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Arthroscopic Harvest of Adipose-Derived Mesenchymal Stem Cells From the Infrapatellar Fat Pad

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Background: The successful isolation of adipose-derived mesenchymal stem cells (ADSCs) from the arthroscopically harvested infrapatellar fat pad (IFP) would provide orthopaedic surgeons with an autologous solution for regenerative procedures.

Purpose: To demonstrate the quantity and viability of the mesenchymal stem cell population arthroscopically harvested from the IFP as well as the surrounding synovium.

Study Design: Descriptive laboratory study.

Methods: The posterior border of the IFP, including the surrounding synovial tissue, was harvested arthroscopically from patients undergoing anterior cruciate ligament reconstruction. Tissue was then collected in an AquaVage adipose canister, followed by fat fractionization using syringe emulsification and concentration with an AdiPrep device. In the laboratory, the layers of tissue were separated and then digested with 0.3% type I collagenase. The pelleted stromal vascular fraction (SVF) cells were then immediately analyzed for viability, mesenchymal cell surface markers by fluorescence-activated cell sorting, and clonogenic capacity. After culture expansion, the metabolic activity of the ADSCs was assessed by an AlamarBlue assay, and the multilineage differentiation capability was tested. The transition of surface antigens from the SVF toward expanded ADSCs at passage 2 was further evaluated.

Results: SVF cells were successfully harvested with a mean yield of $4.86 \pm 2.64 \times 10^5$ cells/g of tissue and a mean viability of 69.03% ± 10.75%, with ages ranging from 17 to 52 years (mean, 35.14 ± 13.70 years; n = 7). The cultured ADSCs composed a mean 5.85% ± 5.89% of SVF cells with a mean yield of $0.33 \pm 0.42 \times 10^5$ cells/g of tissue. The nonhematopoietic cells ($CD45^-$) displayed the following surface antigens as a percentage of the viable population: $CD44^+$ (52.21% ± 4.50%), $CD73^+CD90^+CD105^+$ (19.20% ± 17.04%), and $CD44^+CD73^+CD90^+CD105^+$ (15.32% ± 15.23%). There was also a significant increase in the expression of ADSC markers CD73 (96.97% ± 1.72%; $P < .01$), CD10 (84.47% ± 15.46%; $P < .05$), and CD166 (11.63% ± 7.84%; $P < .005$) starting at passage 2 compared with freshly harvested SVF cells. The clonogenic efficiency of SVF cells was determined at a mean 3.21% ± 1.52% for layer 1 and 1.51% ± 0.55% for layer 2. Differentiation into cartilage, fat, and bone tissue was demonstrated by tissue-specific staining and quantitative polymerase chain reaction.

Conclusion: SVF cells from the IFP and adjacent synovial tissue were successfully harvested using an arthroscopic technique and produced ADSCs with surface markers that meet criteria for defined mesenchymal stem cells.

Clinical Relevance: An autologous source of stem cells can now be harvested using a simple arthroscopic technique that will allow orthopaedic surgeons easier access to progenitor cells for regenerative procedures.

Keywords: infrapatellar fat pad; synovium; adipose-derived stem cell; mesenchymal stem cell; stromal vascular fraction; adipose tissue

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In the past decade, regenerative medicine has quickly emerged into the forefront of orthopaedic research with the hope of cures for musculoskeletal conditions that were not feasible with conventional treatments. There are an increasing number of reports revealing the benefits of mesenchymal stem cell–based therapies in the treatment of osteoarthritis knee and cartilage injuries,[9,18,26,30,34,43,45] which include improved knee motion and decreased visual analog scale scores for pain with improved quality of life.[9,25,26,45]

Adipose-derived mesenchymal stem cells (ADSCs) have been recognized as a relevant cell source for cell-based

therapy because of their abundance and multipotency. Most of the progenitor cells are believed to reside in the stromal vascular fraction (SVF) of adipose tissue; this perivascular location appears to be consistent throughout most mesenchymal tissue.[13] Harvesting the SVF results in a heterogeneous mesenchymal population that includes adipose stromal and progenitor cells, endothelial cells, hematopoietic stem cells, and pericytes.[7] In laboratory cultures, a subset of SVF cells begin to adhere and grow into an elongated and homogeneous cell population known as ADSCs. ADSCs have the capacity to differentiate into adipocytes, chondrocytes, osteoblasts, or myoblasts under appropriate stimuli and culture conditions. In contrast to bone marrow–derived mesenchymal stem cells, ADSCs provide (1) greater availability (up to 10% of nucleated cells from ADSCs[4,52] vs 0.001% to 0.01% of bone marrow–derived mesenchymal stem cells[42]), (2) less donor site morbidity, and (3) rapid expansion and high proliferation potential[23] for various applications in the field of tissue engineering and regenerative medicine.[39,53] In addition, the intra-articular injection of ADSCs has been found to be a safe therapeutic alternative with no significant complications.[18,37,40]

ADSCs have traditionally been harvested via liposuction; however, this technique is unfamiliar to most orthopaedic surgeons. Alternatively, portions of the infrapatellar fat pad (IFP), an intracapsular, extrasynovial structure that fills the anterior knee compartment posterior to the patellar tendon, can be easily harvested through either open or arthroscopic techniques. It is structurally similar to subcutaneous adipose tissue with a rich vascular supply and has been reported to be a reliable source of mesenchymal stem cells.[12,25,29,41,47] Arthroscopic debridement of the IFP has been proved to be safe and provides significant pain relief and function restoration in patients with chronic fat pad inflammation (Hoffa disease).[12,15,28,31]

Previous studies have shown that IFP cells harvested via arthrotomy were multipotent and able to differentiate into chondrogenic and osteogenic phenotypes in cultures, while in vivo implantation led to the appearance of hyaline cartilage and bone.[17] In a side-by-side experiment with lipoaspirate-derived ADSCs, the bone production was equally efficient for engineered implants from either source.[16] In addition, the study published by Anz et al[2] confirmed the presence of viable stem cells in the synovial fluid, fat pad, and waste by-products of arthroscopic cruciate ligament surgery, which further supports this study that characterizes and evaluates the usefulness of the IFP and synovial-derived mesenchymal stem cells in tissue regeneration.

The goals of this study were to evaluate (1) the availability and viability of SVF cells and surrounding synovial cells immediately after arthroscopic harvest from the IFP, (2) the characterization of cell surface markers of the freshly isolated SVF cells by flow cytometry, (3) the consistency of immunophenotypes in culture-expanded ADSCs compared with a defined mesenchymal stem cell population, and (4) the clonogenic capability of SVF cells and multilineage differentiation capacity of expanded ADSCs.

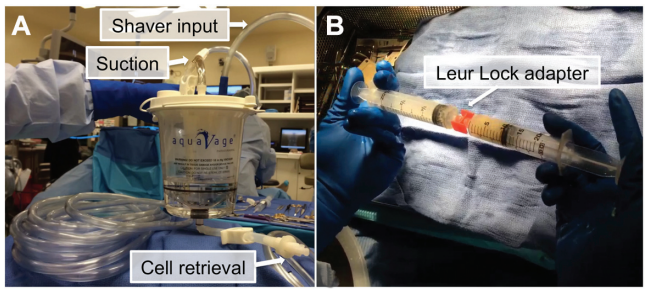

Figure 1. Intraoperative fat processing. (A) Arthroscopic setup of the fat collection system. (B) Fat fractionization using syringe emulsification.

METHODS

Donor Selection and Tissue Collection

After discussions with the Administrative Panel on Human Subjects Research, the posterior border of the fat pad, including the synovial lining that covers the IFP, was arthroscopically harvested from 7 patients undergoing routine anterior cruciate ligament (ACL) reconstruction without evidence of osteoarthritis. The majority of patients were women (6 female, 1 male), with ages ranging from 17 to 52 years (mean, 35.14 ± 13.70 years). Informed consent was obtained only to keep track of their outcome data from ACL reconstruction but not for the removal of a portion of the fat pad, as only fat impeding ACL visualization was removed and the institutional review board categorized the specimens as surgical waste products not requiring separate written consent.

The arthroscopic technique required a standard motorized shaver system with a synovial shaver attachment. Care was taken to keep the back of the shaver against the condyles so as not to overly resect the fat pad. Shaving was discontinued when visualization of the ACL was adequate to perform ACL reconstruction. Resected margins of the fat pad were then cauterized to prevent bleeding and possible arthrofibrosis.

The adipose tissue was collected in a US Food and Drug Administration–approved AquaVage (MD Resource) container system, which separated the fat and synovial tissue from saline (Figure 1A). The fat tissue was then subjected to fractionization with syringe emulsification, which consisted of transferring the adipose tissue between two 20-mL syringes using a luer lock adapter for 25 repetitions (Figure 1B). This step further homogenized the adipose tissue. The tissue was then processed by the AdiPrep system (Harvest Technologies) according to the manufacturer's instructions. After centrifugation, there were 2 layers: layer 1 settled at the bottom (likely synovium plus fragmented adipose tissue), separated from layer 2 (concentrated adipose tissue at the top of the AdiPrep tube) (Figure 2). Both layers were then immediately transferred to the research laboratory for further processing for the isolation of mesenchymal cells.

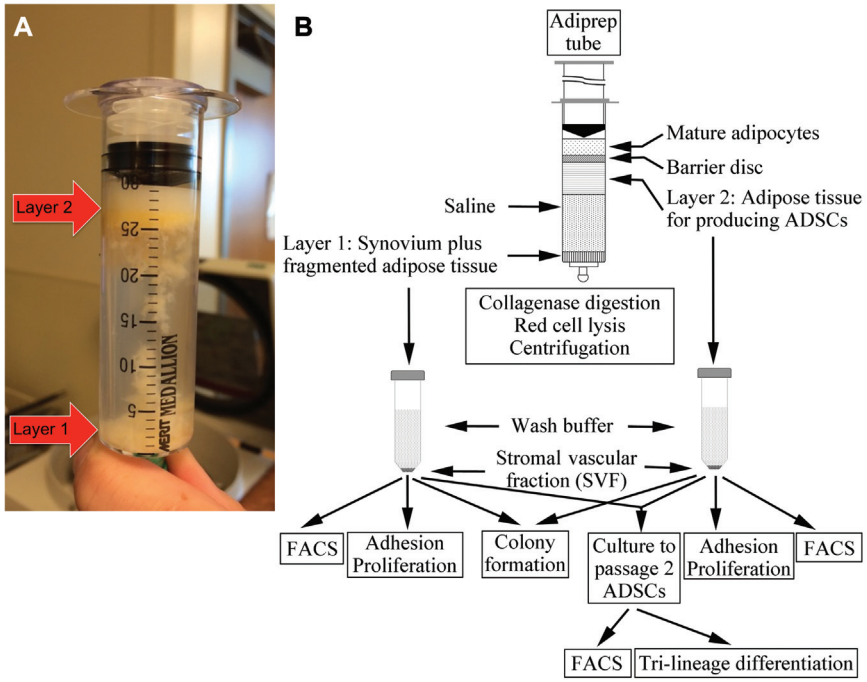

Figure 2. Harvest of the infrapatellar fat pad (IFP) cells. (A) Appearance of harvested tissue in the AdiPrep chamber showing the isolation of the stromal vascular fraction (SVF) from layer 1, synovium plus fragmented adipose tissue (lower arrow), and layer 2, concentrated adipose tissue (upper arrow). (B) Illustration of experimental procedures after the tissue concentration step with the AdiPrep tube. Note that only SVF cells from layer 2 were cultured to passage 2 adipose-derived mesenchymal stem cells (ADSCs) but not layer 1 because of limited tissue.

Cell Isolation and Culture

Cell extraction from adipose tissue has been described previously.[17] Briefly, both layers were transferred into separate tubes for weight measurement and then digested in phosphate-buffered saline (PBS) supplemented with 0.3% type I collagenase (Worthington Biochemical), 1% bovine serum albumin (Sigma-Aldrich), and 2 mM $CaCl_2$ for 2 hours at 37°C with continuous agitation. Released cells were washed in PBS, and red blood cells were removed by incubating in erythrocyte lysis buffer (Sigma-Aldrich). The SVF nucleated cells were obtained by centrifugation at 300*g* for 5 minutes from both layers (Figure 2B). Cell pellets were then resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1× antibiotic/antimycotic (Thermo Fisher Scientific), and 50 μg/mL of ascorbic acid (Sigma-Aldrich). The viability of cells was determined by Trypan Blue exclusion assay (Thermo Fisher Scientific), and the yield was calculated using a hemocytometer. SVF cells from each layer were subjected to flow cytometric analysis immediately. For assays of colony formation, SVF cells were seeded at 125 cells/cm^2 in dishes and cultured for 10 days. Colonies were visualized by 0.05% crystal violet staining. Only colonies with a size of at least 2 mm in diameter were scored. Because of tissue availability, only cells isolated from layer 2 were seeded onto culture plates in Dulbecco's modified Eagle's medium at 1×10^5 cells/cm^2 for expansion. After 24 hours' incubation at 37°C and 5% CO_2, nonattached cells were washed off, and adherent cells were maintained in cultures until they reached 90% confluence and were classified as passage 0. The cultures were trypsinized and subcultured for up to passage 2 before further flow cytometric analysis for the characterization of ADSCs.

Flow Cytometric Analysis

Freshly isolated SVF cells from both layers and trypsin-harvested passage 2 ADSCs (layer 2 only) were washed twice with PBS, followed by one wash with fluorescence-activated cell sorting (FACS) media (1× Hank's balanced salt solution, 0.5% bovine serum albumin, 1% fetal calf serum), and aliquots of 1×10^5 cells were incubated with fluorochrome-conjugated mouse anti-human antibodies. The first panel of antibodies used for the identification of freshly harvested SVF cells was APC-Cy7-CD45 (lymphocyte common antigen; BioLegend), FITC-CD44 (hyaluronan receptor), APC-CD73 (ecto-5-nucleotidase), PE-Cy7-CD90 (Thy-1), and PE-CD105 (endoglin; Becton Dickinson); and the second panel used for both SVF cells and passage 2 ADSCs was APC-Cy7-CD10 (neprilysin; BioLegend), PE-Cy7-CD13 (aminopeptidase N), FITC-CD44, APC-CD73, and PE-CD166 (activated leukocyte cell adhesion molecule; Becton Dickinson). The corresponding fluorochrome-conjugated IgGκ antibodies were used as nonspecific binding controls. Cells were incubated with antibodies for 20 minutes at 4°C and then resuspended in FACS media

TABLE 1
Arthroscopic Harvest of Fat Versus Synovial Tissue From Infrapatellar Fat Pad[a]

	Layer 1	Layer 2	*P* Value
Weight of tissue, g	0.74 ± 0.40	2.95 ± 1.92	NS
Nucleated cell yield per gram of tissue at isolation[b] ($\times 10^5$)	4.86 ± 0.28	4.86 ± 2.64	NS
Adherent cells capable of proliferation from nucleated cells,[c] %	4.05 ± 5.66	5.85 ± 5.89	NS
Adherent cells capable of proliferation per gram of tissue ($\times 10^5$)	0.19 ± 0.23	0.33 ± 0.42	NS

[a]Data are presented as mean ± SD. NS, not significant.
[b]Nucleated cell yield was determined by cell counts with Trypan Blue exclusion assay at isolation.
[c]Proliferation-capable cells were determined by AlamarBlue/proliferation assay with exponentially growing adipose-derived mesenchymal stem cells as standard curves.

and analyzed immediately. To distinguish live from dead cells, 3 μM of DAPI (4',6-diamidino-2-phenylindole; Thermo Fisher Scientific) was added to the staining reaction in the last 5 minutes. For each sample, 10,000 events were collected on a FACScan flow cytometer (Becton Dickinson) using FlowJo Collector's Edition software and analyzed using FlowJo software (Tree Star).

Cell Proliferation Assay

The yield of adherent cells capable of proliferation in culture conditions was assessed by the metabolic activity assay with AlamarBlue reagent (Thermo Fisher Scientific).[35] This assay was used to measure cell viability, or proliferation, by assessing the oxidation-reduction status or metabolic activity resulting from the living cells. The increase of cell metabolic activity during cultivation is related to cell division and proliferation. Briefly, freshly harvested SVF cells from each layer were seeded in 48-well culture plates at 20,000 cells/well in triplicate with culture media and cultured for 14 days with a medium change every other day. In parallel, ADSCs, generously provided by Dr Jeffrey Gimble,[51] were seeded at a 2-fold serial dilution to generate a standard curve for cell proliferation. The assay was performed at days 0, 2, 4, 6, 8, 10, 12, and 14 by adding AlamarBlue reagent to the final 10% in each well. After 3 hours' incubation, 100 μL of the culture medium was transferred into 96-well plates, and the fluorescent signals were determined by using a spectrophotometer plate reader (SpectraMax M2; Molecular Devices) at 540 nm for excitation and 590 nm for emission. The raw fluorescent values were plotted against the standard curve to obtain the number of living cells with SoftMax Pro software (Molecular Devices).

Trilineage Differentiation

The multilineage differentiation potential of the expanded ADSCs toward cartilage, fat, and bone tissue has been described previously.[11,14,24] Chondrogenic differentiation was induced in pellet cultures for 21 days, initiated from 2.5×10^5 cells. Frozen sections (9 μm) from pellets were stained with 0.1% safranin O (Sigma-Aldrich) for aggrecan and hematoxylin for nuclei. Moreover, 80% to 90% confluent cultures were used for adipogenic and osteogenic induction. Lipid droplets in adipogenic differentiation was assessed by 0.2% Oil Red O staining. Calcium deposition was visualized by 2% Alizarin Red S staining. Tissue-specific mRNA expression was determined by quantitative polymerase chain reaction (PCR) as described previously,[24] with TaqMan primers (Applied Biosystems) for type II collagen (Col2A1), aggrecan, SOX-9, PPAR-γ, RUNX-2, Oct4, and 18S RNA as internal reference primers. To reveal the synthesis of glycosaminoglycans during chondrogenesis, the glycosaminoglycan content of pellets was measured by a 1,9-dimethylmethylene blue assay.[24]

Statistical Analysis

Statistical analysis was carried out using SAS 9.4 software (SAS Institute). A P value of $<.05$ was considered significant. For cell proliferation, the standard curve of fluorescent signals was plotted, and linear regression analysis was conducted to validate standard curves. The Student t test (2-tailed) was used for the intergroup comparison of viability, cell yield, proliferation, surface marker expression, gene expression, and glycosaminoglycan content between both layers, induced and noninduced groups, and freshly isolated SVF cells versus cultured ADSCs (passage 2).

RESULTS

SVF and ADSC Yield

The weight of the harvested tissue was variable, but in general, the synovial tissue (layer 1) fraction was about one-fifth that of the adipose fraction (layer 2) (Table 1). The mean SVF cell yield was $4.86 \pm 2.64 \times 10^5$ cells/g from layer 2, similar to $4.86 \pm 0.28 \times 10^5$ cells/g from layer 1. An individual specimen weight of 0.3 g was required to perform both the FACS analysis and adhesion/proliferation assay; therefore, 4 of 7 layer 1 samples qualified and were included in the final analysis, while all 7 adipose samples were used in this study. The mean viability of freshly isolated SVF cells was 69.03% ± 10.75% from layer 2 and 66.88% ± 12.98% from layer 1.

The capacity to adhere and proliferate in regular culture conditions is a hallmark of stem cells, including ADSCs.[7] After we seeded SVF cells into culture vessels, the adherent cells were allowed to proliferate for up to 2

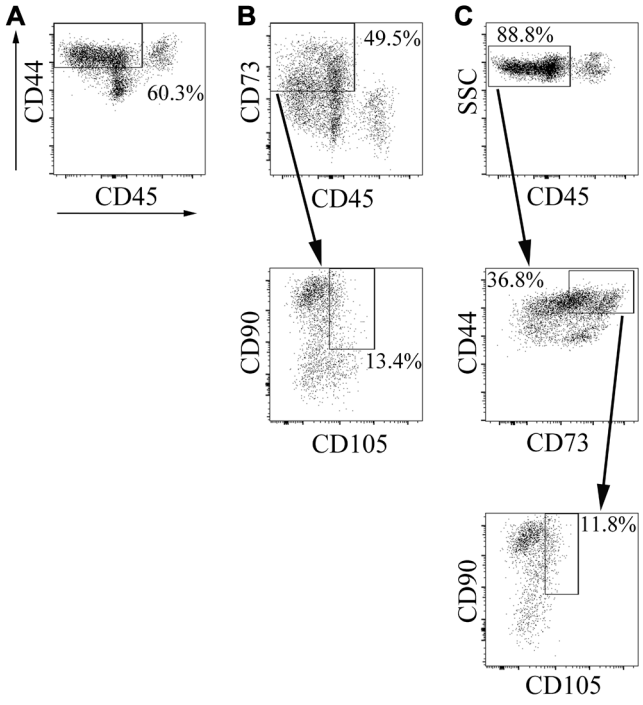

Figure 3. Representative results from the fluorescence-activated cell sorting analysis of freshly isolated stromal vascular fraction (SVF) cells from layer 2 for (A) synovial markers ($CD45^-CD44^+$), (B) mesenchymal markers ($CD45^-CD73^+CD90^+CD105^+$), and (C) final gating ($CD45^-CD44^+CD73^+CD90^+CD105^+$). The numbers represent the percentage of gated cells over live cells as the parental population.

weeks, and the proliferation capacity was monitored by the AlamarBlue assay.[36,50] The results showed that 4% to 6% of SVF cells were capable of adhesion and proliferation under normal culture conditions from both layers. Although the number of ADSCs per gram of tissue was higher in layer 2 compared with layer 1, there was no significant difference.

Flow Cytometric Analyses for SVF Cells

To characterize the immunophenotype of the SVF or culture-expanded ADSC progenitor cells, 2 antibody panels were selected based on the International Society for Cellular Therapy (ISCT) position statement on the criteria for defining progenitor cells derived from adipose tissue[7,33] including CD44, as it is one of the most common synovial surface markers.[8] The first panel of antibodies (CD45, CD44, CD73, CD90, CD105) was chosen for the detection of progenitor cells within the freshly isolated SVF. [6,7,10,27,44] The representative results of the SVF cells that displayed nonhematopoietic and synovial markers ($CD45^-CD44^+$), mesenchymal markers ($CD45^-CD73^+CD90^+CD105^+$), and progenitor cell markers ($CD45^-CD44^+CD73^+CD90^+CD105^+$) are shown in Figure 3.

The viability of the harvested cells was assessed by DAPI staining, followed by the FACS analysis that exhibited a mean viability close to 70% for the SVF from both layers:

TABLE 2
Surface Marker and Viability of Stromal Vascular Fraction Cells by Flow Cytometric Analysis[a]

Marker	Layer 1	Layer 2	*P* Value
$DAPI^-$ (live)	66.88 ± 12.98	69.03 ± 10.75	NS
$CD45^-$	80.28 ± 6.41	80.29 ± 7.45	NS
$CD44^+$	81.25 ± 9.58	71.97 ± 6.77	NS
$CD73^+$	62.50 ± 12.15	60.44 ± 6.39	NS
$CD90^+$	71.95 ± 7.31	65.20 ± 10.25	NS
$CD105^+$	34.30 ± 28.12	33.41 ± 22.03	NS
$CD45^-CD44^+$	63.16 ± 10.91	52.21 ± 4.50	NS
$CD45^-CD73^+CD90^+CD105^+$	22.33 ± 25.79	19.20 ± 17.04	NS
$CD45^-CD44^+CD73^+CD90^+CD105^+$	20.66 ± 25.85	15.32 ± 15.23	NS

[a]Data are presented as mean ± SD of the percentage of cells staining with anti-human mouse IgG antibodies. The matched isotype antibodies are used as negative controls. NS, not significant.

layer 1 = 66.88% ± 12.98% and layer 2 = 69.03% ± 10.75% (Table 2). The freshly harvested SVF cells of layer 2 displayed the percentage of cells staining positive for the following surface markers: $CD45^-$ (80.29% ± 7.45%), $CD44^+$ (71.97% ± 6.77%), $CD73^+$ (60.44% ± 6.39%), $CD90^+$ (65.20% ± 10.25%), and $CD105^+$ (33.41% ± 22.03%). In contrast, isotype control antibodies displayed nonspecific binding ranging from 1.13% to 1.47%, with a mean of 1.31% ± 0.23%. To demonstrate markers for subpopulations, multicolor analysis exhibited the percentage of cells for the following groups: $CD45^-CD44^+$ (52.21% ± 4.50%), $CD45^-CD73^+CD90^+CD105^+$ (19.20% ± 17.04%), and $CD45^-CD44^+CD73^+CD90^+CD105^+$ (15.32% ± 15.23%). Similar to layer 2, the SVF cells isolated from layer 1 revealed the percentage of cells for the following surface markers: $CD45^-$ (80.28% ± 6.41%), $CD44^+$ (81.25% ± 9.58%), $CD73^+$ (62.50% ± 12.15%), $CD90^+$ (71.95% ± 7.31%), and $CD105^+$ (34.30% ± 28.12%); they also showed the percentage for the following subpopulations: $CD45^-CD44^+$ (63.16% ± 10.91%), $CD45^-CD73^+CD90^+CD105^+$ (22.33% ± 25.79%), and $CD45^-CD44^+CD73^+CD90^+CD105^+$ (20.66% ± 25.85%). Among all the individual surface markers, CD105 presented the lowest positive rate, with a noticeable variation that contributed to the higher variation in the multicolor analysis for each subpopulation. Taken together, these results suggested that the processing procedure for cell isolation (1) achieved good initial cell viability, (2) did not prevent cell adherence and eventual expansion to ADSCs, and (3) produced a similar percentage of ADSCs as reported in previous peer-reviewed analyses.[33,49]

Surface Markers of Expanded ADSCs Resemble Mesenchymal Stem Cells

Once the ADSCs expand in cultures, they gradually change their expression of surface markers.[7] We evaluated this surface antigen transition in 3 samples by comparing the original SVF cell population to expanded ADSCs at

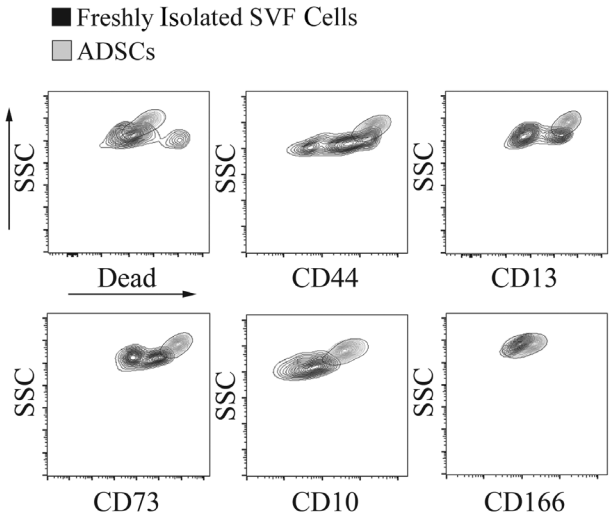

Figure 4. The change of surface markers from freshly isolated stromal vascular fraction (SVF) cells to cultured passage 2 adipose-derived mesenchymal stem cells (ADSCs) resembles the immunophenotype of defined mesenchymal stem cells.

passage 2 with a second panel of antibodies, as suggested by the ISCT position statement and others.[7,32,33,49] This antibody panel, including CD44, CD13, CD73, CD10, and CD166, was applied first to the freshly isolated SVF cells from layer 2 and then to the corresponding ADSCs expanded up to passage 2 in cultures. The representative results of flow cytometric analysis are shown in Figure 4.

The cultured ADSCs exhibited high viability (95.40% ± 3.36%) compared with the lower viability of freshly isolated SVF cells (78.63% ± 10.08%) (Table 3). The freshly harvested SVF cells revealed the percentage of positive cells for the following markers: $CD44^+$ (76.27% ± 10.80%), $CD13^+$ (56.47% ± 21.48%), $CD73^+$ (40.77% ± 8.57%), $CD10^+$ (13.95% ± 11.48%), and $CD166^+$ (not detectable). After the cultures, the expression of all 5 surface markers in passage 2 ADSCs increased to the following: $CD44^+$ (96.60% ± 4.85%), $CD13^+$ (94.33% ± 6.30%), $CD73^+$ (96.97% ± 1.72%), $CD10^+$ (84.47% ± 15.46%), and $CD166^+$ (11.63% ± 7.84%). Among these 5 surface antigens, an increase of 3 markers was statistically significant: CD73 ($P < .01$), CD10 ($P < .05$), and CD166 ($P < .005$). Although CD44 and CD13 were expressed in very high levels in ADSCs after expansion, their moderate expression in SVF cells reduced the statistical results below significant levels. Because the expression of CD166 was not detectable in SVF cells, and the lowest in ADSCs after expansion, the multicolor analysis for all 5 markers resembled the results similar to CD166 alone: not detectable in SVF cells and 10.35% ± 7.76% in ADSCs.

SVF Cell Clonogenic Capacity

To characterize the clonogenic capability of SVF cells, we seeded single cell suspensions from both layer 1 and layer 2 in culture dishes. After 10 days, colonies that arose from

TABLE 3
Flow Cytometric Analysis of Freshly Isolated SVF Cells Versus Passage 2 ADSCs[a]

Marker	SVF Cells	Passage 2 ADSCs	*P* Value
$DAPI^-$ (live)	78.63 ± 10.08	95.40 ± 3.36	NS
$CD44^+$	76.27 ± 10.80	96.60 ± 4.85	NS
$CD13^+$	56.47 ± 21.48	94.33 ± 6.30	NS
$CD73^+$	40.77 ± 8.57	96.97 ± 1.72	<.01
$CD10^+$	13.95 ± 11.48	84.47 ± 15.46	<.05
$CD166^+$	ND	11.63 ± 7.84	<.005
$CD44^+CD13^+CD73^+CD10^+CD166^+$	ND	10.35 ± 7.76	<.05

[a]Data are presented as mean ± SD of the percentage of cells staining positive with anti-human mouse IgG antibodies. The matched isotype antibodies are used as negative controls. ADSCs, adipose-derived mesenchymal stem cells; ND, not detectable; NS, not significant; SVF, stromal vascular fraction.

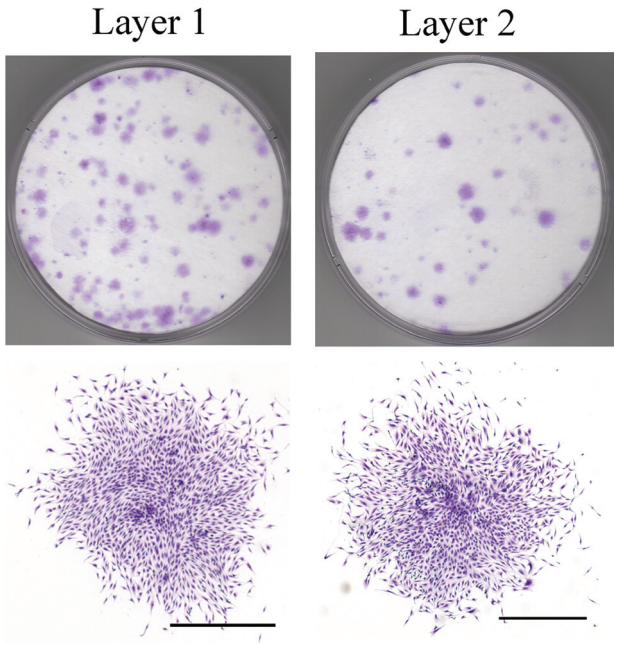

Figure 5. Colony formation of freshly isolated stromal vascular fraction (SVF) cells. Individual colonies are shown in the lower panel. Scale bar = 1 mm.

single cells were visualized by crystal violet staining (Figure 5). The clonogenic efficiency of SVF cells was determined at a mean 3.21% ± 1.52% for layer 1 and 1.51% ± 0.55% for layer 2. These colonies were heterogeneous in size potentially because of the differences in cell proliferation.

Expanded ADSCs Have Trilineage Differentiation Potential

The multilineage differentiation potential of the expanded ADSCs toward cartilage, fat, and bone tissue was

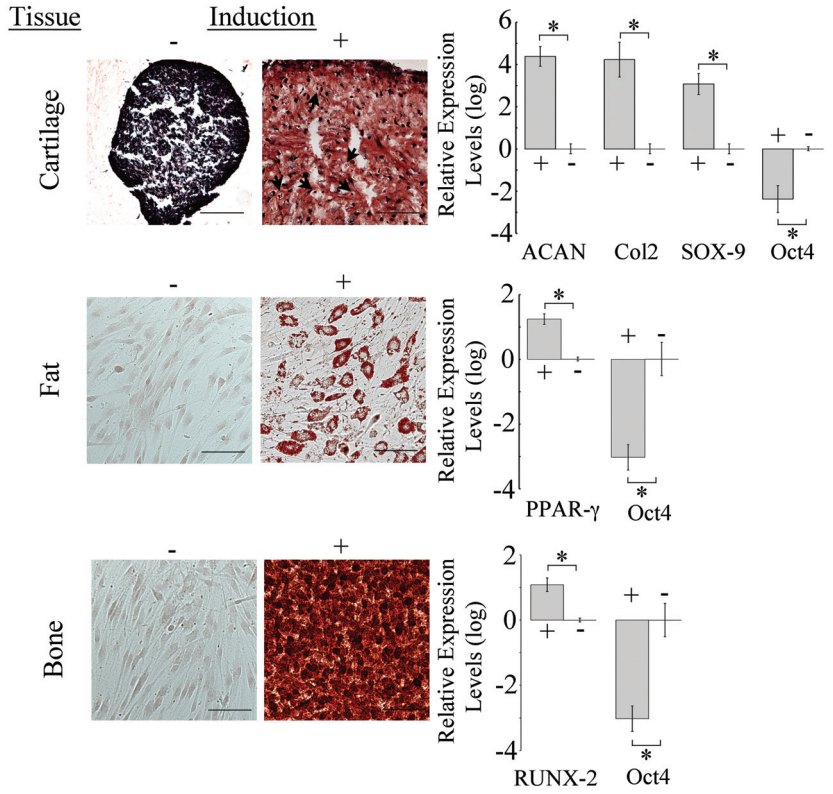

Figure 6. Multilineage differentiation of adipose-derived mesenchymal stem cells (ADSCs). (Left panels) Histological staining and (right panels) quantitative polymerase chain reaction results. Arrows indicate the appearance of cartilage lacunae containing chondrocytes. *$P < .001$. Scale bar = 100 μm.

determined by tissue-specific staining and gene expression. Chondrogenic differentiation was assessed by safranin O staining for the proteoglycan-rich extracellular matrix (Figure 6). The appearance of chondrocyte-specific lacunae revealed the successful differentiation process toward cartilage. Oil Red O staining of lipid droplets revealed adipogenic differentiation, while calcium deposition during bone induction was visualized by Alizarin Red staining. Tissue-specific gene expression was assessed by quantitative reverse transcription PCR analysis to further support lineage-specific differentiation. The expression of the chondrogenic markers aggrecan, type II collagen, and SOX-9 was dramatically increased ($P < .001$) in cartilage induction as well as the glycosaminoglycan content (8.70 ± 1.63; $P < .001$) compared with the non-induced group. As a marker for adipogenesis, the expression of PPAR-γ was significantly elevated ($P < .001$) after induction. In addition, the osteogenic marker RUNX-2 was highly expressed ($P < .001$) after bone induction. In contrast, the stemness marker Oct4 was significantly reduced ($P < .001$) in all differentiation conditions.

DISCUSSION

In this study, we described the successful separation of both the IPF and surrounding synovial cells using standard arthroscopic instruments, with initial processing performed in the operating room. We also showed that this tissue is a reliable source of ADSCs and that the cells were able to adhere and proliferate under normal culture conditions as ADSCs with clonogenic capability, and the expanded ADSCs maintained the surface markers as defined ADSCs with multilineage differentiation potential.

Mesenchymal stem cells derived from different tissue sources share a similar capacity for multilineage differentiation and immunophenotypic characteristics, but there has been a noted difference in growth, apoptosis tolerance, and differentiation tendency in previous studies.[20,46] Adipose tissue proved to be an abundant and accessible source for mesenchymal stem cells, and ADSCs possessed the highest proliferation potential and higher induction capacity than other mesenchymal stem cell sources.[38] By analysis of mesenchymal stem cells from bone marrow taken from the iliac crest, Pittenger et al[42] reported that 1 mL of bone marrow yielded approximately 6×10^6 nucleated cells, with only 0.001% to 0.01% of bone marrow–derived mesenchymal stem cells. On the other hand, adipose tissue yielded approximately 2×10^6 cells/g of tissue, and up to 10% of those were ADSCs.[4,52] As an alternative source of ADSCs, the IFP has particular relevance with respect to the homologous application of stem cells for regenerative medicine procedures in the knee. In addition, expanded ADSCs from the IFP have previously shown a stronger

chondrogenic potential, with a significantly higher expression of SOX-9 expression compared with subcutaneous adipose tissue.[47] Our mean yield of freshly harvested SVF cells from both layers at 4.86×10^5 cells/g was comparable with previously reported results by Jurgens et al[22] with a mean of 4.4×10^5 cells/g (N = 53). Furthermore, our results showed the clonogenic ability of IFP-derived SVF cells at 6986 ± 2797 colonies/g of tissue, which is much higher than the average 486 to 612 colonies/mL of bone marrow from bone marrow–derived mesenchymal stem cells.[5,19]

The antibody panels for surface markers were selected following the recommendation from the ISCT position statement on the criteria for defining mesenchymal stem cells.[7] Our results excluded the hematopoietic cells with CD45 staining and showed medium to high expression of CD44 as a common synovial marker and CD73 and CD90 as mesenchymal stem cell markers in SVF cells, which were comparable with previous studies.[22,33] In those studies, the expression of CD105 was relatively low at 4.3% and 4.9%, respectively, compared with our findings of 20.66% (layer 1) or 15.32% (layer 2) at final gating. In addition, the expression of CD105 has been associated with increased type II collagen, aggrecan, and SOX-9 expression during chondrogenic induction from synovial-derived mesenchymal stem cells,[3] and $CD105^+$ ADSCs also exhibited an enhanced chondrogenic potential.[21] Taken together, these results suggest that the freshly isolated SVF cells extracted from both layers in this study may possess a stronger chondrogenic potential than other reports. We demonstrated the multilineage differentiation of ADSCs, including chondrogenesis, in this study. It has been shown that synovium can produce hyaline cartilage in benign synovial disorders, such as synovial chondromatosis,[1] which suggests its potential for articular cartilage repair. Additionally, synovial-derived mesenchymal stem cells from both the knee and the shoulder have shown a greater potential for self-renewal and chondrogenic differentiation than those derived from bone marrow, periosteum, adipose tissue, muscle, or tendon.[44,48] In this study, we demonstrated the successful isolation of SVF cells from the arthroscopically harvested IFP and surrounding synovium. These cells displayed a similar epitope profile as previously reported SVF cells but with an even higher $CD105^+$ rate, which supports the enhanced chondrogenic potential of synovial-derived mesenchymal stem cells.

As one of the criteria for mesenchymal stem cells by the ISCT position statement,[7] we demonstrated that 3.21% (layer 1) and 1.51% (layer 2) of SVF cells possessed the capability of colony formation. These results were comparable with the fibroblastoid colony-forming unit derived from the IFP that varied between 2.6% and 3.13%.[22,47] The SVF cells gradually changed their cell surface markers while in cultures toward the ADSC immunophenotype. This evolution was demonstrated by the FACS analysis with the second panel of antibodies. Our findings confirmed the increased expression of a synovial marker (CD44) and stromal-mesenchymal markers (CD10, CD13, CD73, CD166) in expanded ADSCs[7,22,47] with lower CD166 signals. It has been shown that CD166 expression is low initially but then becomes significant in expanded ADSCs until passage 4.[33,49]

Overall, these results of the transition of surface markers from freshly isolated SVF cells to expanded ADSCs were comparable with those defined by the ISCT position statement,[7] which suggested that these expanded ADSCs possess multilineage capacity to differentiate into various cell lineages under appropriate stimuli. Although the clinical and therapeutic relevance of these markers remains to be elucidated, these results suggest that the expanded ADSCs resembled well-defined mesenchymal stem cells.

The present study has some limitations. The small number of samples and the individual variation of cell surface marker profiles made a statistical evaluation difficult. In addition, the differentiation potential of mesenchymal stem cells may vary from patient to patient, and the cell yield from each donor may change based on their age, sex, previous surgery, injury, and other conditions. Although we demonstrated the chondrogenic capacity of these cells in vitro and in the animal model,[13] we do recognize the lack of human in vivo studies. We did not compare the results of fat pad harvest with mesenchymal stem cells isolated from bone marrow or other tissue. In addition, we did not investigate in vivo data that are necessary to elucidate the potential of these SVF cells or ADSCs in cell-based therapy.

In summary, we demonstrated the successful harvest and maintenance of the viability of arthroscopically harvested adipose and synovial tissue from the IFP and the clonogenic capacity of SVF cells, which were comparable with published results. The transition of surface markers from SVF cells to expanded ADSCs at passage 2 was documented and resembled defined mesenchymal stem cells. The multilineage differentiation capability of the ADSCs into cartilage, fat, and bone was also demonstrated. The arthroscopic availability and accessibility of the IFP, and its surrounding synovium, may provide an alternative source of homologous stem cells for orthopaedic surgeons to use for tissue regeneration procedures in the knee.

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