Strategies to Identify Mesenchymal Stromal Cells in Minimally Manipulated Human Bone Marrow Aspirate Concentrate Lack Consensus

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Background: There is a need to identify and quantify mesenchymal stromal cells (MSCs) in human bone marrow aspirate concentrate (BMAC) source tissues, but current methods to do so were established in cultured cell populations. Given that surface marker and gene expression change in cultured cells, it is doubtful that these strategies are valid to quantify MSCs in fresh BMAC.

Purpose: To establish the presence, quantity, and heterogeneity of BMAC-derived MSCs in minimally manipulated BMAC using currently available strategies.

Study Design: Descriptive laboratory study.

Methods: Five published strategies to identify MSCs were compared for suitability and efficiency to quantify clinical-grade BMAC-MSCs and cultured MSCs at the single cell transcriptome level on BMAC samples being used clinically from 15 orthopaedic patients and on 1 cultured MSC sample. Strategies included (1) the guidelines by the International Society for Cellular Therapy (ISCT), (2) CD271 expression, (3) the Ghazanfari et al transcriptional profile, (4) the Jia et al transcriptional profile, and (5) the Silva et al transcriptional profile.

Results: ISCT guidelines did not identify any MSCs in BMAC at the transcriptional level and only 1 in 9 million cells at the protein level. Of 12,850 BMAC cells, 9 expressed the CD271 gene. Only 116 of 396 Ghazanfari genes were detected in BMAC, whereas no cells expressed all of them. No cells expressed all Jia genes, but 25 cells expressed at least 13 of 22. No cells expressed all Silva genes, but 19 cells expressed at least 8 of 23. Most importantly, the liberalized strategies tended to identify different cells and most of them clustered with immune cells.

Conclusion: Currently available methods need to be liberalized to identify any MSCs in fresh human BMAC and lack consensus at the single cell transcriptome and protein expression levels. These different cells should be isolated and challenged to establish phenotypic differences.

Clinical Relevance: This study demonstrated that improved strategies to quantify MSC concentrations in BMAC for clinical applications are urgently needed. Until then, injected minimally manipulated MSC doses should be reported as rough estimates or as unknown.

Keywords: bone marrow aspirate concentrate; mesenchymal stromal cells; single-cell RNA sequencing; stem cell marker

Regenerative therapies with autologous bone marrow (BM) have experienced a lot of attention in recent years. Bone marrow aspirate concentrate (BMAC) is considered a rich source for mesenchymal stromal cells (MSCs), among other immunomodulatory components, and is used to treat bone defects, osteoarthritis, tendinopathies, and other orthopaedic conditions.[6,13,14,24,26,32] Even though BMAC has been in use for over a decade,[28,30] it remains poorly characterized and the clinical-translational field has called for more rigorous analysis of its cellular composition, and of its MSC dose specifically.[19,22,23,28] To identify, quantify, and/or isolate MSCs from human BMAC are prerequisites for formulation, optimization, and administration of the right dose of BMAC to the patient, and a large variety of strategies has been used in literature to target MSC identification.[1,4,10,29] Therefore, to provide guidelines for research and clinical use, the International Society for Cellular Therapy (ISCT) has suggested a 3-level approach to confirm the identity of an MSC: (1) MSCs must adhere to plastic; (2)

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MSCs express the surface proteins CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR; (3) MSCs can differentiate into osteoblasts, chondrocytes, and adipocytes in vitro.[10] The clinical practicality of these guidelines has been questioned, as autologous BMAC treatments are typically minimally manipulated—that is, directly reinjected after aspiration and concentration—all within the same surgical procedure.[7] Therefore, it is impossible to perform the suggested culture experiments to quantify plastic-adherent cells and their multipotency before dosing. The usefulness of in vitro multipotency to discriminate a specific MSC population has been challenged further as different bone-derived mesenchymal cell populations show multipotency in vitro in a mouse model.[9] However, less than 1 mL of BMAC contains enough cells to quantify surface marker expression, but these markers may only work for cultured MSCs, but not for fresh, noncultured human MSCs, because cell identity and surface protein expression changes upon culturing.[11] As an example, previous studies have suggested that a majority of fresh MSCs may lack protein expression of CD90,[25] while in contrast, some $CD34^+$ cells and even a small fraction of cells expressing the hematopoietic marker CD45 have demonstrated mesenchymal features in vitro.[16,20] To overcome this issue, researchers have looked for specific markers that identify freshly harvested MSCs immediately after BMAC aspiration and found that CD271 is the most convenient marker to separate MSCs from other BM cell populations.[2] However, CD271 does not qualify as a single marker for MSCs as approximately 44% of $CD271^+$ cells are also positive for the hematopoietic progenitor/endothelial cell marker CD34.[25]

Gene expression analysis represents another potential approach to quantify and characterize MSCs instead of, or in combination with, surface protein expression. Top expressed transcripts of cultured MSCs include FN1, COL1A1, COL1A2, SPARC, TGFBI, CFL1, VIM, and others,[15,27] but like protein markers, many gene expression patterns change immediately upon exposure to culture conditions.[11] Ghazanfari et al[11] found only 2 out of 16 gene clusters remained unchanged in lineage-depleted $CD45^-/CD31^-/CD71^-/CD235a^-/CD271^+$ minimally manipulated bone marrow cells compared with their cultured counterparts. These gene clusters are of particular interest, as they may conveniently identify both cultured and fresh MSCs. However, this hypothesis is yet untested. In addition, there has been some debate about the heterogeneity or multiple MSC subtypes. Single cell transcriptional profiles would allow us to make these observations, unlike any other technique.[17]

Current strategies to identify MSCs have been established in, and focused on, cultured MSCs, and it is unclear whether they are also suited to identify MSCs in a source that is of immediate clinical relevance (ie, fresh, minimally manipulated BMAC). Therefore, the aim of this study was to evaluate the potential of these strategies to identify MSCs in fresh human BMAC that are being used for autologous reinjection. To do so, we assessed the single cell transcriptome of fresh human BMAC by single-cell RNA sequencing (scRNA-seq), identified general BMAC cell populations based on transcript expression, and screened for MSCs using (1) the ISCT guidelines,[10] (2) CD271,[2] (3) the transcript expression pattern by Jia et al,[15] (4) the transcript expression pattern by Silva et al,[27] and (5) the genes found to be expressed before and after culture by Ghazanfari et al.[11] Furthermore, we used the 2 surface marker-based strategies (ISCT and CD271) to quantify MSCs by flow cytometry. We hypothesized that these culture-established strategies will fail to identify fresh MSCs in human BMAC, and that a low overlap between seemingly identified MSCs by the different strategies will leave us unable to conclude about the actual MSC numbers and heterogeneity.

Abbreviations used in this article are defined in Table 1.

METHODS

Participants and Study Approval

Orthopaedic patients aged older than 18 years, free from hematologic diseases, and receiving BMAC injections as their standard of care were recruited from University of California San Diego (UCSD) clinics. For this study, 1 mL of BMAC was used while the remaining volume was injected or mixed with graft and placed into the surgical site. A total of 15 participants were enrolled (Table 2) by giving informed written consent. Approval for this study was obtained from the UCSD Institutional Review Board. Cultured human MSCs were purchased (Lonza AG).

Bone Marrow Aspiration and Concentration

Approximately 52 mL of bone marrow aspirate in 8 mL Acid Citrate-Dextrose anti-coagulant (Citra Labs LLC) was obtained from the iliac crest or the acetabulum using the Angel BMC kit (Arthrex Inc) with the patient under

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TABLE 1
Abbreviations

Abbreviation	Definition
ACK	Ammonium-Chloride-Potassium
APC	Allophycocyanin
BM	Bone marrow
BMAC	Bone marrow aspirate concentrate
BSA	Bovine serum albumin
BV421	Brilliant Violet 421
BV650	Brilliant Violet 650
BV711	Brilliant Violet 711
CD	Cluster of differentiation
CF594	Cyanine-based fluorescent dye 594
cpD	classical & plasmacytoid dendritic
Cy7	Cyanine7
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
GEM	Gel bead emulsion
GEO	Gene Expression Omnibus
Gran	Granulocyte
GRCh38	Genome Reference Consortium human build 38
HBSS	Hank's balanced salt solution
ISCT	International Society for Cellular Therapy
Lin	Lineage
MSCs	Mesenchymal stromal cells
NK	Natural killer
PCs	Principal components
PE	Phycoerythrin
RBCs	Red blood cells
scRNA-seq	Single cell RNA sequencing
UCSD	University of California San Diego

general anesthesia. Bone marrow aspirate was then loaded into the Angel PRP System Centrifuge (Arthrex Inc) and spun according to the manufacturer's protocol with a 2% hematocrit setting. The aspirate of patient 15 was concentrated with the EmCyte system (EmCyte Corp) according to the manufacturer's protocol.

Single-Cell RNA Sequencing

The BMAC of patients 1 to 11 (Table 2) was used for scRNA-seq. A total of 1 mL of BMAC was diluted with 1 mL of Hank's Balanced Salt Solution (HBSS) and transported on ice from the surgery room to the laboratory. The remaining red blood cells (RBCs) were digested in ACK Lysate buffer for 7 minutes and the supernatant was removed after centrifugation at 300 *g* for 5 minutes. Then, RBC digestion was repeated, and the pellet was resuspended in 1 mL HBSS and filtrated through a 40 μm Flowmi tip strainer (Bel-Art). Cell counts and viability were assessed by Trypan blue staining and a single cell suspension at 1000 cells/μL in Dulbecco's phosphate buffered saline (DPBS) containing 0.04% bovine serum albumin was used to prepare the gel bead emulsion (GEM). GEM preparation, reverse transcription, cDNA amplification and subsequent quality control, library construction and subsequent quality control, and sequencing were performed at the Institute for Genomic Medicine core at UCSD, strictly according to the Chromium Single Cell 3′ V2 protocol (10X Genomics). The targeted sequencing depth was 50,000 reads per cell.

Bioinformatics Analysis

Quality control, alignment, and quantification of reads were performed using Cell Ranger V2.2.0 software from 10 × Genomics. Sequencing reads were mapped to the human genome (GRCh38) and annotated with Ensembl release 84. The R package Seurat[5] was used for downstream dimension reduction, clustering, and differential expression analyses. Before downstream analyses, cells with high percentages of mitochondrial genes (≤15%) and low number of unique genes per cell (<750) were removed.[21] After low-quality cells were filtered out, gene expression levels were log-normalized and scaled using Seurat functions NormalizeData and ScaleData, respectively. The FindVariableGenes function was used to find the top 1989 genes by variable dispersion. Principal component (PC) analysis was used on the scaled data and subset of variable genes. Sixteen PCs were deemed significant using the elbow plot method. Subsequently, the 16 PCs were clustered using the shared nearest neighbor algorithm implemented by the Seurat function FindClusters. Differentially expressed genes were calculated using the FindMarkers function, which applies the Wilcoxon rank-sum test with Bonferroni correction. The codes are available on https://github.com/ucsd-ccbb/Ward_scRNAseq_2019.

The following cell populations were determined according to their expression of canonical signature genes: T cells (CD3D, CD3E, CCR70)[12]; CD8 + T cells (CCL5, CD8A, CD8B)[12]; erythroblasts (AHSP, PRDX2, HBM, HBD)[12]; monocytes (S100A9, S100A8, S100A12, VCAN, FCN1)[33]; FCGR3A + monocytes (FCGR3A, MS4A7)[12]; B cells (CD79A, MS4A1, BANK1)[12]; B cell progenitors (TCL1A, IRF4, CD24, PCDH9),[12] CD34 + (hematopoietic) progenitors (CD34, SPINK2, CDK6)[12]; granulocyte progenitors (PRSS57, MPO, AZU1, ELANE, PRTN3)[12]; classical dendritic cells (FCER1A, CLEC10A, CD1C)[33]; plasmacytoid dendritic cells (TCF4, IRF8, JCHAIN)[31,33]; plasma cells (IGHA2, IGHGP, DERL3, SDC1)[12]; pre plasma cells (DNTT, VPREB1, VPREB3)[12]; and natural killer cells (GNLY, NKG7).[12]

Flow Cytometry

The BMAC of patients 12 to 15 (Table 2) was used to detect surface proteins by flow cytometry. After digestion of RBCs (see above), cells were incubated in washing buffer (DPBS + 2.5% fetal bovine serum [FBS]) containing the following antibodies for 30 minutes on ice: mouse anti-human CD271-PE (#560927); CD105-BV421 (#566265); CD90-APC (#561971); CD73-FITC (#561254); CD45-BV711 (#564358); CD34-PE-CF594 (#562383); CD19-PE-Cy7 (#560911); CD14-BV650 (#563420); and HLA-DR-APC-H7 (#561358, all BD Biosciences). After washing, dead cells were stained using the Fixable viability dye eFluor 506

TABLE 2
Patient Characteristics[a]

Patient No.	Sex	Age, y	Height, cm	Weight, kg	BMI, kg/m^2	Ethnicity/Race
1	Male	25	183	74.8	22.3	Not declared/White
2	Male	39	170	61.2	21.2	Non-Hispanic/Asian
3	Male	28	175	117.9	38.5	Non-Hispanic/White
5	Male	35	185	102.5	29.9	Non-Hispanic/White
9	Male	35	178	95.3	30.1	Non-Hispanic/White
10	Male	73	175	75.3	24.6	Hispanic/Other or mixed
12	Male	76	175	103.9	33.8	Non-Hispanic/White
13	Male	57	188	95.3	27.0	Non-Hispanic/White
Male mean		46.0	178.7	90.8	28.4	
Male SD		20.0	6.1	18.7	5.9	
4	Female	63	152	49.0	21.2	Not declared
6	Female	57	163	51.7	19.5	Non-Hispanic/White
7	Female	66	165	86.2	31.7	Non-Hispanic/White
8	Female	68	150	61.2	27.2	Non-Hispanic/White
11	Female	66	163	71.7	27.0	Non-Hispanic/White
14	Female	77	160	65.8	25.7	Non-Hispanic/White
15	Female	79	157	51.3	20.7	Non-Hispanic/White
Female mean		68.0	158.6	62.4	24.7	
Female SD		7.7	5.8	13.4	4.4	

[a]BMI, body mass index.

TABLE 3
Single-Cell RNA Sequencing Quality Control[a]

Patient No.	Sex	Cell No.	Mean Reads per Cell	Median Genes per Cell	No. of Reads	Valid Barcodes, %	Q30 Bases in Barcode, %	Q30 Bases in RNA Read, %	Total Genes Detected, %
1	Male	1159	144,891	991	167,928,775	98.4	97.0	72.0	17,544
2	Male	1769	133,580	1506	236,304,719	95.9	98.4	64.6	19,857
3	Male	1207	64,701	1049	78,095,197	92.4	97.9	64.2	17,865
5	Male	609	167,516	1108	102,017,587	96.7	98.0	64.2	16,982
9	Male	1759	46,468	826	81,737,455	98.2	97.8	82.2	17,727
Male mean		1301	111,431	1096	133,216,747	96.3	97.8	69.4	17,995
Male SD		484	52,819	252	67,996,223	2.4	0.5	7.9	1094
4	Female	810	158,715	1007	128,559,485	97.3	98.1	63.2	17,108
6	Female	1183	74,481	1191	88,112,077	97.2	97.8	83.2	17,917
7	Female	2600	41,652	657	108,296,301	98.8	98.0	81.9	17,336
8	Female	1754	35,579	876	62,406,503	98.6	97.6	80.6	17,493
Female mean		1587	77,607	933	96,843,592	98.0	97.9	77.2	17,464
Female SD		779	56,708	225	28,279,644	0.8	0.2	9.4	341
Cultured MSCs		3001	62,174	3450	186,586,855	98.5	97.8	75.9	20,392

[a]MSC, mesenchymal stromal cell.

(#65-0866-14, eBioscience) in DPBS for another 30 minutes on ice. After washing, the pellet was resuspended in pH-adjusted (7.4) DPBS containing 2.5% FBS and 1 mM EDTA, and run through a 40-μm cell strainer cap into a 5-mL tube. Surface protein expression was assessed on a ZE5 flow cytometer (BioRad). Compensation was established using single stain beads and, if necessary, manually adjusted in the FlowJo software (V.10.6.1, BD Biosciences). Gates were set in FlowJo and double-checked using Fluorescence Minus One stains (Appendix Figure S1, available in the online version of this article).

RESULTS

ISCT Markers, Ghazanfari Genes, Jia Genes, and Silva Genes Identify Cultured MSCs

Of 11 patient samples, 9 passed quality control and were included for analysis. The transcriptomes of 3001 purchased MSCs (62,000 reads per cell) and 1428 ± 606 cells per patient (96,398 ± 54,025 reads per cell) were analyzed (Table 3). Cell cycle/proliferation markers MKI67, CENPF, TOP2A, ASPM, NUSAP1, and TYMS did not significantly

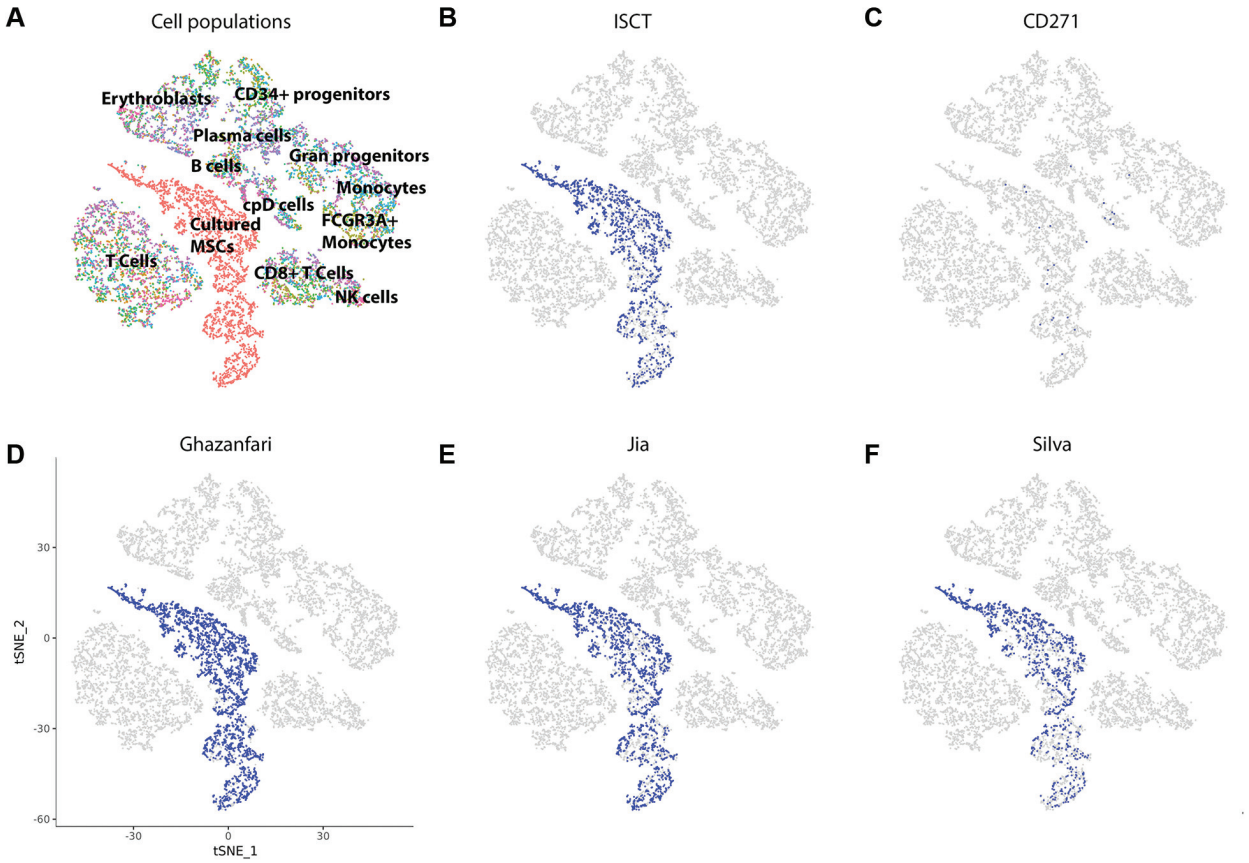

Figure 1. Two-dimensional *t*-distributed stochastic neighbor embedding plots of pooled BMAC cells and cultured MSCs. (A) Cell populations labeled according to cell type–specific expression of key genes. The 9 different BMAC samples and the cultured MSCs are represented by different colors. (B) The ISCT guidelines identified the cluster of cultured MSCs. (C) The NGFR gene (CD271) is barely expressed by cultured MSCs. (D) The cluster of cultured MSCs identified by the ''cells expressing at least 31 of 116 Ghazanfari genes'' criterion. (E) The cluster of cultured MSCs identified by the ''cells expressing at least 18 of 22 Jia genes'' criterion. (F) The cluster of cultured MSCs identified by the ''cells expressing at least 19 of 23 Silva genes'' criterion. BMAC, bone marrow aspirate concentrate; cpD, classical and plasmacytoid dendritic; Gran, granulocyte; ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cell; NK, natural killer.

contribute to clustering, as none of these genes appeared in the top PCs. PC 7 contained TOP2A and NUSAP1 (data not shown).

To first verify that all strategies identify cultured MSCs, we pooled the single cell transcriptomes of cultured MSCs with our patient samples and labeled all cell clusters (Figure 1A). Cultured MSCs were initially divided into 3 separate clusters, which we manually merged because they showed minimal transcriptional variability and presented in their own clade in an unbiased hierarchical clustering diagram. Homogeneous MSC identification across this new single cluster further supported this step.

Of the protein expression-based markers, the ISCT definition[10] labeled 1354 of 3001 cultured MSCs (Figure 1B), while the CD271 transcript was only expressed by 14 cultured MSCs (Figure 1C). From 396 genes that were reported to be expressed before and after culture by Ghazanfari et al,[11] only 116 were detected in our BMAC samples. Of the cultured MSCs, 2624 expressed at least 31 of these 116 genes at the same time (Figure 1D).

The Jia strategy[15] detected 1738 of the cultured MSCs (Figure 1E). Only 23 of the cultured MSCs were positive for all 23 Silva genes[27] (data not shown), but 1128 cells expressed at least 21 of the 23 genes (Figure 1F).

ISCT Markers, Ghazanfari, Jia, and Silva Gene Patterns Do Not Identify ''MSCs'' in Minimally Manipulated BMAC Unless the Criteria Were Liberalized

After removing the cultured MSCs from the analysis and all clusters being labeled (Figure 2A), the ISCT definition failed to identify any MSCs in BMAC. However, when THY1/CD90 positivity was removed from the ISCT

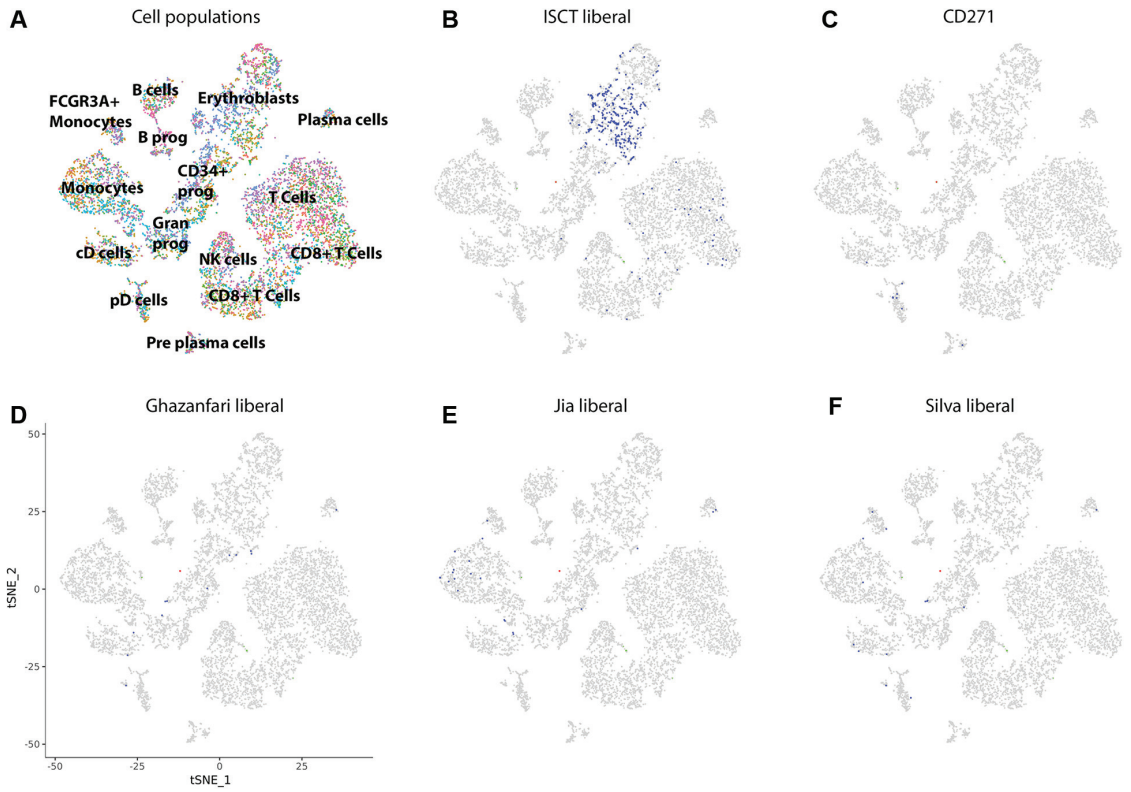

Figure 2. Two-dimensional *t*-distributed stochastic neighbor embedding (tSNE) plots of pooled BMAC cells. (A) Cell populations labeled according to cell type–specific expression of key genes. The 9 different BMAC samples are represented by 9 different colors. (B) BMAC cells expressing NT5E (CD73) or ENG (CD105), or both (liberal ISCT guidelines). (C) BMAC cells expressing NGFR (CD271). (D) BMAC cells expressing at least 9 of 116 Ghazanfari genes. (E) BMAC cells expressing at least 13 of 22 Jia genes. (F) BMAC cells expressing least 8 of 23 Silva genes. (B-F) Blue cells were identified by the respective strategy only, red cells were identified by the respective strategy and clustered with cultured MSCs in Fig. 1, light green cells clustered with cultured MSCs but were not identified by the respective strategy. BMAC, bone marrow aspirate concentrate; cD, classical dendritic; Gran prog, granulocyte progenitors; ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cell; NK, natural killer; pD, plasmacytoid dendritic.

definition, 1 cell matched the criteria of expressing both NT5E (CD73) and ENG (CD105). In total, 353 cells expressed either NT5E or ENG (Figures 2B and 3) or both. Nine cells were positive for NGFR (CD271) (Figures 2C and 3). No BMAC cells expressed at least 31 of 116 Ghazanfari genes, but 17 cells expressed at least 9 of the 116 genes (Figures 2D and 3). Similarly, no cells expressed 18 of 22 Jia genes or 19 of 23 Silva genes, but 25 BMAC cells expressed at least 13 of the 22 Jia genes (Figures 2E and 3), and 19 cells expressed at least 8 of the 23 Silva genes (Figures 2F and 3). Importantly, there was no separate cluster that could have been identified as MSCs independent of failing strategies, indicating that MSCs were either too rare or not unique enough to drive their own cluster. As an additional approach, we identified all BMAC cells that clustered with cultured MSCs and found 5 of these 11 BMAC cells shared features defined by other strategies (Appendix Table S1, available online; Figure 2, B-F). In summary, different cells and cell percentages were called MSCs in patient BMAC samples depending on which strategy was applied (Figure 3, Table 4).

Flow Cytometry–Based ISCT and CD271 Markers do Not Identify the Same Cells

As identification of MSCs at the transcriptional level did not lead to coherent results, BMAC of 4 patients was subjected to flow cytometry to quantify MSCs by surface protein expression. A total of 14.3 million events were recorded (0.7-8.6 million events per patient). Out of 8.9 million live, single cells, only 1 cell was found to be an MSC according to ISCT (Table 4, patient 13; Figure 4). This cell was also positive for CD271 (Figure 4). However, identification of only 1 single cell by flow cytometry is technically not reliable[8]; thus, it does not represent the MSC frequency in the assessed samples. At least 1 of the other 3 MSC markers were expressed in 64.3% of CD73 + cells, 58.7% of CD271 + cells, 31.2% of CD90 + cells, and only 1.3% of CD105 + cells (Figure 4). A total of 113 cells expressed 3 positive MSC markers and were negative for the 5 ISCT lineage markers CD14, CD19, CD34, CD45, and HLA-DR, but were still not considered MSCs by ISCT (Figure 4).

TABLE 4
Percentages of MSCs per Patient Depending on the Used Strategy as Assessed by scRNA-seq (ie, Transcript Expression) or Flow Cytometry (ie, Protein Expression; Last 2 Columns)[a]

Patient No.	Sex	ISCT Strict	ISCT Liberal	CD271$^+$ (NGFR)	Ghazanfari Liberal	Jia Strict	Jia Liberal	Silva Strict	Silva Liberal	Cells That Cluster With Cultured MSCs	ISCT Strict	Lin$^-$CD271$^+$
1	Male	0	2.416	0	0.259	0	0.259	0	0.259	0.173		
2	Male	0	1.809	0.113	0.396	0	0.961	0	0.622	0.170		
3	Male	0	4.474	0.331	0.166	0	0.083	0	0.083	0.166		
5	Male	0	4.105	0	0	0	0	0	0	0		
9	Male	0	1.592	0	0	0	0.114	0	0	0		
12	Male										0	0.015
13	Male										$<10^{-5}$	0.002
Male mean		0	2.879	0.089	0.164	0	0.283	0	0.193	0.102	$<10^{-5}$	0.009
Male SD		0	1.329	0.144	0.171	0	0.390	0	0.262	0.093		0.009
4	Female	0	2.346	0	0.247	0	0.247	0	0.370	0.123		
6	Female	0	2.451	0.254	0.169	0	0	0	0	0.169		
7	Female	0	4.231	0	0.000	0	0	0	0	0		
8	Female	0	1.596	0	0.057	0	0	0	0.057	0.057		
14	Female										0	0.013
15	Female										0	0.017
Female mean		0	2.656	0.063	0.118	0	0.062	0	0.107	0.087	0	0.015
Female SD		0	1.117	0.127	0.111	0	0.123	0	0.178	0.074	0	0.003

[a]Lin$^-$ includes CD14$^-$CD19$^-$CD34$^-$CD45$^-$HLA-DR$^-$ cells as suggested by ISCT.[10] ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cells; scRNA-seq, single-cell RNA sequencing.

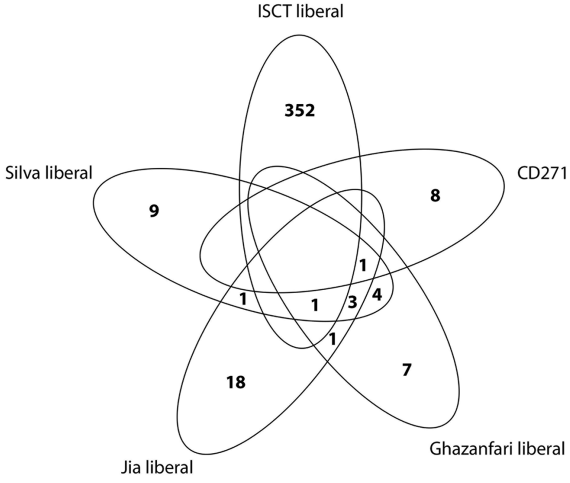

Figure 3. Venn diagram showing the overlap of cells identified as ''MSCs'' at the transcriptional level by the different liberal strategies. A total of 12,850 cells from 9 patients were analyzed. ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cell.

Percentages of Different BMAC Cell Populations Identified by scRNA-seq

Based on classification by canonical use of transcript markers, the predominant cell populations in human BMAC are immune cells. T and CD8 + T cells are the largest cell population, covering 22.0% and 17.3%, respectively, of all BMAC cells, followed by 15.7% erythroblasts, 13.3% monocyte populations, 7.9% CD34 + progenitors, 6.4% dendritic cells, 6.2% B cell populations, 4.5% granulocyte progenitors, 3.6% natural killer cells, and 3% plasma cell populations (Table 5).

The entire scRNA-seq raw data are uploaded to Gene Expression Omnibus (GEO) accession number GSE162692.

DISCUSSION

Aspiration and reinjection of minimally manipulated BMAC during the same surgical procedure is the current state of the art for clinical application of autologous BMAC in orthopaedics in the United States.[7] Therefore, the goal of the current study was to establish the presence, quantity, and heterogeneity of BMAC-derived MSCs in minimally manipulated BMAC. By assessing the single cell transcriptome, we were able to apply the most commonly used strategies from the literature and found that, when strictly applied, no BMAC cells were identified as MSCs using the ISCT definition. Seeking for optimization of these strategies, we liberalized the criteria to evaluate potential bottlenecks and suggest improvements. Unfortunately, consensus between these liberal strategies was relatively low at the single-cell transcriptome level. The cells called "MSCs" by these liberalized strategies were already part of different, annotated clusters; thus, most of them were likely false positives. Analysis of protein expression revealed that the ISCT definitions did not catch MSCs within the expected range and that there are discrepancies between the ISCT versus CD271 expression. Therefore, both transcriptional and protein expression data suggest

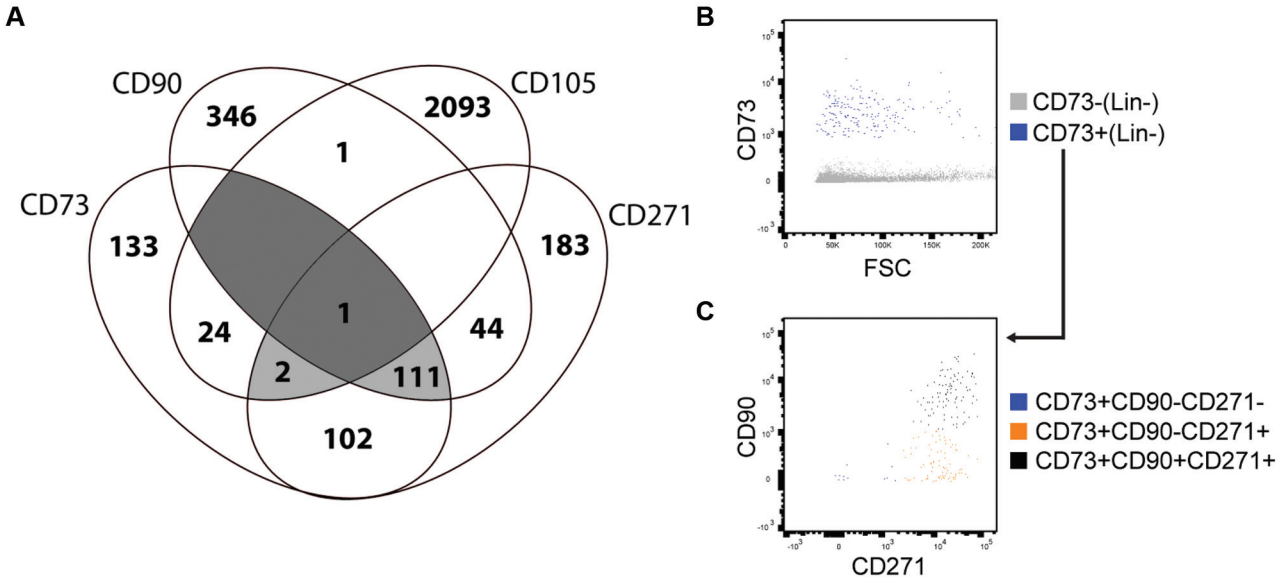

Figure 4. (A) Venn diagram showing the overlap of cells expressing the surface proteins CD73, CD90, CD105, and CD271. The dark gray areas denote ''MSCs'' according to ISCT, the light gray areas denote triple positivity but not being called an MSC by ISCT. A total of 6.7 million live, single cells that are negative for CD14, CD19, CD34, CD45, and HLA-DR (Lin-) from 4 patients were used for this diagram (14.3 million total events recorded by flow cytometry). (B) Forward scatter dot plot of all Lin- cells of patient 15. CD73 + cells were forwarded into (C) as an example of Lin- cell with overlapping surface markers used in (A). ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cell.

TABLE 5
BMAC Cell Populations by Patient as Identified by scRNA-seq[a]

Patient No.	T Cells	CD8 + T Cells	Erythroblasts	Monocytes	FCGR3A + Monocytes	CD34 + Progenitors	B Cells	Pre/Pro B Cells	cD Cells	pD Cells	Gran Progenitors	NK Cells	Pre Plasma Cells	Plasma Cells
1	30.0	26.8	14.4	9.4	0.9	2.2	5.5	1.4	1.4	0.5	1.0	3.8	1.5	1.2
2	13.0	19.4	8.3	19.2	2.6	11.9	4.2	0.1	8.5	4.5	3.3	1.8	0.5	2.8
3	13.6	26.8	19.3	5.6	1.3	11.2	3.3	3.0	3.8	2.1	4.5	2.3	2.7	0.6
4	30.5	18.0	13.2	13.6	1.2	5.0	3.0	0.7	3.6	1.3	5.3	2.7	1.5	0.4
5	21.7	17.2	11.9	11.5	0.0	5.2	7.1	3.2	3.9	3.9	5.7	5.3	2.7	0.7
6	17.7	15.4	6.7	24.6	1.3	8.1	3.1	1.2	5.7	4.2	7.5	1.5	1.3	1.6
7	8.4	2.0	41.8	3.1	2.9	19.2	1.2	1.2	3.9	1.4	8.3	3.1	2.2	1.5
8	32.3	13.7	8.3	12.0	4.3	5.2	3.5	4.5	3.2	2.3	3.2	5.4	2.0	0.2
9	31.1	16.4	17.2	6.2	0.3	3.6	6.3	3.6	2.0	1.4	1.9	7.0	1.6	1.3
Mean	22.0	17.3	15.7	11.7	1.6	7.9	4.1	2.1	4.0	2.4	4.5	3.6	1.8	1.2
SD	9.2	7.4	10.7	6.8	1.4	5.3	1.9	1.5	2.1	1.5	2.4	1.9	0.7	0.8

[a]Data are reported as %. BMAC, bone marrow aspirate concentrate; cD, classical dendritic; Gran, granulocyte; NK, natural killer; pD, plasmacytoid dendritic; scRNA-seq, single-cell RNA sequencing.

that a new gold standard to identify MSCs in this clinically relevant source tissue is needed.

The current gold standard to identify MSCs was proposed by the ISCT and based on flow cytometry using 9 reference markers, of which 3 are positive: CD73; CD90; and CD105.[10] We here confirmed that these markers remain valid in cultured MSCs at the transcriptional level. Interestingly, only 45% of the cultured cells expressed all 3 positive markers, suggesting that a transcriptional heterogeneity exists between these cells. Nevertheless, the number of tagged cells was sufficiently high to confidently identify the cultured MSC cluster within other cell populations. None of the BMAC cells expressed THY1 (CD90), thus the ISCT guidelines were not able to detect noncultured MSCs in patient BMAC. As only 1 of 353 BMAC cells expressing NT5E (CD73) or ENG (CD105) was also identified as an MSC by other strategies, we concluded that liberalizing the positive marker criteria for the ISCT guidelines may be the wrong approach. On the other hand, this cell was clustered with cultured MSCs and complied with the liberal Ghazanfari, Jia, and Silva strategies, and thus could be an MSC. Interestingly, it was negative for NGFR (CD271), underpinning the proposed transcriptional heterogeneity (Appendix Table S1, available online; Figure 3). Furthermore, identification of MSCs in fresh BMAC also failed at the protein level, as only 1 in 8.9

million cells was an MSC according to ISCT, which is not a technically reliable population size in flow cytometry.[8]

CD271 (NGFR) was reported to be a convenient marker to isolate a multipotent cell fraction from human bone marrow.[1,2,25] We found less than 0.1% of BMAC cells expressed NGFR and none of them expressed NT5E or ENG, which supports previous findings of subpopulation-specific expression of NGFR.[3,18] Only 1 $NGFR^+$ cell was also identified by other strategies: it clustered with cultured MSCs and complied with the liberal Ghazanfari, Jia, and Silva gene strategies, and as such, could potentially be an MSC. At the protein level, there is significantly more overlap of $CD271^+$ cells with $CD73^+$ and $CD90^+$ cells, but barely with $CD105^+$ cells.

Ghazanfari et al[11] published a list of 396 genes that were expressed in both fresh and cultured bone marrow-derived MSCs. This list is of high interest for clinical researchers, as it might be a convenient tool to identify both minimally manipulated and cultured MSCs. Only 116 of these genes were expressed in the current BMAC samples, but we highly recommend these genes to identify cultured MSCs, as 87% of them expressed at least 31 of these genes, and thus this strategy is far more precise and efficient than the ISCT-defined positive markers (45%). On the other hand, this list had to be liberalized to 9 of 116 genes to tag a significant number of cells in BMAC. Nevertheless, 10 of 17 tagged cells (59%) were suggested to be an MSC by other strategies, too. Like the ISCT guidelines, this strategy included a 9-marker criterion. At this point, it must be considered that Ghazanfari et al analyzed an NGFR-positive population; thus, it remains to be elucidated whether a gene list of the true, entire multipotent stromal population could lead to different results.

Of the 11 cells that clustered with cultured MSCs, 5 were also identified by other strategies and all 5 cells expressed the liberal Ghazanfari, Jia, and Silva genes. Furthermore, every cell that was identified as potential MSC by more than 1 strategy was identified by Jia, Silva, or both, pointing out a potential accuracy of these strategies. Both publications by Jia et al[15] and Silva et al[27] analyzed the entire adherent cell fraction and, as such, do not miss adherent subpopulations. On the other hand, they lack resolution at the single-cell level, fail to identify subpopulations, and are biased toward cultured MSCs. Therefore, the relevance of these strategies for clinical applications still needs to be evaluated.

This study has several limitations. First, protein expression-based markers do not necessarily need to be mirrored by transcript expression. However, ISCT protein markers were expressed at the transcriptional level. Second, as overlap between strategies to identify noncultured MSCs is relatively low and no gold standard for minimally manipulated BMAC-MSCs has been established yet, there is no verification of whether the identified cells truly are MSCs. Third, as MSCs are only a small cell population, it is open to question whether other cell populations (eg, T cells) should have been depleted before transcriptional analysis to measure more BMAC-derived MSCs. We decided not to do so, as the purpose of this study was the analysis of BMAC that is in the form it can clinically be applied. Furthermore, even when 8.9 million cells from 4 RBC-depleted BMAC samples were analyzed by flow cytometry for ISCT markers, only 1 "MSC" was found, suggesting that the strategy itself, not the cell number being analyzed, is currently the most limiting factor.

In conclusion, this study showed that strict translation from cell culture–defined strategies to quantify MSCs to noncultured, minimally manipulated BMAC fails. When liberalizing these strategies, potential MSCs are detected by several approaches, but due to relatively low overlap, there is too little consensus between these strategies to confidently call a cell an MSC and it has to be expected that most of these "MSCs" were false positives. As such, although this study provides important answers to clinical questions, it raises even more questions, as number and transcriptome of ostensible MSCs are heterogeneous and highly dependent on the applied strategy both at the transcriptional and protein level. Therefore, more effort needs to be put into formulating a gold standard to reliably quantify all MSCs in clinically relevant cell sources, such as BMAC, so that standardized treatments with a known number of MSCs can be prescribed. Until then, clinicians and researchers should consider the applied MSC dose in BMAC injections as rough estimates or even as unknown.

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