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EXPERIMENTAL

Cryopreservation of Stromal Vascular Fraction Cells Reduces Their Counts but Not Their Stem Cell Potency

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Background: Adipose-derived stem cells are derived from the nonfat component of adipose tissue termed the stromal vascular fraction (SVF). The use of freshly isolated autologous SVF cells as an alternative to adult stem cells is becoming more common. Repeated SVF administration for improved clinical outcomes is complicated by the need for repeated liposuction. This can be overcome by cryopreservation of SVF cells. The current study aimed to assess whether SVF cells retain their stem cell potency during cryopreservation.

Methods: SVF cells isolated from lipoaspirates (donor age: 46.1±11.7 y; body mass index: 29.3±4.8 kg/m^2) were analyzed either immediately after isolation or following cryopreservation at −196°C. Analyses included assessment of nucleated cell counts by methylene blue staining, colony-forming unit fibroblast counts, surface marker expression using a flow cytometric panel (CD45, CD34, CD31, CD73, CD29, and CD105), expansion in culture, and differentiation to fat and bone.

Results: While cryopreservation reduced the number of viable SVF cells, stem cell potency was preserved, as demonstrated by no significant difference in the proliferation, surface marker expression in culture, bone and fat differentiation capacity, and the number of colony-forming unit fibroblasts in culture, in cryopreserved versus fresh SVF cells. Importantly, reduced cell counts of cryopreserved cells were due, mainly, to a reduction in hematopoietic CD45+ cells, which was accompanied by increased proportions of CD45−CD34+CD31− stem cell progenitor cells compared to fresh SVF cells.

Conclusions: Cryopreservation of SVF cells did not affect their in vitro stem cell potency and may therefore enable repeated SVF cell administrations, without the need for repeated liposuction. *(Plast Reconstr Surg Glob Open 2019;7:e2321; doi: 10.1097/GOX.0000000000002321; Published online 5 July 2019.)*

INTRODUCTION

Adipose-derived stem cells (ASCs) were first characterized by Zuk et al.[1] and, like other adult mesenchymal stem cells, ASCs have been shown to possess regenerative and immunosuppressive potentials.[2] ASC preparation requires the isolation of nonfat cells from adipose tissue by enzymatic digestion and subsequent centrifugation to separate a floating fat fraction from the pelleted nonfat fraction termed the stromal vascular fraction (SVF). The SVF consists of a heterogeneous mixture of cells including various hematopoietic cell types, endothelial cells, and mesenchymal stem cell progenitor cells.[3,4] The research on and the clinical use of freshly isolated autologous SVF cells are increasing worldwide, and SVF use has been suggested as a simpler and cheaper clinical alternative for ASCs.[5,6] The first use of SVF, administered in a clinical cosmetic setting, was reported in 2007, and since then, has expanded to a broad spectrum of applications in clinical research including for the treatment of multiple sclerosis, diabetes, radiation damage, bone and peripheral nerve regeneration, burn injuries, and so on.[3,5]

Today, SVF is mainly utilized in orthopedic and plastic surgery settings.[6,7] Like mesenchymal stem cells, clinical SVF treatment may benefit from repeated SVF

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administration to achieve optimal results.[8–13] This results in a need for repeated fat harvesting by liposuction to allow SVF isolation for each cell administration. Despite its relatively safe clinical profile, liposuction remains an invasive procedure and its repetition can increase the incidence of morbidity and limit the clinical use of SVF. One way to allow repetitive SVF administration without repeating liposuction procedures is by long-term SVF cryopreservation.

Long-term cryopreservation options would obviate the need for repeated SVF harvesting. Yet, for SVF cryopreservation to be effective and applicable for clinical use, it must preserve the characteristics of fresh SVF cells. Optimally, a cryopreserved population of SVF cells intended for therapeutic applications will maintain its viability and stem cell potency and the ability to form high-quality ASCs when cultured. Maintaining cell viability during freezing and thawing presents various challenges, the most prominent being the formation of intracellular and extracellular ice crystals. The main methods used to minimize the damage inflicted by freezing and thawing are cryoprotectant solutions such as dimethyl sulfoxide (DMSO), and a gradual controlled decrease of temperature during cell freezing.[14] However, DMSO use may lead to adverse effects, limiting its clinical relevance. Importantly, efficient cryopreservation of cultured adult stem cells including ASCs was previously achieved.[15–17] In contrast to cultured stem cells, which form a relatively homogeneous cell population due to their adaptation to culture conditions, freshly isolated cells, such as SVF, are usually composed of a heterogeneous cell population, rendering their efficient cryopreservation challenging because of their different sensitivity to the freezing and thawing processes. Previous works which examined the survival of cryopreserved SVF cells or SVF cells isolated from cryopreserved fat demonstrated mixed results regarding the quality of the surviving SVF cells.[18–20] Using standard laboratory techniques, the current study aimed to determine whether SVF cells isolated from human lipoaspirates retain their quantity and quality following cryopreservation.

METHODS

Experimental Subjects

Abdominal subcutaneous adipose tissue samples were obtained from 8 patients undergoing liposuction. The mean age of the patients was 46.1 ± 11.7 years, and the mean body mass index was 29.3 ± 4.8 kg/m^2 (Table 1). All procedures were performed in accordance with the Declaration of Helsinki guidelines and approved by the Ethics Committee at the Tel Aviv Sourasky Medical Center (approval No. 0369-12-TLV). Written informed consent was obtained from all patients before undergoing surgery.

Adipose Tissue Harvesting

Adipose tissue was subjected to power-assisted liposuction, which involved use of a 3.0-mm diameter, blunt, hollow cannula (length: 30 cm; PAL-200E MicroAire power-assisted lipoplasty device, MicroAire Surgical Instruments LLC, Charlottesville, Va.), which was introduced into the subcutaneous space through a small incision. Gentle suction was performed by applying <1 atm negative pressure. Standard Klein tumescent solution (1 mg adrenaline and 400 mg lidocaine per 1 L saline; ratio of infiltration fluid to aspirate volume, 1:1–2:1) was applied for the superwet liposuction technique. Additional fluids were not needed. The fat was then decanted, and the fat fraction was aspirated into an empty sterile container.

SVF Cell Isolation

SVF cells were extracted from subcutaneous lipoaspirates by incubation with collagenase (0.075% type I collagenase, Sigma-Aldrich, St. Louis, Mo.) for 45 minutes at 37°C, with gentle shaking, followed by fat separation by centrifugation (15 min, 400*g*). The SVF pellet was resuspended, and nucleated cells were stained with a solution of 3% acetic acid and methylene blue (Stemcell Technologies, Vancouver, B.C., Canada) and were counted manually, under a high-power light microscope. SVF cells were divided into 3 groups: (1) Fresh cells: cells were cultured and analyzed immediately. (2) –80°C: SVF cells were immediately transferred to a –80°C freezer for 24 hours, transferred to liquid nitrogen for 6–8 weeks, and then thawed, analyzed, and cultured. (3) Mr. Frosty (MF): SVF cells were immediately transferred to an MF device and frozen in a –80°C freezer for 24 hours, after which, they were transferred to liquid nitrogen for 6–8 weeks, thawed, analyzed, and cultured. MF is a polycarbonate freezing container (Sigma) that provides the critical, replicable, 1°C/min cooling rate required for successful cryopreservation of cells.

Cryopreservation of SVF

Cryopreservation medium included 90% fetal bovine serum (Thermo Scientific HyClone, Tauranga, New Zealand) and 10% DMSO and was cooled to 4°C before cell freezing. SVF cells were frozen in samples of 2 million SVF cells per 1 ml cryopreservation solution. Once aliquoted, cryovials were placed on ice and then either directly stored at –80°C for 24 hours or transferred into an MF freezing container for storage at –80°C. After 24 hours at –80°C, the cryovials were transferred to liquid nitrogen for long-term storage (6–8 wk) before thawing.

Cell Culture

Fresh and cryopreserved SVF cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gib-

Table 1. Patient Summary

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Average
Age	44	51	48	52	58	31	34	25	42.8±11.6
Gender	M	F	F	F	F	M	F	F	
BMI	29	31	31	26	32	28	21	30	28.5±3. 6
Surgery	Abdomino	Abdomin	Abdomino	Apro	Abdomino	Gynecom	Abdomino	Apro	

Abdomino indicates Abdominoplasty; Apro, Apronectomy; Gynecom, Gynecomastia repair; BMI, body mass index.

co, Paisley, Scotland, United Kingdom), supplemented with 10% FBS (Thermo Scientific HyClone, Tauranga, New Zealand), 60 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml kanamycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and nonessential amino acids. The cultures were propagated at 37°C in a 10% CO_2 atmosphere. Medium was changed twice a week, and the cells were passaged once after reaching full confluence.

Colony-forming Unit Fibroblast Assay

SVF cells (2,000 nucleated cells) were seeded in a 6-mm dish and cultured for 21 days under normal culture conditions. Colonies were then fixed with methanol, stained with Giemsa (Sigma), and counted to evaluate the number of colony-forming unit fibroblasts (CFU-Fs) formed. The total number of colonies was quantified using an Olympus IX71 microscope.

Flow Cytometry

The following mouse antihuman antibodies were used for flow cytometry staining: CD31 APC, CD34 PE (PeproTec, London, United Kingdom), CD29 Alexa Fluor 488, CD105 PerCP/Cy5.5, CD73 PE/Cy7 (BioLegend, San Diego, Calif.), and CD45 BD Horizon BV650 (BD Biosciences, San Jose, Calif.). All the antibodies which were IgG1 kappa had their respective isotype controls.

SVF Surface Marker Analysis

For multicolor surface marker analysis by flow cytometry, SVF cells were harvested and simultaneously incubated with the respective antibody panels for 1 hour in the dark at room temperature (RT). The 7-color panel contained antibodies to CD31, CD34, CD29, CD105, CD73, and CD45. To exclude dead cells, the samples were stained with violet viability dye (Molecular Probes, Invitrogen, Eugene, Oreg.), as described in the manufacturer's protocol. All antibodies were used at the dilution recommended by the manufacturer. Appropriate isotype controls were performed. Following staining, the harvested cells were incubated with a red blood cell lysis solution (BD FACS Lysing Solution) and analyzed by flow cytometry (FACSCanto II, BD Biosciences).

ASC Surface Marker Analysis

For surface marker analysis by flow cytometry, ASCs were harvested and incubated with the respective antibodies as above. The 7-color panel contained antibodies to CD31, CD34, CD29, CD105, CD73, and CD45. Dead cells were excluded by violet viability dye. All antibodies were used at the dilution recommended by the manufacturer. Appropriate isotype controls were performed.

Differentiation

Adipogenic Differentiation

Confluent passage 1 ASCs were cultured in adipogenic medium containing high-glucose Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% FCS (Thermo Scientific HyClone, Tauranga, New Zealand), 10 µg/ml insulin, 1×10^{-6} M dexamethasone, 0.5 mM IBMX, and 50 µM indomethacin (all from Sigma). The adipogenic medium was replaced every 3–4 days. After 21 days, the cells were fixed in 4% formalin (20 min, RT) and stained with 0.5% Oil Red O (Sigma) for 10 minutes at RT. Following staining, the cells were photographed (Olympus IX71 microscope, Olympus, Tokyo, Japan) with a DP73 camera and Oil Red O was extracted by 4% IGEPAL (Sigma) in isopropanol and quantified at 520 nm using a TECAN Infinite M200 plate reader (TECAN, Männedorf, Switzerland).

Osteogenic Differentiation

Confluent passage 1 ASCs were cultured in StemPro Osteogenesis Differentiation medium (Gibco). The differentiation medium was replaced every 3–4 days. After 21 days, the cells were fixed in 4% formalin (20 min, RT) and stained with 2% Alizarin Red (Sigma), pH 4.2 (10 min, RT). The photographs were taken using an Olympus IX71 microscope with a DP73 camera. Then, Alizarin Red was extracted by extraction solution (0.5 N HCl/5% sodium dodecyl sulfate (SDS)) and quantified at 415 nm using a TECAN Infinite M200 plate reader (TECAN, Männedorf, Switzerland).

Statistical Analysis

Results are represented as mean ± SD. Differences between the experimental groups were evaluated using the one-way analysis of variance test. Statistical significance was defined as $P < 0.05$.

RESULTS

Reduced Viable Cell Counts Following Cryopreservation

A comparison of viable SVF cell (nucleated cell) counts immediately after cell isolation from fat and following cryopreservation and thawing showed a sharp reduction (>60%) in nucleated cell counts during the cryopreservation and thawing processes using both freezing methods (Fig. 1A).

Cryopreservation Does Not Affect Short-term Expansion Potential of Cultured SVF Cells

Next, we compared the ability of fresh versus cryopreserved SVF cells to expand in culture by seeding equal quantities of viable fresh versus cryopreserved nucleated SVF cells and assessing their counts 10 days thereafter. Counts of the cryopreserved and fresh cells were similar, indicating maintained viability of ASC progenitor cells during the freezing process (Fig. 1B). No significant change in the expansion potential was observed between the 2 different freezing methods examined.

Cryopreservation Does Not Affect Colony-forming Capacities of SVF Cells

Stemness of SVF cells was further assessed by seeding low concentrations of fresh or cryopreserved SVF cells and comparing the number of CFUs that developed after 21 days. No significant difference was measured in the number of colonies formed by fresh versus cryopreserved SVF cells, further verifying retained survival and potency of ASC progenitor cells during the freezing process (Fig. 1C).

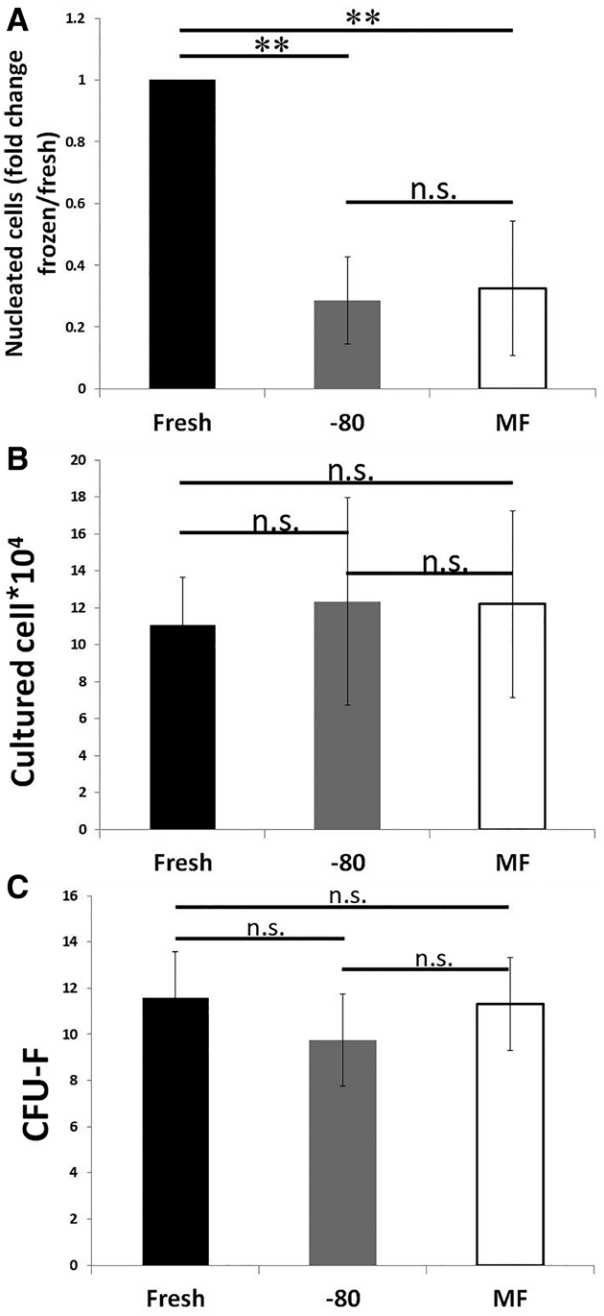

Fig. 1. Cryopreservation of SVF cells leads to a reduction in cell count without affecting short-term expansion capacity and number of CFU-F. A, The quantity of viable nucleated cell was compared between fresh and cryopreserved SVF cells that were frozen at −80°C either in a standard freezing box (−80°C) or in an MF device. Data are presented as the fold change between the quantity of fresh and cryopreserved SVF cells per 1 ml fat. B, Equal quantities of viable nucleated fresh isolated or cryopreserved SVF cells were seeded, cultured for 10–12 days, and then harvested and counted. C, The ability of fresh vs cryopreserved SVF cells to form CFU-Fs was examined by culturing the cells at low densities for 21 days, staining colonies with Giemsa and counting large colony (>100 cells). Data are presented as mean ± SD. $^{**}P < 0.01$. n.s., difference not statistically significant.

Cryopreserved SVF Cells Display Characteristic ASC Surface Marker Expression

To assure that the cultured cells produced from cryopreserved SVF cells are indeed ASCs, we compared the surface marker expression profiles of cell cultured from fresh versus cryopreserved SVF cells. As can be seen in Figure 2, both fresh and cryopreserved ASCs passaged once in culture demonstrated the expected surface marker expression pattern (CD45+, CD34+, and CD31 <2% and CD73+, CD29+, and CD29+ >90%), verifying the sustained ability of cryopreserved SVF cells to form ASCs in culture.

Cryopreserved and Fresh SVF Cells Demonstrate Comparable Adipogenic and Osteogenic Differentiation Potentials

Next, we assessed the multipotency of fresh and cryopreserved ASCs by comparing their adipogenic and osteogenic differentiation potentials. After 21 days in relevant induction medium, ASCs prepared from cryopreserved SVF cells retained their multipotent potential and demonstrated similar fat and bone differentiation compared with ASCs prepared from fresh SVF cells (Fig. 3).

Surface Marker Profiles Demonstrate an Enrichment of Stem Cell Progenitor Cell Populations within SVF Cells Following Cryopreservation

In efforts to understand how cryopreserved SVF cells retained their stem cell potency, despite their reduced numbers, we compared the surface marker expression profiles of cryopreserved versus fresh SVF cells before culturing. As expected, an example fresh SVF sample contained a mixture of CD45+ hematopoietic (~40%) and CD45− cells (Fig. 4A). In both types of cryopreserved SVF samples, the proportion of CD45+ cells dropped to ~23% in Figure 4B (MF: data not shown). Analysis of samples from various patients verified that the reduction in the CD45+ cell fraction in cryopreserved SVF following cryopreservation by both methods was significant (Fig. 5A). Next we identified the proportion of the different hematopoietic cell types within the CD45+ cells. As can be seen in Figures 4B, 5B, cryopreservation led to an almost complete eradication of granulocytes and to a consequent significant increase in the proportion of lymphocytes in the cryopreserved samples. Importantly, the cryopreserved samples showed a significant enrichment of CD34+CD31− stem cell progenitor cells among the CD45− subpopulation as compared to fresh SVF samples (over 90% versus ~80%) (Figs. 4B, 5C). The enrichment of stem cell progenitor cells following cryopreservation was evident also in the increased proportion of CD73+CD29+ subpopulation among the CD45− cells (Figs. 4B, 5D).

DISCUSSION

Appreciation of the clinical potential of autologous SVF cells in a growing list of clinical indications is expanding.[3,5–7] Because of the relative simplicity of their isolation and handling, SVF cells provide a more cost-effective and

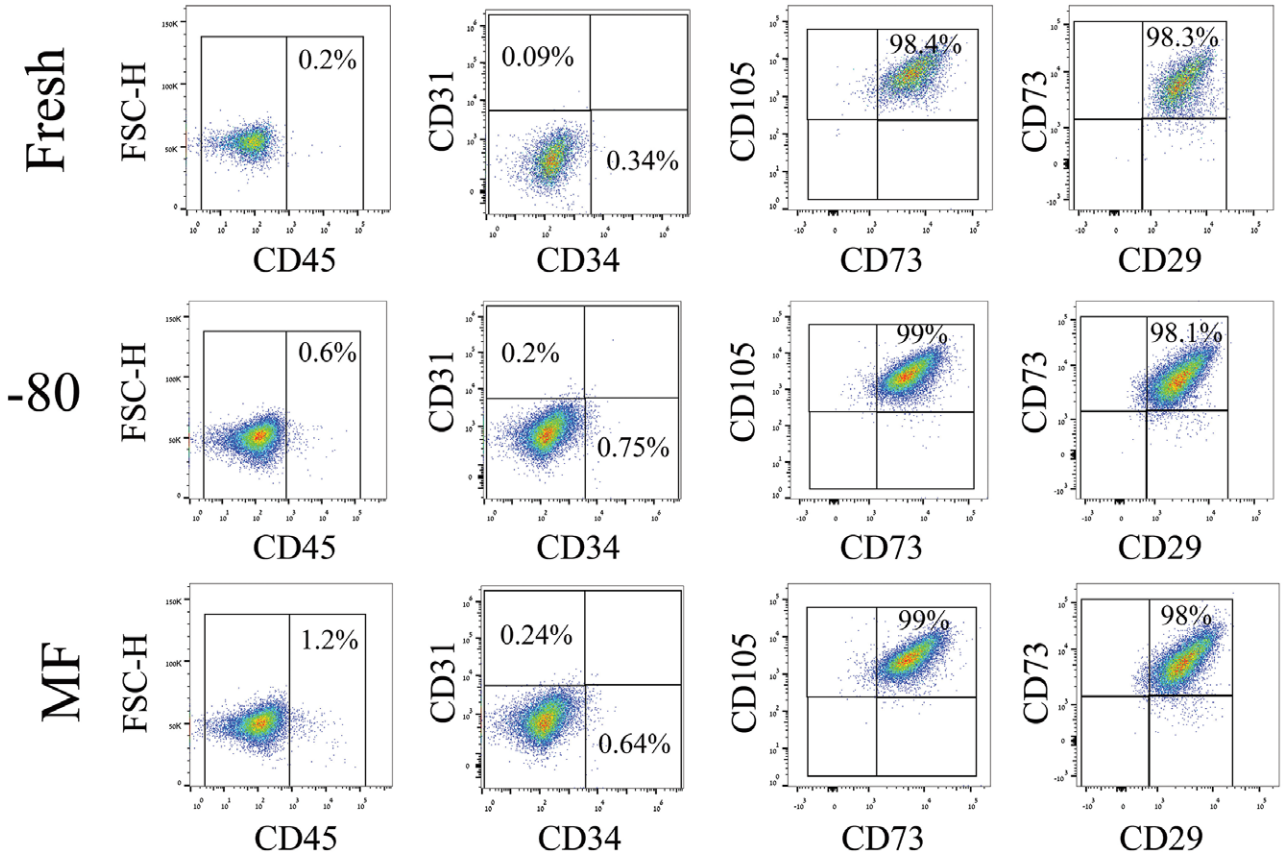

Fig. 2. Cryopreserved SVF cells display characteristic ASC surface marker expression. Passage 1 ASCs prepared from fresh vs cryopreserved SVF cells frozen at −80°C in a standard freezing box (−80°C) or in an MF device were analyzed for their surface marker distribution using a 7-color flow cytometry panel. FSC-H, forward scatter height.

more readily available alternative to cultured ASCs. However, their repeated administration, which may be required to achieve optimal treatment outcomes, is burdened by the need for repeated liposuction, which is a time-consuming, costly, and labor-intensive procedure, requiring the use of surgical facilities each time it is performed. Furthermore, liposuction comes with risks and sequelae such as hematomas and fat emboli.[21] Cryopreservation of a large quantity of SVF cells from a single liposuction session may provide a simple means of avoiding repeated liposuction.

It is generally accepted that the stem cell potency, namely the proportion of stem cell progenitor cells, within SVF samples is an important factor influencing their therapeutic potential. In the current study, we observed that despite a drastic reduction in the quantity of viable cells following SVF cryopreservation, the remaining cells retained their stem cell potency as was evident by their expansion in culture, their ability to form CFU-Fs, their characteristic surface marker expression in culture, and their multipotent phenotype, all of which were similar to their fresh counterparts. Taken together, we hypothesize that when administered at similar doses, cryopreserved SVF cells and fresh SVF cells will be equally clinically beneficial.

In agreement with our results, previous studies demonstrated similar numbers of CFU-Fs[20] and similar bone and fat differentiation[19] when comparing cryopreserved to fresh SVF cells. However, in these studies, the number of surviving cryopreserved SVF cells immediately following lipoaspiration or following SVF cryopreservation was higher than that in the current study.[19,20] A recent study reported a reduction in SVF numbers similar to our results following the cryopreservation of lipoaspirates by 2 different freezing methods.[18] However, in contrast to our findings, the surviving SVF cells demonstrated poor culture expansion and a reduced capacity to differentiate to bone and fat compared to their fresh counterparts.[18] Based on these studies,[18,20] it is hard to conclude whether SVF cells that are isolated from cryopreserved fat retain their viability and stem cell potency.

Despite the wide variety of surface proteins that were previously suggested as markers for mesenchymal stem/stromal cell progenitor cells, the in vivo identity of MSCs remains largely elusive.[22] Similarly to other adult MSCs, ASC identity within adipose tissue also remains obscure, despite the attempts to define their specific properties.[4,23] CD34 is perhaps the most significant SVF surface marker suggested to predict the stem cell potency of SVF cells, with only a portion of CD34+ SVF cells (CD45−CD34+CD31−) suggested to represent the progenitor cells of multipotent ASCs.[24–28] To elucidate the mechanisms enabling cryopreserved SVF to retain their stem cell potency, we compared

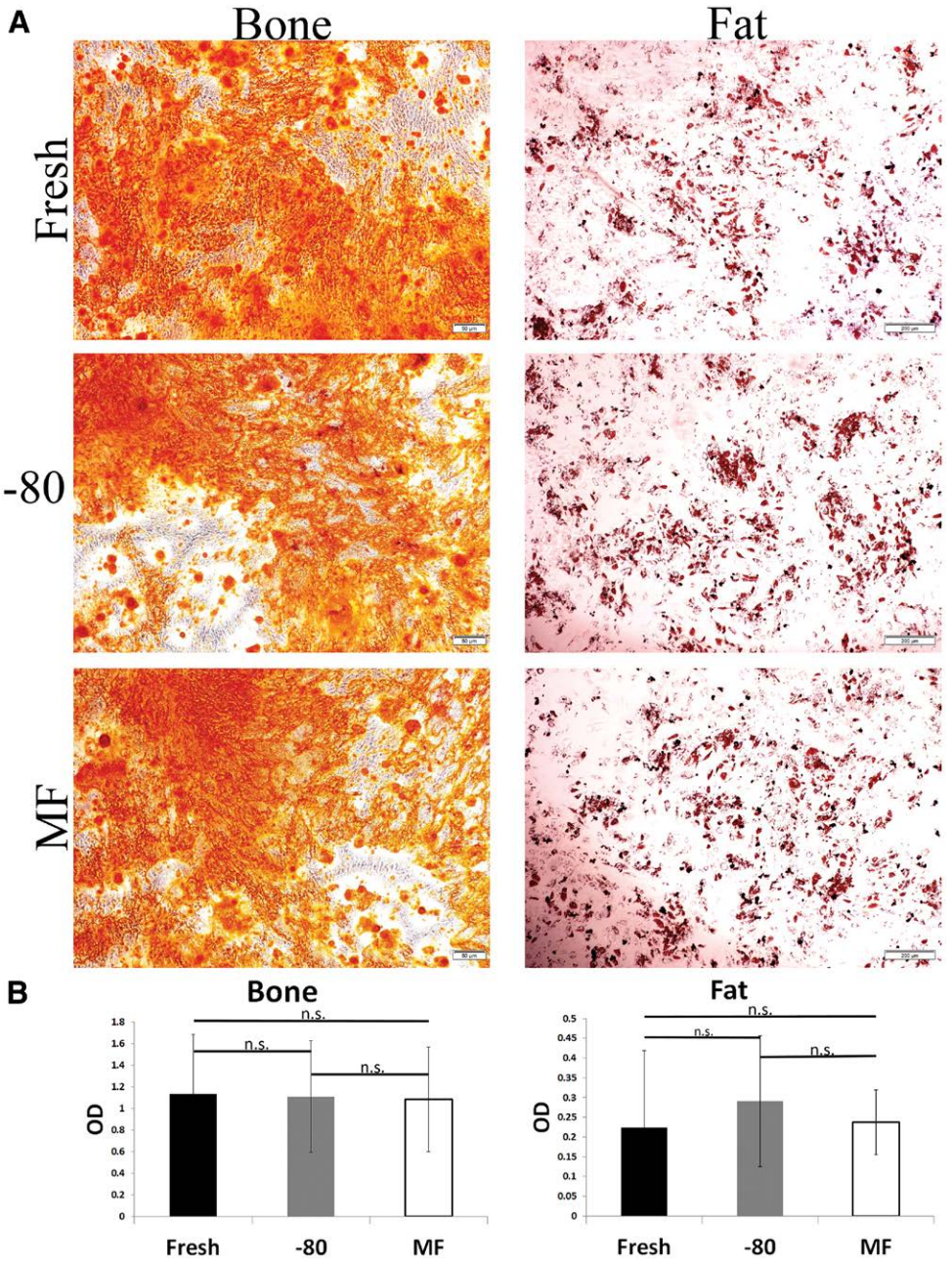

Fig. 3. Cryopreserved and fresh SVF cells demonstrate comparable adipogenic and osteogenic differentiation potential. Fresh and cryopreserved SVF cells were cultured and induced to undergo fat or bone differentiation by incubating them in designated differentiation media at passage 1 for 21 days. Differentiation into bone and fat was detected by Alizarin Red and Oil Red O staining, respectively. Cells were photographed (A) and then the stain was extracted and quantified (BI and BII). Data are presented as mean ± SD (N = 8). OD, optical density.

the different subpopulations within fresh versus cryopreserved SVF samples, using a multicolor flow cytometry panel. We found that cryopreservation of SVF cells led mainly to the death of CD45+ hematopoietic cells, particularly of granulocytes, which were almost entirely eradicated. Indeed, granulocytes were previously shown to be significantly incapacitated or killed upon cryopreservation in DMSO-containing cryopreservation media.[29] At the same time, the CD45(−) nonhematopoietic SVF population showed an enrichment of CD34+CD31− following SVF cryopreservation. Cryopreservation also led to the enrichment of the CD29+CD73+ subpopulation among the CD45− SVF cells. Taken together, SVF cryopreservation leads mainly to the death of granulocytes, although having only a minor effect on the more clinically relevant CD45−CD34+CD31− and CD45−CD29+CD73+ stem cell progenitor cells. A recent study by Zanata et al.[20] examined the effect of cryopreservation on adipose tissue

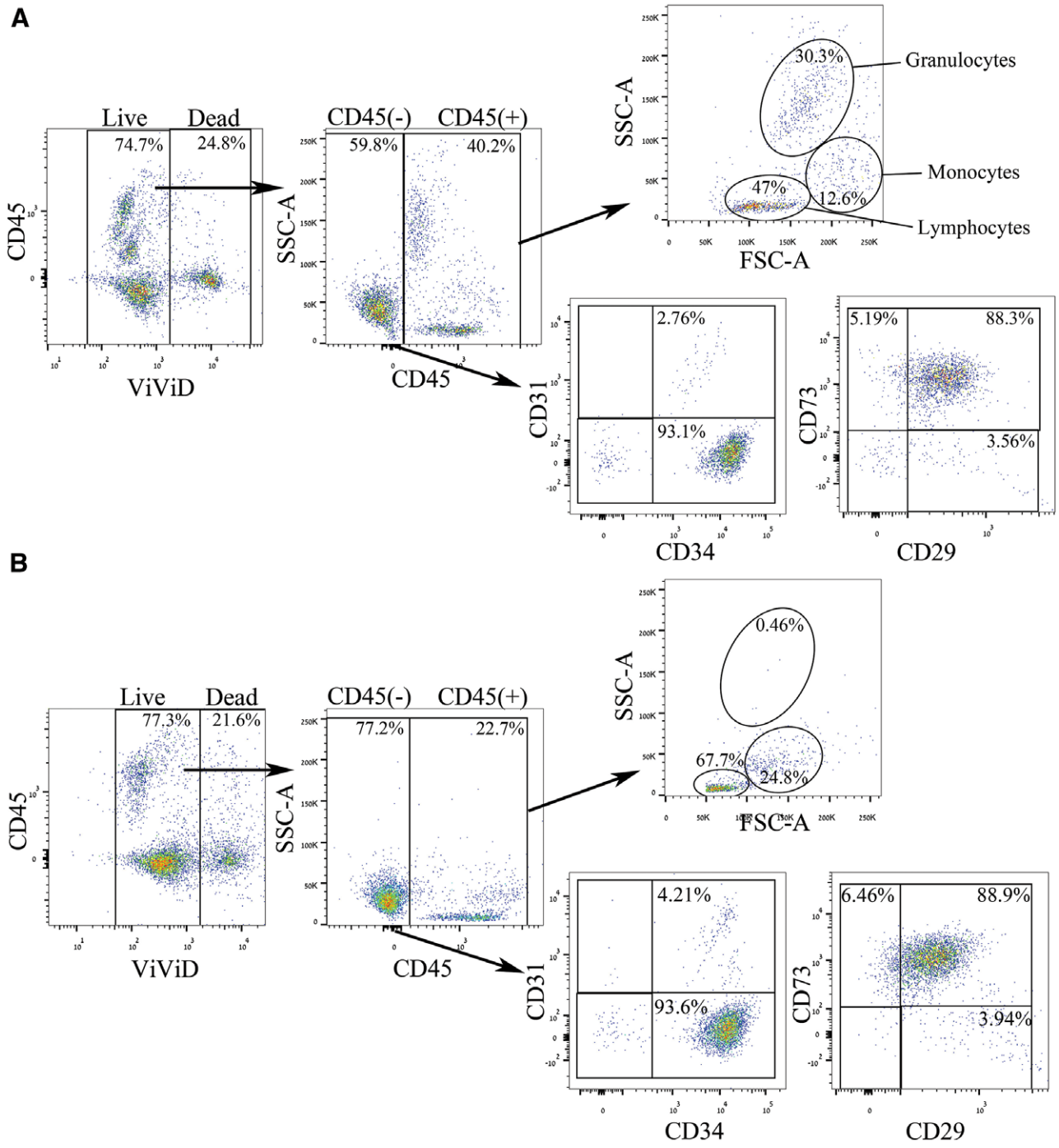

Fig. 4. Multicolor flow cytometry analysis demonstrated a reduced percentage of hematopoietic cells and an enrichment of stem cell progenitor cells in cryopreserved SVF samples. Fresh (A) and cryopreserved (–80°C) SVF cells (B) were stained with a 7-color flow cytometry panel, treated with a fixating red blood lysis buffer and analyzed by flow cytometry. Using the gradual gating strategy shown, only viable cells determined by low ViViD staining (an amine reactive viability dye) while discarding RBCs were analyzed for their surface marker expression. Discrimination between the CD45– and CD45+ cells enabled determination of the percent of the different hematopoietic cell types within the hematopoietic (CD45+) population and the percent of the stem cell progenitor cells (CD31–CD34+ or CD29+CD73+) within the nonhematopoietic (CD45–) population. ViviD indicates violet viability dye. FSC-A, forward scatter area; SSC-A, Side scatter area.

and its constituent SVF cells. In agreement with our findings, they found a reduced proportion of CD45+ cells and enrichment of CD34+ and CD73+ cells in cryopreserved versus fresh cells.[20] However, their analysis relied mainly on a single surface marker analysis, whereas in the current study, characterization was performed by simultaneous analysis of multiple cell markers. The marked ability of CD45–CD34+CD31– and CD45–CD29+CD73+ SVF popu-

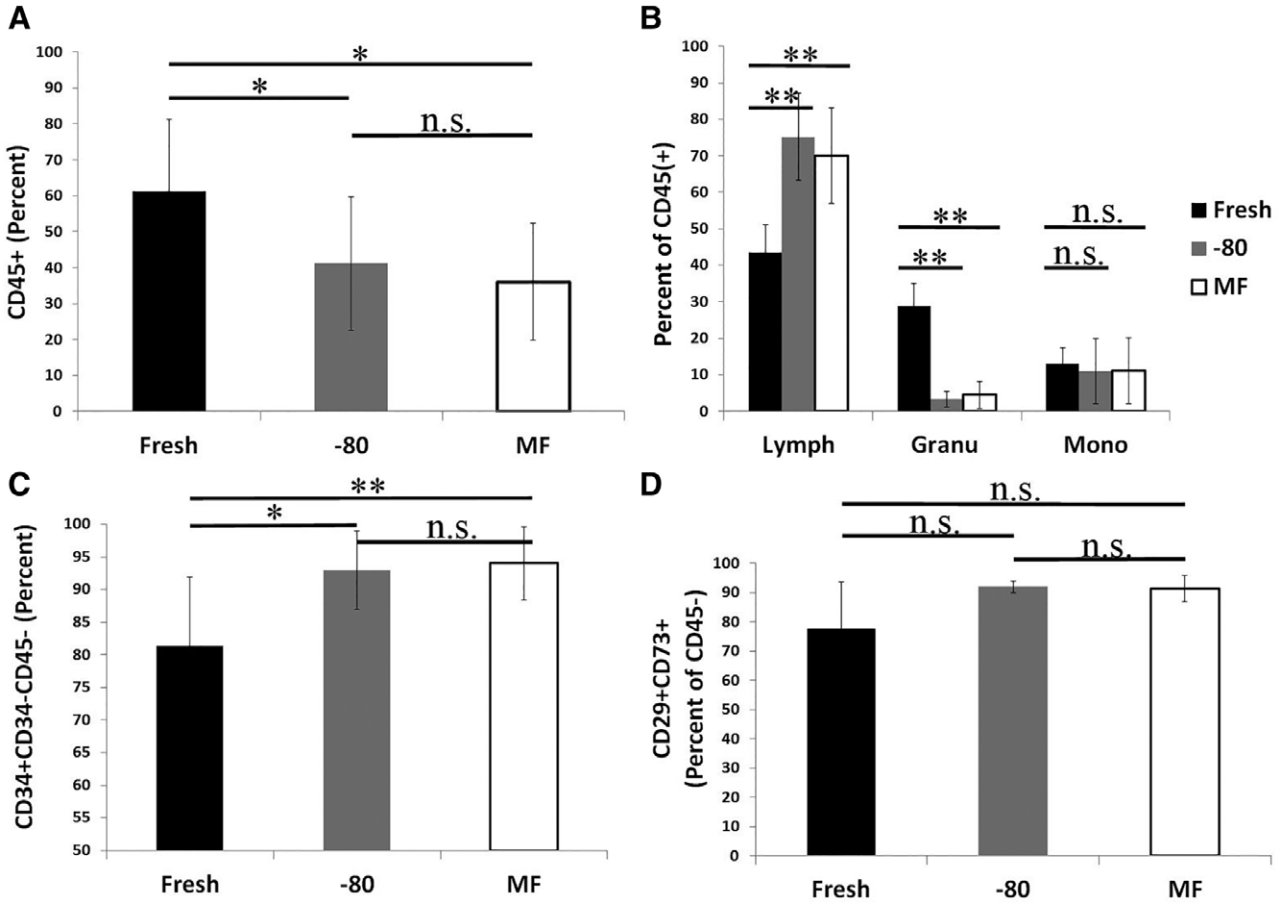

Fig. 5. Surface marker analysis demonstrates a reduction of hematopoietic cells and an enrichment of stem cell progenitor cell populations within SVF cells following cryopreservation. Fresh and cryopreserved SVF cells were analyzed using a 7-color flow cytometry panel, according to the gating strategy presented in Figure 4. The percent of SVF cells that were CD45+ (A), the percent of the different hematopoietic cell types within the hematopoietic (CD45+) population (B), and the percent of CD31−CD34+ (C) or CD29+CD73+ (D) within the nonhematopoietic (CD45−) population are shown. Data are presented as mean ± SD. **P* < 0.05, ***P* < 0.01. n.s., difference not statistically significant.

lations to survive cryopreservation offers a good explanation of how the cryopreserved SVF cells maintain their stem cell potency despite their reduced counts.

The rate of temperature reduction during freezing is considered an important factor in maintaining cell survival during cryopreservation.[14,30] Interestingly, in the current study, we observed no significant difference in the quantity or quality of survival of SVF cells following their cryopreservation by either immediately transferring them to a –80°C freezer in standard vials or freezing them in the MF freezing container, which was reported to provide a replicable, 1°C/min cooling rate required for successful cryopreservation of cells. Thus, according to our results, it seems that a slow and constant freezing rate, as is provided by MF, does not improve the quantity and quality of the cryopreserved SVF cells.

CONCLUSIONS

Long-term preservation of SVF cells will considerably enhance their clinical utility. We found that cryopreservation of SVF cells using simple, cost-effective, and easily accessible methods did not reduce their in vitro stem cell potency despite a significant reduction in their quantities. Further work will be needed to examine the effect of cryopreservation on the stem cell potency of SVF cells in vivo. For the most part, the number of SVF cells isolated from a single liposuction procedure is sufficient for several treatments. While the use of cryopreserved SVF cells for future treatments may require the extraction of a larger quantity of fat cells during the initial liposuction procedure, it will obviate the need for repeat lipoaspirations. Thus, cryopreservation may serve as a safe and low-cost method for SVF cell preservation to enable repeated use in orthopedics and plastic surgery treatments.

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