to equilibration and vitrification solutions (Cho et al., 2002). The equilibration solution was 20% ethylene glycol (EG; Sigma) and the vitrification solution was composed of 40% EG, 18% Ficoll 70 (Sigma) and 0.3M sucrose (Sigma). All solutions were based on DPBS containing 20% FBS. A pellet of ∼1 × 10⁶ HAMs (∼10 μl) was first suspended in 50 μl equilibration solution for 5 min, and then mixed with 500 μl vitrification solution for 40 s. Suspended HAMs were immediately transferred to 1.2 ml cryovial (Nunc) and plunged directly into liquid nitrogen. Two weeks later, the cells were thawed by rapidly immersing the vials in a water bath at 37°C and were suspended serially in 0.5, 0.25 and 0 M sucrose in DPBS containing 20% FBS, through a simple dilution of sucrose solution. After thawing, the survival rate was evaluated by the Trypan Blue staining method. After removing some of the cell pellet and adding 0.4% Trypan Blue (Gibco), the cells were plated onto a slide, and unstained cells were counted as live cells (Zhao et al., 2001). The remaining cells were plated at a density of 1 × 10⁶ cells/ml in a 75 cm² culture flask and subcultured over 7 days.

Flow cytometry analysis

Thawed and non-cryopreserved fresh HAMs (at Passage 3) were examined for expression of surface antigens using a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson, CA, USA) equipped with Cell Quest software. At least 10 000 cells were analyzed per sample. Trypsinized HAM cells were re-suspended in DMEM supplemented with 10% FBS and washed with PBS containing 3% FBS. Cells were incubated with primary antibodies for 1 h at 4°C. Unbound antibodies were removed by washing with PBS. The following specific primary monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC) were used to detect surface antigen expression: CD31-FITC, CD34-APC, CD44-FITC, CD45-FITC, CD49d-PE, CD59-PE, CD90-PE, CD105-FITC, CD106-FITC, CD117-PE, HLA-G-PE, HLA-ABC-FITC and HLA-DR-FITC (all from BD Pharmering, San Diego, CA, USA, http://www.bd-biosciences.com). Table I shows the CD antigens used in this study.

RNA extraction and RT–PCR

RNA extraction and RT–PCR was performed in thawed and non-cryopreserved HAMs (both at 3rd and 7th passage). Total RNA was extracted using Tri-reagent (Sigma). RNA concentrations were determined by absorbance at 260 nm with a spectrophotometer. A total of 5 μg RNA was reverse transcribed using a 1 mM dNTP mixture, 20 U RNase inhibitor (Takara, Japan) and 20 U moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, Canada) for 60 min at 42°C in the presence of 0.5 μg/μl oligo (dT) primer. PCR amplification was performed in a Gene Amp PCR system 2400 (Perkin Elmer, Boston, MA, USA) using 0.25 U Taq polymerase (Takara) and 10 pM gene-specific primers, as described previously (Kim et al., 2007). Amplification was performed using 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s. Annealing temperatures differed depending on the primers used (Table II). PCR products were stained with ethidium bromide on a 2% agarose gel and visualized by UV light using a
Bioprofile image analysis system (Viber Lourmat, Marne la Vallee, France).

**Immunocytochemistry**

Thawed and non-cryopreserved HAMs (each from 3rd passage) were seeded and cultured onto a Laboratory-Teck chamber slide (8 wells, Nunc), and then fixed with 4% paraformaldehyde at 4°C for 2 h and rinsed with PBS. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After three times washes, they were treated with 3% hydrogen peroxidase for 15 min to quench endogenous peroxidase activity and then incubated in 2% bovine serum albumin (BSA) for 1 h at 4°C. Cells were then incubated with mouse monoclonal antibodies; anti-TRA-1-60 (1:50), anti-SSEA-3 (Chemicon, Temecula, CA, USA) and anti-SSEA-4 (both 1:100) (R&D System, Minneapolis, MN, USA) and an-1:100) (R&D System, Minneapolis, MN, USA) for 17 h at 4°C. Cells were then incubated with mouse monoclonal antibodies; anti-TRA-1-60 (1:50), anti-SSEA-3 (Chemicon, Temecula, CA, USA) and anti-SSEA-4 (both 1:100) (R&D System, Minneapolis, MN, USA) and an-1:100) (R&D System, Minneapolis, MN, USA) for 17 h at
Evaluation of the differentiation potential of HAMs

To identify osteogenic differentiation, thawed and non-cryopreserved HAMs (each from 3rd passage) were cultured in a chamber slide (Nunc) for subsequent staining, in 0.01 μM dexamethasone, 100 mM β-glycerol phosphate and 50 μM ascorbic acid-2-phosphate in 400 μl DMEM-LG supplemented with 10% FBS.

During the culture period, medium was changed once per week. After 1 week, calcium accumulation, an indicator of osteogenic induction, was assessed by Von Kossa staining (Sigma). Adipogenic differentiation was induced in 400 μl DMEM-LG with 10% FBS, 1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, 0.05 mg/l human insulin and 200 μM indomethacin in chamber slide (Nunc). After culture for 2 weeks, accumulation of lipid-rich vacuoles within cells, an indicator of adipocyte differentiation, was assessed by Oil-red O staining (Sigma). Chondrogenic differentiation was induced in chamber slide (Nunc) using 400 μL high glucose DMEM, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid-2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline, 10 ng/ml transforming growth factor-β3 and 50 μg/ml ITS-premix (insulin, transferrin and selenious acid at 6.25 μg/ml each, 1.35 mg/ml BSA and 5.35 mg/ml linoleic acid). After culture for 3 weeks, chondrogenesis was evaluated using Alcian Blue staining (Sigma). Each staining was performed after fixing with 4% paraformaldehyde at 4°C for 2 h and washing twice with PBS. The differentiated HAMs were also evaluated by RT–PCR analysis to check appropriate expression of each cell-specific marker; core binding factor α-1 (CBFA-1) for osteogenic differentiation, lipoprotein lipase (LPL) for adipogenic differentiation and decorin for chondrogenic differentiation. Each differentiated HAM cell pellet for RT–PCR was prepared after culture for 2 or 3 weeks in 75 cm² flask (Nunc, Rochester, MN, USA) in 10 ml differentiation media (for osteogenic, adipogenic or chondrogenic induction). We also changed media once per week. The primer sequences used to test for markers of osteogenic, adipogenic or chondrogenic induction were as follows; CBFA-1 (320 bp product, forward: 5’ CTCACTACCAACCTACTTGT 3’, reverse: 5’ TCAATATGGTGCGCAAAACAGATTGTC 3’), LPL (276 bp, forward: 5’ GAGATTCTCTGTAGTGCCAC 3’, reverse: 5’ CTGGAAATGAGACACTTTCTC 3’) and Decorin (300 bp, forward: 5’ CCTTTGGTGAGATTGAGACGG 3’, reverse: 5’ AAGATGTAAATCTCCGTAAGGG 3’) and annealing temperatures were 63°C, 59°C and 55°C, respectively. RT–PCRs were performed as described above.

Statistical analysis

Statistical analysis for comparison of post-thaw survival rate was performed using the χ² test. A P-value of <0.05 was considered to be statistically significant.

Results

Morphology of HAMs and viability of vitrified-thawed HAMs

In primary culture, the HAMs grew in a scattered manner and some cells formed a colony. The morphology of the HAMs appeared rather heterogeneous in primary culture as seen in Fig. 1; however, they progressively showed homogenous fibroblast-like features following subsequent subculture. The viability of HAMs after vitrification and thawing was 84.3 ± 3.2%. The thawed HAMs showed fibroblast-like morphology and growth patterns, similar to non-cryopreserved HAMs (Fig. 2).

Proliferation of vitrified-thawed HAMs

The thawed and non-cryopreserved HAMs were subcultured until Passage 14 (30 or 31 cell doublings) (Fig. 3). Until 13th passage, fibroblast-like morphology was observed consistently

Figure 1: Morphology and growth of HAMs.
(A) Primary (×100), (B) Passage 2 (14 days, ×100), (C) Passage 4 (28 days, ×100) and (D) Passage 4 (28 days, ×200). In primary culture, the cells grew up scattered and some cells formed colony. Following subsequent subculture, the cells changed into spindle-like fibroblast. Bar: A–C = 100 μm, D = 50 μm.

Figure 2: Morphology and growth of vitrified-thawed HAMs.
(A) 1 day after thawing (×100), (B) 7 days after thawing (×100) and (C) 7 days after thawing (×200). After vitrification and thawing, HAMs had similar morphology like fibroblast and were indistinguishable from non-cryopreserved HAMs. Bar: A, B = 100 μm. C = 50 μm.
both in thawed and non-cryopreserved HAMs. At 14th passage, cells in both cultures became large and flat, suggesting senescence. Average doubling time from Passage 3 to 14 in thawed and non-cryopreserved HAMs was 3.4 and 3.3 days, respectively. The cumulative cell number for thawed and non-cryopreserved HAMs was $13 \times 10^{14}$ and $4.6 \times 10^{14}$, respectively.

Expression of surface antigens by flow cytometry
The cell surface antigen profiles of thawed and non-cryopreserved HAMs at 3rd passage were analyzed. Thawed HAMs showed strong positive immunoreactivity for CD44, CD49d, CD59, CD90, CD105 and HLA-ABC, weak positive signals for HLA-G, and cells were negative for CD31, CD34, CD45, CD106, CD117 and HLA-DR (Fig. 4, Table III). These expression patterns were similar for thawed and non-cryopreserved HAMs.

Expression of ESC markers
The vitrified-thawed HAMs at 3rd and 7th passage consistently expressed CK18, fibroblast growth factor-5, GATA-4, neural cell adhesion molecule, Nestin, Oct-4, stem cell factor (SCF), HLA-ABC, Vimentin, bone morphogenetic protein 4 (BMP-4), hepatocyte nuclear factor 4α, Pax-6, alphafetoprotein AFP, Brachyury, BMP-2, as analyzed by RT–PCR (Fig. 5), and showed positive immunoreactivity for TRA-1-60, SSEA-3 and SSEA-4 (Fig. 6). These patterns of expression were consistent with non-cryopreserved HAMs at the same passages. HLA-DR was not detected in any of the cells studied.

Examination of differentiation potential
As shown in Fig. 7, mRNAs of CBFA-1 and LPL were detected by RT–PCR in thawed HAMs after osteogenic and adipogenic differentiation, respectively. These patterns of expression were consistent with non-cryopreserved HAMs. After chondrogenic differentiation, decorin mRNA was detected and the signal was stronger in differentiated thawed HAMs than in the undifferentiated state. This pattern of expression was also observed in non-cryopreserved HAMs.

After culture for osteogenic differentiation, vitrified-thawed HAMs showed mineral accumulation (stained by Von Kossa).
After culture for adipogenic differentiation, numerous neutral lipid droplets were observed in the cytoplasm of vitrified-thawed HAMs cells (stained by Oil Red O). After culture for chondrogenic differentiation, chondrogenic matrices were observed (stained by Alcian Blue) (Fig. 8).

Discussion

The objective of this study was to test the possibility that vitrification is a useful method for cryopreservation of HAMs. Slow freezing has been principally used for cryopreservation of various cells, including human embryos. We successfully vitrified HAMs, and confirmed the morphology, cell surface antigens, gene expression patterns and differentiation capacity of the post-vitrification HAMs compared with non-cryopreserved controls. These observations indicate that vitrification can be an efficient storage method for HAMs without losing the activity and usual properties of MSCs.

Table III. Comparison of phenotype of non-cryopreserved and post-vitrification HAMs assessed using a fluorescence-activated cell sorter.

<table>
<thead>
<tr>
<th>CD no.</th>
<th>Expression</th>
<th>Non-cryopreserved HAMs</th>
<th>Post-vitrification HAMs</th>
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<tr>
<td>CD90</td>
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<td>CD105</td>
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<td>HLA-ABC</td>
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<td>HLA-DR</td>
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<td>HLA-G</td>
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<td>CD117</td>
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+, strong positive; ±, weak positive; −, negative.

Figure 4: Immunophenotypic characteristics of thawed and non-cryopreserved HAMs.

Immunophenotypic characteristics were examined by flow cytometry. A similar pattern of expressions of surface antigens was observed in thawed and non-cryopreserved HAMs; strong positive for CD44, CD49d, CD59, CD90, CD105 and HLA-ABC, weak positive for HLA-G, and negative for CD31, CD34, CD45, CD106, CD117 and HLA-DR. +, strong positive; ±, weak positive; −, negative.

Vitrification of amnion-derived mesenchymal stem cells
Slow freezing is a method used to balance the damage caused by various factors, including ice crystal formation, fracture, toxic and osmotic damage, by decreasing temperature slowly with low concentration of cryoprotectant (Vajta et al., 2006). But it is difficult to eliminate injuries from intracellular ice formation completely, which is the main source of fracture and damage to the cytoplasm (Yousry et al., 2008); it is also time-consuming procedure and requires an expensive programmable freezer. In our preliminary study on HAMs, we observed that slow freezing resulted in lower survival rate compared with the vitrification (data not shown).

Vitrification is a process where glass-like solidification of a solution occurs without the formation of ice crystals inside living cells, by exposing to high concentration of cryoprotectant with higher cooling rate (Chian et al., 2004). This procedure is a simple method to circumvent the obstacles of slow freezing (Rall and Fahy, 1985) without the need for a freezing container to modulate the reduction of temperature in the deep freezer before storing in liquid nitrogen. Also, Kuleshova and Lopata (2002) ascertained advantages of vitrification when compared with previously applied cryopreservation techniques. These advantages include control of solute penetration and dehydration rate, prevention of prolonged temperature shock and damage from ice formation, and inexpensive equipment and running costs. In most vitrification protocols, very high concentrations of cryoprotectants have been used, thus rapidly dehydrating the cells or embryos (Rall et al., 1987). A tight correlation between the concentration of the cryoprotectant and cooling-warming rates during the vitrification process has been previously demonstrated (Chian et al., 2004). However, the vitrification method requires a high concentration of permeable and/or non-permeable cryoprotectants loaded onto cells before plunging into liquid nitrogen, exposing them to extreme osmotic stresses and chemical toxicity (Woods et al., 2004). Therefore, use of a cryoprotectant as a permeating agent is an essential component in all vitrification solutions (Kasai et al., 2004). EG is the most commonly used cryoprotectant for vitrification due to its low molecular weight and low toxicity (Chian et al., 2004). Moreover, it has been reported that cryoprotectant mixtures may have some advantage over solutions containing only one permeable cryoprotectant (Vajta et al., 1998). In addition,
additives with high molecular weights, such as sucrose, can significantly reduce the toxicity by decreasing the concentration of permeating agents required for vitrification solution. In this study, we established a two-step vitrification protocol for the HAMs using EG-based cryoprotectant, and our findings are in line with the previous reports showing the superiority of EG, which has been proven to be less toxic on murine and human embryos compared with permeating agents such as DMSO and propylene glycol (PROH) (Ali and Shelton, 1993; Chi et al., 2002). We chose EFS 40 containing 40% v/v EG for the vitrification solution, which is widely used for successful vitrification of mouse embryos (Kasai et al., 1990; Nagy et al., 2003), human blastocysts (Cho et al., 2002) and ESCs (Fujioka et al., 2004). Our preliminary data demonstrated that the combination of EG with either PROH or DMSO resulted in a very low survival rate of HAMs compared with EFS 40 alone (data not shown). In addition, we used Ficoll 70 as a macromolecule to promote permeation by cryoprotectants, which seems to have advantages of lower toxicity, higher solubility and lower viscosity (Kasai et al., 1990).

In addition, there have also been several reports of the successful vitrification of human oocytes, zygotes and embryos by their direct contact with liquid nitrogen (Park et al., 2000; Isachenko et al., 2003, 2004a). The achievable cooling rate by direct plunging into liquid nitrogen (Palasz and Mapletoft, 1996) was approximately from 2500°C/min to 5000°C/min. But liquid nitrogen used for storage can be a source of contamination by micro-organisms (Tedder et al., 1995; Bielanski et al., 2000), and filtration or ultratreatment of liquid nitrogen cannot guarantee the absence of contamination of biological material by virus (Isachenko et al., 2005b).

Figure 7: mRNA expressions of non-cryopreserved and vitrified-thawed HAMs after induction of differentiation. (A) Non-cryopreserved HAMs, undifferentiated; (B) non-cryopreserved HAMs, differentiated; (C) vitrified-thawed HAMs, undifferentiated; (D) vitrified-thawed HAMs, differentiated. The vitrified-thawed HAMs expressed lineage specific markers in the same manner as non-cryopreserved HAMs after induction of differentiation for adipogenesis, osteogenesis and chondrogenesis.

Figure 8: Differentiation potency of vitrified-thawed HAMs. (A) Before differentiation (×200), (B) after osteogenic induction (Von Kossa staining, ×200), (C) after adipogenic induction (Oil Red O staining, ×200) and (D) after chondrogenic induction (Alcian Blue staining, ×200). After induction of differentiation for 2–3 weeks in respective induction media, post-vitrification HAMs were stained positively for each staining, which indicate appropriate osteogenic, adipogenic and chondrogenic induction, respectively. Bar = 50 μm.
Some authors suggested the aseptic vitrification method, using open-pulled straw or reliable sealing, to avoid direct contact with liquid nitrogen (Kuleshova et al., 2000; Isachenko et al., 2005b), although this isolation method results in a decreased speed of cooling, which can decrease the efficacy of the vitrification protocol (Isachenko et al., 2005b). They also obtained the equally high survival rates of mouse 8-cell embryo after vitrification (Isachenko et al., 2005a) at cooling rates ranging from \( \sim 15^\circ\text{C/}2500^\circ\text{C/min} \) and similar post-thaw characteristics of human vitrified spermatozoa by fast \( (20\,000^\circ\text{C/min}) \) or relatively moderate \( (200^\circ\text{C/min}) \) cooling (Isachenko et al., 2004b). Rall (1987) reported cooling rates of \( 10^\circ\text{C/min} \) as sufficient for the vitrification of mouse embryos, whereas the elevated warming rate is the most important parameter for increasing viability of vitrified cells after thawing (Isachenko et al., 1998). We achieved vitrification of a bulk solution and HAMs using a cryovial and abrupt cooling at very high speed by plunging into liquid nitrogen without direct contact.

In our study, HAMs showed typical features of spindle-shaped cells and underwent successful passage over ten times, with 30 cell doublings. The cells uniformly expressed cell surface antigens (positive for CD44, CD59, CD90 and CD105, negative for CD31, CD34, CD45, CD106 and CD117), consistent with the antigen profile previously reported (Pittenger et al., 1999). HAMs are known to share many surface antigenic properties with other cells (Pittenger et al., 1999) as well as gene expression profiles; SCF (Majumdar et al., 2003), CK18 (Lee et al., 2004a,b) and Nestin (Vogel et al., 2003), which are similar to MSCs derived from bone marrow. However, HAMs were different from MSCs derived from bone marrow with regard to immunopositivity for TRA-1-60, and SSEA-3 and -4 (Xu et al., 2001). In addition, Yen et al. (2005) reported that placenta-derived stem cells share ESC markers including SSEA-4 and TRA-1-80. In our study, we could confirm the immunopositivity for TRA-1-60, SSEA-3 and -4 in HAMs before and after vitrification. This might indicate that HAMs originating from the amnion have different MSCs characteristics, thus suggesting that HAMs are a new source of pluripotent stem cells.

In addition, a similar expression profile for surface antigens were observed in HAMs before and after vitrification in our study and this finding suggests that vitrification may be a useful method for cryopreservation of HAMs. Post-vitrification HAMs still expressed multilineage specific genes such as Oct-4, and ESC markers (Tsai et al., 2004); CK 18, one of the endodermic lineage-specific genes (Wells et al., 1997); Nestin, a specific marker for neural stem cells (Lendahl et al., 1990); BMP-4, a marker for chondrogenesis (Kuroda et al., 2006); GATA-4, a cardiac specific marker (Grepin et al., 1997); AFP, a hepatocyte marker (Engelhardt et al., 1984); and HLA-ABC, similar to those of the non-cryopreserved HAMs. These results indicate that HAMs still have the potential for multilineage differentiation after vitrification. In addition, the findings from immunocytochemical staining of the post-vitrification HAMs were similar to the non-cryopreserved HAMs with respect to the positive immunoreactivity against TRA-1-60, SSEA-3 and -4.

When the HAMs were thawed after vitrification, they were recovered with acceptable viability (84.3 ± 3.2%), even after relatively short duration (2-week storage). After thawing, the HAMs retained their typical morphology, resembling the fibroblast immunophenotype and their proliferation/differentiation potential was similar to their non-cryopreserved counterparts. Moreover, the vitrified HAMs showed a prolonged growth capacity for >10 passages without any changes in their morphology, proliferation potential or immunophenotype. In addition, post-vitrification HAMs expressed the mesenchymal tissue-specific genes such as CBFA-1 (a marker for osteoblast), LPL (a marker for adipocyte) and Decorin (a marker for chondrocyte) after induction of differentiation. These findings indicate that HAMs consistently maintain the potential to differentiate into mesodermal tissues after vitrification. Although further studies on prolonged duration of cryopreservation and consideration of other cryoprotectant solutions for vitrification are needed, we have shown that HAMs which are vitrified using sequential exposure to EG 20 and EFS 40 could maintain the characteristics of MSCs and differentiation potential into mesenchymal derivatives, including osteoblast, adipocyte and chondrocyte cell types.

In conclusion, the results of our study indicate that vitrification is an effective and highly conservative cryopreservation method for HAMs. We believe that vitrification might be useful for banking HAMs for future research and clinical applications.

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**References**


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