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Research article

Rapid enrichment of adipose derived mesenchymal stromal cells for use in cellular therapies

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Abstract: *Background:* Mesenchymal stromal cells (MSC) are multipotent cells that can be isolated from many tissues and are the subject of multiple clinical investigations. While adipose tissue is a reliable source of MSC, it still requires enrichment to increase potency and decrease volume prior to use in autologous cellular therapy. Prolonged *in vitro* culture can enrich and expand MSC but may also induce functional changes and stem cell senescence. *Methods:* The stromal vascular fraction (SVF) obtained by enzymatic digestion of lipoaspirated human adipose tissue was plated at a maximum density of 100,000–200,000 cells/cm^2 onto tissue culture flasks. A method which involved washing every 24 hours to remove non-adherent cells (Method A) was compared to a method that involved harvesting and re-plating cells in fresh cell culture flasks (Method B). Adherent cell populations containing the purified MSC could then be harvested and assayed at various time points. *Results:* MSC from the SVF were enriched from 16% of cells positive for CD73 at day 0 to 39% at day 1 to 59% within 2 days, with minimal (1–2 cell doublings) cellular expansion for Method B. Flow cytometric analysis demonstrated co-expression of known MSC phenotypic markers (CD73, CD105, and CD90) along with maintenance of functional capabilities (e.g., cell growth, colony formation and directed differentiation). After enrichment MSC can be harvested and concentrated for resuspension in minimal volumes for clinical use. *Discussion:* The maximum density plating and short-term culture approach described herein represents a simple and novel method to rapidly isolate

MSC for clinical therapies, with minimal costs and time, which can also be performed in a closed culture system.

Keywords: cell therapy; MSC; purification; short-term panning

Abbreviations: FACS: fluorescence activated cell sorter; MSC: mesenchymal stromal cells, mesenchymal stem cells; SVF: stromal vascular fraction

1. Introduction

Mesenchymal stromal cells (MSC), also called mesenchymal stem cells or multipotent stromal cells, can be isolated from many tissues and are the subject of multiple clinical investigations [1–5]. While adipose tissue is a reliable source of MSC, these cells may constitute less than 1% of the total nucleated cells found in the stromal vascular fraction (SVF). Therefore, the SVF requires enrichment to increase the purity of the MSC cell population and decrease infusion volumes prior to use in autologous cellular therapy [2,4,5]. Prolonged *in vitro* culture enriches and expands MSC but may also induce functional changes, as well as cellular senescence [3–5]. MSC can be purified through fluorescence-activated cell sorting (FACS) or magnetic bead enrichment; however, both methods are expensive, require specialized equipment, and can be more difficult to perform in a manner suitable for clinical grade applications. Additionally, traditional cell culture protocols of purification and expansion of MSC can lead to contamination, cell senescence, and typically require weeks of cell culture.

We are interested in the use of autologous adipose-derived MSC for potential cellular therapies. Our goal is to make this process rapid, economical, and efficient, yet as efficacious as approaches using more costly methodology. To achieve this goal, a maximum density, short-term panning procedure was developed in which the lipoaspirate was enzymatically digested to obtain the SVF, and plated at a maximum density of 100,000–200,000 cells/cm^2 onto tissue culture flasks. Two methods were compared, a more traditional method (Method A) which involved washing every 24 hours to remove non-adherent cells and a method (Method B) that involved harvesting and re-plating cells in fresh cell culture flasks after 24 hours. The cells from both methods were harvested, counted, and analyzed at various time points. Method A the enriched for the SVF-derived MSC up to 46% purity within 2 days and method B enriched for MSC by up to 59% purity within 2 days, with minimal cellular expansion. Flow cytometric analysis of the panned cell population demonstrated co-expression of known MSC phenotypic markers (CD73, CD105, and CD90) along with maintenance of functional capabilities (e.g., cell growth, colony formation, and directed differentiation), but minimal (one to two doublings) cell expansion [3]. After enrichment by short-term panning, MSC could be harvested and concentrated for resuspension in minimal volumes

for clinical use. This panning approach is a simple and novel method that can also be adapted to a closed culture system to rapidly isolate and purify MSC for clinical therapies, with minimal costs and time, and without need for specialized, expensive reagents or equipment.

2. Materials and Methods

1.1. Human subjects

Consent was obtained from all human donors before the liposuction procedures. All protocols were approved by the local Institutional Review Board (IRB). Human adipose tissue was obtained from elective liposuction procedures performed under local or general anesthesia. All adipose tissue samples were processed under the same conditions.

1.2. Lipoaspirate harvesting and stromal vascular fraction production

Fresh or cryopreserved lipoaspirate was processed using a previously described method [6]. Cells from adipose tissue samples were isolated by enzymatic digestion. Tissue was prepared by placing 5 mL of thawed adipose tissue in a 50 mL conical tube, which was then washed vigorously three times with 10 mL of phosphate-buffered saline (PBS). Cells in the wash fraction (primarily non-MSC, composed of adipocytes) were retained. The adipose tissue was treated with an equal volume (5 mL) of 0.2% collagenase type IV (Sigma) at 37 ℃ for 15 minutes. Complete medium (20 mL Minimal Essential Medium; Thermo Scientific, USA, supplemented with 10% fetal bovine serum [FBS; Hyclone] and 1% each of non-essential amino acids, sodium pyruvate, glutamine and streptomycin/penicillin solution [Hyclone]) was added to the digested tissue to neutralize the collagenase, passed through a 40 μm filter, and centrifuged at 150× g for 10 minutes at room temperature. Cells from the wash fraction and the digested fraction were suspended in complete medium and counted manually using both trypan blue (viability dye) and Turk's solution (total nucleated cell counts), and with an automated cell counter (Cellometer) using acridine orange and propidium iodide (AO/PI). Cells were plated in 25 cm^2 culture flasks and maintained at 37 ℃/5% CO^2 in complete medium with 95% relative humidity. MSC adhered to the culture flasks whereas other cells were depleted by replacing the spent medium with fresh medium. The medium was changed daily thereafter, or as indicated by the panning method. Cells were harvested with 0.05% trypsin-EDTA for use in subsequent assays.

1.3. Panning procedure

In preliminary experiments, multiple plates were seeded with cells from the SVF at densities of 10,000 cells/cm^2, 20,000 cells/cm^2, 40,000 cells/cm^2, 80,000 cells/cm^2, 150,000 cell/cm^2,

200,000 cell/cm^2, 300,000 cell/cm^2, 400,000 cell/cm^2, and 500,000 cell/cm^2. It was found that regardless of the density of cells plated, the maximum percentage of cells recovered after 24 hours of being plated was 50% at 100,000–200,000 cells/cm^2 (data not shown).

The stromal vascular fraction (SVF) containing the MSC population was isolated by enzymatic digestion of the lipoaspirate as described above, and the cell viability and yield in the SVF was determined as described above. A portion of the SVF was designated for characterization by the expression of typical cell surface markers by flow cytometry to determine the proportion of MSC within the sample. The remaining SVF was plated at maximum density (100,000–200,000 cells/cm^2) for 24 hours at 37 ℃. All panning experiments were conducted in alpha-MEM media with FBS [6–7]. To enrich the MSCs, two methods (A and B) were tested.

In method A, the cultures were depleted of non-adherent cells every 24 hours by changing the media in the culture flasks. At each time point (1, 2, 5, and 7 days of culture) the MSC were harvested, and viability and cell counts were determined. MSC were stained for flow cytometric analysis to determine purity, and functionality was assessed by directed differentiation as described below.

In method B, the cultures were washed by media exchange after 24 hours to remove all non-adherent cells, then the adherent cells were harvested using trypsin/EDTA and then re-plated in new flasks for an additional 1–7 days without any subsequent media changes. At each time point (1, 2, 6, and 7 days after the second plating) the MSC were harvested, and viability and cell counts were determined. Cells were stained for flow cytometric analysis to determine purity, and directed differentiation was used to determine functionality.

1.4. Flow cytometric analysis

Cells were stained with a panel of antibodies for flow cytometric analysis. A minimum of 1×10^5 cells were re-suspended in flow buffer (phosphate buffered saline PBS with 1% FBS) and incubated at 4 ℃ with IgG block (Invitrogen) for 5 minutes to block non-specific antibody binding. The following antibodies were used: FITC-conjugated CD45 (BD Pharmingen, USA), BV421-conjugated CD73 (BD Pharmingen, USA), BV510-conjugated CD90 (Biolegend, USA), and PE-conjugated CD105 (Biolegend, USA). Cells were stained for 30 minutes at 4 ℃. After washing with flow buffer, samples were fixed with 1% paraformaldehyde and then analyzed using a LSR II Flow Cytometer (BD, USA), with at least 1×10^4 events acquired for each population. Data acquisition and analysis were performed using FACS DIVA software (BD Biosciences, USA). Unstained cells and single color controls were used to establish flow cytometer settings. Debris and cells/particles with auto-fluorescence were removed by using a threshold on the forward scatter plot.

1.5. Directed differentiation

1.5.1. Adipogenic differentiation

Adipose-derived MSCs (obtained from the enrichment cultures) were seeded in triplicate in 12 well plates at a cell density of 9×10^3 cells per cm^2 in complete medium. 24 hours later, which was designated as day 1, directed differentiation was initiated using adipogenic induction medium (ThermoScientific, USA), as per the manufacturer's instructions [7].

1.5.2. Oil Red O staining

Adipogenesis was confirmed four weeks after induction by Oil Red O staining to visualize accumulated cytoplasmic lipid rich vacuoles according to the manufacturer's instructions (IHC World, USA). Cells were counterstained with haematoxylin solution for 1 minute and visualized using a compound light microscope [3,5,7,9].

1.5.3. *In vitro* osteogenic differentiation

For osteogenic differentiation 3.6×10^4 MSC per well (obtained from the enrichment cultures) were seeded in 6 well plates in complete medium. After 24 hours osteogenic differentiation was promoted by treating MSC cultures with osteogenic induction medium (ThermoScientific, USA) for 3 weeks [7].

1.5.4. Alizarin Red S staining

Osteoblasts were detected using Alizarin Red S staining, which complexes with extracellular matrix calcium deposits. The stain was prepared by dissolving 2 g of Alizarin Red S powder in 100 mL of distilled water and adjusting the pH to between a range of 4.1 and 4.3 using 0.1% ammonium hydroxide. The cells were fixed with 10% neutral buffered formalin for 5 minutes and then washed with distilled water before staining with the Alizarin Red S solution for 45 minutes. Excess staining solution was removed with distilled water and the cells were visualized with a compound light microscope.

3. Results

As expected, during the panning procedures most of the non-MSC nucleated cells in the initial SVF were lost, while the MSC were retained through adherence. Over the next several days, cell numbers were shown to increase (Figure 1). By day 7 post-replating, when MSC purity exceeded 90%, the cells had doubled to approximately 200% of the initial cell numbers, indicating maintenance of viability and functional activity as shown by cell proliferation (Figure 1) and colony formation (data not shown).

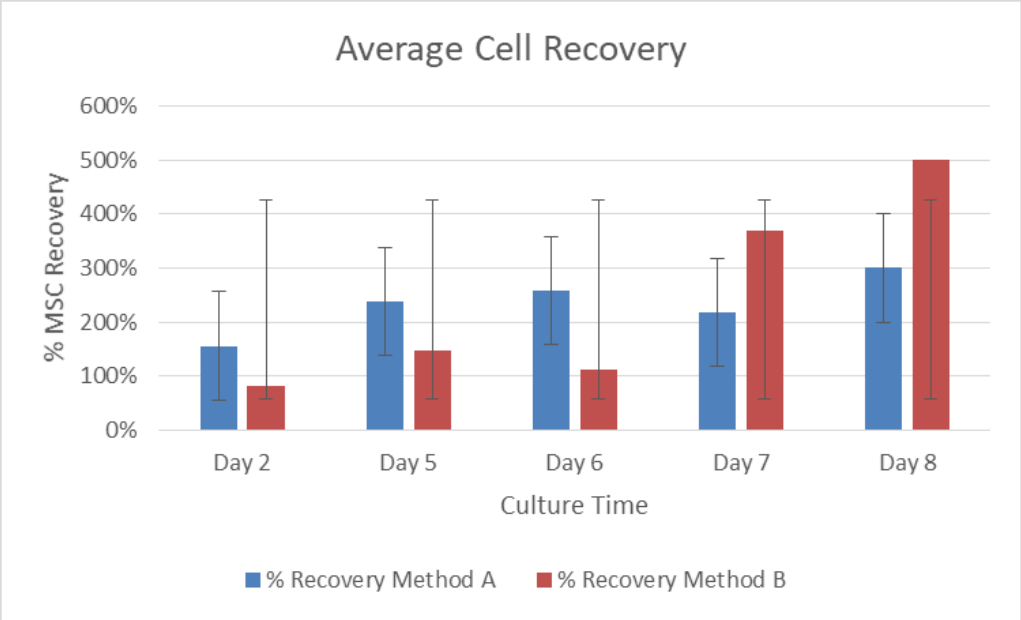

Figure 1. Cell Recovery and Expansion of MSC during Short-term Panning.

Note: Adipose-derived SVF was obtained as described and plated in cell culture flasks. After 24 hours, the cells were washed (Method A) or harvested and re-plated (Method B). At days 2, 5, 6, 7 and 8 following each manipulation the cells were harvested and viable cell numbers enumerated. A total of 8 independent cell cultures were analyzed. Data is shown as the mean +/− SD for viable MSC recovered compared to initial cells plated.

As shown in Table 1, the starting SVF population from freshly digested adipose tissue (day 0) contained a mean of 16% of the gated cells positive for the MSC marker CD73, which was enriched after 1 day of plastic adherence to a mean of 39% of cells positive for the MSC cell surface markers. Analysis of CD73, CD90, CD105 and CD45 expression is shown in Table 1**,** demonstrating that cells co-expressing prototypical MSC markers were a minority of the initially plated cell population. However, the percentage of cells positive for co-expression of CD73, CD90 and CD105 continued to increase in culture such that by day 2 of culture in Method A (washing every 24 h), as many as 46% ± 34% of cells were positive for prototypical MSC markers, up from an average of 22% found in the SVF. By day 7 of culture with Method A, as many as 99% ± 1% of cells were enriched for the CD105 marker. Similar results were seen for CD73 and CD90 with a mean of 98% and 96% positive, respectively, for these markers by day 7. For Method B (replating without washing), a similar increase in cells positive for prototypical MSC markers was observed (Table 1). In that regard Figure 2 shows a qualitative population shift towards more cells positive for the MSC marker CD73 with time in culture. Typical leukocytes were lost from culture, as exhibited by a decrease in CD45 expression from 21% initially to 0% by day 7 of culture for both methods.

Directed differentiation of the adherent cells in both Methods A and B demonstrated that cells enriched using both techniques were capable of both adipogenic and osteogenic differentiation after plastic adherence enrichment (Figure 3), as would be expected of true multipotent MSC.

Table 1. Phenotypic Analysis of MSCs Isolated with Short Term Panning.

FACS Analysis (mean %+)				
	CD73	CD90	CD105	CD45
Day 0	16 (13, 3, 45, 4)	20 (15, 5, 60, 2)	22 (22, 7, 59, 0)	21 (45, 1, 38, 0)
Day 1	39 (59, 54, 61, 16, 4)	38 (45, 24, 73, 36, 13)	54 (91, 78, 64, 36, 1)	10 (9, 31, 0, 0)
Method A				
Day 2	46 (80, 12)	22 (37, 6)	95 (95)	11 (20, 3)
Day 5	66 (51, 81)	47 (47, 48)	71 (71)	2 (2, 3)
Day 7	80 (99, 100, 21, 98)	76 (99, 100, 8, 96)	99 (100, 100, 98)	0 (0, 0)
Method B				
Day 2	59 (33, 85)	38 (17, 60)	21 (36, 5)	4 (8, 0)
Day 5	73 (62, 84)	56 (53, 60)	37 (37)	2 (4, 0)
Day 7	84 (99, 99, 63, 75)	76 (99, 99, 41, 67)	89 (99, 100, 67)	0 (0, 0)

Note: Adipose-derived SVF was obtained as described and plated in cell culture flasks. After 24 hours, the cells were harvested and re-plated. At total culture days 1, 2 and 6 following the re-plating the cells were analyzed by flow cytometry. A total of 8 independent cell cultures were analyzed. Data is presented as the mean percentage positive cells when more than one experiment was performed.

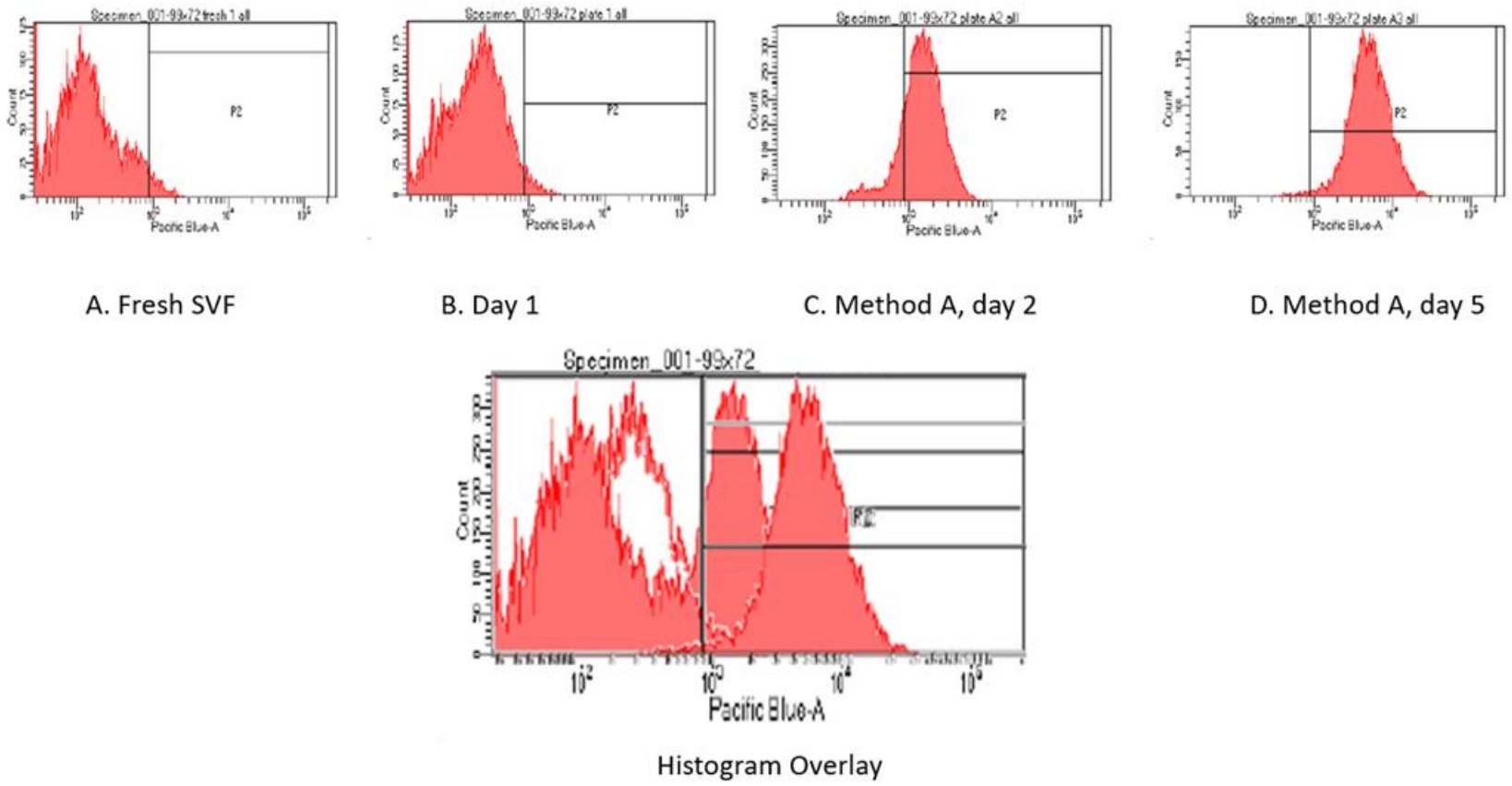

Figure 2. Increased Enrichment of MSC Purity by Short-Term Panning.

Note: Adipose-derived SVF was obtained as described and plated in cell culture flasks. After 24 hours, the cells were washed (Method A) or harvested and re-plated (Method B). At total culture days 2, 5, and 7 the cells were analyzed by flow cytometry. A representative experiment is shown for increased expression of the MSC cell surface antigen, CD73, where the fresh SVF was 4% positive for the CD74 cell surface antigen, increasing to 81% positive by day 5.

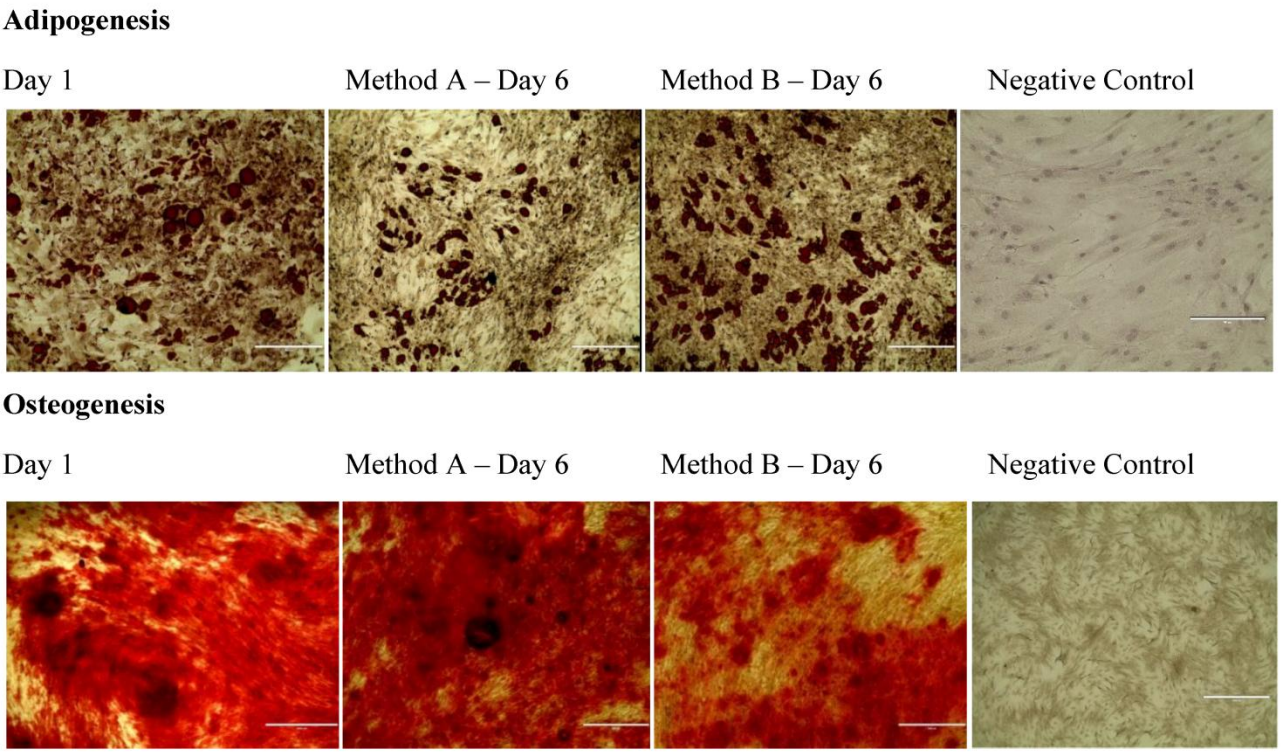

Figure 3. Directed Differentiation of Adherence – Derived MSC.

Note: Adipose-derived SVF was obtained as described and plated in cell culture flasks. The cells marked as day 1, were harvested after 24 hour and replated for differentiation induction the after an additional 24 hours. The cells were washed every 24 hours (Method A) or harvested at the indicated time point and re-plated (Method B). At the indicated time point, cells from each method were harvested and placed into directed differentiation cultures. At the end of 28 days the cultures were analyzed as described in Methods for adipocytes and osteoblasts.

4. Discussion

Purification and/or enrichment of a stem cell population is often more important than cell expansion for many cell therapy applications. Although most stem cell sources such as bone marrow, cord blood, and adipose tissue can be harvested in large quantities or volumes, the stem cells contained within these sources are present only at very low concentrations (comprising as little as 0.01% of the total nucleated cells). Generally, cell expansion can be used to increase the total cell numbers and simultaneously enrich the stem cell population, though this process may take as long as several weeks to months, with increasing costs and increasing risks of microbial contamination. If an enrichment procedure could be performed quickly it would help minimize risks of contamination, possible loss of critical cell populations, and overall processing costs. Although adipose tissue collections can be as large as 5000 cc, the MSC population represents a minority of

cells present in the tissue, being interspersed with larger numbers of adipocytes, red blood cells, white blood cells, endothelial cells and other cell types. While the MSC could be purified using FACS and/or enriched with magnetic beads, these processes are more complex, more expensive, and can require specialized equipment.

High-density plating and short-term panning of the stromal vascular fraction of human adipose tissue is a rapid method to enrich for adipose tissue-derived MSC at low costs within 7 days. Within 7 days a 90% or greater, purified MSC population could be obtained that is suitable for use in cell therapy. Interestingly, it was possible to enrich the MSC population with minimal cell expansion, minimizing the risk of culture-induced cellular senescence [8], which could negatively affect the efficacy of cellular therapies. Both methods (A and B) of purification showed similar cell number recovery ($p = 0.26$), purification ($p > 0.5$), and differentiation capabilities.

The results of the flow cytometric analyses showed that panning the SVF for 7 days in culture is sufficient to purify a high percentage of cells positive for commonly accepted MSC markers (CD90, CD105, and CD73) with both methods when compared to the whole stromal vascular fraction. During that time the cell numbers increased minimally (1–2 cell doublings maximum), alleviating worries of cell senescence that might be a consequence of excessive cell division. Additionally, the enriched MSC demonstrated conserved multilineage capabilities towards adipogenesis and osteogenesis differentiation after short-term panning.

The methods described in this study can be applied to autologous stem cell therapies where both time and starting stem cell numbers may be limited and minimal infusion volumes are required. The novelty of this procedure is a high density of cells adhering to plastic as is normally performed by most laboratories, followed by washing with media every 24 hours. Historically, cells are plated at lower densities to prevent contact inhibition for cell expansion [10]. High density, short term panning does not require cell expansion, just sufficient time for stem cells to recover from digestion and thawing (if applicable). This method demonstrates that 5–7 days of panning in tissue culture flasks (or on any suitable plastic surface) as described is all that is required to enrich the MSC population found in human adipose tissue. Washing the cells daily (Method A) depletes the debris, red blood cells, and adipocytes present after digestion, and requires little interaction. While method B may reduce adherent cells that are non-stem cells, it requires multiple harvests. Therefore method B requires more disruption to the cell recovery from enzymatic processing and may induce additional unknown changes. The ability of method A to purify large volumes of an unenriched stem cell source, make it amenable for a clinically administered cell dose. Furthermore, this approach could also be adapted to closed culture systems with minimal expense making it readily available for clinical trial applications.

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Conflict of interest

None of the authors have any conflicting interests to disclose.

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