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Proliferative and phenotypical characteristics of human adipose tissue–derived stem cells: comparison of Ficoll gradient centrifugation and red blood cell lysis buffer treatment purification methods

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Abstract

Background aims. Adult human subcutaneous adipose tissue harbors a multipotent stem cell population, the so-called human adipose tissue–derived mesenchymal stromal cells (AT-MSCs). These cells are able to differentiate *in vitro* into various cell types and possess immunomodulatory features. Yet procedures to obtain AT-MSCs can vary significantly. The two most extensively used AT-MSC purification techniques are (i) density gradient centrifugation using Ficoll and (ii) red blood cell (RBC) lysis buffer treatment of the stromal vascular fraction. In the context of potential clinical cell therapy, the stem cell yield after purification and upon consecutive passages, as well as the purity of the obtained cell population, are of utmost importance. *Methods.* We investigated the expansion capacity and purity of AT-MSCs purified by both procedures immediately after isolation and upon consecutive passages. We also investigated possible purification-dependent differences in their expression of immune-inhibitory factors and cell adhesion molecules. *Results.* We found that RBC lysis buffer treatment is a more robust and easier method to purify AT-MSCs than density gradient fractionation. However, the resulting AT-MSC-RBC population contains a significantly higher number of $CD34^+$ cells, particularly during the first passages after plating. From passage 4 onward, no significant differences could be observed between both populations with respect to the immunophenotype, expansion capacity and expression of immune inhibitory factors and cell adhesion molecules. *Conclusions.* Our data show that RBC lysis buffer treatment may be a good alternative to density fractionation, providing a faster, more robust and easier method to purify AT-MSCs with biologically preserved characteristics.

Key Words: *adipose tissue, adult stem cell, density gradient centrifugation, Ficoll, mesenchymal stromal cell, red blood cell lysis, stromal vascular fraction*

Introduction

Postnatal stem cells have been identified in most human tissues/organs (1,2). Mesenchymal stromal cells (MSCs) represent, together with hematopoietic stem cells, the most widely used class of postnatal stem cells. According to the International Society for Cellular Therapy, MSCs can be identified by their plastic adherent properties, immunophenotype ($CD73^+$, $CD90^+$, $CD105^+$, $CD11b/14^-$, $CD19/CD73b^-$, $CD34^-$, $CD45^-$ and $HLA\text{-}DR^-$) and differentiation potential into adipocytes, chondrocytes and osteoblasts (3). MSCs have been isolated from various human adult sources including, but not limited to, bone marrow (4), liver (5), adipose tissue (6) and umbilical cord (7,8). Moreover, MSCs are able to differentiate *in vitro* into various cell types and possess immunomodulatory features (9,10). These properties make MSCs an attractive tool for regenerative medicine in the context of cell therapy.

The best characterized MSCs are derived from bone marrow (BM). However, the technique to isolate BM is invasive and yields a low quantity of MSCs. Adipose tissue (AT), on the other hand, has emerged over the years as an alternative source for human MSCs (11). AT, like BM, is derived from the embryonic mesoderm and contains a stromal

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vascular fraction (SVF) that harbors a stem cell population termed adipose tissue–derived mesenchymal stromal cells (AT-MSCs) (6,12). These AT-MSCs can be easily isolated from routine liposuction (lipoaspirate) and reconstructive surgery (lipectomy) waste materials using a collagenase-based digestion procedure (13). Under proper conditions, cells within the SVF subsequently adhere to plastic tissue culture dishes and exhibit a fibroblast-like appearance (6,14). It is generally agreed that the SVF is a heterogeneous cell population that harbors not only AT-MSCs but also fibroblasts, endothelial cells, pericytes, smooth muscle cells and circulating cell types, such as immune cells and hematopoietic stem cells (15,16). For example, early passages of AT-MSCs often express the hematopoietic stem cell marker $CD34^+$ (17,18). As a consequence, several distinct purification techniques are currently being used to enrich the AT-MSC subpopulation from the SVF. The most extensively used purification techniques are based on either density gradient centrifugation or red blood cell (RBC) lysis buffer treatment of the SVF (19). Other techniques include mechanical dissociation (20) and more advanced and expensive methods such as the use of immunomagnetic beads coated with specific antibodies, including CD34, CD105 and CD271 (21,22), fluorescence-activated cell sorting (FACS) (23) and approaches based on high aldehyde dehydrogenase activity (17,24). However, in the context of clinical cell therapy, the stem cell yield after purification and upon consecutive passages, as well as the purity of the obtained cell population, are of key importance. Therefore, we investigated the proliferative capacity and the purity of the resulting AT-MSCs purified by the two most frequently used methods.

Besides their multi-lineage differentiation potential, AT-MSCs also exhibit immunomodulatory properties. More specifically, AT-MSCs are able to inhibit the activation and proliferation of immune cells (25) due to cell-cell contacts and release of soluble immunosuppressive factors, such as hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF) and prostaglandin (PG) E2 (9,10,25). As such, AT could represent an alternative, easily accessible source of MSCs to BM for clinical cell therapy, in particular for the treatment of graft-versus-host disease and autoimmune disorders (26).

Within this context, we also investigated whether the purification procedure affects the gene expression of the major regulatory factors reported to be involved in AT-MSC inhibitory effects including the cell adhesion molecules (CAMs) intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1. The immune inhibitory factors HGF, LIF, heme oxygenase (HMOX) 1, prostaglandin-endoperoxide synthase [illegible] 1 and PTGS2 were also studied.

To the best of our knowledge, no work has thus far been reported that evaluates whether the proliferation capacity of AT-MSCs and their expression of CAMs and immune inhibitory factors are affected by the purification method used.

Methods

AT collection

AT (lipoaspirate) was collected after obtaining informed consent from three male and nine female patients (age range 25–45 [37 ± 8] years) undergoing elective liposuction in cooperation with the Department of Plastic Surgery of the UZ-Brussels (Brussels, Belgium) and the ATLAS clinic (Brussels, Belgium).

Isolation and culture of AT-MSCs

Human AT-MSCs were isolated from 12 patients according to a modified protocol from Oedayrajsingh-Varma *et al.* (13). Importantly, both purification methods were applied to all patients in parallel. In brief, 125 mL of liposuction material was extensively washed with equal volumes of phosphate-buffered saline (PBS) to remove erythrocytes. Centrifugation was carried out for 3 min at 600*g*. The adipose tissue samples were incubated for 45 min at 37°C with dissociation medium (1:1), that is, 125-mL aliquots of fat + 125 mL of dissociation medium. The latter consisted of 1% (v/v) bovine serum albumin (BSA) (Sigma-Aldrich, Diegem, Belgium) and 1 mg/mL collagenase A (Roche Applied Science, Vilvorde, Belgium) in PBS. The digested tissue was then passed through a mesh filter to remove connective tissue debris. Subsequently, the filtrate was centrifuged for 10 min at 600*g* (4°C), and the supernatant was removed. In the classical Ficoll gradient protocol, the cells were suspended in 50 mL PBS supplemented with 1% (v/v) BSA and centrifuged again for 10 min at 600*g* (4°C), after which the pellet was resuspended in 30 mL of PBS, supplemented with 1% (v/v) BSA. The cell suspension was then carefully brought on top of 15 mL of Ficoll gradient solution (Sigma-Aldrich) and centrifuged for 20 min at 1000*g* (4°C). Upon centrifugation, the top layer was removed and the AT-MSC-Ficolls (Fs) collected in 50 mL PBS supplemented with 1% (v/v) BSA. The cell suspension was centrifuged for 10 min at 600*g* (8°C), after which the supernatant was removed and total AT-MSC-Fs were counted using a hematocytometer. Cell viability was assessed using a 0.4% (w/v) trypan blue dye solution (Sigma-Aldrich).

In the RBC lysis protocol, the cells were incubated for 10 min with 10 mL 1× RBC lysis buffer at room temperature (20–25°C; ImTec Diagnostic, Antwerp, Belgium) and centrifuged for 5 min at 600*g* (20–25°C) to remove the contaminating RBCs. After removal of the supernatant, the cell viability was assessed using a 0.4% (w/v) trypan blue dye solution (Sigma-Aldrich), and the total number of cells was counted using an automated cell counter (Bio-Rad, Nazareth, Belgium).

Purified AT-MSC-Fs and-RBCs were both cultured at a density of 1×10^4 cells/cm^2 in T75 flasks in Dulbecco's Modified Eagle Medium (Lonza, Braine-l'Alleud, Belgium) supplemented with 10% (v/v) fetal bovine serum (HyClone, Perbio Science, Erembodegem-Aalst, Belgium), 7.33 IU/mL benzyl penicillin (Continental Pharma, Brussels, Belgium), 50 µg/mL streptomycin sulphate (Sigma-Aldrich), 2.5 µg/mL Fungizone (Invitrogen, Merelbeke, Belgium). After 24 h of incubation at 37°C, under an atmosphere of 5% CO_2 and 95% air, non-adherent cells were removed with PBS at 37°C and maintained in the same medium until 90% confluence was reached. Throughout the culture period, the growth medium was changed every 2 days. Both cell populations were expanded and cultured until passage 4.

Morphology

The morphology of AT-MSCs was examined by phase-contrast light microscopy (Nikon, Brussels, Belgium). When a confluence of 80%–90% was reached, cells obtained by both methods (Ficoll and RBC) were harvested and expanded through successive passages.

Adipogenic differentiation

AT-MSCs from three donors at passage 4 were cultivated at 100% confluence in STEMPRO adipogenic differentiation medium (Life Technologies, Ghent, Belgium) for 2 weeks. The medium was changed every 3 days. To evaluate the adipogenic differentiation, cells were fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature and stained with 0.5% (v/v) oil red O in isopropanol (Sigma-Aldrich, Belgium) for 30 min at room temperature. The plates were thoroughly washed with water and the lipid accumulation was identified as bright red droplets within the cells.

Osteogenic differentiation

AT-MSCs from three donors at passage 4 were cultivated at 60% confluence in STEMPRO osteogenic differentiation medium (Life Technologies) for 3 weeks. The medium was changed every 3 days. To evaluate the osteogenic differentiation, cells were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature and stained with 2% (v/v) alizarin red, pH 4.1 (Sigma-Aldrich) for 30 min at room temperature. The plates were washed three times with water and the mineral deposits were visualized as a dark red staining.

Flow cytometry analysis of AT-MSCs

Freshly purified and cultured AT-MSC-Fs and -RBCs (1×10^5) from six donors were examined for changes in their hematopoietic ($CD34^+$) and stromal cell surface ($CD105^+$) marker expression using flow cytometry at four consecutive passages (P) 0, 1, 2, 3 and 4. In addition, P4 cultured AT-MSC-Fs and -RBCs were further characterized for their expression of other hematopoietic, endothelial and stromal cell surface markers as well as human leukocyte antigens. As such, cells were incubated with fluorochrome-conjugated monoclonal antibodies directed against cluster of differentiation CD34 (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA), CD45 (Miltenyi Biotec, Leiden, the Netherlands), CD73, CD105 (Ancell Corporation, Bayport, MN, USA), CD90 (R&D Systems, Minneapolis, MN, UK), CD14, CD19 and HLA-DR (Beckman Coulter, Indianapolis, IN, USA). After cell labelling, data were acquired with a MACS Quant analyzer (Miltenyi Biotec).

Growth kinetics

Growth kinetics were analyzed by determining different parameters. When 80–90% confluence was reached, cells obtained from six donors by both methods were passaged, and the cumulative number of expanded cells after each passage (from P0 through P4) was evaluated. The total number of cells at each passage was calculated as a ratio of the total number of cells harvested to the total number of cells seeded multiplied by the total number of cells from the previous passage.

Population doublings were calculated using the following formula (NI is the inoculum cell number and NH the harvested cell number):

$$X = (\log NH - \log NI)/\log 2$$

To estimate the cumulative population doublings, the population doubling for each passage was calculated and added to the population doubling levels of the previous passages.

The population doubling time (DT) between P1/P2 was determined by the following formula

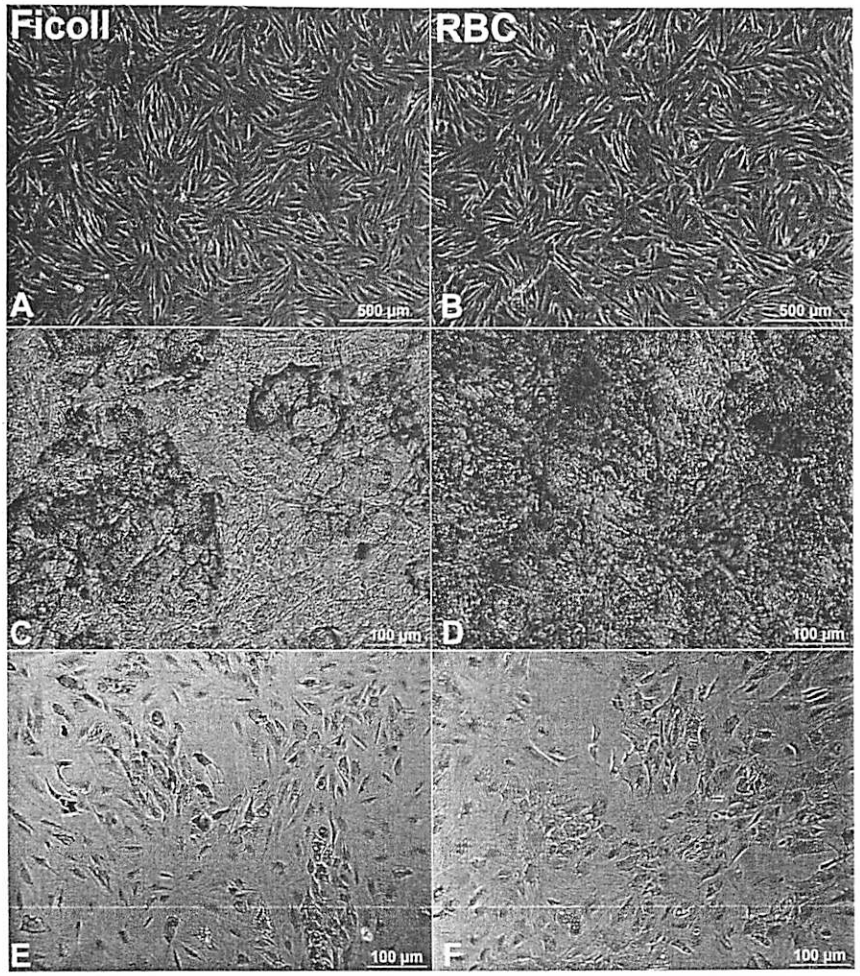

Figure 1. Morphology and mesodermal differentiation capacity of AT-MSCs. (A, B) Phase contrast micrographs at P0. (C, D) Alizarin red S staining of calcium deposits 3 weeks after osteogenic differentiation. (E, F) Oil red O staining of lipid droplets 2 weeks after adipogenic differentiation of AT-MSCs purified by Ficoll density gradient centrifugation or RBC lysis buffer treatment, respectively ($n = 3$).

(TP (time period) represents the time period of the culture in hours):

$$DT = TP \log 2/(\log NH - \log NI)$$

Quantitative polymerase chain reaction

Extraction, quantification and reverse transcription of total RNA (three donors), complementary DNA purification and the quantitative polymerase chain reaction (qPCR) reaction were performed as previously described (27). The gene expression assays used are listed in supplementary Table I. The estimation of the qPCR efficiency and the selection of the most stable reference gene(s) were conducted as previously described (27). According to geNorm, the minimal optimal number of reference targets to be used in this experiment was 3 ($V < 0.15$). As such, the geometric mean of beta-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hydroxymethylbilane synthase (*HMBS*) was further used to normalize the qPCR data.

Statistical analysis

The data are reported as mean ± SD. The statistical analyses of the qPCR data were performed using qBase Plus (Biogazelle, Ghent, Belgium). In addition, Student's *t*-test was used to determine significant differences between both isolation techniques for the expression of surface markers obtained by flow cytometry. The significance level was set at 0.05.

Results

Cell morphology and differentiation potential

AT-MSCs were purified from AT using either Ficoll gradient centrifugation or RBC lysis buffer treatment of the SVF based on their ability to adhere to plastic culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. As shown in Figure 1, the P0 cultures from both AT-MSC populations were heterogeneous and exhibited a large, flattened, fibroblast-like

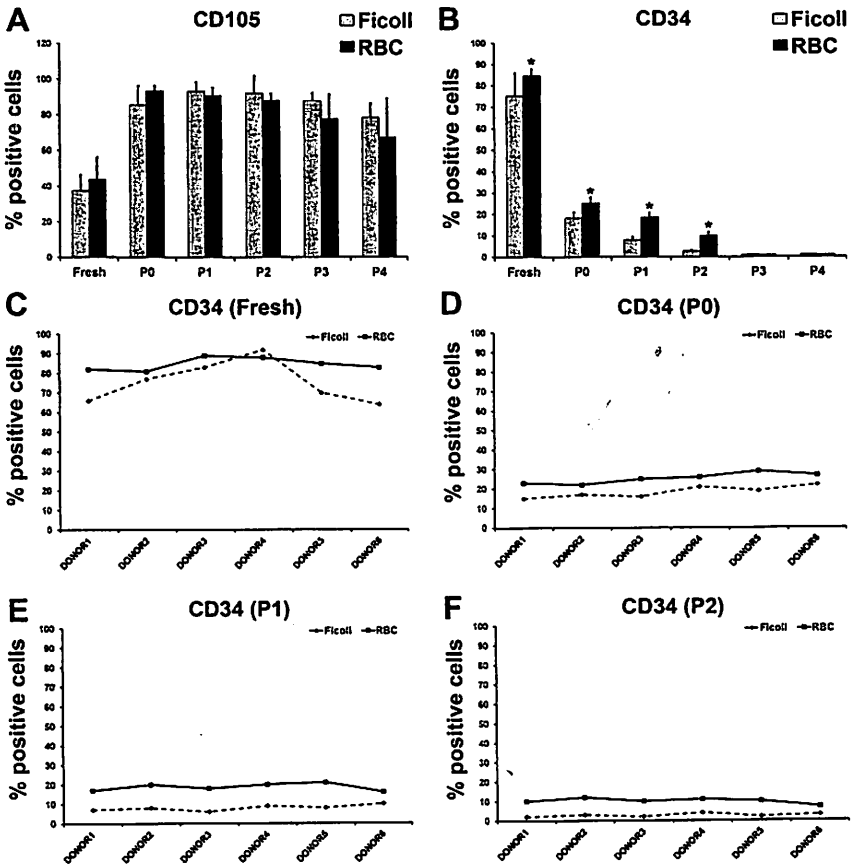

Figure 2. RBC lysis buffer treatment is a more robust purification method but shows a higher percentage of $CD34^+$ cells on isolation and during first passages. Flow cytometry was performed on six donors ($n = 6$). (A) Percentage of $CD105^+$ cells and (B) $CD34^+$ cells in AT-MSCs purified by Ficoll gradient centrifugation or RBC lysis buffer treatment on isolation and during four consecutive passages. (C–F) variation in percentage of $CD34^+$ cells between six individual isolations using Ficoll gradient centrifugation or RBC lysis buffer treatment in samples of freshly purified AT-MSCs, P0, P1 and P2 cultures, respectively.

morphology. There were no relevant morphologic differences observed between AT-MSCs purified by both methods, and their fibroblast-like shape was consistently maintained (Figure 1A,B). In addition, both AT-MSCs were able to differentiate into mesodermal cell types (e.g., osteoblasts and adipocytes). Three weeks after osteogenic induction, AT-MSC-Fs and AT-MSC-RBCs were able to deposit calcium (Figure 1C,D). Adipocytes that exhibited the ability to accumulate lipid droplets were obtained from both AT-MSC-Fs and -RBCs after 2 weeks of adipogenic stimulation (Figure 1E,F).

Phenotypical properties and population purity

Flow cytometric analysis of freshly purified AT-MSC-Fs and -RBCs, as well as of their consecutive cultures, revealed the presence of $CD34^+$ and $CD105^+$ expressing cell types in both purified cell populations (Figure 2, Table I). More specifically, in freshly isolated AT-MSC-Fs the $CD34^+$ cell population represented 75.3 ± 10.8% of the total cell population whereas a significantly higher percentage (84.7 ± 3.3%) was found for freshly purified AT-MSC-RBCs (Figure 2B). In addition, we observed a significantly

Table I. Characterization of freshly isolated and cultured human adipose–derived stem cells using flow cytometry purified by Ficoll gradient centrifugation or RBC lysis buffer treatment.

	Marker	Freshly isolated	P0	P1	P2	P3	P4
Ficoll	CD105	37.5 ± 8.9	85.3 ± 10.8	92.8 ± 5.4	91.8 ± 9.9	87.3 ± 4.5	78.2 ± 7.7
	CD34	75.3[a] ± 10.8[a]	18.3[a] ± 2.8[a]	8.0[a] ± 1.4[a]	2.7[a] ± 0.8[a]	1.0 ± 0.0	1.0 ± 0.0
RBC	CD105	43.7 ± 12.5	93.2 ± 3.1	90.3 ± 4.8	87.7 ± 3.9	77.3 ± 13.8	67.0 ± 21.9
	CD34	84.7[a] ± 3.3[a]	25.3[a] ± 2.6[a]	18.7[a] ± 2.0[a]	10.0[a] ± 1.7[a]	1.0 ± 0.0	1.0 ± 0.0

Positive cells for each marker are presented as a percentage (mean ± SD) for $n = 6$ donors.
[a]Significant difference between Ficoll and RBC lysis methods ($P < 0.05$).

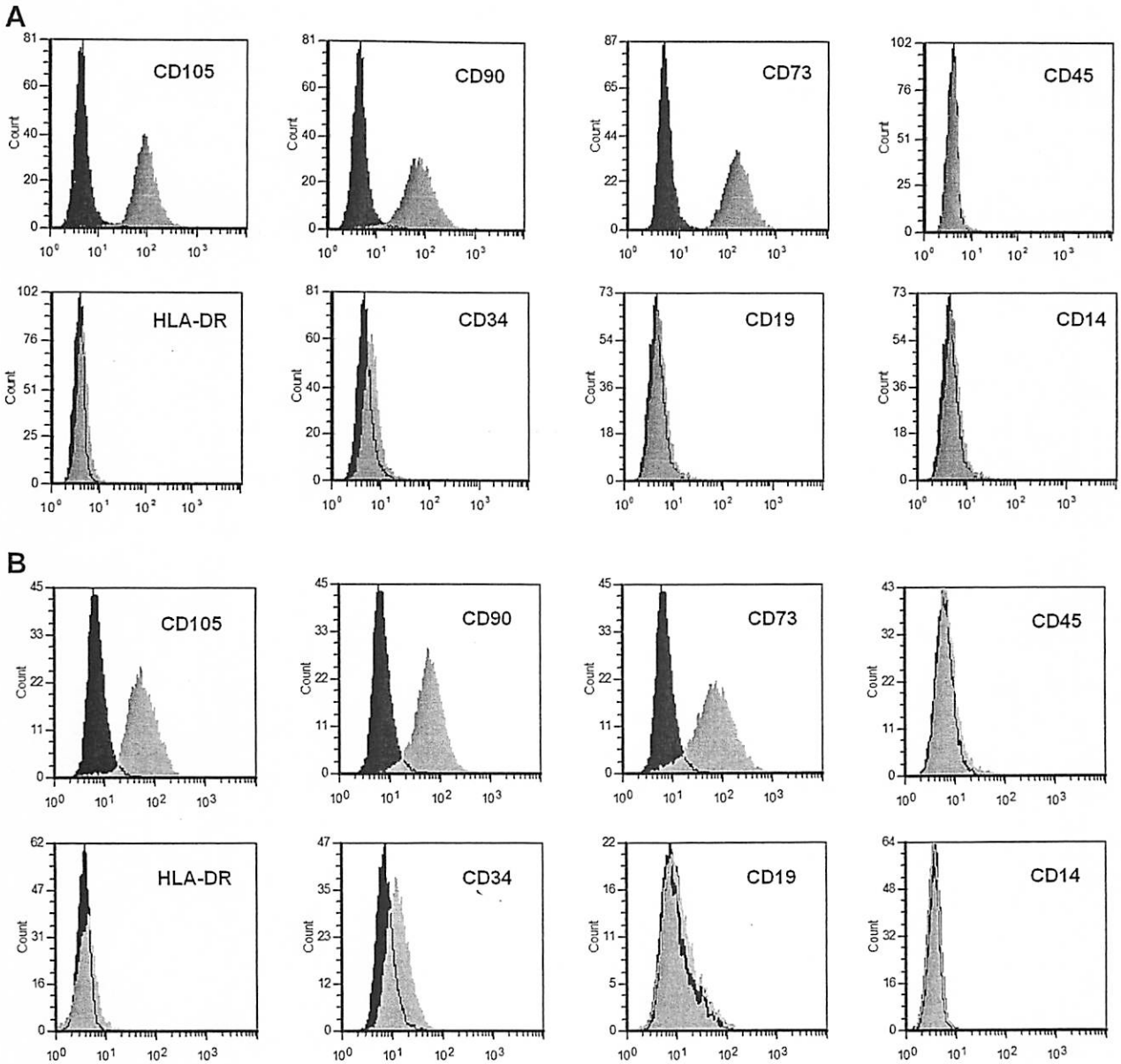

Figure 3. Immunophenotype of AT-MSCs. Representative immunophenotype of AT-MSCs at P4 purified by (A) Ficoll density fractionation or (B) RBC lysis buffer treatment ($n = 6$).

higher variation between the purified cell populations using Ficoll gradient centrifugation ($\sigma^2 = 116.7$) than RBC lysis buffer ($\sigma^2 = 10.7$) with respect to their yield of $CD34^+$ cells (Figure 2C). In contrast, no significant difference could be observed for $CD105^+$ expressing cells. Indeed, 37.5 ± 8.9% of the cells purified by Ficoll gradient centrifugation expressed CD105, whereas 43.7 ± 12.5% $CD105^+$ cells were obtained after RBC lysis buffer treatment of the SVF (Figure 2A, Table I). After plating, however, the MSC cell surface marker CD105 increased significantly to approximately 90% in cell cultures derived from both purified cell populations. By passage 4, approximately 80% of both cell populations stably expressed CD105 (Figure 2A, Table I). However, no significant differences could be observed between both purification procedures for the expression of this marker. In contrast, CD34 expression significantly decreased after plating (P0 ≤ 25%) and dramatically diminished (1 ± 0%) by P3 in AT-MSC-Fs and RBCs (Figure 2B, Table I). It was found, however, that the cell population obtained by the Ficoll-based purification procedure contained significantly less $CD34^+$ cells during the first passages after plating (Figure 2B, Table I) compared with the RBC lysis buffer treatment technique. In contrast to freshly purified AT-MSCs, significant differences in variation between donors could no longer be observed for either purification techniques with respect to the $CD34^+$ cell yield (Figure 2D–F). More specifically, the variance

between donors for $CD34^+$ cells after plating (P0) was 7.9 and 6.8 for the Ficoll- and RBC-based purification methods, respectively. From P1 onward, the variance was less than 4 for both purification techniques.

From P4 onward, significant differences between purification procedures could no longer be observed, and both populations yielded an equivalent phenotype (Figure 3). More specifically, AT-MSC-Fs and AT-MSC-RBCs expressed the MSC markers CD73, CD90 and CD105, but not the endothelial marker CD34 and the human leukocyte antigens CD14, CD19, CD45 and HLA-Dr (Figure 3). In addition, no significant differences could be observed for the expression of the CAMs *ICAM1* and *VCAM1*, and for the immune regulatory factors *HGF*, *HMOX1*, *LIF*, *PTGS1* and *PTGS2* (Figure 4).

Proliferation capacity and doubling time

AT-MSCs, obtained by both purification methods, presented a sustained proliferation capacity that was maintained during consecutive passages (Figure 5). Compared with AT-MSCs, purified by Ficoll gradient centrifugation (75.75 ± 27.51), the population doubling time of AT-MSC-RBCs was found to be slightly higher (86.65 ± 23.06; Figure 5D). However, no significant differences in population doublings were found over consecutive passages between both purification methods (Figure 5B). This resulted in approximately 12 population doublings over four passages for both cell populations and a maximal cumulative cell number of 1.36×10^{10} and 2.35×10^9 for AT-MSC-RBCs and -Fs, respectively (Figure 5A,C).

Discussion

There is a growing number of reports showing that MSCs can be obtained from human subcutaneous AT (6,28,29). In these studies, different approaches were used to purify the AT-MSCs from the SVF. Because MSCs possess immunomodulatory features (9,10), which is of interest in the context of immune cell therapy, a phenotypic comparison between AT-MSC-Fs and -RBCs could be of key importance for further cell therapy design (30). Here, we investigated whether the applied purification procedure has an impact on the properties of AT-MSCs with respect to their morphologic and proliferative features, the purity of the cultures and the expression of CAMs and immune regulatory molecules.

Our results show that, independent of the purification method used, AT-MSCs exhibited several characteristics typical of MSCs. More specifically, both purified cell populations could adhere to plastic culture flasks and maintain their fibroblast-like shape.

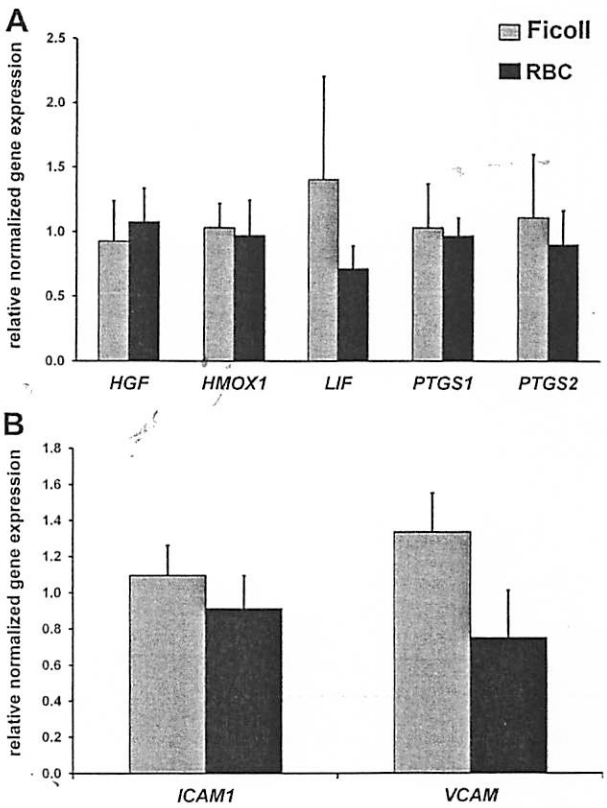

Figure 4. Expression of immune inhibitory factors and cell adhesion molecules in AT-MSCs. AT-MSCs express several genes involved in (A) immunomodulation and (B) cell adhesion. No significant differences could be observed between AT-MSCs purified by Ficoll density fractionation and RBC lysis buffer treatment ($n = 3$).

Furthermore, the proliferative potential of the AT-MSC-F and -RBC populations was quite similar. Expansion of AT-MSCs upon consecutive passaging was not affected by the purification method, and both methods allowed collection of a high number of AT-MSCs. Indeed, *ex vivo* cell amplification is essential to explore potential clinical applications of MSCs; expansion to a quantity of more than 10^8 cells is a minimal prerequisite for therapeutic use (31,32). Importantly, our findings are in accordance with previously reported results for BM-MSCs in which a similar morphology and only a slightly higher number of harvested cells was obtained when the cells were purified by using RBC lysis buffer treatment compared with density gradient centrifugation (Ficoll) (33).

To be valuable candidates for cell therapy, AT-MSCs must meet several criteria. First, we investigated the endothelial/stromal cell profile of both AT-MSC populations because it defines the cell population and determines their purity (30). We found that the purity of the obtained AT-MSCs significantly differs between both purification methods. This is particularly true during the first passages after plating in which a higher degree of $CD34^+$ cells was found using the RBC lysis buffer

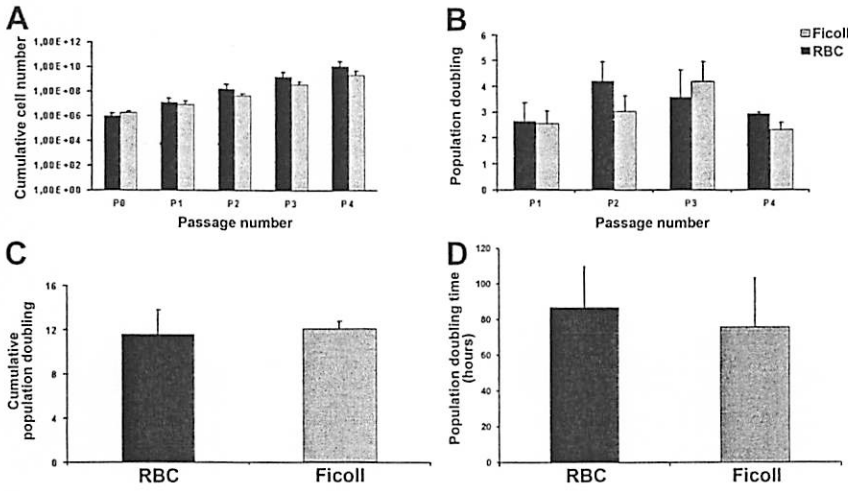

Figure 5. AT-MSCs purified by Ficoll density fractionation or RBC lysis buffer treatment exhibit equivalent expansion potential. No significant differences could be observed between both purification methods with respect to their (A) cumulative cell number, (B) population doubling, (C) cumulative population doubling and (D) population doubling time in hours ($n = 6$).

treatment technique compared with the Ficoll-based method. As such, this higher level of impurity within the cell population needs to be taken into account when AT-MSCs are used for clinical applications directly after purification or during their first passages after plating. This was particularly the case for cells purified by RBC lysis buffer treatment of the SVF. Compared with more advanced yet time-consuming and expensive purification methods, such as immunomagnetic beads or fluorescence-activated cell sorting, the purity of the obtained AT-MSCs population remains significantly lower (12,21–23). We also found that the variation in the purity of the obtained cell population between donors was higher when density gradient centrifugation was used in compared with RBC lysis buffer treatment. This observation confirms that density fractionation (Ficoll) is not only time-consuming but also difficult to standardize. It highlights the advantages of RBC lysis buffer–based methods. In addition, the reproducibility of the cell population obtained was much higher for the RBC lysis buffer purification protocol. Therefore, as previously reported for BM-MSCs (33), RBC lysis may represent an efficient and more robust method for the purification of human AT-MSCs. Finally, we could demonstrate that from P4 onward, morphologic, proliferative and phenotypical features of AT-MSCs purified by RBC lysis buffer treatment did not differ from those purified by Ficoll density gradient centrifugation. Furthermore, they meet the internationally established criteria of MSCs. Taken together, our observations suggest that RBC lysis buffer treatment may be a good alternative to density fractionation, offering a faster, more robust and easier method to purify AT-MSCs with preservation of their biological characteristics.

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Supplementary data

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