

Isolation and Characterization of Human Mesenchymal Stromal Cell Subpopulations: Comparison of Bone Marrow and Adipose Tissue

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Preparations of mesenchymal stromal cells (MSCs) are generally obtained from unfractionated tissue cells, resulting in heterogeneous cell mixtures. Several markers were proposed to enrich these cells, but the majority of these markers are defined for bone marrow (BM). Moreover, the surface markers of freshly isolated MSCs also differ from those of cultured MSCs in addition to a phenotypic variation depending on the MSC source. For tissue engineering applications, it is crucial to start with a well-defined cell population. In this study, we performed immunomagnetic selections with five single surface markers to isolate MSC subpopulations from BM and adipose tissue (AT): CD271, SUSD2, MSCA-1, CD44, and CD34. We determined the phenotype, the clonogenicity, the proliferation, the differentiation capacity, and the immunoregulatory profile of the subpopulations obtained in comparison with unselected cells. We showed that native BM-MSCs can be enriched from the positive fractions of MSCA-1, SUSD2, and CD271 selections. In contrast, we observed that SUSD2 and MSCA-1 were unable to identify MSCs from AT, meaning they are not expressed *in situ*. Only the CD34⁺ selection successfully isolated MSCs from AT. Interestingly, we observed that CD271 selection can define AT cell subsets with particular abilities, but only in lipoaspiration samples and not in abdominoplasty samples. Importantly, we found a population of clear CD34⁺ fresh BM-MSCs displaying different properties. A single marker-based selection for MSC enrichment should be more advantageous for cell therapy and would enable the standardization of efficient and safe therapeutic intervention through the use of a well-identified and homogeneous cell population.

Introduction

MESENCHYMAL STROMAL CELLS (MSCs) were first isolated from the bone marrow (BM) by Friedenstein and coworkers in the 1960–70s [1]. Since then, they have been isolated from various human tissues, such as adipose tissue (AT) [2], umbilical cord blood and Wharton's jelly [3,4], skin [5], and some other organs [6]. They are currently the focus of particular attention because of their properties: (1) they are multipotent, (2) they can support hematopoiesis, and (3) they have trophic capacity and immunomodulatory potential thanks to their ability to secrete cytokines and chemokines [7]. Altogether, these properties make them interesting candidates for numerous clinical uses [8].

So far, no universal specific marker has been described to identify or isolate MSCs. Thus, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define

MSCs in 2006 [9]. These cells are fibroblast-like cells in standard culture conditions and must be plastic adherent. They are positive for mesenchymal surface markers (CD105, CD73, and CD90) and they lack the expression of endothelial and hematopoietic markers (CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR). They are multipotent and they should be able to differentiate into osteoblasts, adipocytes, and chondroblasts in an appropriate medium. It is important to note that this minimal set of rules defined with regard to MSCs in *in vitro* culture does not reflect what their native status might be [10]. Little is known about native MSCs, from their location to their isolation and to the nature of their surface marker expression [11].

Moreover, culture conditions can modify the pattern of molecules expressed on the MSC surface [12]. In fact, both a decline and an increase in expression of some molecules have been observed [13]. The expression of CD73 and

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CD105 appears to be constitutive regardless of the environment. In contrast, the expression of CD44, CD271, CD146, and CD106 changes during culture [14,15]. CD146 expression varies with time and the oxygen rate [16], and CD105 enrichment increased significantly after 1 week of culture [17]. The time-based variation of culture is also described for CD34 expression on adipose-derived stromal cells (ADSCs) [17,18], which starts decreasing within a few hours of adhesion [19,20]. The proliferation and differentiation of MSCs during in vitro culture can thus alter the natural characteristics that these cells have in vivo. For tissue engineering applications, it is crucial to start with a well-defined cell population, including well-characterized cell functionality. Achieving this will enable the standardization of efficient and safe clinical protocols.

Several MSC purification techniques are currently being used to isolate and enrich MSCs and their related subpopulations, including conventional plastic adherence, immunomagnetic beads (IMBs), and fluorescence-activated cell sorting (FACS) [21]. Currently, the most commonly employed method for MSC isolation is based on plastic adherence. This technique is efficient, but yields heterogeneous cell populations with different lineage commitments. Thus, more effective strategies to purify MSCs are needed.

Based on phenotypic analysis, alternative isolation strategies were proposed, including negative and positive selection or a combination of both. Antibodies against CD45 or other hematopoietic markers can be used to negatively select MSCs [22]. IMB-positive or negative isolation procedures are based on the selection or exclusion of cells expressing an antigen of interest. This method can select subpopulations and reduce cross-contamination with unwanted cells. Several cell surface antigens have been tested to improve the efficiency of MSC isolation, mostly for BM-MSCs. These markers include STRO-1, CD271, CD73, GD2, CD146 [23], etc., for the positive selection of MSCs. Concerning negative isolation, depleting selection through RosetteSep[®] has been successfully shown to be an easy method of rapidly obtaining MSCs from BM with preserved characteristics for therapeutic uses [22].

Although CD271 has been proposed as one of the most specific markers for BM-MSCs, this marker has failed to isolate MSCs from umbilical cord blood [24]. Taken together, these results highlight the fact that MSCs are not a homogeneous population, but consist of different subpopulations of cells bearing different cell surface markers and properties.

The pattern of expression of these markers also varies among these subpopulations and, most importantly, could not be applied to each type of MSC from different sources. In this study, we therefore aimed to isolate and characterize these subpopulations of MSCs from BM and AT based on their particular expression of a marker. Once obtained, the phenotype (of fresh and cultured MSCs), the ability to form colony-forming unit-fibroblast (CFU-F), the proliferative capacity, the differentiation potential, and the immunoregulatory profile of these immunoselected subpopulations were evaluated in comparison with the total MSC population purified by plastic adherence. We have chosen to deal with BM and AT as they represent the two main sources for MSC isolation in clinical trials [25]. AT seems to be an alternative source of MSCs for cell-based therapy as some disadvantages are associated with BM [26,27].

In our study, we demonstrated that MSC enrichment from a fresh sample was possible thanks to an appropriate cell selection. Importantly, the choice of the cell surface marker with which to proceed with the selection depended on the source of the sample as well as the therapeutic use of the MSCs. To obtain large amounts of BM-MSCs, selection of the MSCA-1 positive fraction seemed to be the most effective selection and the CD34 positive fraction was the only positive fraction that made ADSC enrichment possible. In contrast to ADSCs, whose CD271 expression depends on the AT sample (abdominoplasty vs. lipoaspiration), only fresh BM-MSCs expressed MSCA-1, CD271, and SUSD2. Moreover, BM-MSCs displayed both positive and negative CD34 and CD44 subpopulations. However, all fresh ADSCs are CD34⁺ and CD44⁻.

Particular immunophenotype, distinct growth and clonogenic capacities, various multilineage potentials, and specific immunoregulatory profile were pointed out for all these isolated and characterized subpopulations. Collectively, our results demonstrate that different subpopulations of MSCs with distinct characteristics might be isolated from the whole MSC population and should be used for cell-targeted therapy.

Materials and Methods

Tissue samples

Ethical approval was delivered by the institutional ethics committee. BM aspirates were obtained from healthy volunteers or from donors in the context of allogeneic transplantation after informed written consent ($n=26$). ADSCs were obtained from lipoaspirates and from abdominoplasties of healthy volunteers for esthetic surgery after informed written consent as well ($n=94$).

Choice of cell surface markers for positive MSC selection

For all our immunoselections, a chosen marker was used to isolate the corresponding subpopulation. Once obtained, all subpopulations were characterized according to the previously described goals and always compared with the whole MSC population.

CD44

CD44, also known as Pgp1, is a receptor for hyaluronic acid (HA). Other known ligands are collagens, matrix metalloproteinases, and osteopontin (OPN). This marker is implied in adhesion function, cell-cell interaction, homing, hematopoiesis, and tumor metastasis [28]. Qian et al. described fresh BM-MSCs as negative for the presence of CD44 and its expression is acquired in vitro [14].

CD34

The CD34 expression on other cells than hematopoietic progenitors is still a matter of some debate [29,30]. In a statement article published in 2006, the ISCT describes BM-MSCs as negative for CD34 expression, but this criterion is only appropriate for BM-MSCs under in vitro conditions [9]. ADSCs are described as positive for CD34 and its expression decreases in culture until its disappearance [19]. Little is known about CD34 expression on MSCs in situ [31] and, moreover, about its function [32].

MSCA-1

The MSCA-1 cell surface marker has been identified as a tissue-nonspecific alkaline phosphatase [33], which catalyzes the hydrolysis of phosphomonoesters with the production of inorganic phosphate (Pi). This enzyme is known to be important for bone remodeling. The MSCA-1 antibody selectively recognized the CD271^{bright} (see CD271 section) BM cell population, which contains CFU-Fs, but no other BM cells [34].

SUSD2

The membrane protein, sushi domain containing 2 (SUSD2), was recently described to selectively identify BM-MSCs, and only SUSD2⁺ BM cells were able to form CFU-Fs [35]. The SUSD2 function is not yet clearly defined, but the different domains of the protein are involved in a wide range of functions, such as cell adhesion and cell migration.

CD271

The CD271 antigen is also known as the low-affinity nerve growth factor receptor (L-NGFR). In 2002, Quirici et al. showed that the CD271 antibody labeled BM-MSCs with high specificity and purity, suggesting it as an enrichment marker [36].

Isolation and culture of BM-MSCs and ADSCs

BM aspirates were diluted in equivalent volumes of HBSS. Mononuclear cells (MNCs) were collected with a Ficoll-Hypaque density gradient centrifugation and washed with PBS (Lonza) supplemented with 0.05% SSPP (Stable Solution of Plasmatic Proteins) (C.A.F.-D.C.F, Belgium).

AT aspirates were directly digested in an equivalent volume of collagenase D (0.1%; Roche) during a period of 30 min at 37°C under stirring. Abdominoplasty samples were cut into small pieces ($\pm 5 \text{ mm}^3$) before collagenase D digestion (Roche) (30 min, 37°C, stirring). Floating AT and the aqueous phase were discarded after centrifugation (800 g, 5 min) to keep the pellet, called the stroma-vascular fraction (SVF). Red cells were lysed with ammonium chloride solution (StemCell Technologies) (10 min, 37°C) before cell washing with PBS supplemented with 0.05% SSPP.

Total MNCs and SVF were either directly plated in culture medium to select MSCs by plastic adherence or immunoselected as described below.

Immunomagnetic selection of the different cell fractions from BM-MNCs and AT-SVF. MNCs and SVF were incubated with microbeads coated with anti-CD34, -CD44, or -MSCA-1 antibodies for direct positive selection and with APC-conjugated antibodies, anti-CD271, or -SUSD2 for indirect positive selection according to the manufacturer's instructions (MicroBead Kit; Miltenyi Biotec). For CD271 and SUSD2 indirect selection, cells were first labeled with the appropriate conjugated antibody: SUSD2-APC (BioLegend) and CD271-APC (Microbead Kit (APC); Miltenyi Biotec), and indirectly labeled with anti-APC microbeads (MACS Miltenyi Biotec).

The cell suspension was then loaded onto a MACS column separator (MACS Miltenyi Biotec). Negative and positive fractions were collected, counted, assessed for viability, and plated in culture. Purity was determined by flow cytometry. When a sufficient number of cells was obtained

from positive fractions, native surface markers were studied (see phenotype section).

Culture and passages. BM-MNCs, AT-SVF, and isolated cells were cultured in a flask (Greiner) in culture medium (Dulbecco's modified Eagle's medium with 1.0 g/L glucose, without L-glutamine (DMEM; Lonza) supplemented with 15% fetal bovine serum (Sigma), 2 mM L-glutamine (Lonza), and 1% Pen/Strep Amphotericin B solution (Lonza). After 5 days of culture (humidified atmosphere, 5% CO₂, 37°C), we eliminated the nonadherent cells of the primary cultures (PM) through gentle washing with culture medium. Cultures were pursued until 80–90% of confluency with the medium renewed weekly.

When one fraction reached subconfluency, cells from all the fractions were harvested (TrypLE Select; Gibco) (10 min, 37°C), washed in PBS, counted, and replated at a lower density (1,000 cells/cm²). Growth, clonogenicity, and phenotype were analyzed for each passage.

Growth and clonogenicity

Cell growth was evaluated for each passage by Trypan blue (Gibco) exclusion assay. Cumulative cell number was calculated with cumulative addition of the total cell number obtained at each passage.

The number of mesenchymal progenitors obtained in different fractions and at each passage was evaluated by CFU-F assay. Briefly, 10⁵ unselected cells or 1,000–5,000 selected cells were plated in a Petri dish (100 mm diameter; Greiner) with culture medium for 10 days (humidified atmosphere, 5% CO₂, 37°C). After May-Grünwald/Giemsa staining, colonies were defined as more than 50 fibroblastic cells and scored using an inverted microscope.

Phenotype

The cell phenotype was determined immediately after sample collection (on BM-MNCs and AT-SVF), after column collecting (before any culture), and during cell passaging. Cells were incubated with adequate monoclonal antibodies (Table 1) for 30 min at room temperature in the

TABLE 1. MONOCLONAL ANTIBODIES USED FOR FLOW CYTOMETRY ANALYSIS

Antigens	Fluorochromes	Companies
CD31	FITC	Miltenyi Biotec
CD34	PC7	Becton Dickinson
CD34	PC5	BD Pharmingen
CD44	FITC	Miltenyi Biotec
CD45	PC7	BD Pharmingen
CD45	PerCP	BD Pharmingen
CD73	PE	Miltenyi Biotec
CD90	PE	Miltenyi Biotec
CD90	FITC	Miltenyi Biotec
CD105	FITC	Ancell corporation
CD146	PC5	Beckman Coulter
CD166	PE	BD Pharmingen
SUSD2	APC	BioLegend
CD271	PE	BD Pharmingen
HLA-DR	PerCP	Miltenyi Biotec
HLA-DR	PC5	BD Pharmingen
MSCA-1	APC	Miltenyi Biotec

dark. Regarding the phenotype of uncultured fractions, remaining erythrocytes were lysed and cells were fixed with the Uti-Lyze kit (Dako), according to the manufacturer's instructions. Data acquisition was realized on the MacsQuant analyzer (MACS Miltenyi Biotec) and the analysis performed with FCS 4 Express software (DeNovo Software).

In vitro differentiation assays

The multipotential capacity of MSCs was checked on cells obtained at P2 by inducing osteogenic and chondrogenic differentiation *in vitro*. The cells were cultured in appropriate differentiation medium for 2 weeks.

Osteogenic differentiation. Two thousand cells/well and 150,000 cells/well were seeded in culture medium for staining and gene expression experiments, respectively. After 5 days, the culture medium was totally discarded and replaced by osteogenic medium (NH OsteoDiff Medium; Miltenyi Biotec). The osteogenic medium was changed weekly by complete replacement. On day 14 of differentiation, the cells were fixed with 8% formaldehyde (PAF) and mineralization was qualitatively demonstrated by Alizarin Red staining. For gene expression, cells were detached (TrypLE Select; Gibco) (10 min, 37°C), centrifuged, and lysed in TriPure Isolation Reagent (Roche Applied Science). TriPure samples were then frozen before mRNA extraction (see quantitative real-time PCR section).

Chondrogenic differentiation. The cells were plated in culture as described previously. After 5 days, the culture medium was totally discarded and replaced by chondrogenic medium (NH ChondroDiff Medium; Miltenyi Biotec). The chondrogenic medium was changed weekly by complete replacement. On day 14, the chondrocyte differentiation was verified by staining of proteoglycans with Alcian Blue after PAF fixation. For gene expression, cells from six-well plates were treated as reported for osteogenic differentiation samples.

Immunoregulatory profile

The immunoregulatory profile of unselected BM-MSCs and ADSCs and their different subpopulations was established by determining the hepatocyte growth factor (*HGF*) gene expression under constitutive conditions and after inflammatory priming. Briefly, 150,000 cells/w were seeded in a six-well plate with culture medium. After 24 h, we checked whether the cells were adherent and replaced the culture medium with culture medium without FBS supplemented or not by a cocktail of proinflammatory cytokines as previously described by our group [37]. After 18 h of induction, the cells were detached (TrypLE Select; Gibco) (10 min, 37°C), centrifuged, and lysed in TriPure Isolation Reagent (Roche Applied Science). TriPure samples were then frozen before ARNm extraction (see quantitative real-time PCR section).

Quantitative real-time PCR

Total RNA was extracted from BM MSCs and ADSCs after differentiation induction or under inflammatory conditions from each fraction using the TriPure Isolation Reagent (Roche Applied Science). Then, a reverse transcription was performed to obtain cDNA from 1 µg of RNA with qScript™ cDNA SuperMix (QUANTA bioscience).

To quantify transcripts, a quantitative real-time PCR (qRT-PCR) was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). We used cDNA equivalent of 25 ng of ARN in a qRT-PCR with Fast SYBR® Green Master Mix (Applied Biosystems) and 0.20 µM of gene-specific forward and reverse primers.

Primer sequences were designed using the primer designing tool Primer-BLAST from NCBI or Primer Express 2.0 (Applied Biosystems) or from the RTprimerDB database. For each set of primers, one primer at least was designed to span an interexonic junction to avoid the amplification of contaminant genomic DNA. qRT-PCR negative controls were used with the reverse transcription of RNA samples without RT polymerase (Table 2 for primer sequences)(5'-3' forward and reverse, respectively).

The results are expressed as a relative fold change normalized to *GAPDH* gene expression and calibrated with the control value in undifferentiated/before priming MNCs for BM and undifferentiated/before priming SVF for AT. Several housekeeping genes were tested (*actin*, *18S rRNA*, *HPRT*) and *GAPDH* was chosen as its expression was not affected by our culture conditions as previously published by our group [38].

Statistical analyses

A total of 26 BM aspirates and 94 AT lipoaspirates and abdominoplasties were analyzed. Data are presented as mean ± standard error of the mean (SEM). Comparison between sorted fractions and total fraction from the same source was evaluated with the Wilcoxon matched pairs test (two tailed), and comparisons between the different sources were performed with the Mann-Whitney test (unpaired, two tailed). Differences were significant (*) for $P \leq 0.05$ ("**" stands for $0.01 < P \leq 0.05$, "***" for $0.005 < P \leq 0.01$, and "****" for $P \leq 0.005$). All analyses were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com).

Results

Patients and samples

Healthy volunteers were 16.0 ± 2.9 years old when BM aspirates were realized with a ratio of 12 men for 14 women, $n = 26$. The AT samples were obtained from 94 patients undergoing esthetic surgery, 42.6 ± 1.6 years old on the day of surgery, with a ratio of 6 men for 70 women (and 18 not specified).

TABLE 2. OLIGONUCLEOTIDE PRIMERS USED FOR QRT-PCR

<i>Transcripts</i>	<i>Forward</i>	<i>Reverse</i>
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
OPN	TGTCAGTGATTGCTTTTGC	GCCACAGCATCTGGGTATTT
COMP	CAGGGAGATCACGTTCTGTA	GGCCGGTGCGTACTGAC
HGF	CAATGCCTCTGGTTCCCCTT	AGGCAAAAAGCTGTGTTTCGTG

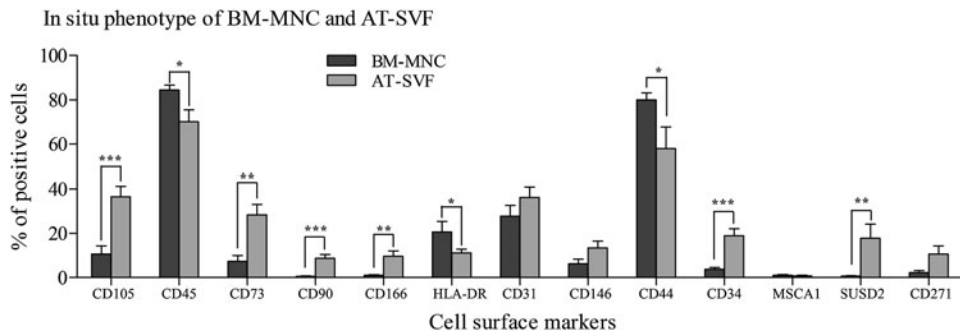


FIG. 1. Single-cell suspensions of freshly isolated bone marrow-mononuclear cells (BM-MNCs) (dark gray) and adipose tissue stroma-vascular fraction (AT-SVF) (light gray) were phenotypically characterized by flow cytometry. Data are from 20 BM and 23 AT samples. Results are expressed as the mean percentage \pm SEM of positive cells. * $0.01 < P \leq 0.05$, ** $0.005 < P \leq 0.01$, and *** $P \leq 0.005$.

BM-MNCs and AT-SVF present a different phenotype in situ

After collection, the phenotype of total BM-MNCs and total AT-SVF was analyzed by flow cytometry. Both populations presented a different phenotype as shown in Fig. 1 with several markers that are significantly higher in AT-SVF compared with BM-MNCs (CD105: $37\% \pm 5\%$ in AT-SVF and $11\% \pm 4\%$ in BM-MNCs; CD45: $70\% \pm 5\%$ and $84\% \pm 2\%$; CD73: $28\% \pm 5\%$ and $7\% \pm 3\%$; CD90: $9\% \pm 2\%$ and $0.5\% \pm 0.2\%$; CD166: $10\% \pm 2\%$ and $1\% \pm 0.2\%$, respectively). Interestingly, the percentage of CD34 positive cells is also significantly greater in AT-SVF than in BM-MNCs ($19\% \pm 3\%$ for AT-SVF vs. $4\% \pm 1\%$ for BM-MNCs). In contrast, the percentage of cells expressing CD45 (71% \pm 5%), CD44 (58% \pm 10%), and HLA-DR (11% \pm 2%) is less important in AT-SVF than in BM-MNCs (84% \pm 2%, 80% \pm 3%, and 20% \pm 5%, respectively).

Importantly, the percentages of cell expression for MSCA-1, SUSD2, and CD271 are more important in AT-SVF with mean values not exceeding 20% ($0.7\% \pm 0.3\%$, $18\% \pm 6\%$, and $11\% \pm 4\%$ for AT-SVF and $1\% \pm 0.4\%$, $0.7\% \pm 0.2\%$, and $2\% \pm 0.9\%$ for BM-MNCs, respectively). These differences in phenotype indicate that initial cell preparation is heterogeneous independently of the cell origin and may contain several distinct subpopulations.

Total cell recovery varies among selections

To evaluate the efficiency of immunoselections, we determined the total cell recovery (Fig. 2) for each cell selection according to the following formula:

$$\text{Cell recovery} = \frac{\text{number of cells obtained in the positive fraction}}{\text{total number of cells engaged in sorting}} \times 100$$

Finding MSCs in BM and ADSCs in AT are rare events [8,39,40]. To obtain the best MSC enrichment, the total cell recovery should be low to avoid the dilution of MSCs by contaminant cells. The chosen surface markers used for purifying cells lead to different cell recovery for the same source. Thus, for BM samples, CD44-based immunoselection gave the highest cell recovery ($28\% \pm 5\%$), while the other markers (CD34, MSCA-1, SUSD2, and CD271) presented a cell recovery below 5% ($2.6\% \pm 0.5\%$, $0.6\% \pm 0.2\%$, $0.7\% \pm 0.2\%$, and $0.7\% \pm 0.2\%$, respectively). The cell recovery for the different selections obtained from the AT samples is different from that of BM (CD44: $14\% \pm 4\%$; CD34: $11\% \pm 2\%$; MSCA-1: $3\% \pm 1\%$; SUSD2: $4\% \pm 1\%$; CD271: $3\% \pm 1\%$).

Purity of cell fractions: differences between direct and indirect selection

Based on the profile established in Fig. 1, and in accordance with the literature, we chose CD44, CD34, MSCA-1, SUSD2, and CD271 as potential cell surface markers to

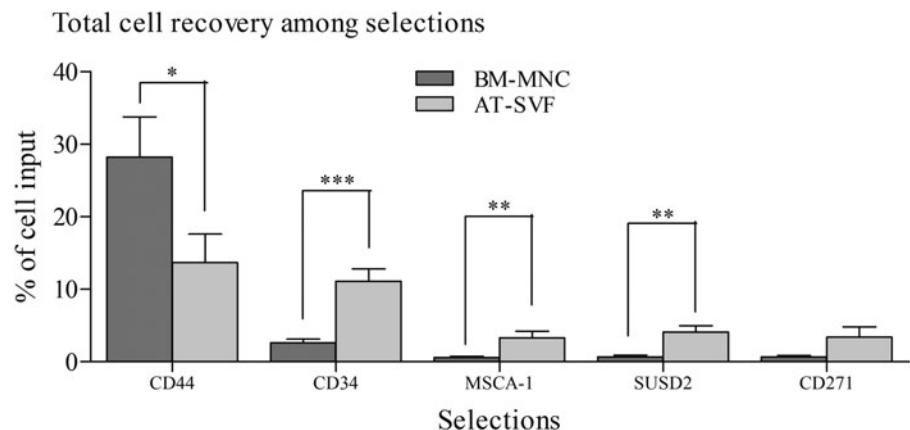


FIG. 2. The total cell recovery was calculated for each selection as the percentage of cells obtained in the positive fraction compared with the total of cells engaged in the sorting. Results are expressed as the mean percentage of cell input \pm SEM. *: $0.01 < p \leq 0.05$, **: $0.005 < p \leq 0.01$ and ***: $p \leq 0.005$.

TABLE 3. PURITY OF CELL FRACTIONS

Selection purity	CD34	CD44	CD271	SUSD2	MSCA-1
Bone marrow	85.6 ± 5.2	78.0 ± 5.4	38.8 ± 10.2	61.8 ± 5.6	72.1 ± 25.5
Adipose tissue	82.4 ± 3.5	98.5 ± 1.0	29.3 ± 5.3	66.1 ± 14.9	90.3 ± 36.6

The purity of the fractions is expressed as mean ± SEM of the percentage of positive cells and was determined on single-cell suspensions of freshly isolated cells by flow cytometry.

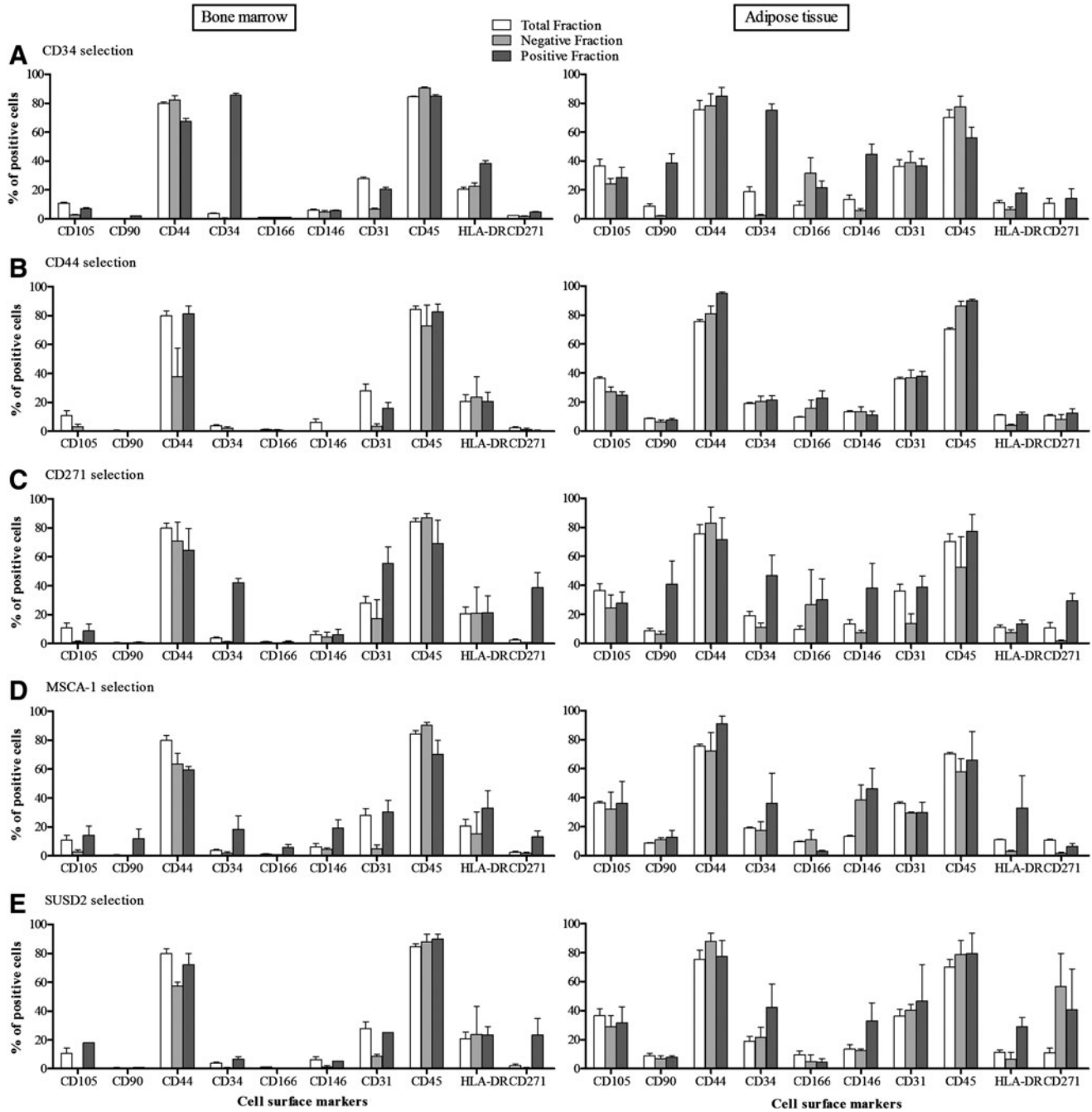


FIG. 3. Phenotypic study of fresh mesenchymal stromal cell (MSC) subsets. Single-cell suspensions of freshly isolated fractions were phenotypically characterized by flow cytometry after sorting. Positive (*dark gray*) and negative (*light gray*) fractions of each sorting are compared with the total unselected BM-MNCs or AT-SVF (*white*). Results are expressed as the mean percentage ± SEM of positive cells. **A, B, C, D** and **E** correspond respectively to CD34, CD44, CD271, MSCA-1 and SUSD2 selection phenotypes; *left panel*: bone marrow; *right panel*: adipose tissue.

immunoselect MSC subpopulations. Table 3 summarizes the cell purity of the different fractions determined by flow cytometry. Purity levels were at least above 70% for CD34, CD44, and MSCA-1 selections for BM and AT cells. CD271 and SUSD2-based selections were less efficient, possibly due to an indirect cell selection and no previous negative cell selection using CD45 and CD235 microbeads to enrich MSCs.

Phenotypic study of fresh immunoselected populations

After selection, the phenotype of the positive and the negative fractions was studied by flow cytometry before putting them into culture and compared with unselected cells (Fig. 3). In general, there is no significant difference in terms of phenotype between the different immunoselected populations from the BM samples. However, CD34, CD31, and CD271 appeared to be differently expressed among the different fractions. We observed CD34⁺ cell enrichment in the positive fractions of the CD271, MSCA-1, and SUSD2 selections, with CD271 selection displaying the greatest enrichments ($P < 0.0001$ for the three selections). There is also CD31⁺ cell enrichment in those fractions, but only significant for the CD271⁺ fraction ($P < 0.0001$).

On the other hand, MSCA-1 and SUSD2-positive fractions yielded a significant enrichment in CD271⁺ cells ($P = 0.001$ and $P = 0.004$, respectively). Moreover, the MSCA-1⁺ fraction and, in a lower proportion, the CD34⁺ fraction showed a slight decrease in CD44⁺ cells (both, $P < 0.0001$).

In general, the phenotype of the different immunoselected populations from the AT samples is quite similar. However, CD90, CD34, and CD146 appeared to be differently expressed among the different fractions. In AT, the CD34 and CD271-positive fractions displayed a significant enrichment in CD90 ($P = 0.0005$) and CD146 ($P = 0.03$), which was not observed in BM samples. Enrichment in CD34⁺ cells may be observed in the CD271⁺ fraction, which was similar to what was observed in BM. Contrary to the BM samples, CD44 expression did not substantially vary among selections.

Regarding the CD44 selection, it is important to note that both positive and negative fractions presented a CD44 expression. In both sources, two populations of CD44⁺ cells were observed with different levels of expression: a population strongly expressing the CD44 (MIF of 68.28 ± 8.51

and 103.04 ± 8.31 for BM and AT, respectively) and another with low expression of CD44 (MIF of 35.19 ± 7.04 and 74.79 ± 8.69 for BM and AT, respectively). We considered the population expressing low levels of CD44 as CD44⁻.

CFU-F assay: distinct enrichment of the immunoselected MSCs in the MSCA-1⁺, SUSD2⁺, and CD271⁺ fractions in BM and only in the CD34⁺ fraction for AT

The CFU-F assay is the most frequently used test to analyze the clonogenic potential of isolated MSCs and to demonstrate MSC enrichment. Thus, we performed a CFU-F assay directly on sorted cells before proceeding with culture (Fig. 4). In BM, cells from the MSCA-1⁺ fraction ($3,205 \pm 754$ CFU-F, $P = 0.004$), the CD271⁺ fraction ($1,334 \pm 390$ CFU-F, $p = 0.02$), and the SUSD2⁺ fraction ($1,018 \pm 497$ CFU-F, $P = 0.02$) displayed a high enrichment level as they have a significantly greater number of CFU-Fs compared with the unsorted MNCs (153 ± 43 CFU-F). The CFU-F enrichment came out as 50 ± 20 times, 39 ± 18 times, and 32 ± 23 times for the MSCA-1⁺, CD271⁺, and SUSD2⁺ fractions, respectively. The CD34⁺ selection yielded 2.7 ± 0.7 times more CFU-Fs than unselected cells. In addition, no residual CFU-F activity was observed in the MSCA-1 and CD271-negative fractions. A residual CFU-F activity was observed in SUSD2 and CD34-negative fractions.

In contrast, the CD44⁺ cells displayed little or no CFU-F activity, whereas the CD44⁻ fraction contained almost all CFU-Fs, but no enrichment was observed in comparison with unselected cells (1.04 ± 0.34 -fold).

In contrast to BM, only the AT-derived CD34⁺ fraction presented an enrichment as it had a CFU-F number of 834 ± 198 compared with the unsorted SVF (296 ± 93 CFU-F, $P = 0.009$). The CFU-F enrichment is 6 ± 2 times higher than in the corresponding SVF.

Proliferation ability of immunoselected MSCs in primary culture: opposite results for BM-MSCs and ADSCs

We compared the number of cells (MSCs and ADSCs) obtained in primary culture when one of the fractions reached 80–90% of confluency and calculated the cell number obtained for each fraction from 1 million plated

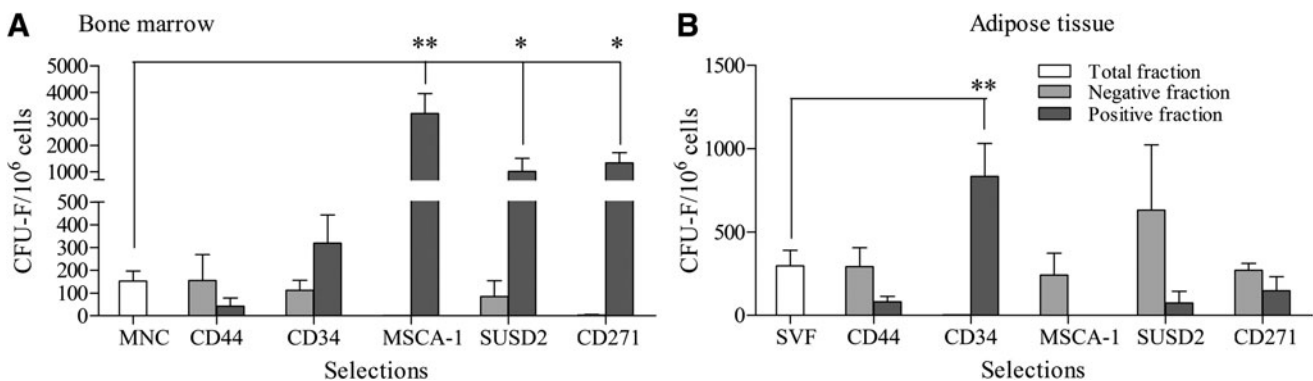


FIG. 4. Colony-forming unit-fibroblast (CFU-F) enrichment in BM (A) and AT (B) fractions. CFU enrichment was determined for each fraction from 1,000,000 cells plated. Positive (dark gray) and negative (light gray) fractions of each sorting are compared with the total unselected BM-MNCs or AT-SVF (white). Results are expressed as the mean \pm SEM of CFU-Fs observed after 10 days of culture. * $0.01 < P \leq 0.05$, ** $0.005 < P \leq 0.01$.

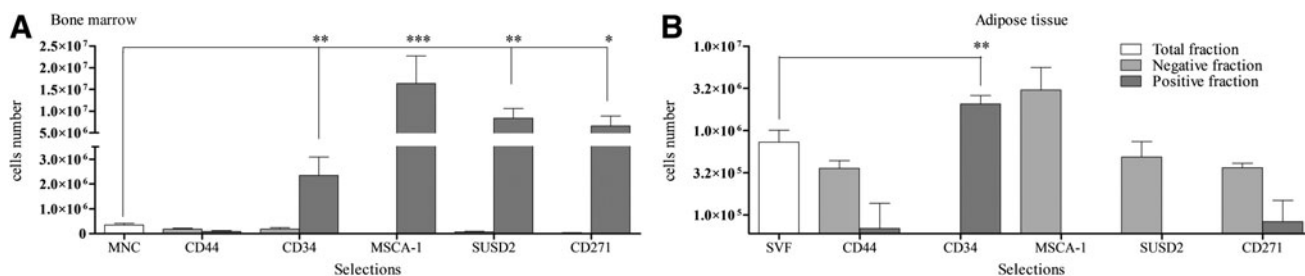


FIG. 5. MSC growth in primary culture. Proliferation ability of immunoselected fractions. The number of MSCs obtained in primary culture was calculated for 10^6 cells initially plated. (A) Bone marrow. (B) Adipose tissue. Negative (light gray) and positive (dark gray) fractions of each sorting are compared with the total unsorted BM-MNCs or AT-SVF (white). Results are expressed as the mean cell number \pm SEM. * $0.01 < P \leq 0.05$, ** $0.005 < P \leq 0.01$, and *** $P \leq 0.005$.

cells (Fig. 5). For BM-MSCs, all positive selections showed high cell numbers in cell proliferation compared with the nonsorted MNCs ($3.56 \pm 0.65 \times 10^5$ cells), except for the CD44⁺ fraction ($1.3 \pm 0.5 \times 10^5$ cells, $P = 0.03$). This increment in the cell number is observed in a marker-dependent manner. The selection, which yields the greatest amount of cells, is the MSCA-1⁺ fraction ($1.6 \pm 0.6 \times 10^7$ cells, $P = 0.001$), followed by the SUSD2⁺ fraction ($8.3 \pm 2.2 \times 10^6$ cells, $P = 0.008$), the CD271⁺ fraction ($6.6 \pm 2.3 \times 10^6$ cells, $P = 0.03$), and finally the CD34⁺ fraction ($2.3 \pm 0.7 \times 10^6$ cells, $P = 0.001$).

The proliferation ability of the different AT fractions was clearly different from that of BM. Only the CD34 selection gave a significantly greater number of ADSCs in the positive fraction ($4.2 \pm 0.9 \times 10^6$ cells, $P = 0.0001$) versus the nonsorted cells from the SVF ($7.0 \pm 2.7 \times 10^5$ cells).

Phenotype of MSCs in primary culture: only the BM-MSCs from the MSCA-1⁺ and CD271⁺ fractions already fulfill ISCT criteria

For each fraction, we compared the phenotype of cells able to adhere to a plastic surface after the primary culture and at each following passage. After primary culture, mesenchymal markers (CD105, CD90, CD166, and CD146) are already present on all cell populations (Table 4 for BM-MSCs and Table 5 for ADSCs). The CD271⁺ fraction and MSCA-1⁺ fraction from BM already fulfilled the phenotypic criteria for MSCs as described by the ISCT. The nonsorted MNCs, the CD34-negative and positive fractions, and the

SUSD2⁺ fraction still contained more than 2% of positive cells for at least one hematopoietic or endothelial marker (CD31, CD34, CD45, and/or HLA-DR).

Concerning cells from AT, all cells able to adhere and grow on a plastic surface after primary culture showed low percentages of positive cells for endothelial and hematopoietic markers. None of the different fractions of cells already met the ISCT criteria in terms of phenotype.

Growth and clonogenicity of cultured MSCs at passage 2: Increased cumulative number of BM-MSCs in MSCA-1⁺ fraction and ADSCs in CD34⁺ fraction

Unselected and selected cell populations from BM and AT were expanded in vitro and evaluated for their proliferation ability and clonogenic potential. Figure 6 shows the cumulative number of cells obtained from 1 million initially plated cells after 2 passages used in clinical applications [41].

For BM, cells from the positive fractions of the MSCA-1 ($2.15 \pm 1.96 \times 10^{10}$ cells, $P = 0.02$), CD271 ($1.00 \pm 0.67 \times 10^{10}$ cells, $P = 0.01$), SUSD2 ($3.99 \pm 1.68 \times 10^9$ cells, $P = 0.03$), and CD34 selections ($1.28 \pm 0.62 \times 10^9$ cells, $P = 0.04$) yield a greater amount of cells than the nonsorted MNCs ($3.10 \pm 1.41 \times 10^8$ cells).

Concerning AT, only the CD34-positive fraction gave a greater number of ADSCs after 2 passages compared with the nonsorted SVF ($2.32 \pm 0.79 \times 10^8$ and $7.92 \pm 1.87 \times 10^7$, respectively, $P = 0.01$).

TABLE 4. PHENOTYPE OF CULTURED BM-MSC

PM	MNC	Fr CD34 ⁻	Fr CD34 ⁺	Fr CD44 ⁻	Fr CD44 ⁺	Fr CD271 ⁺	Fr SUSD2 ⁺	Fr MSCA-1 ⁺
CD105	82.71 \pm 3.27	81.66 \pm 4.93	81.28 \pm 4.25	92.33 \pm 1.71	86.00 \pm 7.13	97.4 \pm 0.4	64.33 \pm 19.34	92.65 \pm 1.89
CD90	82.62 \pm 3.15	79.37 \pm 6.46	77.15 \pm 3.81	91.37 \pm 1.52	83.17 \pm 5.64	93.33 \pm 2.67	80.63 \pm 6.33	82.00 \pm 5.08
CD44	90.63 \pm 2.95	87.76 \pm 5.72	85.61 \pm 7.97	96.60 \pm 0.86	93.5 \pm 2.91	93.33 \pm 3.71	82.75 \pm 5.27	85.57 \pm 5.52
CD34	7.38 \pm 6.17	0.51 \pm 0.20	0.70 \pm 0.35	0.45 \pm 0.14	0.54 \pm 0.16	0.15 \pm 0.05	1.40 \pm 0.31	0.23 \pm 0.05
CD166	85.9 \pm 2.71	77.00 \pm 10.90	83.50 \pm 8.27	92.63 \pm 1.93	74.67 \pm 5.31	95.8 \pm 1.60	87.75 \pm 3.35	69.75 \pm 13.31
CD146	87.62 \pm 2.45	85.14 \pm 10.81	92.79 \pm 4.15	90.94 \pm 0.95	93.20 \pm 1.50	95.00 \pm 2.00	94.67 \pm 2.40	84.00 \pm 1.79
CD31	4.04 \pm 1.20	6.07 \pm 2.90	3.99 \pm 1.63	2.40 \pm 0.69	5.73 \pm 1.68	0.60 \pm 0.4	5.33 \pm 4.33	1.02 \pm 0.29
CD45	9.03 \pm 3.84	6.60 \pm 4.67	6.68 \pm 1.97	2.57 \pm 1.54	3.74 \pm 1.37	0.97 \pm 0.24	1.63 \pm 0.50	1.64 \pm 0.73
HLA-DR	13.05 \pm 4.73	10.42 \pm 7.94	9.97 \pm 7.57	2.66 \pm 2.09	11.42 \pm 5.77	1.50 \pm 0.5	1.65 \pm 0.47	0.85 \pm 0.23

Cell surface marker profile of BM-MSC obtained in different fractions after the primary culture. Results are expressed in percentages of positive cells (mean \pm SEM).

PM, primary cultures; MNC, mononuclear cell.

TABLE 5. PHENOTYPE OF CULTURED ADSC

PM	SVF	Fr CD34 ⁺	Fr CD44 ⁻	Fr CD271 ⁻	Fr CD271 ⁺	Fr SUSD2 ⁻	Fr MSCA-1 ⁻
CD105	57.69 ± 5.21	79.11 ± 6.14	70.03 ± 15.75	69.48 ± 8.00	81.12 ± 8.04	51.77 ± 8.33	7.30 ± 3.71
CD90	56.61 ± 5.09	55.90 ± 8.29	57.73 ± 8.12	54.59 ± 10.22	84.37 ± 5.39	66.68 ± 10.03	50.31 ± 40.93
CD44	95.80 ± 0.76	98.02 ± 0.36	97.85 ± 0.63	93.35 ± 2.60	88.67 ± 6.51	96.97 ± 1.24	50.22 ± 47.05
CD34	12.55 ± 2.31	17.40 ± 6.22	9.48 ± 4.32	10.67 ± 3.69	4.49 ± 1.71	11.53 ± 4.97	11.90 ± 3.43
CD166	45.82 ± 5.06	59.42 ± 10.98	63.76 ± 16.11	36.23 ± 8.75	23.68 ± 9.76	45.51 ± 9.20	44.36 ± 38.01
CD146	10.65 ± 2.24	13.01 ± 3.88	13.48 ± 6.51	17.18 ± 4.68	5.97 ± 3.14	5.05 ± 1.54	6.81 ± 2.21
CD31	5.17 ± 1.58	6.93 ± 1.79	7.31 ± 2.97	4.44 ± 1.16	1.32 ± 0.30	3.16 ± 1.15	9.33 ± 8.85
CD45	3.71 ± 0.60	5.80 ± 1.88	6.27 ± 3.41	2.99 ± 0.67	0.95 ± 0.43	3.16 ± 1.32	24.58 ± 5.65
HLA-DR	3.89 ± 0.79	4.93 ± 2.25	7.74 ± 4.75	3.43 ± 1.04	0.48 ± 0.20	2.10 ± 0.92	9.53 ± 9.53

Cell surface marker profile of ADSC obtained in different fractions after the primary culture. Results are expressed in percentages of positive cells (mean ± SEM).

PM, primary culture; SVF, stroma-vascular fraction.

The clonogenic potential of expanded MSCs at passage 2 was determined in all the cell fractions by calculating the mean cumulative CFU-F number (Fig. 7). In BM, the CD44 selection showed two populations displaying differences in terms of growth and clonogenicity. For the CD34 selection, cells with a greater clonogenicity were originally found in the CD34⁺ fraction with a significant difference compared with the unselected fraction ($P=0.04$). Selections based on CD271, MSCA-1, or SUSD2 surface markers enabled the MSCs in the positive fractions to be enriched and a greater cumulative number of clonogenic cells to be obtained compared with the nonsorted fraction. Moreover, no cell proliferation was observed for the negative fractions (CD271⁻, MSCA-1⁻, and SUSD2⁻ fractions).

In AT, the CD44⁺ fraction cells hardly multiplied and displayed low clonogenic potential. For the CD34 selection, cells with the greatest significant clonogenicity (and growth capacity, Fig. 6) were in the CD34⁺ fraction compared with the unselected fraction ($P=0.01$), but cells from the AT CD34⁻ fraction were neither able to proliferate (Fig. 6) nor able to generate CFU-Fs. Surprisingly, the MSCA-1, SUSD2, and CD271 selections showed totally opposite results in AT in comparison with BM. In fact, ADSC prolif-

eration and CFU-Fs were only observed in the negative fractions, whereas growth and clonogenic ability were only seen in positive fractions for those selections in BM.

It should be noted that the CD271-positive selection in AT can define cell subsets with high proliferative (insert Fig. 6C.) and clonogenic (insert Fig. 7C.) abilities, but only in the case of lipoaspiration samples. For abdominoplasty samples, no cell proliferation was observed in the CD271-positive fraction ($P=0.01$).

Phenotypic study of cultured MSCs: phenotype at passage 2 fulfills ISCT criteria

In BM, cells obtained in the different fractions, with a clonogenic capacity and able to grow on several passages, presented the phenotypic profile of MSCs (Fig. 8) as described by the ISCT (positivity for mesenchymal markers and negativity for hematopoietic and endothelial markers). There is no significant phenotype modification whatever the cell selection.

Concerning AT selections, the percentage of positive cells for hematopoietic and/or endothelial markers is under 2%, except for the CD34 marker for all fractions. The expression of mesenchymal markers was also confirmed, but the levels of

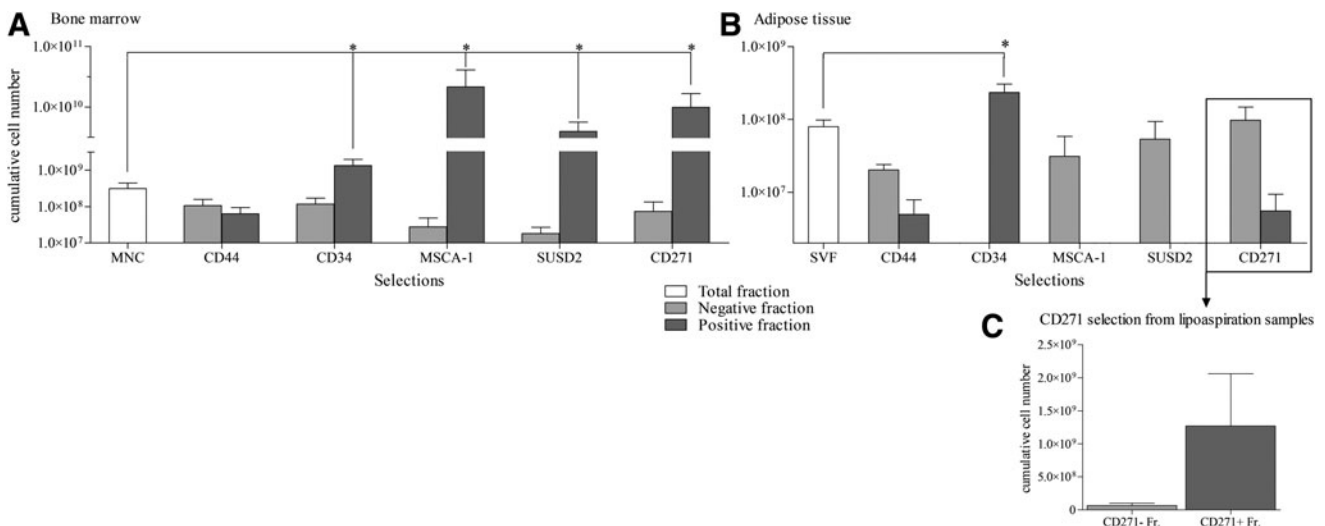


FIG. 6. Growth of MSCs at passage 2. Growth was determined for each fraction from 1,000,000 cells plated for BM (A) and AT (B). Negative (light gray) and positive (dark gray) fractions of each sorting are compared with the total unselected BM-MNCs or AT-SVF (white). Insert (C) details CD271 selection for lipoaspiration samples. Results are expressed as the mean of the cumulative number of cells ± SEM. *0.01 < P ≤ 0.05.

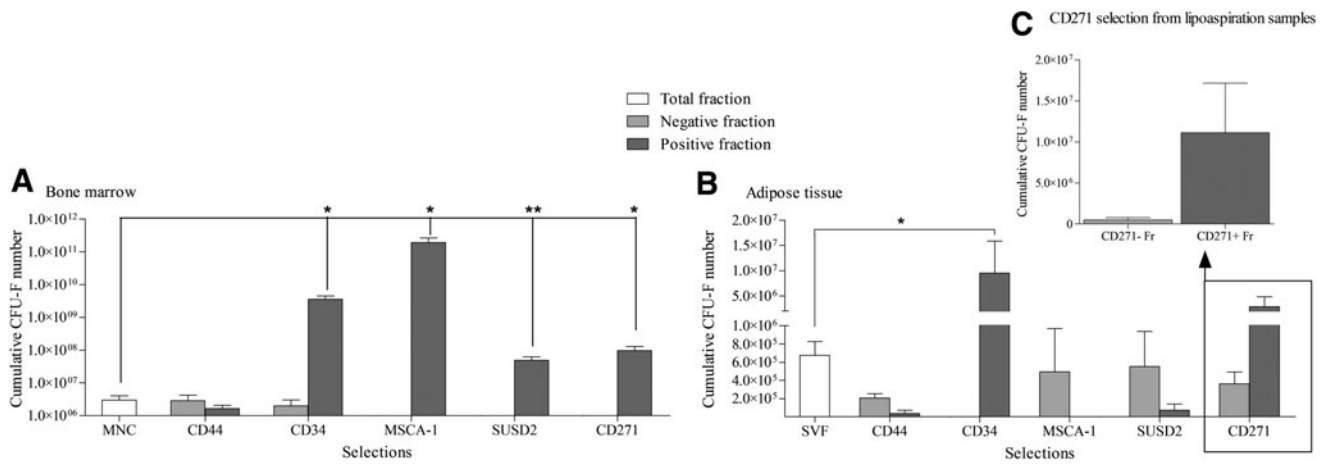


FIG. 7. Clonogenicity of MSCs after passage 2. Cumulative clonogenicity was evaluated after 2 passages for each fraction from 10^6 cells initially plated. Negative (*light gray*) and positive (*dark gray*) fractions of each sorting are compared with the total unselected BM-MNCs or AT-SVF (*white*). Insert (C) details CD271 selection for lipoaspirate samples. Results are expressed as the mean \pm SEM of CFU-F. * $0.01 < P \leq 0.05$ and ** $0.005 < P \leq 0.01$.

some positive markers were lower in ADSCs than for BM-MSCs and particularly CD166 and CD146. Other groups have already described the close but distinct phenotypic profile that BM-MSCs and ADSCs display [42–44].

The differentiation ability of cultured MSCs depends on the fractions

We evaluated the differentiation ability of cultured MSCs by specific staining and by determining the expression of specific related lineage genes. For osteogenic differentiation, we evidenced extracellular calcified matrix deposition by Alizarin Red staining and we followed the expression of the

OPN, which is an osteoblast marker gene. *OPN* expression increased to a maximum after 21 days under osteogenic conditions [45]. For chondrogenic differentiation, we evaluated the cartilaginous structure by Alcian Blue staining of proteoglycans and we followed the expression of the cartilage oligomeric matrix protein (*COMP*), which is a chondrogenic marker gene. *COMP* expression rapidly increases under chondrogenic conditions, reaches a peak at day 12, and decreases slightly to reach a second peak at day 21 [46].

BM MSCs showed a differentiation profile depending on the subset (Figs. 9 and 10). qRT-PCR analysis for specific osteogenic marker demonstrated a higher expression for *OPN* in BM-MSCs from CD271⁺, followed by those from

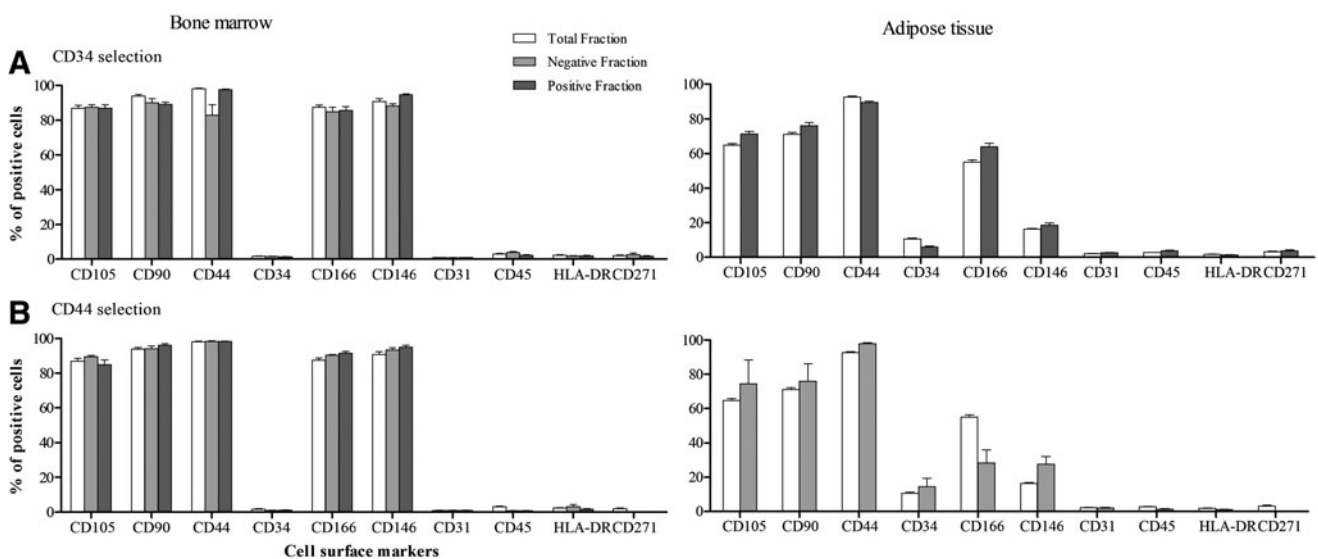


FIG. 8. Phenotypic study of cultured MSC subsets after passage 2. Single-cell suspensions of the different fractions were phenotypically characterized by flow cytometry after passage 2. MSCs and adipose-derived stromal cells (ADSCs) from negative (*light gray*) and positive (*dark gray*) fractions of each sorting are compared with the MSCs from total unselected fractions (*white*). (A) and (B), respectively, represent CD34 and CD44 selection fraction results. The other fractions (MSCA-1⁺, SUSD2⁺, and CD271⁺ fractions for BM-MSCs and MSCA-1⁻, SUSD2⁻, and CD271⁻ fractions for ADSCs) displayed the same phenotypic profile as the MSCs and ADSCs from the total fractions (data not shown). Results are expressed as the mean percentage \pm SEM of positive cells.

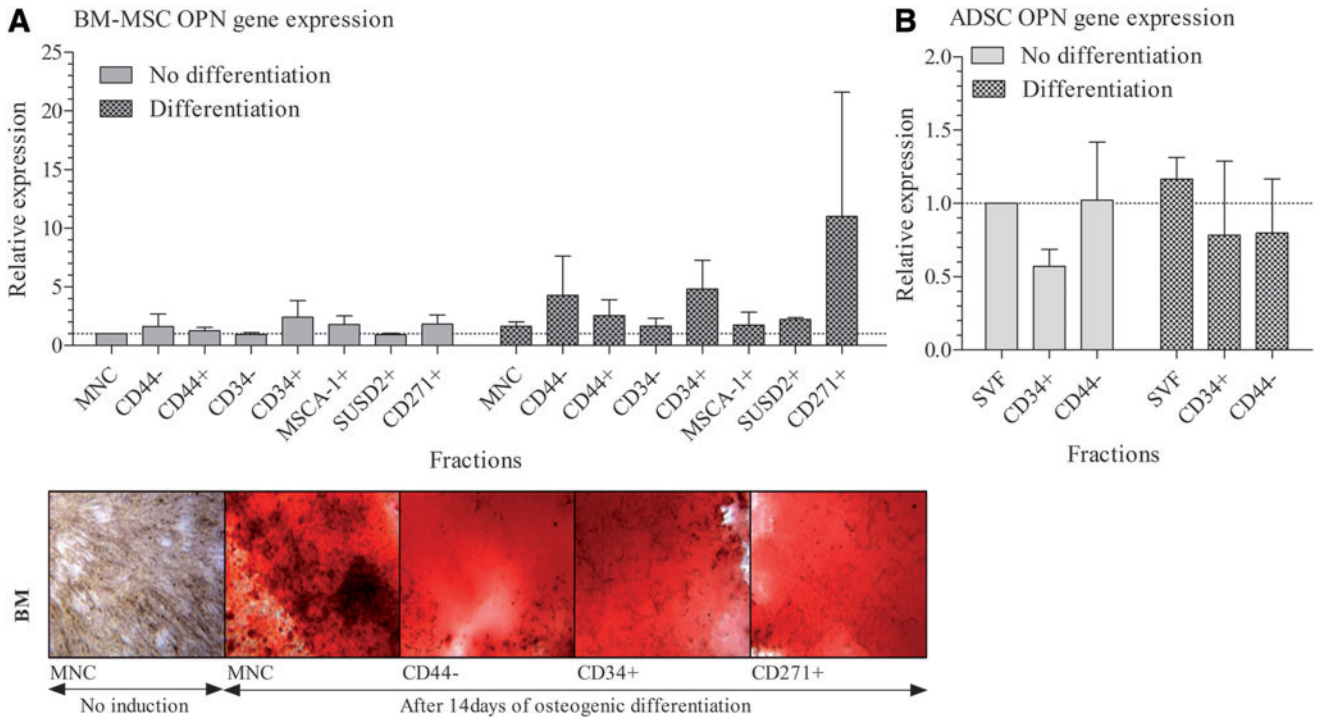


FIG. 9. Osteogenic differentiation ability. Osteogenic ability of BM-MSCs and ADSCs was assessed before and after osteogenic differentiation by the mineralization evaluation with Alizarin Red staining and by the determination of osteopontin expression by qPCR. The gene expression values were normalized to those of *GAPDH* and data are presented as mRNA levels relative (fold change) to the control: (A) undifferentiated MNCs for BM and (B) undifferentiated SVF for AT. The results represent the mean \pm SEM of three different experiments. OPN: osteopontin; optical microscope 25 \times . Color images available online at www.liebertpub.com/scd

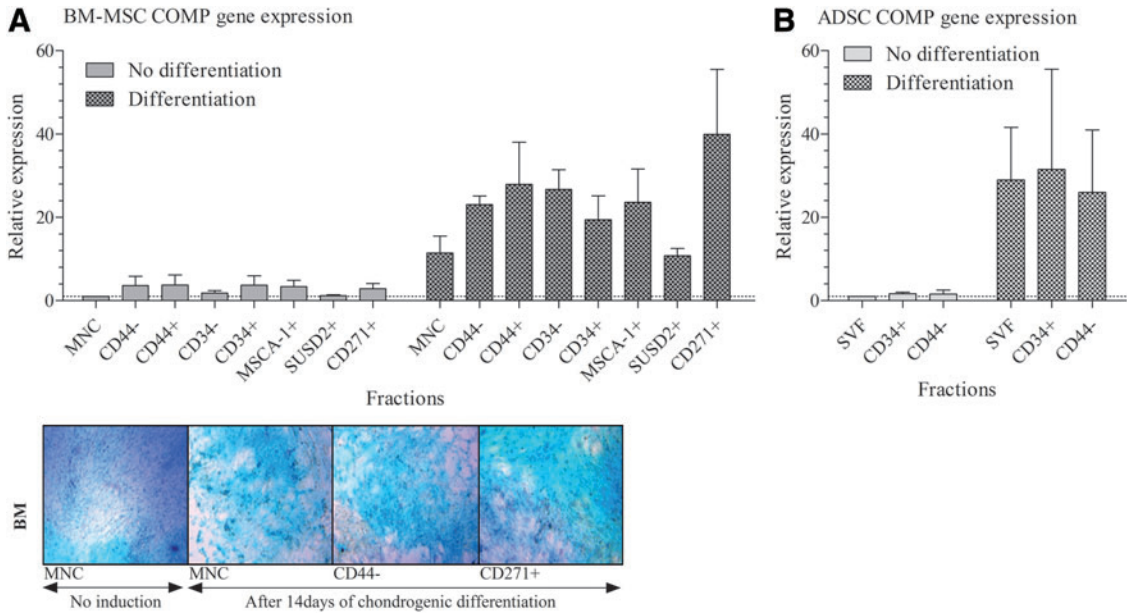


FIG. 10. Chondrogenic differentiation ability. Chondrogenic ability of BM-MSCs and ADSCs was assessed before and after chondrogenic differentiation by the proteoglycan evaluation with Alcian Blue staining and by the determination of *COMP* expression by qPCR. The gene expression values were normalized to those of *GAPDH* and data are presented as mRNA levels relative (fold change) to the control: (A) undifferentiated MNCs for BM and (B) undifferentiated SVF for AT. The results represent the mean \pm SEM of three different experiments. COMP, Cartilage Oligomeric Matrix Protein; optical microscope 25 \times . Color images available online at www.liebertpub.com/scd

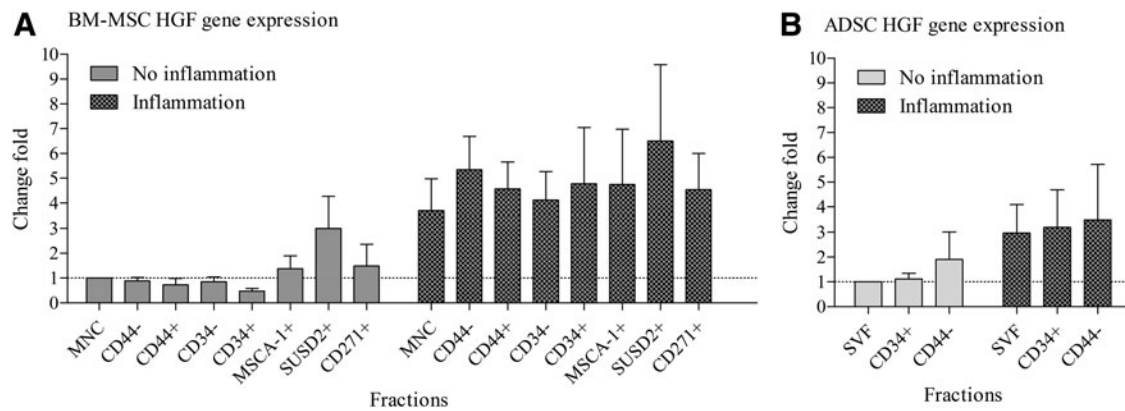


FIG. 11. *HGF* gene expression immunoregulatory profile of (A) BM-MSCs and (B) ADSCs based on *HGF* gene expression was assessed before (No inflammation) and after inflammatory priming (Inflammation) of each fraction by qPCR. The gene expression values were normalized to those of *GAPDH* and data are presented as mRNA levels relative (fold change) to the control in noninflammatory conditions for (A) BM-MNCs and (B) AT-SVF. The results represent the mean \pm SEM of three different experiments. *HGF*, hepatocyte growth factor.

the CD34⁺ and CD44⁻ fractions when compared with the total unselected MSCs from the MNC fraction. Such upregulation of *OPN* gene expression indicates that CD271⁺, CD34⁺, and CD44⁻ fractions have a greater osteogenic potential than the total unselected MNCs one. BM-MSCs from the CD44⁺, SUSD2⁺, MSCA-1⁺, and CD34⁻ fractions showed osteogenic differentiation potential compared with the nonselected MNC total fraction. These results were confirmed by Alizarin Red staining (Pictures Fig. 9).

qRT-PCR analysis for specific chondrogenic markers demonstrated the highest expression of *COMP* for BM-MSCs from the CD271⁺ fraction. BM-MSCs from the CD44⁺, CD34⁻, MSCA-1⁺, CD44⁻, and CD34⁺ also displayed a higher *COMP* expression compared with the unselected MNC fraction. This upregulation indicated that these fractions had a greater chondrogenic potential than the unselected MNCs one. BM-MSCs from the SUSD2⁺ fraction showed an equivalent chondrogenic potential to the MSCs from the unselected fraction. Those results were confirmed by Alcian Blue staining (Pictures Fig. 10).

Concerning ADSCs, selection did not seem to improve the capacity of osteogenic and chondrogenic differentiation in comparison with the total SVF.

The immunoregulatory profile of cultured MSCs depends on the fractions

We studied the immunomodulation profile of the different fractions in both sources based on *HGF* gene expression with and without the presence of proinflammatory cytokines by qRT-PCR. *HGF* is an immunomodulating factor known to be secreted by MSCs in inflammatory conditions [37]. MSCs are able to inhibit lymphocyte proliferation by releasing soluble factors such as *HGF* (among others) [38].

In BM-MSCs, a constitutive expression of *HGF* is observed (Fig. 11). The profile of *HGF* expression depended on the fraction studied. MSCs from the CD34⁺ fraction (0.48 ± 0.11 -fold) expressed less *HGF* than the total fraction. The MSCs from the SUSD2⁺, the CD271⁺, and the MSCA-1⁺ fractions seemed to constitutively express more *HGF* than the total fraction (3.00 ± 1.28 , 1.49 ± 0.88 , and 1.38 ± 0.53 -fold more, respectively). Under proinflammatory

conditions, the total population and all fractions of BM-MSCs presented an increased *HGF* gene expression. The greatest *HGF* expression was reached by the MSCs from the SUSD2⁺ fraction (6.50 ± 3.08 -fold), followed by those from the CD44⁻ (5.34 ± 1.35) versus the total MNC fraction (3.70 ± 1.29), in comparison with the control without inflammation. The *HGF* gene expression for the other fractions was very similar. Thus, fractions, SUSD2⁺ and CD44⁻, may display a higher immunomodulatory potential than the total unselected MNC fraction.

For AT, *HGF* expression was constitutively observed in all fractions and more expressed than in the total fraction: 1.90 ± 1.11 -fold more expressed in ADSCs from the CD44⁻ fraction and 1.12 ± 0.22 -fold more in ADSCs from the CD34⁺ fraction. Under inflammatory conditions, the *HGF* gene expression was upregulated (2.97 ± 1.12 -fold) in ADSCs from the unselected fraction. An upregulation was also observed in the CD44⁻ fraction (3.50 ± 2.21 -fold greater) as well as in the CD34⁺ fraction (3.19 ± 1.5 -fold).

In general, the level of *HGF* gene expression was more important in all fractions from the BM-MSCs in comparison with those of ADSCs under proinflammatory conditions (data not shown).

Discussion

Unfractionated tissue cells are generally used as the starting population for the culture of MSCs. The resulting MSC preparations, especially at the beginning of the culture, consist of a heterogeneous mixture of cells, including MSCs, monocytes, endothelial cells, and reticular cells [47,48]. Different markers have been developed to identify and enrich MSCs, but the majority of these markers are described for BM. Moreover, several studies show that the surface markers of freshly isolated MSCs differ from those of cultured MSCs and there is a large difference in their expression in various sources of MSCs [49]. In this study, we evaluated and compared the potential of 5 single surface markers to identify and enrich MSCs from two different sources: BM and AT. We also determined the phenotype, the clonogenic efficiency, the proliferation, the multilineage differentiation ability, and the immunoregulatory profile of

the subpopulations obtained by magnetic selection in comparison with the unselected cells.

Our results showed that native BM-MSCs can be enriched from the positive fractions of MSCA-1, SUSD2, and CD271 selections. Moreover, the negative cell population for these markers is not capable of forming CFU-Fs. The total cell recovery obtained for these selections is very similar ($\pm 0.6\%$), and compared with unfractionated cells, CFU-Fs were more than 30-fold enriched in the positive fractions. However, a higher enrichment was observed for the MSCA-1 selection (50 ± 20 -fold).

Flow cytometry demonstrated an increased percentage of CD34 and CD271 after selection, but the expression of typical MSC phenotypic markers (CD105, CD90, CD166, and CD146) is very low, confirming that these selected populations remain heterogeneous. These selective markers are not exclusively expressed by MSCs, but are also found on other cell types, such as neutrophils, endothelial cells, or hematopoietic progenitors.

The purity of the isolated population following MACS separation typically reaches 70%, but for indirect cell sorting (particularly CD271), the purity was around 40–50% and lower than that previously reported in the literature. The other groups generally used FACS separation [14,16] and/or several purification columns with negative and/or positive cell sorting(s) before targeted cell sorting. Quirici et al. [36] obtained a cell purity of $90.5\% \pm 3.5\%$ for BM CD271⁺ cells after the purification of CD45⁻ α -glycophorin A⁻ BM cells. In other studies, the percentage of CD271 was evaluated by flow cytometry after CD45⁻ gating of the selected population [50].

Higher isolation purity could be also achieved by a second run of purification. However, the aim of our study was to evaluate and to compare the potential enrichment of MSCs from BM and AT directly after one isolation run, with the least possible handling to preserve cell viability, to save time, to ensure reproducibility, and to avoid contamination. A single marker-based selection for MSC enrichment should be more advantageous for clinical practices.

Previous studies have shown that these cell surface markers isolate the majority of CFU-Fs present in the BM. CD271 stains primitive MSCs with high specificity and purity in BM, defining a subset of cells with higher clonogenicity, proliferative, and differentiative potential in comparison with an unselected MSC population [36]. However, the majority of CD271⁺ cells did not coexpress CD90 and CD73, two mesenchymal markers, but coexpressed CD34, confirming the expression of CD271 on hematopoietic progenitor cells. In a comparative study, Jones and McGonagle demonstrated that the CD271 antigen was one of the most selective markers for enriching BM-MSCs [51].

In contrast, SUSD2 is not expressed in hematopoietic cells and appears to be the most suitable target for MSC isolation because of its superior selectivity for MSCs [35]. In our study, we also observed that SUSD2 selection can enrich MSCs (32 ± 23 -fold), which were able to expand and to differentiate into osteoblasts and chondrocytes, but a residual CFU-F activity was observed in the negative fraction. In our opinion, MSCA-1 appears to be the best single marker to enrich primary MSCs since we can reach up to a 70-fold CFU-F enrichment and an expansion rate of 20×10^9 after two passages (versus 0.2×10^9 for an unselected fraction).

We also explored whether these markers successfully used to positively select BM-MSCs were also effective to enrich MSCs from AT. MSCs resident in different tissues are not the same and the surface markers used for BM-MSC selection are not universally expressed by various MSCs. Only a few markers have been established to identify these cells in their respective tissue. The MSCA-1 antigen is expressed at a high level on BM-MSCs, but only at near-background levels on the placental counterpart [52].

Several studies confirm the specificity of CD271 in different tissues. This antigen is expressed at a high level in BM and AT, but at a low level in placenta-derived MSCs and not expressed in umbilical cord MSCs [52–54]. Two recent studies observed that CD271 is not an adequate marker for the identification of MSCs before culture from umbilical cord blood and Wharton's Jelly [24,55]. SUSD2 has been reported to enrich CFU-Fs from the endometrium, but its efficiency in purifying MSCs from other sources such as AT remains to be determined [56].

In our study, we observed that SUSD2 and MSCA-1 were unable to identify and enrich MSCs from AT, meaning that ADSCs do not express these antigens in situ, in contrast to BM-MSCs.

Recently, CD271 has been described as the optimal selective marker for the purification and isolation of ADSCs [54]. CD271⁺ cells have higher multipotency and a higher proliferative capability when compared with a whole population of unselected ADSCs [57]. In these two studies, CD271 selection was performed on lipoaspiration samples. Interestingly, we also observed that CD271 selection can define cell subsets with high proliferative and clonogenic abilities, but only in the case of lipoaspiration samples. No CFU-F activity was detected in the CD271⁺ fraction of abdominoplasty samples obtained from the resection of subcutaneous fat portions. Lipoaspirations contain fatty and fluid portions comprising a saline solution, peripheral blood and cells, or AT fractions [17]. For abdominoplasty, the waste material is dissected to remove fibrous structures and visible blood vessels, and then minced into pieces before enzymatic treatment. This difference of processing methods could account for the opposite results obtained for the CD271 selection. Moreover, recent evidence suggests that ADSCs originate from perivascular cells localized around blood vessels [58,59] and the phenotype of native ADSCs can be specific to tissue localization [40,60].

Hong Qian et al. [14] previously described BM-MSCs as initially CD44 negative. They suggest the use of CD44 as a negative marker for prospective isolation of BM-MSCs. We wanted to verify whether this was also true for ADSCs. Our results showed that ADSCs were initially in the CD44⁻ fraction of the SVF, even though we sometimes observed residual CFU-F activity in the CD44⁺ fraction. However, in the few cases where cells from the positive counterpart were isolated, they hardly grew and not in a sufficient amount to study them.

Like Qian et al., we found a higher CFU-F level, without enrichment, in the CD44⁻ fraction, but we obtained BM-MSC growth in the positive and the negative fractions of the CD44 sorting. After 2 passages, a higher amount of cells was obtained for the negative fraction and these cells had a greater ability to differentiate into osteoblasts compared with BM-MSCs from the positive counterpart. They also seemed to have a higher *HGF* expression than the positive fraction. As

previously reported for BM, we observed that ADSCs also lack CD44 expression. However, the CD44 selection of BM-MNCs and AT-SVF did not allow CFU-F enrichment.

In this study, we also evaluated whether the CD34⁺ selection can be useful in identifying and enriching MSCs from BM and AT.

According to the ISCT, CD34 is a negative marker for MSCs, but some reports suggest that the negativity of CD34 expression is an artifact of culture [29]. Moreover, some authors have confirmed the presence of CD34⁺ in the SVF freshly isolated from AT [18,20,61]. The isolation of CD34⁺CD31⁻ cells makes it possible to obtain a cell population able to differentiate in mesodermal lineages [62,63]. Recently, Quirici et al. reported that the immunomagnetic sorting of ADSCs by CD34 antibodies makes it possible to select a subpopulation with more efficient clonogenic and differentiation abilities compared with the whole population [54].

We confirmed that native ADSCs are CD34⁺ and, among all selections performed, only the CD34⁺ selection allowed CFU-F enrichment without residual CFU-Fs in the negative fraction. Importantly, we found a population of BM-MSCs, which was clearly positive for the CD34 cell surface marker. This population is rare, but it has a high proliferative rate and clonogenicity capacity compared with the total unselected population or the CD34⁻ population. The expression of the CD34 of this population dropped once in culture, until its total disappearance, as previously described for ADSCs. Some studies reported that BM-MSCs also express CD34 [29,30,64]. Moreover, the Stro-1 antibody, which is one of the best MSC markers, was generated using CD34⁺ BM cells as immunogen [65].

A more recent study of Kaiser et al. indicated that although the majority of MSCs derive from a CD34⁻CD45⁻ subpopulation, a small fraction of CD34⁺ can give rise to MSCs [66]. MSCs from these two fractions could be differentiated into adipocytes and osteoblasts. The CD34⁺ population isolated in our study showed a great proliferative capacity, increased osteogenic capacity, and HGF expression, but displayed similar chondrogenic potential to the CD34⁻ population or unselected cells. Studying the kinetic expression of the CD34 and carrying out a deeper functional study of those two subsets would be of the greatest interest for the becoming of those populations as well as for a better understanding of this cell surface marker.

In summary, our study demonstrated that only one selection is able to successfully isolate native ADSCs either from abdominoplasties or lipoaspirates with the CD34-positive sorting. Although CD271 selection can enrich MSCs with high clonogenic and proliferative capacities from lipoaspirates, this positive selection failed to enrich MSCs from abdominoplasties. Concerning BM-MSCs, a CD34⁺ subset with a higher proliferative capacity does exist in situ. We also found MSCs in the negative and positive fractions of the CD44 selection, although it displayed different properties in terms of proliferation, clonogenicity, and differentiation.

Collectively, our results highlight the fact that several markers may be used to selectively enrich MSCs. However, selection efficiency differs between the markers and depends on the source. In our opinion, MSCA-1 seems to be the best marker to isolate BM-MSCs since the greatest enrichment in MSCs with the highest proliferative and clo-

nogenic potentials, rapidly fulfilling ISCT criteria, as well as a great ability to differentiate into chondroblasts, was observed for this surface marker. This being said, the greatest ability to differentiate into osteoblasts was seen for BM-MSCs expanded from the CD271⁺ fraction, but this needs to be confirmed. Our results showed that in situ BM-MSCs are MSCA-1⁺, CD271⁺, SUSD2⁺, CD34[±], and CD44[±] and that native ADSCs express CD34, but are negative for MSCA, SUSD2, and CD44. Concerning the expression of CD271 in AT, ADSCs from subcutaneous AT (ie, from abdominoplasty resections in our study) seemed to be CD271⁻, whereas a CD271⁺ population exists in the lipoaspirate samples, probably from a pericyte origin.

Due to the great value of MSCs in many clinical applications, from regenerative medicine and tissue engineering to immunomodulation, it is important to find markers allowing optimal selection and identification of MSCs before culture. Our findings highlight that selective markers described for BM-MSCs are not necessarily adequate markers for identification and enrichment of MSCs from other tissues such as AT.

Acknowledgment

This work was supported by a grant provided by the F.R.S.-FNRS (Fonds National de la Recherche Scientifique) of Belgium (Grant-Télévie 7.4.517.09 and 7.4610.14).

Author Disclosure Statement

No competing financial interests exist.

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Received for publication May 15, 2015

Accepted after revision June 16, 2015

Prepublished on Liebert Instant Online June 18, 2015