WHITE PAPER

Comparison of Stromal Vascular Fraction to Expanded MSCs

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Abstract

Multipotent mesenchymal stromal/stem cells (MSCs) have the potential to repair and regenerate damaged tissues, making them attractive candidates for cell-based therapies. Expanded and well-characterized MSCs have application in regenerative medicine and have been used in several clinical trials including osteoarthritis and other conditions, while the crude preparation of MSCs, the "stromal vascular fraction" (SVF) has also been subject to clinical trials. Here, we provide results of a comparative study of SVF and expanded MSCs from fat, placenta, and umbilical cord involving determination of phenotype by flow cytometry analysis and cellular potency by quantitative assessment of mitochondrial function and immunosuppression. Our results show substantial differences indicating that SVF does not achieve international standards of the stem cell phenotypic definition, nor cellular potency comparable to expanded and purified MSCs derived from fat, umbilical cord, or placenta.

Introduction

Mesenchymal stem cells (MSCs) are multipotent, non-differentiated cells that have the ability of self-renewal, proliferation, and conversion into differentiated cells and regenerated tissues. Applications of regenerative medicine technology may offer novel therapies for patients with injuries, end-stage organ failure, degenerative diseases, and several other medical conditions. Transplanted MSCs have shown potential therapeutic benefits and safety in myocardial, musculoskeletal, neurological, autoimmune disorders, and several other disorders (1, 2, 3, 4). MSCs are isolated from several tissues including lipoaspirates, perinatal tissues, cord blood, teeth, etc and have considerable capacity for *in vitro* expansion and broad regenerative potential. These properties make MSCs attractive candidates for cell-based therapies.

In the United States, stem cells derived from expanded MSCs are presently excluded from clinical use since the FDA classifies this product as a drug requiring a New Drug Application (NDA) submission, multiple clinical trials, and approval prior to marketing. No MSC-based therapies are yet approved for clinical application in the US while hematopoietic stem cells are FDA-approved for clinical use. Other countries have different regulatory requirements for commercial approval of stem cell therapies.

Several clinics in the US provide a same surgical procedure whereby fat, bone marrow aspirate, or placental tissue is collected, followed by extraction of MSCs through enzymatic treatment and centrifugation to generate the stromal vascular fraction (SVF). Therapy consists of injection of autologous or allogeneic SVF, while that generally has a favorable safety record, also has associated severe adverse events including blindness (5). SVF collection, processing, and administration are conducted on an out-patient basis.

Clinical trials based on expanded MSCs are common internationally, although there are variations in the degree of regulation including the requirements for adherence to cGMP standards and rigorous quality control (QC) release criteria. Here, we report on phenotypic and functional characterization of the SVF from adipose and placental tissues compared to expanded MSCs derived from adipose tissue, placenta,

and umbilical cord. Our results show superior performance of expanded MSCs compared to SVF on a cellular basis. We also discuss clinical findings using SVF and expanded MSCs.

Materials and Methods

Adipose SVF Isolation and Expansion

Lipoaspirate of subcutaneous fat was obtained from ZenBio (Cat. No. T-SQ-50ML). Stromal vascular fraction (SVF) was separated and collected using a modified procedure (6). Lipoaspirate was diluted (1:2) with 10mM HEPES-buffered saline (HBS), pH 7.4, and centrifuged at 430 x g for 10 min at 4°C. The supernatant was digested in 0.2PZ U/mL collagenase (Serva, Cat. No. 17458.03) for 90 min at 37°C. Mature adipocytes and connective tissues were separated from the cell pellet by centrifugation at 600 × g, for 4 min at 4°C. The cell pellet was resuspended in 5% dextrose lactated ringer solution (Spectrum, Cat. No. DE140). The cell suspension was then filtered through a 40 µm mesh filter (Corning, Cat. No. 431750). Total and viable mononucleated cells (MNCs) were measured using acradine orange (Life Technologies, Cat. No. 83568) and propidium iodide (Invitrogen, Cat. No. P3566) exclusion test in an automated cell counter (Life Technologies, Countess FL II Cell Counter). A portion of the cells were used for flow cytometry analysis, selecting only MNCs, γ-IFN-induced IDO activity analysis, and mitochondrial quantitation. For the isolation and expansion of AD-MSCs, the remaining cells of the SVF were plated in TC-coated T75 flasks (B.D. Falcon, Cat. No. 353136) in MSC-Gro™ low serum medium (Vitro Biopharma, Cat. No. SC00B1) plated at 15,000/cm^2 viable MNCs. This initial passage of the primary cell culture was referred to as passage 0 (P_0). Cells were maintained in media until they achieved 75%–90% confluency. The cells were passed until passage 2 (P_2) and were analyzed for cell count, viability, flow cytometry, potency by immunosuppression quantitation of γ-IFN-induced IDO activity (7), and cell-specific ATP, an independent measure of stem cell potency (8). The identity of AD-MSCs were defined by using the criteria published by the ISCT (10).

Isolation and Expansion of Placenta Decidua Basalis and Umbilical Cord Wharton's Jelly

A fresh portion of placenta and entire umbilical cord were obtained from women with healthy pregnancies during caesarean or natural deliveries at the end of gestation, and with signed informed consent. Umbilical cord (UC) and placenta decidua basalis (P-DB) were collected in phosphate-buffered saline (PBS) (Cryopoint LLC), supplemented with 1x penicillin/streptomycin and transported to the laboratory at 2° to 8 °C. UC and P-DB were washed with PBS (Fisher Scientific, Cat. No. BP655-1) within a sterile laminar flow biological safety cabinet and cut into 5 cm^2 segments. The segments were cut longitudinally, blood vessels were removed, and segments transferred to 50mL conical tubes. The segments were suspended in 0.2PZ U/mL collagenase digest (Serva, Cat. No. 17458.03) and incubated at 37°C for 120 min. UC and P-DB digest was filtered through a 120-µm Buchner funnel. Collected cells were centrifuged at 450 x g for 15min at 4°C. The UC-SVF and P-SVF cell pellets were resuspended in 10-ml phosphate buffered saline (PBS) and filtered through a 40-µm mesh filter (Corning, Cat. No. 431750). Total and viable mononucleated cells (MNCs) were determined using acradine orange (Life Technologies, Cat. No. 83568) and propidium iodide (Invitrogen, Cat. No. P3566) exclusion test and in an automated cell counter (Life Technologies, Countess FL II Cell Counter). A portion of the P-SVF was used for flow cytometry analysis, immunomodulatory analysis, and cell-specific ATP determination. For the isolation and expansion of UC-MSC and P-MSC, the remaining cells of the UC-SVF and P-SVF were plated in T-75 flasks in MSC-Gro™ low serum medium (Vitro Biopharma, Cat. No. SC00B1) at 15,000/cm^2 viable MNCs. This initial passage of the primary cell culture was referred to as passage 0 (P_0). Cells were maintained in media at 5% CO_2, 5% O_2 in a humidified cell culture incubator (HeraCell, Model 240i) until

they achieved 75%–90% confluence. The cells were passed until passage 2 (P_2) and then analyzed for cell count, viability, flow cytometry, immunomodulatory potency, and mitochondrial functional quantitation. The identity of UC-MSC (11) and P-MSC (12) cells were determined using the ISCT proposed phenotypic criteria, i.e. CD_{11b}-; CD_{14}-; CD_{19}-; CD_{34}-; CD_{45}-; CD_{73}+; CD_{79A}-; CD_{90}+; CD_{105}+; HLR-DR- (10).

Phenotypic Analysis by Flow Cytometry

AD-SVF and P-SVF were blocked with 1.5% HSA and stained with anti-human monoclonal antibodies for 1hr at room temperature against the following antigens (PE-Conjugated, Miltenyi Biotech): CD_{11b} (Cat. No. 5160831415), CD_{14} (Cat. No. 5160623066), CD_{19} (Cat. No. 5160426628), CD_{34} (Cat. No 5160609355), CD_{45} (Cat. No. 5160621562), CD_{73} (Cat. No. 5160831420), CD_{79a} (Cat. No. 5170106069), CD_{90} (Cat. No. 5160426634), CD_{105} (Cat. No. 5160607470), and HLA-DR (Cat. No. 51609015188). For separation against red blood cells (RBC) and white blood cells (WBC), the cells were stained with the nuclear stain, Hoechst 33258 (Sigma, Cat. No. 94403), to determine total MNCs during flow cytometry and were used as controls.

AD-MSCs, UC-MSCs, and P-MSCs were blocked with 1.5% HSA and stained with anti-human monoclonal antibodies for 1hr at room temperature against the following antigens: CD_{11b}, CD_{14}, CD_{19}, CD_{34}, CD_{45}, CD_{73} CD_{79a}, CD_{90}, CD_{105}, and HLA-DR (all PE conjugated). All cells had a non-stained sample for controls. All PE-conjugated antibodies were purchased through Miltenyi Biotech. The flow cytometric analysis was performed on a Gallios cytofluorimeter (Beckman Coulter) and data were analyzed with Kaluoza software (CU Cancer Center Flow Cytometry Core Laboratory).

Immunomodulatory Potency Analysis

The γ-IFN-induced indoleamine 2,3-dioxygenase (IDO) cell-based assay (7) was performed on the AD-SVF, P-SVF, AD-MSCs, UC-MSC, and P-MSCs. Cells were plated at 5,000/cm^2 per well based on viable MNCs (SVF preparations) or expanded MSC cell counts cultured in TC-coated 96 well plates (Fisher Scientific, Cat. No. 130188) in MSC-Gro™ low serum media (Vitro Biopharma, Cat. No. SC00B1) and incubated at 37°C for 24-hrs in a humidified, hypoxic atmosphere (1% O_2, 5%CO_2, 94% N_2). After a 24-hr incubation, cells were washed three times with 1x PBS (Fisher Scientific., Cat. No. BP655-1) and then cultured in quiescent, serum-free media (Vitro Biopharma, Cat. No. SC00B17) containing 10μM tryptophan (Sigma, Cat. No. T8941) and accelerating dosages of γ-IFN (0, 2.5, 5, 10 ng/ml). Cells were incubated at 37°C for a 72-hr period in humidified, hypoxic conditions (1% O_2, 5%CO_2, 94% N_2). Conditioned media was collected and plated in a new black 96 well plate (B.D. Falcon, Cat. No. 353219) with a kynurenine standard curve (0 to 100 μM) and stained with 0.5mM methylene blue for 60 min at 37°C. Fluorescence (E_x=365nm E_m> 430nm) was determined using the Modulus Microtiter Plate Reader.

Mitochondrial Functional Potency Assay

Cell potency was also measured by cell-specific ATP measurement in the luciferin-luciferase assay as previously used and validated for potency determination of hematopoietic stem cells (HSC) (8) and MSCs (9). The adenosine triphosphate (ATP) assay was performed on the AD- SVF, P-SVF, AD-MSCs, UC-MSC, and P-MSCs. Cells were plated at different concentrations per well based on viable MNCS in SVF samples, and MSC cell counts in expanded MSCs in TC-coated 96 white well-plate (Fisher Scientific, Cat No. 130188) with an ATP standard curve in a quiescent serum-free media (Vitro Biopharma, Cat. No. SC00B17). Following culture in a humidified atmosphere of 1% O_2, 5%CO_2, 94% N_2 for 24 hours, 100 μl

Cell Titer Glo™ (Promega, Cat. No. G9241) was added to all wells and incubated for 30-min at room temperature. Luminescence was determined on a Modulus microtiter plate reader.

Results

Cell Count and Viability

Acradine orange and propidium iodine exclusion was used to determine the number of viable MNCs present in the cell preparation. For AD-SVF, P-SVF, and UC-SVF the MNC counts were 2.2E6 total MNCs at 45.08% viability out of 2.87E8 total cell count (0.0035% of viable MNCs in AD-SVF), 1.13E7 total MNCs at 44.16% viability out of 3.87E8 total cell count (0.013% of viable MNCs in P-SVF), and 1.31E7 total MNCs at 47.33% viability of 5.3E8 total cell count (0.012% viable MNCs in UC-SVF). Once the MSCs were isolated and expanded to P_2 the AD-MSC, P-MSC, and UC-MSC counts were 1.6E8 total AD-MSCs at 96.89% viability, 1.66E8 total P-MSCs at 94% viability, and 1.9E8 total UC-MSCs at 93% viability respectively. Thus, the mononuclear content is lower in SVF preparations than expanded MSCs. MNCs are less than 0.02% of the total cells in SVF.

Cell Line	Total Cell Count	Percent Viability	Viable Mononuclear Cells	Viable Mesenchymal Stem Cells	Percent of Viable MNCs to Total Cell Count
AD-MSC	1.60E+08	96.89%	N/A	1.55E+08	N/A
UC-MSC	1.90E+08	93%	N/A	1.77E+08	N/A
P-MSC	1.66E+08	94%	N/A	1.56E+08	N/A
AD-SVF	2.87E+08	45.08%	9.92E+05	N/A	0.003%
P-SVF	3.87E+08	44.16%	4.99E+06	N/A	0.01%

Table 1 Summary of total cell count and viability between SVF and MSCs.

Characterization of AD-SVF, P-SVF, AD-MSCs, P-MSCs, and UC-MSCs

The AD-SVF and P-SVF were investigated for MSC phenotype by staining for cell surface markers, which were detected using flow cytometry according to the ISCT standards for the definition of MSCs (10). The AD-SVF and P-SVF did not express the typical MSC markers CD_{90}, CD_{73}, and CD_{105}. In addition, the cells showed higher expression of the hematopoietic markers CD_{11b}, CD_{14}, CD_{19}, CD_{34}, CD_{45}, and the MHC class II molecule HLA-DR than those of isolated and expanded AD-MSCs, P-MSCs, and UC-MSCs.

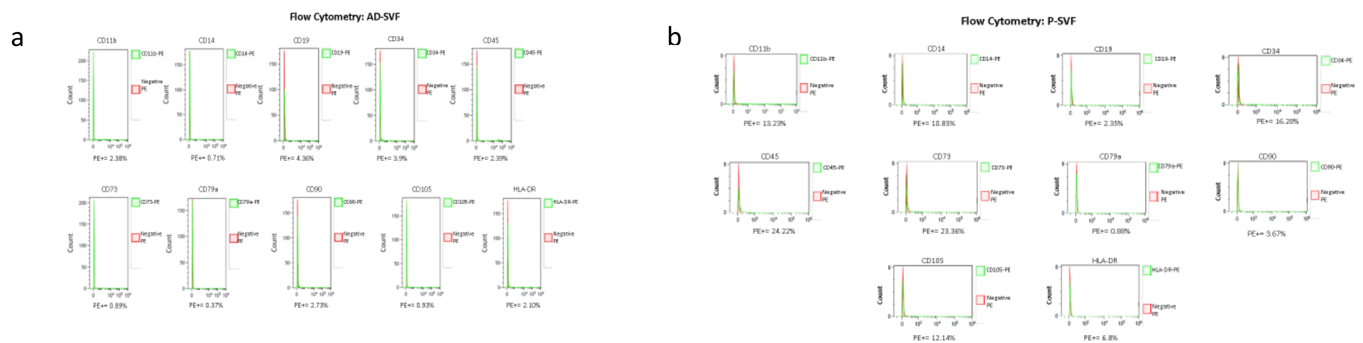

Fig. 1 Flow cytometric analysis of the expression of surface markers on (a) AD-SVF and (b) P-SVF. The immunofluorescence analysis was conducted within an hour after processing and showed no expression of the ISCT markers CD_{73}, $CD_{90,}$ nor CD_{105}.

The isolated and expanded AD-MSCs, P-MSCs, and UC-MSCs were investigated for MSC phenotype at P_2 by staining for cell surface markers, which were detected using flow cytometry according to the ISCT standard (10). The AD-MSCs (Fig. 2a) and UC-MSCs (Fig. 2c) expressed the typical MSC markers CD_{90}, CD_{73}, and CD_{105}. In addition, the cells showed low expression of hematopoietic markers $CD11_b$, CD_{14}, CD_{19}, CD_{34}, CD_{45}, and the MHC class II molecule HLA-DR (11). Similar results have been seen in several replicates (N=4). However, the P-MSCs (Fig. 2b) expressed a high level of CD_{73} and failed to pass ISCT standards for CD_{90} and CD_{105}. The average of replicates showed 97.2% for CD_{105} that is within the ISCT standard, while the average for CD_{90} was 73.5% that is less than the ISCT standard. The mean of replicates of P-MSCs showed low expression of hematopoietic markers CD_{11b}, CD_{14}, CD_{19}, and CD_{34} but showed elevated levels of CD_{45}. (The deviations from ISCT standards for the phenotype of P-MSCs is discussed below.)

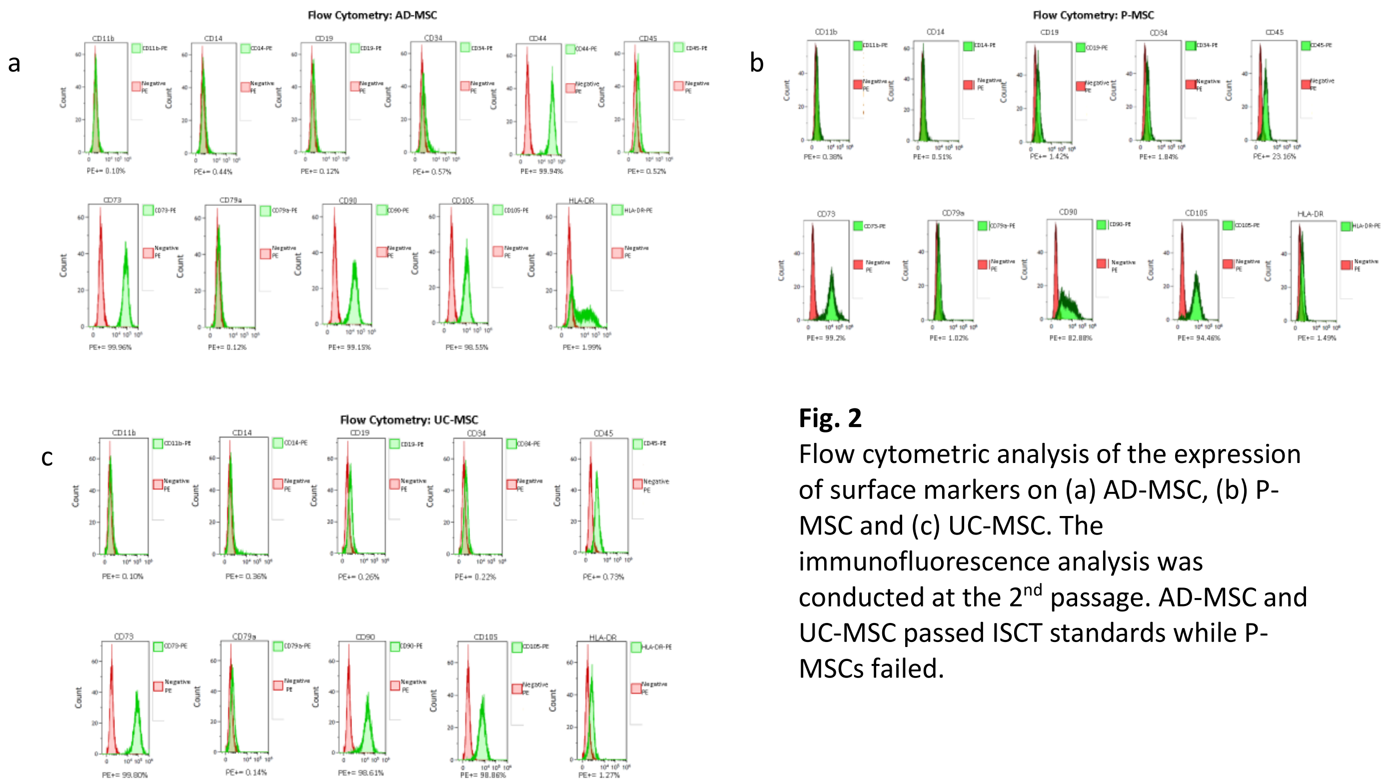

Fig. 2
Flow cytometric analysis of the expression of surface markers on (a) AD-MSC, (b) P-MSC and (c) UC-MSC. The immunofluorescence analysis was conducted at the 2nd passage. AD-MSC and UC-MSC passed ISCT standards while P-MSCs failed.

Immunomodulatory Potency Measures of AD-SVF, P-SVF, AD-MSCs, P-MSCs, and UC-MSCs

To compare immunomodulatory properties of AD-SVF, P-SVF, and MSCs from various sources, the activation of IDO by exposure to γ-IFN was determined (7) on an equivalent cellular basis. The γ-IFN-induced IDO activity was quantified by the conversion of tryptophan to kynurenine. The conversion of tryptophan to kynurenine by AD-SVF and P-SVF were minimal and relatively equal across the γ-IFN dose-response range. However, maximal IDO activity at 10 ng/ml γ-IFN was $\sim$4 fold greater in the isolated and

expanded UC-MSCs, ~1.5 fold greater than in expanded AD-MSCs, and ~1.3 fold greater in expanded P-MSCs relative to AD-SVF and P-SVF. These results show maximal immunomodulatory cellular potency in expanded UC-MSCs followed by AD-MSCs and P-MSCs, while AD-SVF and P-SVF show only minimal immunosuppressive potency. There was a significant difference in γ-IFN-induced IDO activity between the AD-SVF and P-SVF compared to the isolated and expanded UC-MSCs with a p-value<0.0001 by one-way ANOVA analysis (Graph Pad Prism™) of variance for significance of slope difference. There was a significant difference in γ-IFN-induced IDO activity between the AD-MSCs and P-MSCs compared to the isolated and expanded UC-MSCs with a p-value<0.005 by one-way ANOVA analysis (Graph Pad Prism™) of variance for significance of slope difference.

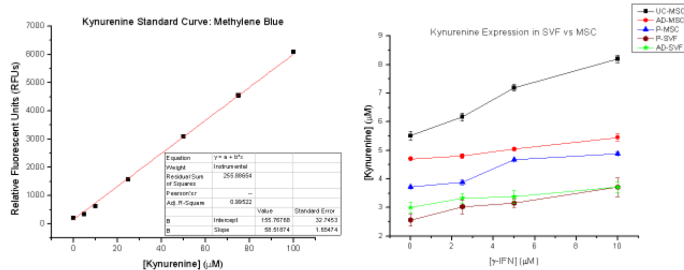

Fig. 3
Umbilical cord MSCs showed robust immunosuppressive potency while AD-MSCs and P-MSC were less potent, and SVF preparations showed minimal potency.

Mitochondrial Function Analysis of AD-SVF, P-SVF, AD-MSCs, P-MSCs, and UC-MSCs

Potency was also measured by cell-specific ATP determination as previously used to determine potency of human HSCs & MSCs (8,9). Relative luminescent units were converted to [ATP] using the ATP standard curve (Left panel, Figure 4) and cellular ATP is shown as a function of cells per well (Right panel, Figure 4). Cellular potency is measured by the slope of this relation (8,9) and UC-MSCs also showed greater potency than expanded AD-MSCs or P-MSCs, while results with AD-SVF and P-SVF show no appreciable ATP, suggesting minimal cellular content. There was a significant difference between the AD-SVF and P-SVF compared to the isolated and expanded UC-MSCs with a p-value<0.0002 by one-way ANOVA analysis (Graph Pad Prism™) of variance for significance of slope difference. There was no significant difference between the AD-MSCs and P-MSCs compared to the isolated and expanded UC-MSCs with a p-value>0.05 by one-way ANOVA analysis (Graph Pad Prism™) of variance for significance of slope difference.

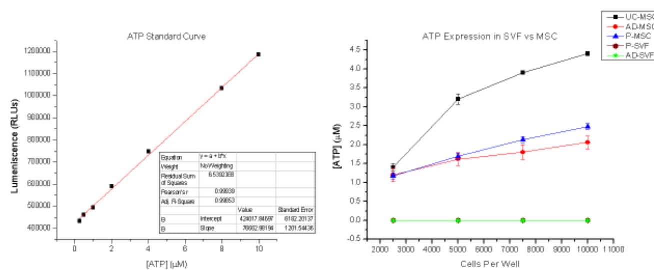

Fig. 4
Umbilical cord MSCs showed a significantly higher ATP expression than the other MSCs. AD-SVF and P-SVF had no expression of ATP.

Discussion

In the present study, we compared the cellular phenotype and potency of SVF and expanded MSCs. We derived SVF from lipoaspirate and perinatal tissues. Expanded MSCs were derived from lipoaspirate, placental decidua basalis, and Wharton's jelly of the umbilical cord. SVF is a crude extract of tissues consisting of low content of MSCs. Our results showed <0.02% viable MNC in SVF at viabilities of 44.2% to 45.1% (Table I). Stem cell content is lower and estimated to be < 10% of the total mononuclear cellular content of adipose SVF (16). Stem cell content of SVF preparations is thus estimated to be 50,000 to 100,000 at most, or 0.002% of the total cellular content. Our results showed that SVF has only minimal similarity to expanded MSCs including an absence of phenotypic identity to MSCs according to the ISCT standard for stem cell definition and substantially reduced cellular potency by immunomodulatory and mitochondrial functional assays. Although expanded MSCs share universal properties, such as morphology, plastic adherence, and multi-lineage differentiation potential, we found variations between AD-MSCs, P-MSCs, and UC-MSCs in terms of growth rate, phenotypic characterization, and potency measurements. The increased expression of CD_{45} in P-MSCs may be due to associated RBCs while the reduced CD_{90} expression may reflect differential presence of amnion-derived contribution as opposed to decidua-derived MSCs (19).

We used quantitative assays to determine cell counts, viability, phenotype, and potency by immunomodulatory and mitochondrial function (8,9). Variability in measurement was minimized by careful adherence to standard procedures including processing, analysis, and expansion. Additionally, each assay was performed at the same passage to avoid variation due to differences in passage number (13).

We found significant variation between SVF preparations and expanded MSCs. The assay to measure the cell count and viability showed the amount of viable MNCs in AD-SVF and P-SVF. We found 0.0035% of viable MNCs in AD-SVF and 0.013% of viable MNCs in P-SVF. Once isolated and expanded, MSCs show a higher viability and can achieve high cell counts typical of other expanded MSC preparations.

International criteria of MSC identity was determined by flow cytometry according to ISCT standards (10). We found that AD-SVF and P-SVF expressed higher levels of hematopoietic markers than mesenchymal stem cell markers, indicative that SVF preparations do not achieve the definition of stem cells by the ISCT criteria. Placental MSCs did not achieve ISCT criterion values of CD_{45}, CD_{90} and CD_{105}, indicating that these products are not stem cells according international standards of stem cell definition.

Cellular potency is an important assessment of stem cells for clinical applications. We used quantitative assessment of mitochondrial function and immunosuppression as measures of cellular potency. While expanded MSCs showed measurable potency, comparable to previous reports (9), SVF preparations yielded significantly reduced cellular potency based on MNC counts. Since MSCs are intrinsically immunosuppressive in nature, they can support graft survival and other clinical immunomodulatory effects. We thus determined cellular potency by quantitation of γ-IFN induced IDO activity. The results showed maximum immunomodulatory potency in UC-MSCs, which was significantly greater than the reduced potency seen in SVF preparations (14) and AD-MSCs.

From the reduced cellular viability in SVF preparations, we anticipated reduced cellular expression of ATP in SVF preparations. No detectable ATP expression was seen with AD-SVF and P-SVF, suggesting an

absence or substantially reduced level of viable MNCs in these cellular fractions. All isolated and expanded MSCs showed higher levels of cell-specific ATP content. UC-MSCs showed the highest ATP expression supporting the assertion that they are the most potent type of MSC.

Numerous clinical trials have been conducted and are presently ongoing for both SVF (17) and various MSC preparations (1, 2, 3, 4). From the results reported here it would be expected that that expanded MSCs exhibit greater therapeutic benefit than SVF preparations. There is more evidence supporting regeneration of cartilage from MSCs than SVF in OA patients (2). However, SVF showed safety and efficacy in pain reduction and restoration of function in a study of over 1000 OA patients (18) without extensive evidence of cartilage regeneration. Direct clinical comparisons of SVF and MSCs are lacking. Mechanisms of stem cell therapies include paracrine effects from stem cell-derived biological factors eliciting anti-inflammatory, neural protective effects, differentiation of stem cells into other lineages and intercellular communication through tunneling nanotubes. From the paucity of MSCs within SVF preparations it is likely that paracrine effects occur in SVF therapies with minimal true cellular therapy (17). However, several other cell types are likely in SVF besides MSCs and these other cells may also elicit therapeutic benefits.

Conclusion

We compared the stromal vascular fraction extracted from lipoaspirate and placental decidua basalis to expanded MSCs. The former preparation is commonly used in same surgical procedures in the United States, while clinical trials relying on expanded MSCs are more common in non-US clinical trials. Here, we compared SVF preparations to expanded MSCs and found that SVF products do not achieve the definition of stem cells by the ISCT standard definition of MSCs and exhibit significantly less cellular potency by measures of mitochondrial and immunomodulatory function. Our working hypothesis is that SVF products provide anti-inflammatory/analgesic effects predominantly through enrichment of the stem cell secretome, while MSC products provide additional clinical benefit of a cellular therapy by multi-lineage differentiation and intercellular regeneration.

Abbreviations

AD-SVF	Adipose Stromal Vascular Fraction
P-SVF	Placental Stromal Vascular Fraction
AD-MSCs	Mesenchymal Stem Cells Derived from Adipose Tissue
UC-MSCs	Mesenchymal Stem Cells Derived from Umbilical Cord
P-MSCs	Mesenchymal Stem Cells Derived from Placenta
P-DB	Placenta-Decidua Basalis
MNCs	Mononucleated Cells
MSCs	Mesenchymal Stem Cells
ATP	Adenosine Triphosphate
PBS	Phosphate Buffered Saline
γ-IFN	Gamma Interferon
RBC	Red Blood Cells
WBC	White Blood Cells

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