Letter to the Editor

Immunophenotypic analysis of human adipose-derived stem cells through multi-color flow cytometry

Paolo Giuseppe Limoli[1]^, Marcella Nebbioso[2]^

[1]Low Vision Research Centre of Milan, Milan, Italy; [2]Department of Sense Organs, Rare Retinal Diseases and Ocular Electrophysiology Centre, Umberto I Policlinic, Sapienza University of Rome, Rome, Italy

Correspondence to: Marcella Nebbioso, MD. Department of Sense Organs, Rare Retinal Diseases and Ocular Electrophysiology Centre, Umberto I Policlinic, Sapienza University of Rome, Viale del Policlinico 155, 00161, Rome, Italy. Email: marcella.nebbioso@uniroma1.it.

Received: 06 February 2023; Accepted: 15 May 2023; Published online: 09 June 2023.

doi: 10.21037/sci-2023-010

View this article at: https://dx.doi.org/10.21037/sci-2023-010

Nowadays, it is established in the scientific literature that adipose tissue is highly bioactive, and it is at the center of regenerative medicine and tissue engineering. Adipose-derived stem cells (ASCs) are regarded as one of the most promising sources of adult or somatic stem cells, identified thus far for cell therapy and regenerative medicine across a wide range of diseases (1-3). ASCs are included in and isolated from the adipose tissue's stromal vascular fraction (SVF), which consists of a heterogeneous mesenchymal cell array, including not only ASCs but also preadipocytes, fibroblastic cells, endothelial cells, pericytes, vascular smooth muscle cells, hematopoietic cells (4,5), macrophages, and lymphocytes (6). It is also largely recognized by the researchers the necessity to establish a common isolation method and a standard characterization of human stromal and stem cells from SVF and ASCs to facilitate the comparison of the study outcomes and so the clinical research advancement within the field of adipose science.

In this study, we proposed an innovative and non-enzymatic protocol to collect clinically useful ASCs, within freshly isolated SVF from adipose tissue, by centrifugation of the infranatant portion of lipoaspirate in order to determine the characteristic cytofluorimetric pattern prior to *in vitro* culture.

Thirty-two patients affected by dry age-related macular degeneration with a mean age of 69.4 years, after having read and signed the informed consent, were recruited taking into account inclusion and exclusion criteria according to the study protocol of the Ethical Committee. With the patients under local anesthesia, adipose tissue was suctioned and the SVF was isolated according to the Lawrence and Coleman technique (7-10). Characterization of the cell composition of SVF has been accomplished through multi-color flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Brea, CA, USA) that allows the identification of the surface marker expression of the cells *in vitro*. The panel of cell surface antigens was chosen in agreement with the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy recommendations (3). The immunophenotypic analysis was performed to confirm the mesenchymal nature of isolated cells. The following fluorochrome-labeled monoclonal antibodies were used: CD31-PE, CD34-PC, and CD45-APC. The markers were used in combination with ViaKrome, which determines cell viability, excluding debris and dead cells induced by the isolation protocol. Cells were incubated with specific mAbs for 15 min. The software CytExpert Version 2.2.0.97, CytoFLEX (Beckman Coulter) was used to create dot plots and to calculate the cell composition percentages according to the profile of the surface marker expression. The SVF yielded a mean of 73.32%±10.89% cell viability evaluated with CALCEINA-FITC, i.e., cell-permeant dye. ASCs were positive for PC7-labeled mAb anti-CD34 and negative for both PE-labeled mAb anti-CD31 and APC-labeled mAb anti-CD45. The frequency of ASCs, estimated according to the panel of cell surface markers used, was 51.06%±5.26% versus the unstained ASCs subpopulation which was 0.74%±0.84% ($P<0.0001$). The ASCs events/µL

^ ORCID: Paolo Giuseppe Limoli, 0000-0003-4338-9274; Marcella Nebbioso, 0000-0002-5512-0849.

were 1,602.13±731.87/μL (Appendix 1).

The findings suggested that ASCs found in freshly isolated adipose SVF obtained by centrifugation of lipoaspirate can be immunophenotypically identified with a basic panel of cell surface markers. Therefore, we used a combination of positive and negative markers in the same multiparameter flow cytometric analysis to phenotype the cells and one viability dye to exclude apoptotic cells and other debris, indicating the viability of ≥70% for ASCs, according to IFATS recommendations.

To date, it is challenging to define a common isolation method and characterization profile of ASC found in adipose tissue (11,12). To the best of our knowledge, we proposed a basic cytofluorimetric pattern of *in vivo* ASC population found in freshly isolated SVF obtained by centrifugation of the infranatant portion of lipoaspirate from adipose tissue without purifying them by adherent culture isolation and expansion *in vitro*.

This paper has limitations and is only an indication for future studies. The flow cytometry pattern of SVF includes several clusters of differentiation (CD) that need to be analyzed. Indeed, CD34 alone is not an indication of SVF stem cell positivity and CD90, CD73, CD105, and CD275 should be associated. Furthermore, to be sure of having identified the SVF cells, these should be able to differentiate in an osteogenic, chondrogenic, and adipogenic sense.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the editorial office, *Stem Cell Investigation*. The article has undergone external peer review.

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://sci.amegroups.com/article/view/10.21037/sci-2023-010/coif). MN serves as an unpaid editorial board member of *Stem Cell Investigation* from August 2022 to July 2024. The other author has no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Prunet-Marcassus B, Cousin B, Caton D, et al. From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res 2006;312:727-36.
2. Bateman ME, Strong AL, Gimble JM, et al. Concise Review: Using Fat to Fight Disease: A Systematic Review of Nonhomologous Adipose-Derived Stromal/Stem Cell Therapies. Stem Cells 2018;36:1311-28.
3. Bourin P, Bunnell BA, Casteilla L, et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy 2013;15:641-8.
4. Cawthorn WP, Scheller EL, MacDougald OA. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. J Lipid Res 2012;53:227-46.
5. Han J, Koh YJ, Moon HR, et al. Adipose tissue is an extramedullary reservoir for functional hematopoietic stem and progenitor cells. Blood 2010;115:957-64.
6. Astori G, Vignati F, Bardelli S, et al. "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med 2007;5:55.
7. Traktuev DO, Merfeld-Clauss S, Li J, et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res 2008;102:77-85.
8. Limoli PG, Vingolo EM, Limoli C, et al. Regenerative Therapy by Suprachoroidal Cell Autograft in Dry Age-related Macular Degeneration: Preliminary In Vivo Report. J Vis Exp 2018;56469.
9. Lawrence N, Coleman WP 3rd. Liposuction. J Am Acad Dermatol 2002;47:105-8.

10. Coleman SR. Structural fat grafting: more than a permanent filler. Plast Reconstr Surg 2006;118:108S-20S.
11. Sheykhhasan M, Wong JKL, Seifalian AM. Human Adipose-Derived Stem Cells with Great Therapeutic Potential. Curr Stem Cell Res Ther 2019;14:532-48.
12. Brianna, Ling APK, Wong YP. Applying stem cell therapy in intractable diseases: a narrative review of decades of progress and challenges. Stem Cell Investig 2022;9:4.

doi: 10.21037/sci-2023-010
Cite this article as: Limoli PG, Nebbioso M. Immunophenotypic analysis of human adipose-derived stem cells through multi-color flow cytometry. Stem Cell Investig 2023;10:13.

Appendix 1

Materials and methods

Patient characteristics

A total of 32 patients affected by dry age-related macular degeneration (dryAMD), 15 males (46.8%) and 17 females (53.2%), with mean age 69.4 years (range, 50–83 years), undergoing elective surgery were recruited for the study. They were identified and enrolled according to several characteristics. The inclusion criteria were as follows:

(I) Participants of whites classified as well-nourished and Caucasian race;
(II) dryAMD diagnosis with spectral domain optical coherence tomography (SD-OCT), fundus autofluorescence imaging, and fluorescein angiography in the presence of drusen, atrophy, and irregularities of retinal pigment epithelium in at least 1 eye;
(III) Good storage extrafoveal areas;
(IV) Measurable visual acuity;
(V) Normal intraocular pressure;
(VI) Acceptance of the clinical protocol by signing the informed consent.

The exclusion criteria from the study for possible cross-related interference with the test were as follows:

(I) Patients with signs of exudative AMD;
(II) Myopia with spherical equivalent >6 diopters;
(III) Other ocular disorders such as cataract, macular pucker or neovascular membrane-associated, glaucoma, optic neuritis, ocular trauma, and high refractive errors;
(IV) Insufficient compliance in individuals affected by medical problems, such as hypovitaminosis, multiple sclerosis, epilepsy, Parkinson's disease, diabetes, hypertension, vasculitis, renal and hepatic diseases, gastrointestinal malabsorption, hypothyroidism, malignant tumors, and other systemic chronic diseases.

For each patient, we evaluated the best corrected visual acuity (BCVA) for far and near distances. BCVA was measured with early treatment diabetic retinopathy study charts at 4m in the logarithm of the minimum angle of resolution (logMAR) and visual acuity for near vision (close-up) in points (Pts). Therefore, we recorded the electrical cell activity through electroretinography (electromedical system of ocular electrophysiology, Retimax; C.S.O. Srl, Scandicci, Italy). The diagnosis was confirmed by confocal scanning laser ophthalmoscope Nidek F10 (Nidek Inc, Fremont, CA, USA), SD-OCT Cirrus (Carl Zeiss Meditec AG, Jena, Germany), and microperimetry Maia 100809 (CenterVue S.p.A., Padova, Italy) or standard automated perimetry Octopus 900 (Haag-Streit AG, Koeniz, Switzerland).

Cell harvesting and processing

The study was performed at Vista Vision between September 2018 and December 2019. Human adipose-derived stem cells (ASCs) included in stromal vascular fraction (SVF) were obtained from patients under the full approval of Low Vision Academy's Institutional Review Board and under the tenets of the 1964 Declaration of Helsinki (No. 2017/MC057, 4 May 2017). Informed consent was obtained from all subjects involved in the study. The harvest of the adipose tissue of the abdomen was surgically removed from patients during elective ocular surgery by PL, MD, in the operating room of Vista Vision (8). The harvest and the post-harvest fat processing method were conducted according to the Coleman technique by two trained doctors, CL and MT (9,10). Briefly, 10 mL of fat is harvested from the abdominal subcutaneous layer of each patient using a 3-mm blunt cannula connected to a locking syringe. The lipoaspirate is then centrifugated at 1,500 g of speed for 5 min at 20 ℃ to eliminate nonviable components, including cellular debris and oil (9,10). Finally, the bottom fluid portion of lipoaspirate from the centrifugated fat was drained, discarding blood, water, and aqueous components (10). This method has been demonstrated to obtain a higher concentration of adipose-derived mesenchymal stem cells within aspirate, reporting no damage to the structural integrity of adipose tissue and increased content of growth factors (9,10).

https://dx.doi.org/10.21037/sci-2023-010

Cell isolation

SVF was obtained from patients affected with eye diseases who underwent elective surgery and were isolated under fresh conditions. With the patient under local anesthesia, adipose tissue was suctioned using a cannula with a 3-mm inner diameter connected to a locking syringe, according to the Lawrence and Coleman technique (9,10). The collected liposuction aspirate, to which was added 30 mL balanced salt solution, was used for isolation of the SVF.

The isolation of SVF was manually performed from each patient's lipoaspirate in a clean room near the operating room, according to a previously described method. Briefly, the adipose portion of the lipoaspirate was washed with phosphate-buffered saline (PBS; Biological Industries, Milan, Italy) and mixed with 2.5 mg/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) for the enzymatic digestion. The collagenase/adipose mixture was placed in a 37 ℃ water bath for 30 min. to create a single-cell suspension and then filtered through a 100 µm cell strainer and finally to a 45 µm mesh. It was centrifugated for 5 min at 1,200 ×g to collect the cellular SVF as a pellet.

Flow cytometry

Once isolated, characterization of the cell composition of freshly-isolated SVF has been accomplished through multi-color flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Brea, CA, USA) that allows the identification of the surface marker expression of the cells *in vitro*. The panel of cell surface antigens was chosen in agreement with International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) recommendations (3). The immunophenotypic analysis is performed to confirm the mesenchymal nature of isolated cells. The following fluorochrome-labeled monoclonal antibodies were used for SVF analysis: CD31-PE, CD34-PC, and CD45-APC (Beckman Coulter) (*Table S1*).

- CD31, or platelet endothelial cell adhesion molecule (PECAM-1), is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes, megakaryocytes, and osteoclasts.
- CD45 or protein tyrosine phosphatase, receptor type, C (PTPRC), leukocyte common antigen (LCA), panhematopoietic marker expressed on all human white blood cells.
- CD34, cell-cell adhesion molecule, a marker for pluripotent stem cells, expressed on all hematopoietic progenitor cells.

The markers were used in combination with ViaKrome (Beckman Coulter), which determines cell viability, excluding debris and dead cells induced by the isolation protocol. Cells were incubated with specific mAbs for 15 min. At least 10^5 cells were acquired from each sample. The software CytEpert Version 2.2.0.97, CytoFLEX (Beckman Coulter) was used to create dot plots and to calculate the cell composition percentages according to the profile of the surface marker expression.

Statistical analysis

All statistical analyses were performed using the software SPSS Statistics (version 20.0, SPSS Inc., Chicago, IL, USA). Data were summarized with the mean ± standard deviation and minimum and maximum (min-max) values were also reported. The student's paired *t*-test was used to compare values between stained cells and unstained controls. A P value <0.001 was considered statistically significant.

Results

ASCs immunophenotypic characterization

The liposuction procedure was successfully performed in all cases. For immunophenotypic characterization of ASCs within the SVF, freshly isolated cells were examined for surface antigen expression using flow cytometry. Flow cytometry data were gated (*Figure S1*) to identify possible stromal cell subpopulations. The SVF yielded a mean of 73.32%±10.89% cell viability evaluated with CALCEINA-FITC, i.e., cell-permeant dye, to reduce the analysis distortion caused by the possible presence of dead or apoptotic cells. The ASCs were positive for CD34 and negative for CD31 and CD45. The frequency of ASCs estimated according to the panel of cell surface markers used was 51.06%±5.26% versus the unstained ASCs subpopulation which was 0.74%±0.84% (P<0.0001). The ASCs events/µL were 1602.13/µL±731.87/µL. The results of this analysis are shown in *Figures S1-S3* and *Table S2*.

https://dx.doi.org/10.21037/sci-2023-010

Table S1 Antibodies used in the study

Fluorochrome-labeled monoclonal antibodies	Target protein	Supplier	Catalog No.
CD31-PE	PECAM-1	Beckman Coulter, CA, USA	1F11, 100 Tests, RUO IM2409
CD34-PC7	gp105-120		581, k 100 Tests, CE, A21691
CD45-APC	LCA		J33, 100 Tests, CE IM2473

The following fluorochrome-labeled monoclonal antibodies were used for stromal vascular fraction (SVF) analysis: CD31-PE, CD34-PC, and CD45-APC. CD31, or platelet endothelial cell adhesion molecule (PECAM-1), is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes, megakaryocytes, and osteoclasts. CD45 or protein tyrosine phosphatase, receptor type, C (PTPRC), Leukocyte common antigen (LCA), panhematopoietic marker expressed on all human white blood cells. CD34, cell-cell adhesion molecule, a marker for pluripotent stem cells, expressed on all hematopoietic progenitor cells. CD, cluster of differentiation.

Table S2 Phenotype characterization by flow cytometry of cell fractions in the stromal vascular fraction

Population	Staining	Events, mean ± SD	Percentage (%)	Events/μL	P value
CD34	No	31.86±20.27	0.69±0.36	2.33±1.73	*<0.0001
	Yes	5,702.33±1,158.78	80.16±1.73	1,644.98±724.57	
CD31	No	7,034.00±927.88	98.95±0.76	4,771.37±1,086.62	0.01877
	Yes	5,516.50±1,347.28	84.69±14.57	3,043.13±429.95	
CD45	No	6,888.46±899.32	99.30±0.63	1,938.61±935.47	0.9860
	Yes	6,155.43±1,264.57	99.27±0.59	2,084.74±793.71	
ASCs	No	83.10±91.82	0.74±0.84	26.675±29.25	*<0.0001
	Yes	3,095.00±815.46	51.06±5.26	1,602.13±731.87	

Values were compared by Student's paired *t*-test. *, statistical significance with P=0.0001. All events: 10,000. Live cells in %: 73.32±10.89.

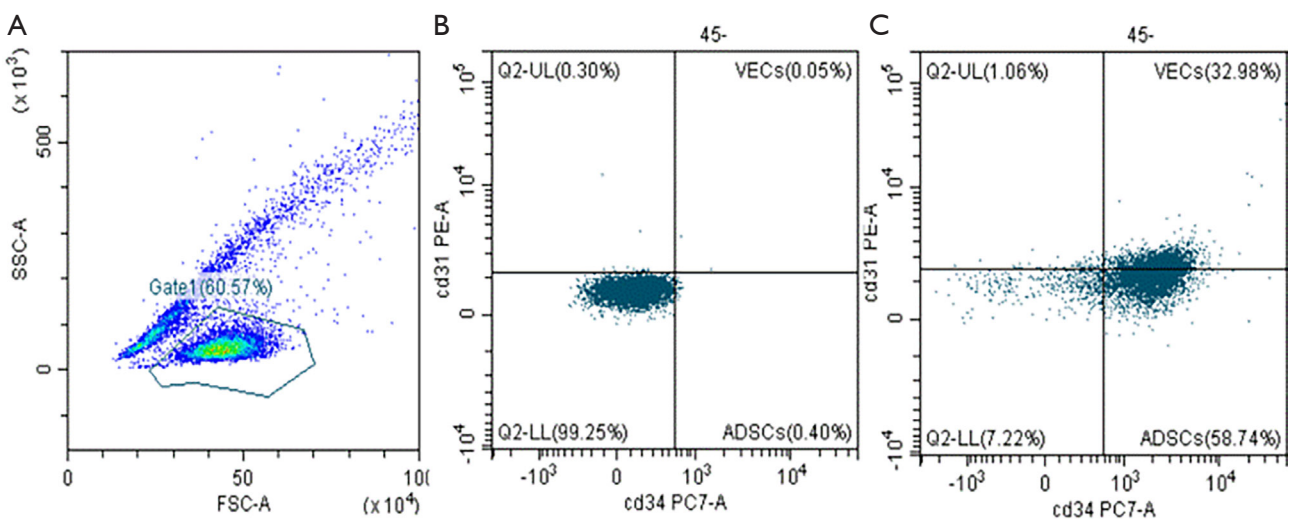

Figure S1 Representative flow cytometric histograms of adipose-derived stem cells within the stromal vascular fraction. Fluorescent dot plot showing cells within the freshly-isolated lipoaspirate (A). Dot plot showing cells unstained (B) and stained by antibodies directed against CD45, CD31, and CD34 (C). The results are presented in brackets as a percent of positively stained cells.

https://dx.doi.org/10.21037/sci-2023-010

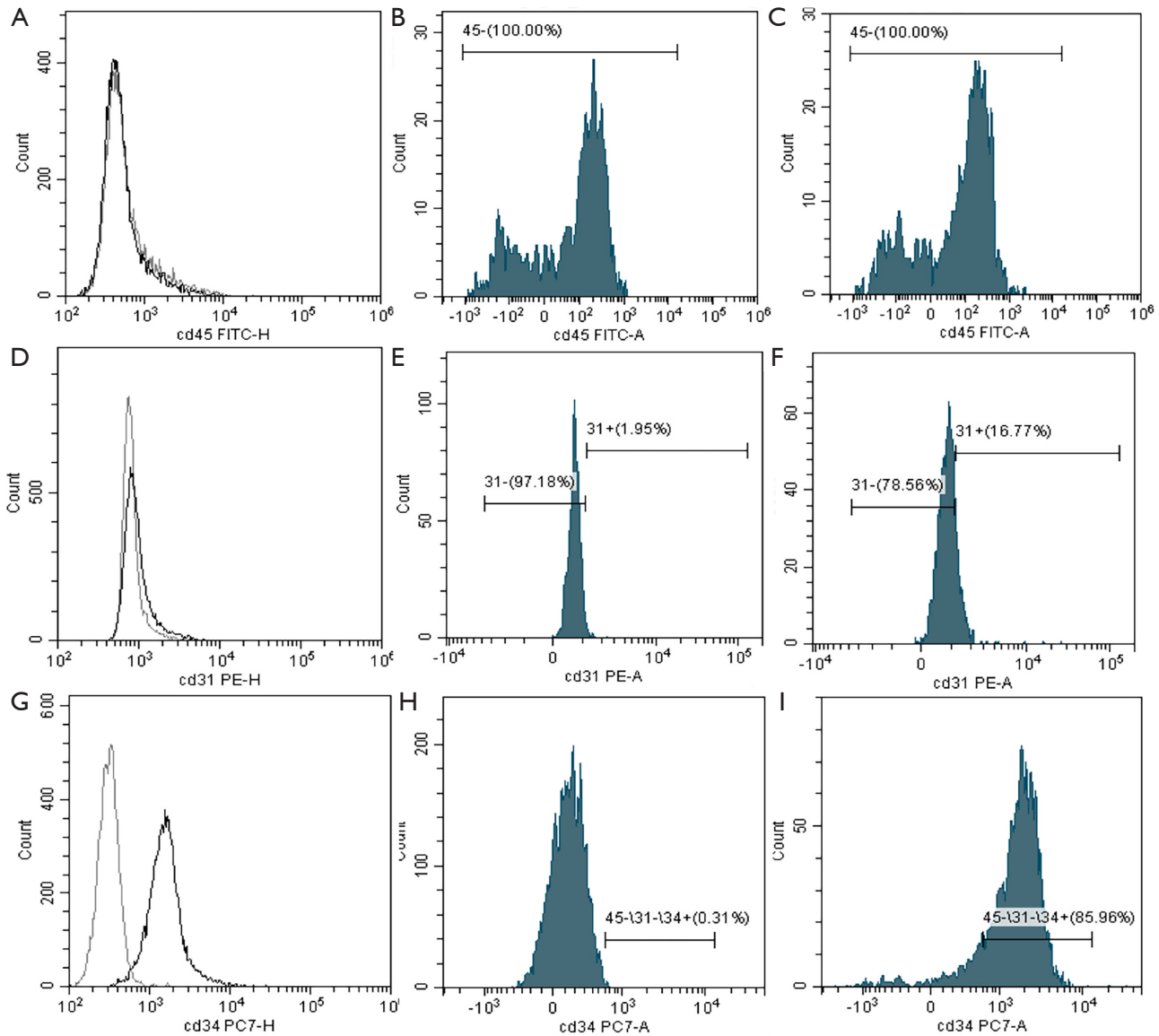

Figure S2 Immunophenotype characterization of ASCs represented by two-dimensional flow cytometry histograms. (A,D,G) Histograms represent an isotype-matched negative Ab control (gray line) and Abs against CD45, CD31, and CD34 respectively (black line); (B,E,H) histograms represent control antibody staining; Histograms displaying staining for CD45-FITC, the panhematopoietic marker (C), CD31-PE, found on endothelial cells, platelets and leukocytes (F) and CD34-PC, a marker for pluripotent stem cells (I). Insets indicate markers and percentages of positively stained cells. Specific-marker fluorescence intensity and cell counts are indicated in abscissa and ordinate respectively.

https://dx.doi.org/10.21037/sci-2023-010

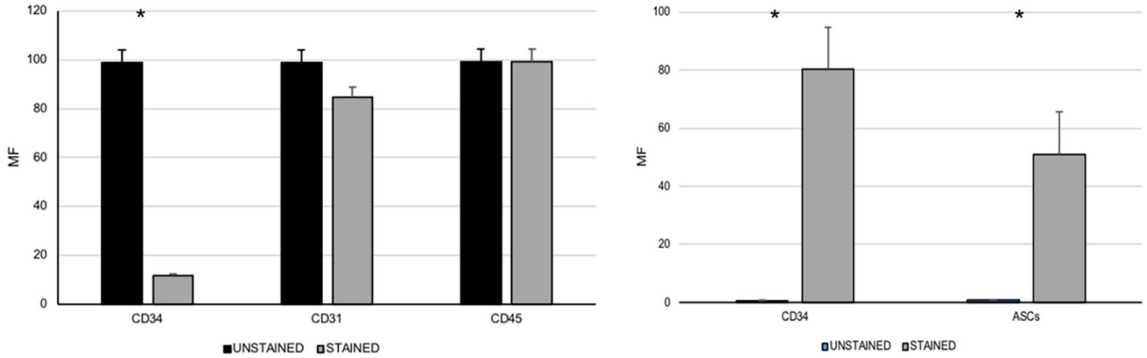

Figure S3 Phenotype characterization by flow cytometry of cell fraction in the stromal vascular fraction containing ASCs. Freshly isolated SVF cells were analyzed using three specific mAb antibodies labeled with three different fluorochromes: PE-labeled mAb anti-CD31, PC7-labeled mAb anti-CD34, APC-labeled mAb anti-CD45. The $CD31^{-}$ $CD45^{-}$ cell fraction was gated to characterize the ASCs population that was $CD34^{+}$. Data are expressed as mean and standard error. N=32. *, P value >0.0001 Unstained *vs.* Stained. MF, mean fluorescence.

https://dx.doi.org/10.21037/sci-2023-010