

Rapid Isolation of Adipose Tissue-Derived Stem Cells by the Storage of Lipoaspirates

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Purpose: This study examined a rapid isolation method decreasing the time and cost of the clinical application of adipose tissue-derived stem cells (ASCs). **Materials and Methods:** Aliquots (10 g) of the lipoaspirates were stored at 4°C without supplying oxygen or nutrients. At the indicated time points, the yield of mononuclear cells was evaluated and the stem cell population was counted by colony forming unit-fibroblast assays. Cell surface markers, stem cell-related transcription factors, and differentiation potentials of ASCs were analyzed. **Results:** When the lipoaspirates were stored at 4°C, the total yield of mononuclear cells decreased, but the stem cell population was enriched. These ASCs expressed CD44, CD73, CD90, CD105, and HLA-ABC but not CD14, CD31, CD34, CD45, CD117, CD133, and HLA-DR. The number of ASCs increased 1×10^{14} fold for 120 days. ASCs differentiated into osteoblasts, adipocytes, muscle cells, or neuronal cells. **Conclusion:** ASCs isolated from lipoaspirates and stored for 24 hours at 4°C have similar properties to ASCs isolated from fresh lipoaspirates. Our results suggest that ASCs can be isolated with high frequency by optimal storage at 4°C for 24 hours, and those ASCs are highly proliferative and multipotent, similar to ASCs isolated from fresh lipoaspirates. These ASCs can be useful for clinical application because they are time- and cost-efficient, and these cells maintain their stemness for a long time, like ASCs isolated from fresh lipoaspirates.

Key Words: Lipoaspirates, adipose tissue, mesenchymal stem cell, proliferation, differentiation

INTRODUCTION

Mesenchymal stem cells (MSCs) have two properties; the ability to self-renew^{1,2} and the ability to differentiate into multiple tissue type lineages, such as cartilage, bone, muscle, ligament, tendon, adipocytes and stromal cells.³⁻⁵ MSCs derived from bone marrow were first recognized by Friedenstein and co-workers,⁶⁻⁹ and many studies have since used bone marrow-derived stem cells (BMSCs). Recently,

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MSCs have been isolated from adipose tissue, umbilical cord blood, peripheral blood, brain, lung, liver, dermis, and skeletal muscle.¹⁰⁻¹⁸ MSCs reside in various tissues but can be isolated from bone marrow aspirates, adipose tissues, and the umbilical cord with 100% efficiency.¹⁹ Adipose tissue can be obtained with less invasive procedures than other tissues. More importantly, adipose tissue-derived stem cells (ASCs) can be recovered in high quantities because adipose tissues are an abundant reservoir of MSC approximately >100-fold higher than bone marrow.²⁰ Therefore, adipose tissue represents an abundant, practical, and appealing stem cell source for regenerative medicine.

ASCs are isolated using a combination of enzymatic digestion with collagenase and methods that take advantage of their adherence properties.²¹ It was reported that $2-6 \times 10^8$ mononuclear cells could be obtained from 300 mL of adipose tissue.²¹ The average frequency of ASCs from lipoaspirates was 0.1-1% of mononuclear cells, and the yield of ASCs was approximately 500-5,000 stem cells per 1 gram of adipose tissue.^{20,22} Most research groups use the isolation method described by Zuk, et al.²¹ In brief, adipose tissue or lipoaspirates are treated with collagenase immediately after surgical resection, and the mononuclear cells are plated in cell culture medium at a density of 5×10^6 mononuclear cells/100-mm culture dish. Subconfluent growth can be observed within two weeks of initial plating. Therefore, to isolate ASCs from 300 mL of lipoaspirates, approximately one hundred 100-mm cell culture dishes are required. This laborious procedure can cause many problems, including cell contamination, increased costs and long isolation times. To improve ASC isolation procedures, we focused on the characteristics of MSCs that were more resistant to unsuitable environments than other somatic cells.^{23,24} Mylotte, et al.²⁴ reported that MSCs were more resistant to ischemia than cardiomyocytes and that exposure to ischemia did not impair MSC differentiation potential. They further demonstrated that MSCs were resistant to hypoxia (0.5% O₂) or inhibitory conditions of mitochondrial respiration with 2,4-dinitrophenol for 72 hours. These results indicate that in the absence of oxygen, MSCs could survive using anaerobic ATP production. Matsumoto, et al.²⁵ reported that aspirated fat could be stored or transported overnight if preserved at 4°C and that adipose tissue-derived stem cell yield was not reduced by preservation at 4°C for one day, but they did not describe the effects of local anesthetics on the quantity and quality of viable preadipocytes. Generally, to obtain human lipoaspirates, elective liposuction procedures are performed under lo-

cal anesthesia. Local anesthetics such as articaine/epinephrine and lidocaine strongly impair preadipocyte viability.²⁶ Keck, et al. reported a marked influence of local anesthetics on not only the quantity but also the quality of viable preadipocytes as determined by their ability to differentiate into mature adipocytes.

In our procedures, lipoaspirates were extensively washed with phosphate-buffered saline (PBS) to remove contaminating blood cells and local anesthetics prior to preservation, and in order to exclude the effects of local anesthetics on the viability of preadipocytes and their differentiation into adipocytes. Therefore, in this study, we evaluated the optimal isolation conditions for ASCs from lipoaspirates stored at 4°C without local anesthetic and a supply of oxygen and nutrients. Our results show that the numbers of mononuclear cells gradually decreased during storage in a time dependent manner, but that, in contrast, ASC isolation was enhanced when lipoaspirates were stored for 24 hours.

MATERIALS AND METHODS

Isolation of adipose tissue-derived stem cells

Human adipose tissue was obtained from three healthy female donors with a mean age of 30.7 ± 7.8 and body mass index of 23.7 ± 3.7 at Park's Cosmetic and Plastic Surgery (Seoul, Korea). The women underwent elective liposuction procedures under anesthesia according to procedures approved by the Institutional Review Board of Ajou University Hospital. Informed consent was obtained from all donors. Mononuclear cells were isolated using a modified protocol described by Zuk, et al.²¹ In brief, lipoaspirates were extensively washed with PBS to remove contaminating blood cells and local anesthetics. Then, aliquots (10 g) of the washed lipoaspirates were preserved at 4°C without supplying oxygen or nutrients. At the indicated time points, an aliquot was enzymatically digested at 37°C for 40 minutes with 0.075% type IA collagenase (Sigma, St. Louis, MO, USA) in PBS. The red blood cell lysis step was omitted to reduce the isolation time because omission of this step caused no difference in yield of mononuclear cells and isolation efficiency of mesenchymal stem cells. The digested lipoaspirates was centrifuged at 1,200 g for 5 minutes, and the pellet was resuspended and passed through a 100- μ m mesh filter (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ, USA) to remove debris. Cells were plated in 100-mm culture dishes at a density of 5×10^6 mononuclear cells with

low glucose Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin). After two days, the medium was changed to remove nonadherent cells. The adhered cells were expanded for seven days, then trypsinized and counted.

Culture and expansion

ASCs were maintained in DMEM supplemented with 10% FBS and 1×penicillin/streptomycin (GibcoBRL, Rockville, MD, USA) in a humidified incubator at 37°C/5% CO₂. The medium was changed twice weekly, and cells were passaged with 0.25% trypsin/0.1% EDTA (GibcoBRL) upon reaching 90% confluency. Experiments for proliferation and differentiation were performed at passage 1 to 3. The number of population doublings was calculated using the following formula: $\log N_0/\log N_1$, where N_0 is the number of seeding cells and N_1 is the number of recovered cells when they were passaged. Doubling time was determined by dividing the total number of hours in culture by the number of doublings.

CFU-F assay

For colony forming unit-fibroblast (CFU-F) assays, cells isolated from lipoaspirates were seeded at 5,000 cells/well in triplicated 6-well plates and fed twice weekly for two weeks. For direct visualization of the colonies, the cells were washed with PBS and fixed in 95% ethanol for five minutes, and then the cells were incubated for 30 minutes at room temperature in 0.5% crystal violet in 95% ethanol. Excess stain was removed by washing with distilled H₂O. The plates were dried and the CFU-F units counted. We defined a CFU-F unit as consisting of more than 100 cells using a microscope.

Immunophenotype analysis

ASCs were stained with combinations of saturating amounts of antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD14-FITC, CD31-PE, CD34-PE, CD44-FITC, CD45-FITC, CD73-PE, CD90-FITC, CD117-PE, CD133-PE, HLA-ABC-FITC, HLA-DR-PE (BD Biosciences, San Jose, CA, USA), and CD105-PE (Ansell, Bayport, MN, USA). A total of 5×10^5 cells were resuspended in 0.2 mL PBS and incubated with FITC- or PE-conjugated antibodies for 20 minutes at room temperature. FITC- or PE-conjugated mouse IgGs were used as the control isotype at the same concentration as specific prima-

ry antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (FC 500; Beckman Coulter) and the data were analyzed with the CXP software (Beckman Coulter).

Differentiation assays

Adipogenic differentiation

Cells were plated at 2×10^4 cells/cm² in 6-well plates and cultured for one week. The medium was then changed to an adipogenic medium [10% FBS, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL insulin and 100 µM indomethacin in high glucose (HG)-DMEM] for an additional three weeks. In order to determine adipogenic differentiation, the cells were fixed in 4% paraformaldehyde for 10 minutes and stained with fresh Oil red-O solution (Sigma) to show lipid droplets in induced cells.²⁷

Osteogenic differentiation

Cells were plated at 2×10^4 cells/cm² in 6-well plates, then induced in the following osteogenic medium for two to three weeks: low glucose (LG)-DMEM medium supplemented with 10% FBS, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and 0.2 mM ascorbic acid (all from Sigma).²⁸ In order to determine osteogenic differentiation, the release of p-nitrophenol from p-nitrophenyl phosphate by the ALP enzyme was observed.²⁹

Neuronal differentiation

Cells were plated at 8×10^3 cells/cm² in 6-well plates. After 24 hours, the cells were preinduced for one day with HG-DMEM supplemented with 20% FBS, and then the medium was changed to neurogenic medium (200 µM BHA, 5 mM KCl, 2 mM valproic acid, 10 µM forskolin, 1 µM hydrocortisone, 5 µg/mL insulin in serum-free HG-DMEM).³⁰ Neuron-specific genes were determined by RT-PCR and immunocytochemistry.

Myogenic differentiation

Cells were plated at 1×10^3 cells/cm² in 6-well plates. After 24 hours, cells were preinduced for one day with LG-DMEM supplemented with 10% FBS, 3 µM 5-azacytidine, 10 ng/mL fibroblast growth factor-2 (FGF-2), and 0.25 µg/mL amphotericin B. The medium was then changed to a myogenic medium (10% FBS and 10 ng/mL FGF-2 in LG-DMEM)^{31,32} and muscle cell specific genes were determined by RT-PCR and immunoblotting as previously described.³³

Immunoblotting

The cells were washed with ice-cold DPBS and lysed in RIPA buffer [50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate] with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice. The lysate was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was transferred to a new tube, and its protein concentration was measured by using a protein assay kit (Bio-Rad, Hercules, CA, USA). 30 µg proteins were resolved by 10% SDS reducing gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) using a trans-blot system (Bio-Rad, Hercules, CA, USA). Blots were probed using anti-actin (1 : 1000 dilution; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-myogenin (1 : 1000 dilution; Santa Cruz Biotech, CA, USA), and anti-MyHC (1 : 1000 dilution; Santa Cruz Biotech, CA, USA). The next day, bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (1 : 2000 dilution; Santa Cruz Biotech, CA, USA), and visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunocytochemistry

The cells were fixed in 10% formalin solution (Sigma), permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and blocked with 2% FBS in PBS for 30 min at room temperature. They were incubated with a primary antibody specific to NF-L (1 : 100 dilution; Santa Cruz Biotech, CA, USA) for 4°C overnight, and then labeled with rhodamine-conjugated secondary antibodies (1 : 100, Santa Cruz Biotech) for 1 h at room temperature after primary incubation. The cells were also stained with 1 µg/mL 4', 6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) in order to visualize their nuclei. The slides were

observed and photographed under a fluorescent microscope (IX-71, Olympus, Shinjuku-ku, Tokyo, Japan).

RT-PCR

Total RNA was extracted from the cells using the TRIzol Reagent (GibcoBRL, Rockville, MD, USA). A total of 2 µg of RNA was reverse-transcribed with AMV reverse transcriptase XL (TaKaRa, Otsu, Shiga, Japan) for one hour at 42°C in the presence of oligo-dT primer. PCR was performed using Taq DNA polymerase (BioQuest, Seoul, Korea). Amplified products were electrophoresed on a 2% agarose gel and photographed under an ultraviolet light transilluminator (Bio-Rad, Hercules, CA, USA). The sequences of oligonucleotide primers used for RT-PCR and the expected transcript sizes are listed in Table 1.

Statistical analysis

Data are expressed as mean±standard deviation. Statistical significance was estimated by the Student's t-test and a paired t-test. Significance was defined as *p*-value of ≤0.05.

RESULTS

Yield of mononuclear cells by processing time

The lipoaspirates were extensively washed with phosphate-buffered saline (PBS) to remove contaminating blood cells and local anesthetics, and then aliquots (10 g) of the washed lipoaspirates were stored at 4°C until needed. An aliquot of lipoaspirates was digested with type IA collagenase after a total storage period of between 3 and 36 hours, and the recovered mononuclear cells were counted. Interestingly, although the total yield of mononuclear cells from preserved lipoaspirates gradually decreased from 3.06×10^6 cells/g at three hours post-storage to 0.4×10^6 cells/g at 36 hours post-storage (Fig. 1A), that of CFU-F and expanded cells during

Table 1. RT-PCR Primers for Validation of Gene Expression

	Forward primer (5'-3')	Reverse primer (5'-3')	Product size, bp
GAPDH	CAAGGCTGAGAACGGGAAGC	AGGGGGCAGAGATGATGACC	194
MyoD	AATGTAGCAGGTGTAACCGT	GCCTTTATTTTGATCACCTG	230
Myogenin	CACTACTTCTGTAGCAGGGG	TCTCTCAAACCGTTTCACTT	305
Dystrophin	CAGTAGCCCCATCACATTTG	ATAACGCAATGGACAAGTGG	566
MCK	GGCACAATGACAACAAGAGC	GAAAAGAAGAGGACCCTGCC	721
NeuroD	TGACCAAATCGTACAGCGAGAG	AGAAGTTGCCATTGATGCTGAGCG	848
NF-L	ACCCGACTCAGTTTCACCAG	TCAGCCTTAGACGCCTCAAT	200
Nestin	GCCCTGACCACTCCAGTTTA	GGAGTCCTGGATTCCTTCC	200

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MyoD, class I myosin; MCK, muscle creatine kinase; NeuroD, neurogenic differentiation; NF-L, neurofilament light polypeptide.

seven days peaked from aliquots preserved for 24 hours (Fig. 1B and C). The ability of mononuclear cells isolated from an aliquot preserved for 24 hours (yield of $2.8 \pm 0.08\%$) to form a CFU-F unit was 4.7 times higher than those stored for only three hours (yield of $0.6 \pm 0.2\%$). Also, the expanded cell number after seven days was 3.9 times higher in the aliquots that were preserved for 24 hours.

Cell surface markers

In order to characterize the surface phenotype of ASCs isolated from an aliquot preserved for 24 hours, cell surface markers were examined at the third passage. Flow cytometric results showed that ASCs isolated after preservation for 24 hours were positive for CD44, CD90, CD73, CD105, and HLA-ABC. In addition, expression of CD14, CD31, CD34, CD45, CD117, CD133, and HLA-DR was not observed (Fig. 2). This phenotype is similar to the phenotype of stem cells isolated from the bone marrow, umbilical-cord blood, and umbilical cord tissue.³⁴ It is also consistent with the ASC phenotype reported by Gronthos and Zuk.^{35,36}

Proliferation capacity

In order to examine the *in vitro* proliferative potential of ASCs isolated from an aliquot preserved for 24 hours at 4°C, we replated the cells at 1,000 cells/cm² every 8-10 days until their initial population doubling (PD) time increased by more than three times. The proliferative potential was retained even at passage 12 (Fig. 3), and cell numbers increased 1×10^{14} fold. The population doubling time at early passages was maintained from about 39 to 50 hours until passage 6, and then gradually increased until passage 12. The PD times of passages 12 and 13 were 104 and 175 hours, respectively.

Differentiation capacity

Previous studies have demonstrated the osteogenic, chondrogenic, adipogenic, myogenic, cardiomyogenic, and neurogenic potential of ASCs. In order to examine the differentiation potentials of ASCs isolated from an aliquot preserved for 24 hours at 4°C, osteogenic, adipogenic, myogenic, or neurogenic differentiation of ASCs was induced. In Fig. 4, alkaline phosphatase activity, a marker of

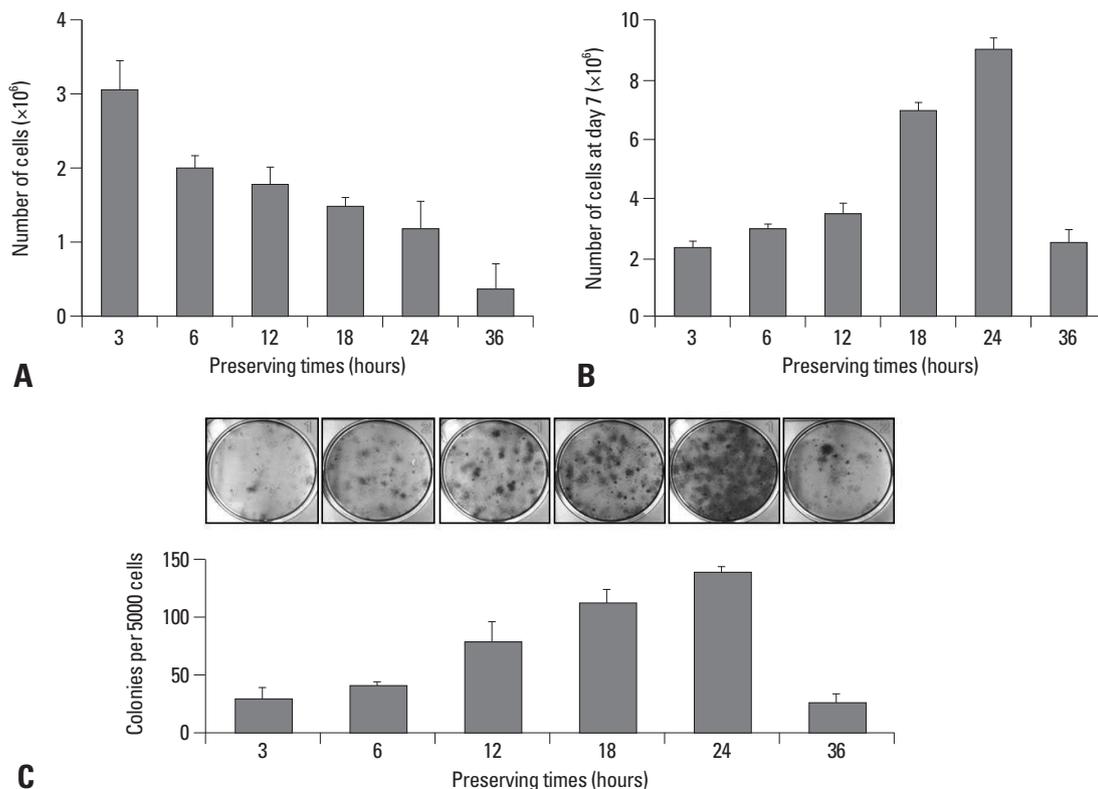


Fig. 1. Isolation of ASCs from lipoaspirates preserved at 4°C. (A) Yields of mononuclear cells isolated from lipoaspirates. Mononuclear cells were isolated from lipoaspirates preserved at 4°C for the indicated times as described in the Materials and Methods section, and then viable mononuclear cells were counted by staining with trypan blue. (B) Recovery of cells at day 7. Each set of mononuclear cells was isolated at the indicated times and cultured in 100-mm culture dishes at a density of 5×10^5 cells. On day 7, the expanded cells were counted. (C) Clonogenic ability of mononuclear cells isolated from lipoaspirates. Five thousand mononuclear cells were plated in 6-well plates. They were cultured for two weeks, and then colony forming unit-fibroblasts (CFU-F) were stained with crystal violet. A colony consisting of more than 100 cells was counted microscopically. Representative histograms of three independent experiments are shown, and error bars represent the standard deviation. ASCs, adipose tissue-derived stem cells.

osteogenesis, was detected at high levels (Fig. 4A), and Oil-Red-O stain accumulated in intracellular lipid-filled droplets of adipocytes differentiated from ASCs (Fig. 4B). In myogenic or neurogenic differentiation of ASCs, markers of skeletal muscle cells (MyoD, Myogenin, dystrophin, MCK, and MyHC) or neuronal cells (neuroD, NF-L, nestin) were expressed in mRNA (Fig. 4C and E) and protein levels (Fig. 4D and F).

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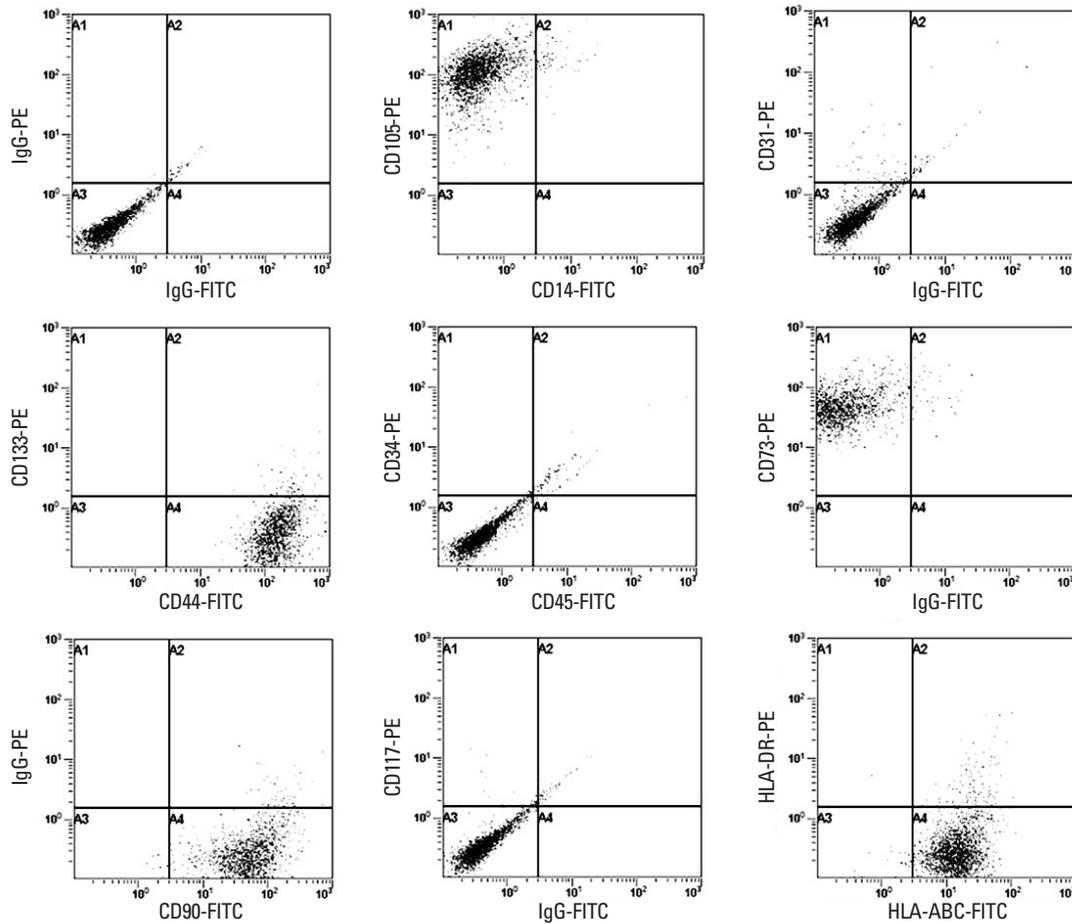


Fig. 2 Expression of cell surface markers of ASCs isolated from lipoaspirates preserved for 24 hours. Expression of cell surface markers of ASCs isolated from lipoaspirates preserved for 24 hours was determined by flow cytometry as described in the Materials and Methods section. CD14-FITC and CD105-PE, CD44-FITC and CD133-FITC, CD45-FITC and CD34-PE, and HLA-ABC-FITC and HLA-DR-PE were double stained. ASCs, adipose tissue-derived stem cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

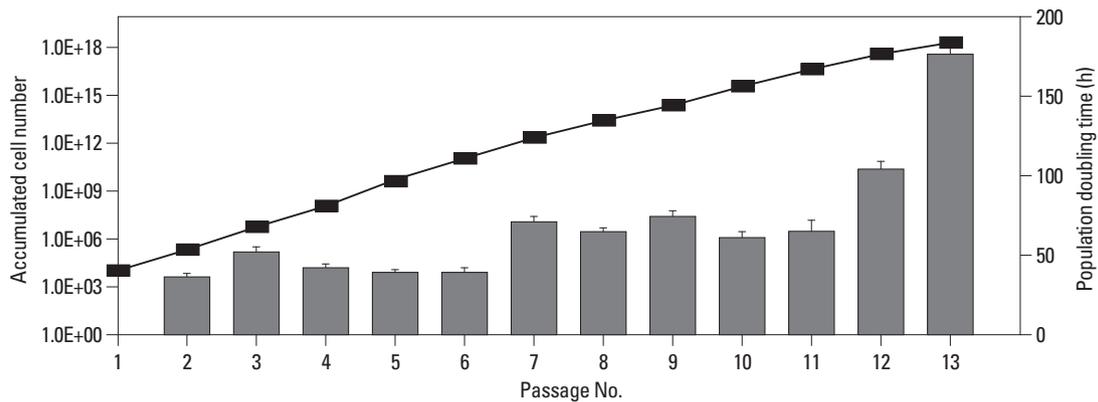


Fig. 3 Proliferation potentials of ASCs isolated from lipoaspirates preserved for 24 hours. ASCs were cultured in 6-well plates at a density of 1000 cells/cm². When the cells grew confluent, the total cell numbers were counted and then re-cultured at the same density. These procedures were repeated until cell growth stopped. Numbers of cells (-■-) and population doubling times (histogram) were represented at each passage. Representative histograms of three independent experiments are shown, and error bars represent the standard deviation. ASCs, adipose tissue-derived stem cells.

DISCUSSION

We report an optimal ASC isolation method that reduces cell contamination or cost and time required by preserving lipoaspirates at 4°C for 24 hours. We carefully evaluated the phenotype, proliferation and differentiation potentials of ASCs isolated from lipoaspirates preserved at 4°C for 24 hours, and our results are in agreement with previous reports³⁴⁻³⁷ that state that the phenotype of ASCs isolated from lipoaspirates preserved at 4°C for 24 hours was similar to that of ASCs isolated immediately after surgery or BMSC. In fact, although we directly compared the characteristics of stem cells isolated at 3- and 24-hour time points from eight donors, we found no differences (data not shown). Our procedure reduced the required time for isolating stem cells from about 14 to 7 days and increased the total yield of stem cells from adipose tissue. We suggest that of the re-

duced time required for isolation and the increased yield of stem cells might be the results of enrichment by increased storage time and the resistance of stem cells to environments unsuitable for somatic cells. In fact, Mylotte, et al.²⁴ reported that MSCs were more resistant to unsuitable environments, such as ischemia, hypoxia (0.5% O₂), and the inhibitory conditions of mitochondrial respiration. These results highlighted the fact that mesenchymal stem cells from lipoaspirates could be enriched by an unsuitable environment such as cold preservation without oxygen or nutrients.

In order to gain the optimal therapeutic effects of stem cell for clinical application, some issues must be considered carefully. First, stemness, as evaluated by proliferation and differentiation potentials, must be maintained during *ex vivo* expansion of stem cells. It is well established that the stemness of stem cells is gradually lost after prolonged cell culture and, thus, that maintenance of stemness may play a pivotal role in the regeneration of damaged cells or tissues.

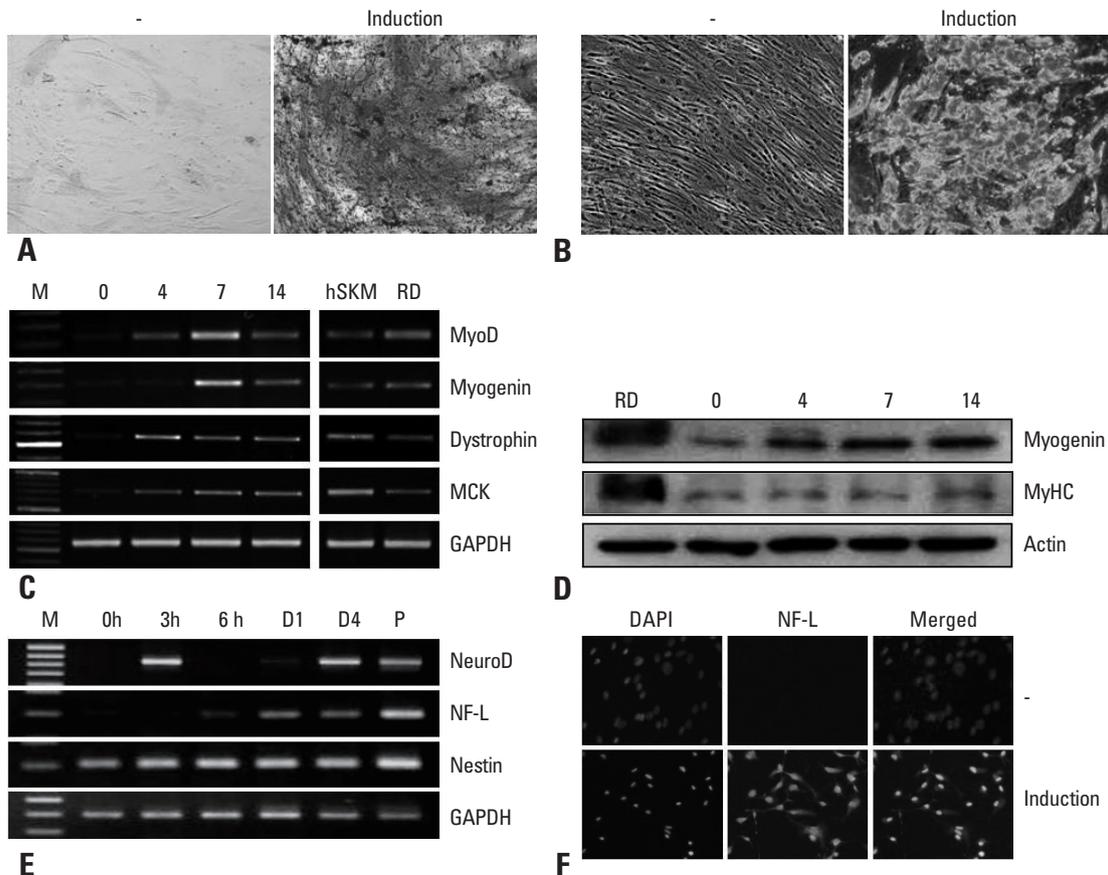


Fig. 4. Differentiation potentials of ASCs isolated from lipoaspirates preserved for 24 hours. Passage 1 cells were seeded and differentiated into adipocytes, osteoblasts, muscle cells, or neuronal cells as described in the Materials and Methods section. Osteogenic (A) or adipogenic differentiation (B) were evaluated by assaying the alkaline phosphatase activity or Oil-Red O staining, respectively. To evaluate differentiation potentials of ASCs into muscle (C and D) or neuronal cells (E and F), we investigated the expression of several markers (myogenic; MyoD, Myogenin, Dystrophin, MCK and neurogenic; NeuroD, NF-L, Nestin) by RT-PCR, immunoblotting (myogenic; Myogenin and MyHC), and immunocytochemistry (neurogenic; NF-L). ASCs, adipose tissue-derived stem cells; MyoD, class I myosin; MCK, muscle creatine kinase; NeuroD, neurogenic differentiation; NF-L, neurofilament light polypeptide; DAPI, 4', 6-diamino-2-phenylindole; MyHC, myosin heavy chain.

In our results, PD time of ASCs isolated from lipoaspirates preserved at 4°C for 24 hours was below 75 hours up to passage 11. Further, expression of stemness-related transcription factors, Klf4, Nanog, Sox2, and Oct4 was not decreased (data not shown). Taken together, ASCs isolated by our protocols were able to expand sufficiently for a relatively long time in culture without loss of stemness. Second, abundant quantities of stem cells are required for clinical trials. To obtain sufficient stem cells within a short period of time, it is important that stem cell sources have high reservoirs of stem cells. In addition, these stem cells must be isolated as rapidly as possible. Therefore, if MSCs were isolated rapidly from adipose tissue using our procedure in numbers approximately 100+ fold than from bone marrow,²⁰ ASCs may provide the best stem cells for clinical trials.

In summary, we found that the required time for the isolation of stem cells decreased and the yield of stem cells increased after preservation of lipoaspirates at 4°C for 24 hours. The characteristics of ASCs isolated by our protocol were similar to MSCs of bone marrow, adipose tissue, and umbilical cord blood; this was in accordance with previous reports.³⁴⁻³⁷ In conclusion, we have demonstrated that stem cells derived from adipose tissue could be isolated more rapidly by preservation at 4°C, and that these stem cells had similar stemness and characteristics to other stem cells. These results provide important information regarding the optimal isolation of mesenchymal stem cells from adipose tissue for enhancing clinical utility.

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