Preserved Proliferative Capacity and Multipotency of Human Adipose-Derived Stem Cells after Long-Term Cryopreservation

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Background: Human adipose-derived stem (stromal) cells are promising as a regenerative therapy tool for defective tissues of mesenchymal lineage, including fat, bone, and cartilage, and blood vessels. In potential future clinical applications, adipose-derived stem cell cryopreservation could be an indispensable fundamental technology, as has occurred in other fields involving cell-based therapies using hematopoietic stem cells and umbilical cord blood cells.
Methods: The authors examined the proliferative capacity and multipotency of human adipose-derived stem cells isolated from lipoaspirates of 18 patients in total before and after a 6-month cryopreservation following their defined protocol. Proliferative capacity was quantified by measuring doubling time in cell culture, and multipotency was examined with differentiation assays for chondrogenic, osteogenic, and adipogenic lineages. In addition, expression profiles of cell surface markers were determined by flow cytometry and compared between fresh and cryopreserved adipose-derived stem cells.
Results: Cryopreserved adipose-derived stem cells fully retained the potential for differentiation into adipocytes, osteoblasts, and chondrocytes and for proliferative capacity. Flow cytometric analyses revealed that surface marker expression profiles remained constant before and after storage.
Conclusions: Adipose-derived stem cells can be cryopreserved at least for up to 6 months under the present protocol without any loss of proliferative or differentiation potential. These results ensure the availability of autologous banked adipose-derived stem cells for clinical applications in the future. (*Plast. Reconstr. Surg.* 121: 401, 2008.)

Human adipose-derived stem (stromal) cells[1,2] are promising as a tool of regenerative therapies for tissue defects of mesenchymal lineage, including fat,[3] skeletal and cardiac muscles,[4,5] bone,[6–10] cartilage,[11–13] and blood vessels.[14–16] They have already been clinically used for enhancing regeneration of fat,[17,18] vasculature,[19] and bone.[20] In addition, they are reported to be differentiated into other cell types deriving from different germ layers such as neural cells[21] and hepatocytes.[22] Several studies have demonstrated that human adipose-derived stem cells can differentiate into vascular endothelial cells[14–16] and augment tissue neovascularization in a paracrine manner in an ischemic environment.[23]

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Thus, it is currently thought that adipose-derived stem cells contribute to bioengineering and transplantation of tissues not only by acting as tissue-specific precursor cells but also through reinforcing vascularization of regenerating tissue, which underlines the importance of adipose-derived stem cells in general applications of tissue engineering.

As emphasized for other types of stem/progenitor cells,[24] harvested autologous adipose-derived stem cells will most probably be preserved for multiple clinical applications in the future. Cryopreservation confers many advantages for practitioners engaged in cell-based therapies, including transportability of stem cells, pooling of cells to reach a therapeutic dose, and time for the completion of safety and quality control testing.[24]

Disclosure: *The authors declare that they have no competing financial interests.*

Therefore, cryopreservation of adipose-derived stem cells should be intensively investigated for optimization as an indispensable fundamental technology. As the first step to achieving this aim, we examined the proliferative capacity and multipotency of human adipose-derived stem cells before and after long-term (6 months) cryopreservation under our defined protocol.

MATERIALS AND METHODS

Human Tissue Samples

Adipose-derived stem cells were harvested at surgery from lipoaspirates of 18 female patients, aged 20 to 51 years (mean, 32.3 years). Donor sites were the abdomen and/or thighs. Before liposuction, patients received external ultrasound pretreatment at the areas to be suctioned with External Ultrasonic Assist (Silberg, Santa Rosa, Calif.) after epidural anesthesia. After saline solution supplemented with 0.1% lidocaine and 0.001% epinephrine was infiltrated into the adipose compartment, liposuction was performed manually with a hollow blunt-tipped cannula (diameter, 2 to 4 mm).

Doubling times were determined for adipose-derived stem cells from six patients before cryopreservation and from another five patients after 6 months of cryopreservation; therefore, the cell lines for the assays before and after cryopreservation were not identical. Similarly, five lines of adipose-derived stem cells were assayed for determination of chondrogenic potential with the micromass culture system (see below) before cryopreservation, and another six lines were examined after cryopreservation. For osteogenic and adipogenic differentiation assays, six lines of adipose-derived stem cells were assayed before cryopreservation, whereas a distinct five lines were examined after cryopreservation. Of these six and five lines, one in the before and after groups was from the same patient, and the others were all from separate patients. As for flow cytometry, we analyzed the expression of a set of surface markers (see below) of five lines of adipose-derived stem cells before cryopreservation and six lines after cryopreservation; one patient both before and after cryopreservation; four other patients before cryopreservation, and five other patients after cryopreservation.

Ideally, cell lines subjected to each assay before and after cryopreservation should be identical, derived from the same donor patient. In determining the experimental design, however, we found that adipose-derived stem cell yield from a single patient was not large enough to perform all the intended assays using both fresh and frozen cells, unless a long-term cell culture was conducted before carrying out the assays. Therefore, in some assays in this study, different cell lines were used.

Cell Isolation and Culture

All protocols used for these procedures have been described previously.[25] Briefly, the suctioned fat was digested with 0.075% collagenase in phosphate-buffered saline for 30 minutes on a shaker at 37°C. Mature adipocytes and connective tissues were separated from pellets by centrifugation (800 *g* for 10 minutes). Pellets were resuspended in erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM ethylenediaminetetraacetic aced) and incubated for 5 minutes at room temperature. The pellets were resuspended and passed through a 100-μm mesh filter (Millipore, Billerica, Mass.), yielding stromal vascular fraction containing adipose-derived stem cells. Freshly isolated cells were plated in medium at a density of 5×10^6 nucleated cells per 100-mm gelatin-coated dish, and cultured at 37°C, 5% carbon dioxide, in humid air. The culture medium was M-199 containing 10% fetal bovine serum, 100 IU of penicillin, 100 mg/ml streptomycin, 5 μg/ml heparin, and 2 mg/ml acidic fibroblast growth factor. Primary cells were cultured for 7 days and were defined as passage 0. The medium was replaced every 3 days, and cells were subcultured every week. After primary culture for 7 days, attached cells were subcultured by trypsinization and plated in the same medium at a density of 2000 cells/cm^2.

Cryopreservation of Cells

Adipose-derived stem cells were counted and resuspended in cryoprotective medium, Cell Banker 1 (Wako Chemicals Co., Ltd., Osaka, Japan) at a density of 10^6 cells/ml. The manufacturer does not disclose its ingredients but states that it contains fetal bovine serum. They were then frozen under temperature control using a programmed freezer (PROFREEZE TNP-87S2Q; Nihon Freezer Co., Ltd., Tokyo, Japan) under a protocol of 4°C for 5 minutes, then decreasing −1°C per minute until the temperature reached −50°C, followed by a decrease of −5°C per minute until −80°C was reached. Samples were then transferred to a liquid nitrogen tank (Isothermal Vapor Storage System V-1500 with Series 2300 Auto-fill/Monitor System; Custom Biogenic Systems, Shelby Township, Mich.) for long-term storage at −196°C.

Cells before cryopreservation were subjected to each assay at passage 1 or 2, whereas those after 6 months of cryopreservation underwent the assays at passage 2 or 3. Cells that had been cryopreserved for 6 months were rapidly thawed in a warm water bath set at 37°C, spun to remove the cryoprotective agent, and seeded onto appropriate culture plates for each experiment.

Measurement of Doubling Time

Fresh or cryopreserved adipose-derived stem cells were seeded onto six-well plates at a density of 1×10^5 cells per well and allowed to grow until they reached logarithmic growth phase. It usually took 3 days for cells to start proliferating logarithmically, but sometimes (especially at passage 0) it took 5 to 6 days. The cells were then sequentially trypsinized at intervals of 48 hours and counted with a cell counter (NucleoCounter; Chemo Metec, Allerød, Denmark). Doubling time was calculated according to the following formula: doubling time = 48 hours/$\log_2(N_2/N_1)$, where N_1 is the first cell count and N_2 is the cell count 48 hours later.

Induced Differentiation of Cultured Adipose-Derived Stem Cells

The protocol for chondrogenic differentiation (micromass culture system) has been described previously.[26] Dulbecco's Modified Eagle Medium and 10% fetal bovine serum was used for the culture medium as negative control. Quantification was performed by measuring the micromass sizes on microphotographs with a scale using image analysis software (Scion v.3.53; Scion Corp., Frederick, Md.). Safranin O staining was performed for qualitative analysis.

To test osteogenic differentiation, calcium deposition was evaluated based on the orthocresolphthalein complexone method with the Calcium C-Test Wako Kit (Wako Chemicals) according to the manufacturer's instructions. After 5×10^4 cells/60-mm dish were cultured in M-199 containing 10% fetal bovine serum for 6 days, the medium was replaced with osteogenic differentiation medium (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 50 μM ascorbate-2-phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate) or control medium (Dulbecco's Modi-

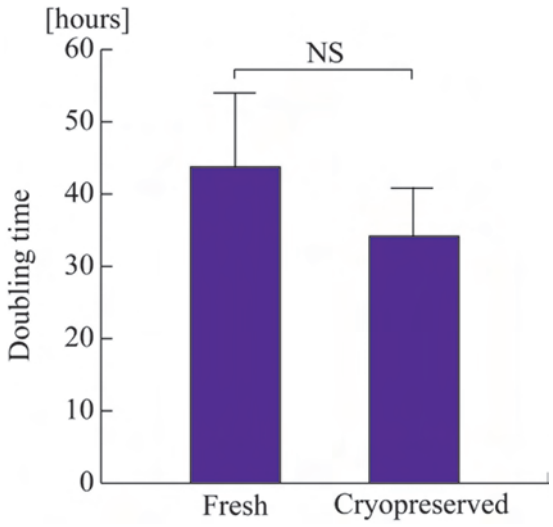

Fig. 1. Doubling time of fresh and cryopreserved human adipose-derived stem cells. Six lines of human adipose-derived stem cells before the freezing and thawing process (*Fresh*) and five lines harvested after 6 months of cryopreservation (*Cryopreserved*) were assayed for cell proliferation rate, and their doubling times were determined. *NS*, no significant difference. Values are mean ± SD.

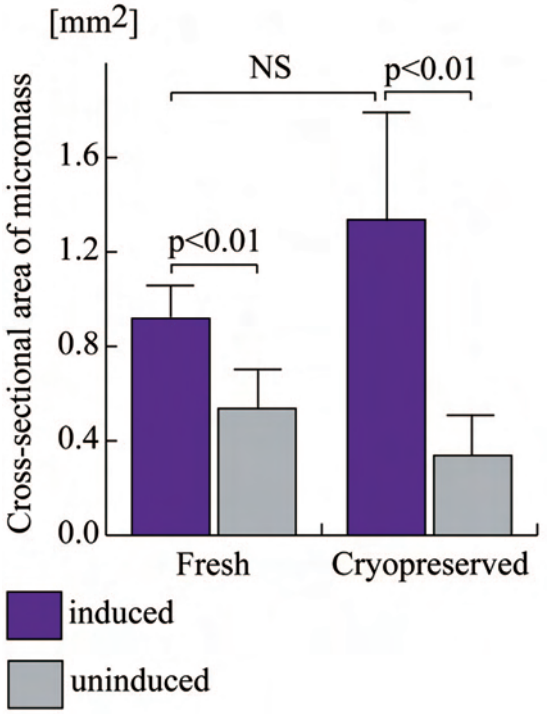

Fig. 2. Chondrogenic potentials of fresh and cryopreserved human adipose-derived stem cells. Five lines of human adipose-derived stem cells before the freezing and thawing process (*Fresh*) and six lines harvested after being cryopreserved for 6 months (*Cryopreserved*) were subjected to micromass culture in the defined chondrogenic differentiation medium (*purple columns*) or control medium (*gray columns*) to assess chondrogenic potential. Micromass sizes were determined using image analysis software on microphotographs with scale as an area (in millimeters squared). *NS*, no significant difference.

fied Eagle Medium with 10% fetal bovine serum). Cells were then assayed after 1, 2, 3, and 4 weeks of differentiation culture.

For assessment of adipogenic differentiation, cells were seeded onto 96-well plates at a density of 1×10^4 cells/well. After 4 days of culture, medium was replaced with adipogenic differentiation medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 0.5 mM isobutyl methylxanthine, 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin) or control medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum). Cells were further cultured for 1, 2, 3, or 4 weeks and stained with 5 μl/well of AdipoRed-Lipid Assay Reagent (Cambrex Corp., East Rutherford, N.J.). After 10 minutes of incubation, the fluorescence with excitation at 485 nm and emission at 535 nm was measured with a microplate spectrophotometer reader Model 680 (BioRad, Hercules, Calif.).

Flow Cytometry

Flow cytometric analyses were performed as described elsewhere.[25] The following monoclonal antibodies conjugated to fluorochromes were used: anti-CD29(β1-integrin)-PE, CD34-PE, CD44-PE, CD49d-PE, CD90(Thy-1)-PE, CD117-PE, and Tie-2-PE (BD Biosciences, San Diego, Calif.); CD71-PE and CD105-PE (Serotec, Oxford, United Kingdom); and CD144-PE (Beckman Coulter, Fullerton, Calif.).

Statistical Analysis

Measured values were expressed as means ± SD; *t* tests were used to compare the population means between groups when their population variances were assumed to be equal, whereas Welch's *t* test was used when variances were unequal. F tests were used to assess variances of the two groups being compared.

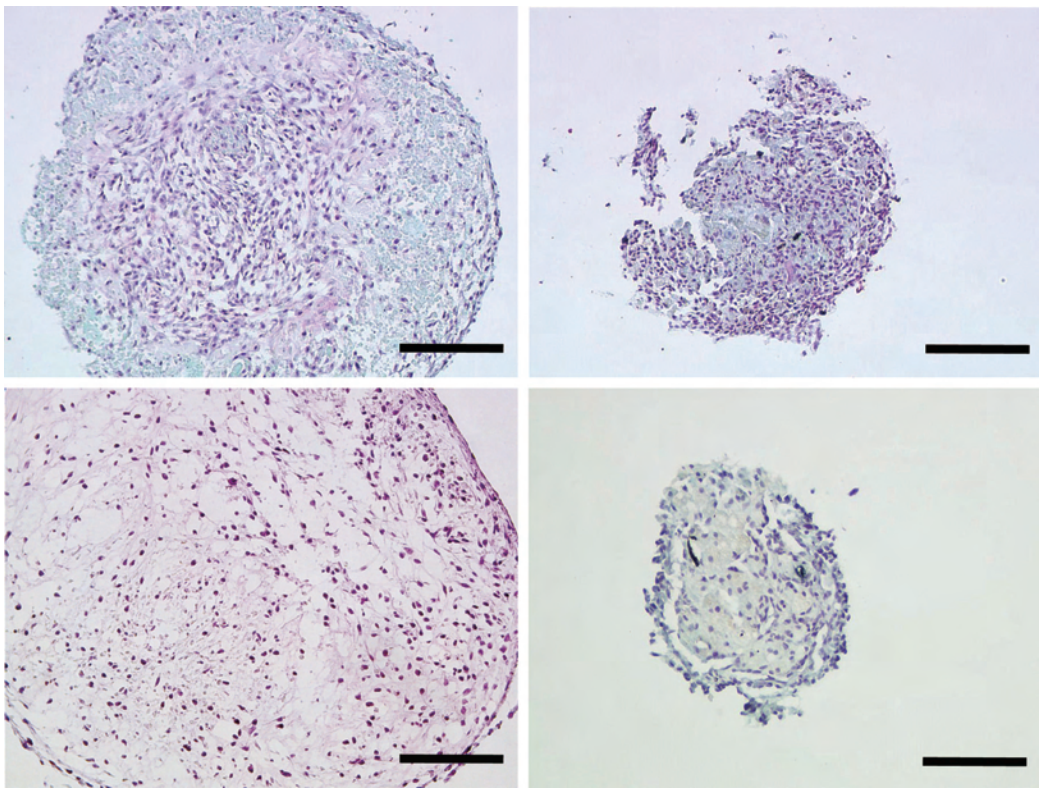

Fig. 3. Chondrogenic potentials of fresh and cryopreserved human adipose-derived stem cells. Photomicrographs of representative sections of micromass. Paraffin-embedded sections were stained with safranin-O and counterstained with hematoxylin: fresh adipose-derived stem cells in chondrogenic medium (*above*, *left*), fresh adipose-derived stem cells in control medium (*above*, *right*), cryopreserved adipose-derived stem cells in chondrogenic medium (*below*, *left*), and cryopreserved adipose-derived stem cells in control medium (*below*, *right*). Bar = 250 μm.

RESULTS

Proliferative Capacity of Fresh and Cryopreserved Adipose-Derived Stem Cells

Doubling times were comparable between fresh and cryopreserved adipose-derived stem cells (Fig. 1), indicating that cryopreservation did not affect the proliferative capacity of the adipose-derived stem cells.

Chondrogenic Potential of Fresh and Cryopreserved Adipose-Derived Stem Cells

The chondrogenic potential of nine lines of adipose-derived stem cells, consisting of four fresh cell lines and five cryopreserved lines, was assayed with the micromass culture protocol, followed by morphometry for quantification (Fig. 2) and safranin O staining for qualitative analysis (Fig. 3). Sizes of micromass, determined by measuring the areas of the masses using image analysis software, could be assumed to reflect the capability of chondrogenic matrix production. Judged based on the micromass sizes, chondrogenic potentials of fresh and cryopreserved adipose-derived stem cells were not significantly different. The SD of micromass sizes of cryopreserved adipose-derived stem cells cultured in chondrogenic medium was larger than that of fresh cells, suggesting variable but definite effects of long-term cryopreservation on their chondrogenic potential. Microphotographs of safranin O–stained sections of micromass showed higher production of proteoglycan matrix in cells with chondrogenic induction than in controls in both fresh and cryopreserved adipose-derived stem cells (Fig. 3). As with the observation from the quantification assay above, micromasses showed comparable degrees of chondrogenic matrix production in both groups.

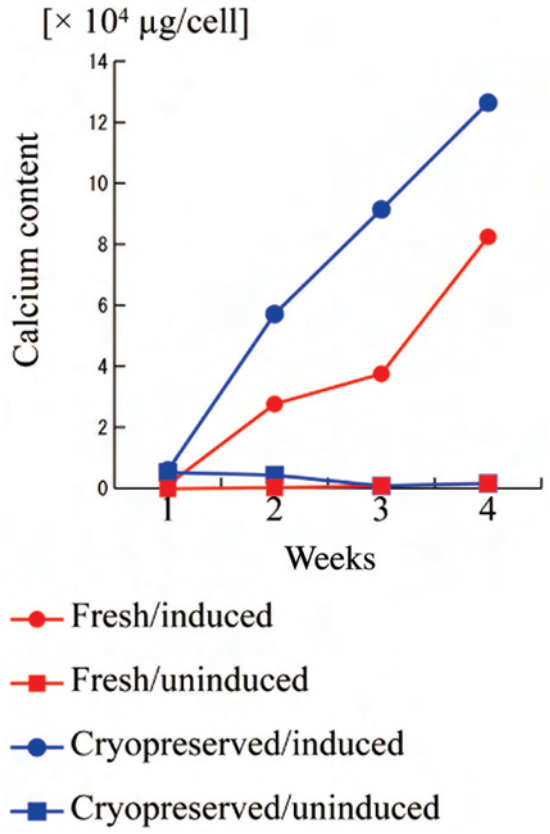

Fig. 4. Osteogenic differentiation potentials of human adipose-derived stem cells before and after long-term cryopreservation. Fresh (*red lines*) and cryopreserved (*blue lines*) adipose-derived stem cells derived from a single patient were assayed for calcium deposition after 1, 2, 3, and 4 weeks of cell culture with osteogenic differentiation medium (*filled circles*) or control medium (*filled rectangles*). Fresh adipose-derived stem cells with (*Fresh/induced*) or without (*Fresh/uninduced*) osteogenic induction are indicated with *red circles* and *red rectangles*, respectively; cryopreserved adipose-derived stem cells with (*Cryopreserved/induced*) or without (*Cryopreserved/induced*) osteogenic induction are indicated with *blue circles* and *blue rectangles*, respectively.

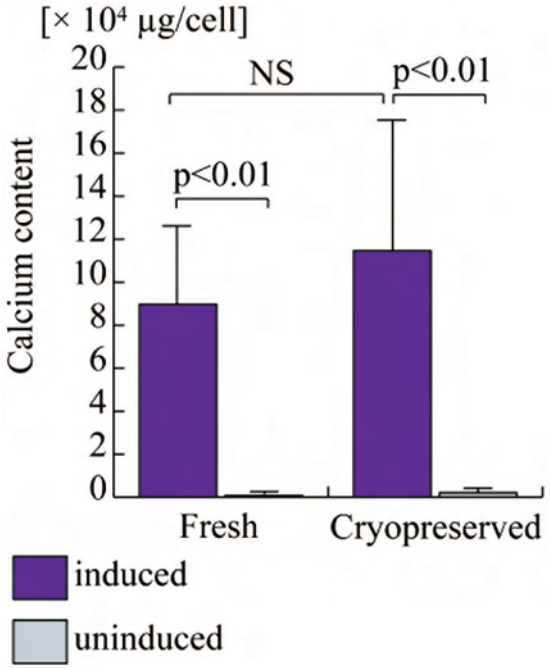

Fig. 5. Osteogenic differentiation potentials of human adipose-derived stem cells before and after long-term cryopreservation. Six lines of human adipose-derived stem cells before the freezing and thawing process (*Fresh*) and five lines after cryopreservation for 6 months (*Cryopreserved*) were assayed for calcium deposition to assess osteogenic potential with (*purple columns*) or without (*gray columns*) osteogenic induction. *NS*, no significant difference.

Osteogenic and Adipogenic Potential of Adipose-Derived Stem Cells before and after Cryopreservation

Fresh and cryopreserved adipose-derived stem cells were assayed for osteogenic (Figs. 4 and 5) and adipogenic (Figs. 6 and 7) potential. Osteogenic potential, determined as calcium deposition in intracellular and extracellular spaces, was comparable between fresh and cryopreserved adipose-derived stem cells; in fact, cryopreserved cells surpassed fresh cells. Adipogenic potential, determined based on spectrophotometric absorbance using AdipoRed staining, showed no apparent difference between fresh and cryopreserved adipose-derived stem cells. These results indicate that long-term cryopreservation up to 6 months does not affect the osteogenic and adipogenic potential of adipose-derived stem cells.

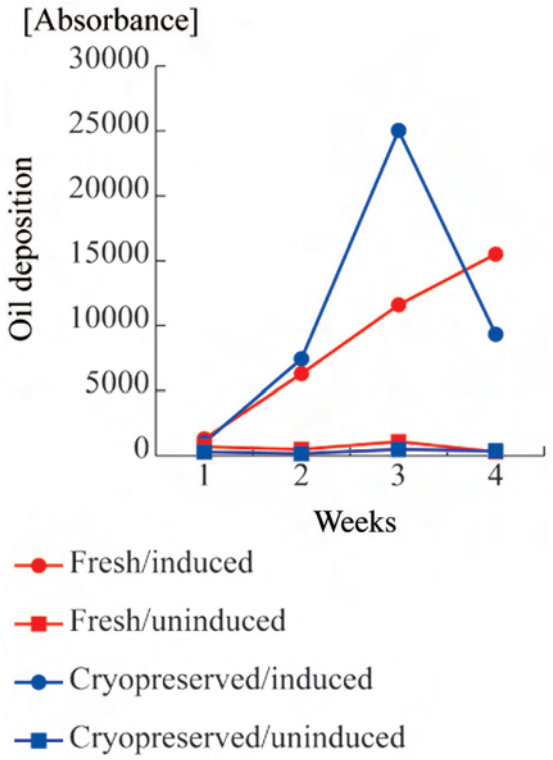

Fig. 6. Adipogenic differentiation potentials of human adipose-derived stem cells before and after long-term cryopreservation. Fresh and cryopreserved adipose-derived stem cells derived from the same patient were assayed for oil deposition as a quantification of adipogenic differentiation at the indicated time point. Fresh adipose-derived stem cells with (*Fresh/induced*) or without (*Fresh/uninduced*) osteogenic induction are indicated with *red circles* and *red rectangles*, respectively; cryopreserved adipose-derived stem cells with (*Cryopreserved/induced*) or without (*Cryopreserved/induced*) osteogenic induction are indicated with *blue circles* and *blue rectangles*, respectively.

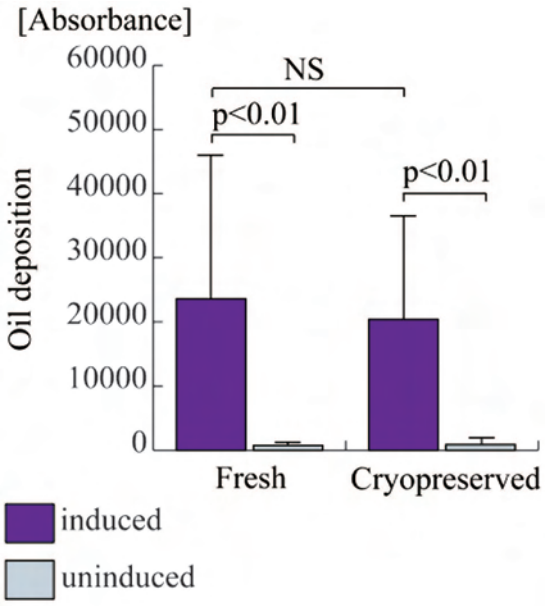

Fig. 7. Adipogenic differentiation potentials of human adipose-derived stem cells before and after long-term cryopreservation. Six lines of human adipose-derived stem cells before the freezing and thawing process (*Fresh*) and five lines after cryopreservation for 6 months (*Cryopreserved*) were assayed for lipid deposition to assess adipogenic potential with (*purple columns*) or without (*gray columns*) adipogenic induction. *NS*, no significant difference.

Flow Cytometric Analysis of Cell Surface Markers on Adipose-Derived Stem Cells

Cell surface marker expression is closely related to cell lineage and biological properties, including multipotentiality. Expression profiles of cell surface markers, and their change over passages, were analyzed with flow cytometry. Adipose-derived stem cells before and after 6 months of cryopreservation showed similar expression patterns of the cell surface markers selected for evaluation at all passage numbers examined (i.e., passages 0, 1, 2, 3, and 7). Also, sequential changes in the expression patterns were quite similar between the two groups of adipose-derived stem cells. Only CD34 declined with increased passage number; the other markers generally remained constant in both groups (Fig. 8 and Table 1). Therefore, as far as those analyzed marker subsets are concerned, the expression profile of cell surface markers of adipose-derived stem cells underwent little change through cryopreservation.

DISCUSSION

In this study, we demonstrated that human adipose-derived stem cells preserve their proliferative capacity, mesenchymal multipotency (chon-

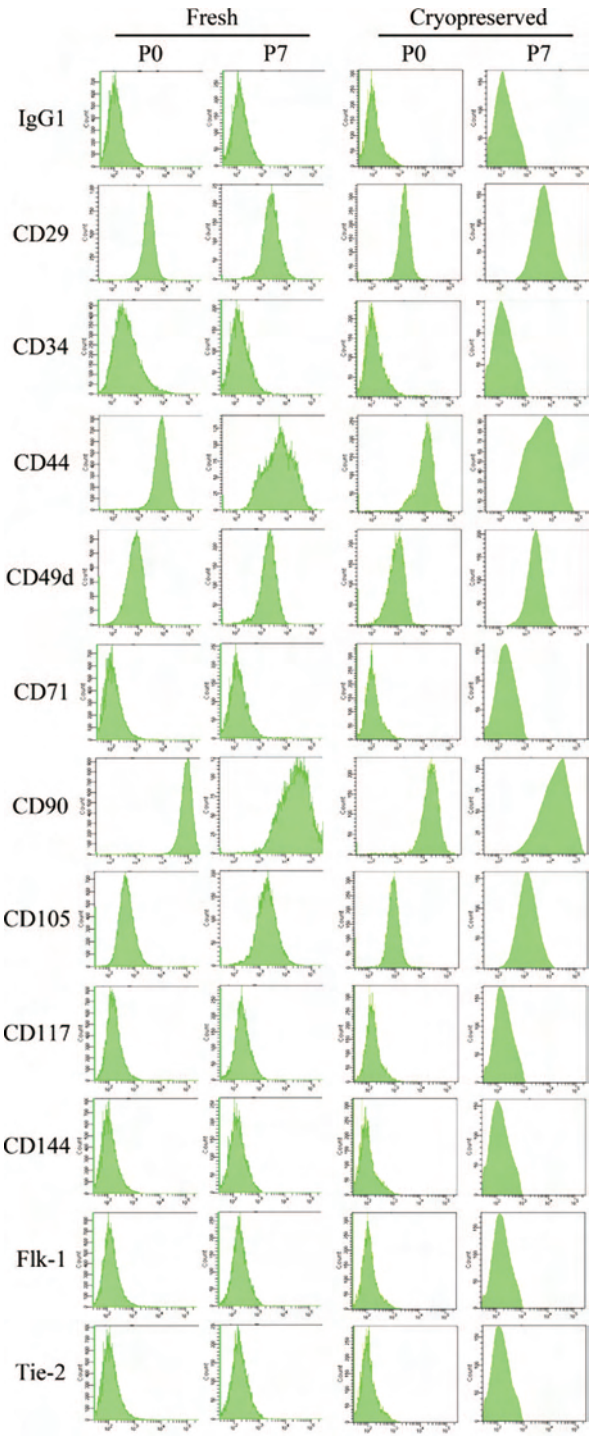

Fig. 8. Representative data of cell surface marker expression in fresh and cryopreserved adipose-derived stem cells (passages 0 and 7) obtained from a single patient. Adipose-derived stem cells before (*Fresh*) and after 6 months of cryopreservation (*Cryopreserved*) were analyzed at passages 0 and 7 with flow cytometry for expression of a selected set of cell surface markers. The representative data from fresh and cryopreserved adipose-derived stem cells (passages 0 and 7) obtained from a single patient are shown. *IgG1* indicates the negative control using a nonspecific mouse immunoglobulin G1 species as an antibody to determine background fluorescence.

drogenic, osteogenic, and adipogenic lineages), and surface marker expression profiles after 6 months of cryopreservation. The only difference we detected between fresh and cryopreserved adipose-derived stem cells in this study was the larger variability in chondrogenic differentiation potentials of cryopreserved adipose-derived stem cells. Although the results do not guarantee the validity of preservation longer than 6 months for adipose-derived stem cells, it is clinically of great importance that at least a single cycle of freezing, thawing, and storage at −196°C up to 6 months does not affect the biological characteristics of human adipose-derived stem cells. Overall, the results of the present study would encourage practicing application of cryopreserved adipose-derived stem cells for potential cell-based therapies.

Human fat is readily obtained from liposuction and frequently used as a filler material for soft-tissue augmentation.[18,27,28] Because multiple lipoinjection is frequently necessary for maximizing cosmetic results, some cosmetic surgeons are strongly inclined to store and repeatedly use the harvested fat. Thus, cryopreserved human fat tissue has been investigated,[29–31] but there have been few reports about the effects of cryopreservation on adipose-derived stem cells.[32–35] The three reports by Thirumala et al.[32–34] studying human adipose-derived stem cells as isolated cells focused mainly on the physical effect of freezing on cell membrane integrity; another report[35] focused on adipose-derived stem cell yield from cryopreserved fat. To our knowledge, before the present study, there have been no reports investigating the potential of cryopreserved human adipose-derived stem cells as a tool for cell therapy. One of the advantages of adipose-derived stem cells compared with other adult stem cells is a large cell yield; a large volume of adipose tissue can be obtained easily, with minimal morbidity. Accordingly, adipose-derived stem cells can be theoretically clinically used without cell expansion, but if adipose-derived stem cells can be cultured and stored for clinical use, the value of adipose-derived stem cells in cell-based therapy would be greatly enhanced. The potential of clinical application of adipose-derived stem cells in plastic surgery has been reviewed elsewhere.[18,36]

The effects of cryopreservation have been investigated using other types of stem cells. Cryopreserved cord blood stem cells retain clonogenic capacity and immunophenotypic composition comparable to fresh cells,[37] whereas cryopreservation does not markedly affect the rate of proliferation and multipotency of murine neural pre-

Table 1. Expression of Cell Surface Markers in Fresh and Cryopreserved Human Adipose-Derived Stem Cells*

	P0	P1	P2	P3	P7	Mean
CD29						
β1-Integrin						
Fresh	98.6 ± 1.0	99.1 ± 0.3	99.3 ± 0.2	99.4 ± 0.3	99.5 ± 0.4	99.2 ± 0.6
Cryo.	91.1 ± 11.3	84.6 ± 35.2	98.3 ± 1.7	99.2 ± 0.5	90.7 ± 13.8	92.8 ± 17.3
CD34						
Fresh	52.6 ± 30.3	26.7 ± 26.5	8.9 ± 14.9	25.7 ± 20.5	9.9 ± 4.2	25.4 ± 25.4
Cryo.	45.1 ± 32.8	31.8 ± 18.7	10.5 ± 7.6	15.3 ± 11.8	10.5 ± 14.2	22.6 ± 22.6
CD44						
Pgp-1						
Fresh	98.2 ± 0.9	99.2 ± 0.3	99.6 ± 0.2	99.6 ± 0.2	99.7 ± 0.2	99.3 ± 0.7
Cryo.	96.5 ± 4.9	99.2 ± 0.8	98.6 ± 1.8	99.7 ± 0.2	97.1 ± 3.8	98.2 ± 3.0
CD49d						
α4-Integrin						
Fresh	86.1 ± 2.8	92.4 ± 5.5	97.9 ± 1.8	98.3 ± 1.0	98.9 ± 0.8	94.6 ± 5.8
Cryo.	81.0 ± 14.6	95.5 ± 5.1	96.0 ± 3.0	98.2 ± 1.2	86.8 ± 17.6	91.5 ± 11.8
CD71						
Transferrin receptor						
Fresh	2.7 ± 3.2	11.7 ± 23.6	2.3 ± 2.1	4.7 ± 7.6	0.9 ± 1.0	4.6 ± 11.2
Cryo.	1.8 ± 2.6	6.1 ± 9.6	3.5 ± 4.8	5.6 ± 5.4	4.4 ± 5.8	4.3 ± 5.8
CD90						
Thy-1						
Fresh	98.8 ± 1.3	99.7 ± 0.2	99.8 ± 0.2	99.8 ± 0.2	99.9 ± 0.0	99.6 ± 0.7
Cryo.	99.1 ± 0.7	99.7 ± 0.3	99.8 ± 0.2	99.8 ± 0.2	99.3 ± 0.7	99.5 ± 0.5
CD105						
Endogrin						
Fresh	89.4 ± 11.1	90.7 ± 9.6	97.6 ± 2.1	92.4 ± 14.2	97.6 ± 1.0	93.4 ± 9.2
Cryo.	88.3 ± 20.7	97.8 ± 1.9	93.1 ± 13.6	96.9 ± 3.2	91.4 ± 9.9	93.5 ± 11.7
CD117						
c-kit						
Fresh	13.6 ± 17.2	19.6 ± 18.7	13.4 ± 10.1	11.4 ± 15.5	24.3 ± 26.0	16.6 ± 17.6
Cryo.	4.6 ± 6.2	10.1 ± 9.2	16.2 ± 12.8	16.0 ± 16.3	14.4 ± 20.5	12.3 ± 13.7
CD144						
VE-cadherin						
Fresh	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.5 ± 1.0	0.5 ± 0.9	0.3 ± 0.6
Cryo.	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	0.7 ± 1.5	0.2 ± 0.3	0.3 ± 0.7
Flk-1						
VEGFR-2 (KDR)						
Fresh	5.4 ± 2.4	7.2 ± 5.3	5.7 ± 3.3	5.6 ± 6.2	10.9 ± 11.5	7.0 ± 6.4
Cryo.	1.8 ± 1.2	4.0 ± 3.6	4.3 ± 2.8	6.4 ± 6.5	7.6 ± 7.5	4.8 ± 5.0
Tie-2						
Fresh	12.9 ± 27.5	6.0 ± 12.3	6.7 ± 11.9	13.7 ± 26.4	21.3 ± 40.3	12.0 ± 23.7
Cryo.	8.1 ± 19.1	7.2 ± 14.6	6.5 ± 8.9	18.1 ± 29.2	16.0 ± 33.2	11.2 ± 21.8

*Adipose-derived stem cells before (Fresh) and after 6 months of cryopreservation (Cryo.) were analyzed at passages 0, 1, 2, 3, and 7 (P0, P1, P2, P3, and P7) using flow cytometry for expression of a selected set of cell surface markers. Percentages of positive cells for each surface marker are shown. Data were collected from five fresh lines and six cryopreserved lines; one line from the fresh and one from the cryopreserved were derived from the same patient; the others were derived from different patients. Values are mean ± SD.

cursor cells.[38] These results and our present results indicate that the freeze-and-thaw process and long-term storage in liquid nitrogen do not critically alter the phenotype of stem/precursor cells. A recently emerging concept of gene activity is that gene expression status is regulated by epigenetic modifications to the genomic DNA and its related histone proteins, in the form of DNA methylation and covalent modifications of histone tails.[39,40] These epigenetic modifications are covalent and therefore are presumably unaffected by a cryopreservation process, resulting in no essential changes in cell gene expression profile and phenotype. Although cryopreservation could cause some damage to the structural and functional proteins of the cells and reduce their viability, immunophenotypic changes in the cells might scarcely occur.

The only positive finding in this study is that the SD of the chondrogenic potential of cryopreserved adipose-derived stem cells cultured in chondrogenic medium, based on micromass sizes, was much larger than that of fresh cells. One explanation for this finding is that individual differences were simply greater in the cryopreserved adipose-derived stem cell group than in the fresh adipose-derived stem cell group. (Note that none of the cell lines was identical between the two groups.) Another explanation is that cryopreservation affected the chondrogenic differentiation

potentials of some of the adipose-derived stem cell lines both positively and negatively.

With advances in tissue engineering and regenerative medicine, use of adult stem cells may be a solid therapeutic option in the near future, and our data can contribute to developing protocols for regenerative therapies with cryopreserved adipose-derived stem cells. As for other types of adult stem cells (e.g., hematopoietic stem cells and umbilical cord blood cells), standard cryopreservation protocols have been established, and the safety of long-term storage has been demonstrated.[41,42] Based on the findings regarding these antecedent stem cells, it is likely that we can reasonably extend the period of cryopreservation of adipose-derived stem cells, although the safety of doing so should be examined further.

Before the present study, we had not fully optimized the protocol for cryopreservation of adipose-derived stem cells. Among the various parameters relating to cryopreservation of cells, cryoprotective agents could significantly affect the survival rate and proliferation potential of the frozen cells after thawing.[43–46] We used the cryoprotective medium Cellbanker 1 because it is widely used in Japan and our previous pilot study suggested that adipose-derived stem cell viability after 1 month of cryopreservation was better when stored with Cellbanker 1 compared with other well-known cryopreservation agents such as dimethyl sulfoxide, trehalose, dextran, methylcellulose, and their combinations. The cryoprotective medium used in this study contains fetal bovine serum as a supplement, which contributes to prevention of viability loss of adipose-derived stem cells during freezing and thawing. Given the point of view that animal-derived biological ingredients should be completely eliminated from the process for cells to be used clinically in cell therapy, and to pursue conditions for better survival of the cryopreserved cells, the cryoprotective agent may have to be further optimized for adipose-derived stem cell storage. The effect of cooling rate on cell freezing is another significant factor affecting cell viability after cryopreservation and should be determined for each cell species respectively, depending on physiochemical properties, especially membrane permeability as a major parameter.[24] We have adopted the standard protocol of cooling rate for many cultured cells, but the specific protocol for adipose-derived stem cells should again be worked out for better survival rate, proliferation rate, and differentiation potentials after cryopreservation.

CONCLUSIONS

A primary purpose of the present study was not to define the best protocol for the cryopreservation of adipose-derived stem cells by comparing various protocols or to assess the significance of each parameter relating to the cryopreservation of adipose-derived stem cells but to determine the effects of cryopreservation on the phenotypes, proliferative potentials, and differentiation potentials of adipose-derived stem cells, which indicate their potentials for cell-based therapy. Thus, our results might well be regarded as having been obtained under specific conditions of cryopreservation that still remain to be optimized. Even under such storage conditions, however, we showed that adipose-derived stem cells still retain their proliferative and differentiation potentials after long-term cryopreservation.

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