



## Obesity Determines the Immunophenotypic Profile and Functional Characteristics of Human Mesenchymal Stem Cells From Adipose Tissue

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### ABSTRACT

Adipose tissue is a major source of mesenchymal stem cells (MSCs), which possess a variety of properties that make them ideal candidates for regenerative and immunomodulatory therapies. Here, we compared the immunophenotypic profile of human adipose-derived stem cells (hASCs) from lean and obese individuals, and explored its relationship with the apparent altered plasticity of hASCs. We also hypothesized that persistent hypoxia treatment of cultured hASCs may be necessary but not sufficient to drive significant changes in mature adipocytes. hASCs were obtained from subcutaneous adipose tissue of healthy, adult, female donors undergoing abdominal plastic surgery: lean ( $n = 8$ ; body mass index [BMI]:  $23 \pm 1 \text{ kg/m}^2$ ) and obese ( $n = 8$ ; BMI:  $35 \pm 5 \text{ kg/m}^2$ ). Cell surface marker expression, proliferation and migration capacity, and adipogenic differentiation potential of cultured hASCs at two different oxygen conditions were studied. Compared with lean-derived hASCs, obese-derived hASCs demonstrated increased proliferation and migration capacity but decreased lipid droplet accumulation, correlating with a higher expression of human leukocyte antigen (HLA)-II and cluster of differentiation (CD) 106 and lower expression of CD29. Of interest, adipogenic differentiation modified CD106, CD49b, HLA-ABC surface protein expression, which was dependent on the donor's BMI. Additionally, low oxygen tension increased proliferation and migration of lean but not obese hASCs, which correlated with an altered CD36 and CD49b immunophenotypic profile. In summary, the differences observed in proliferation, migration, and differentiation capacity in obese hASCs occurred in parallel with changes in cell surface markers, both under basal conditions and during differentiation. Therefore, obesity is an important determinant of stem cell function independent of oxygen tension. *STEM CELLS TRANSLATIONAL MEDICINE 2016;5:464–475*

### SIGNIFICANCE

The obesity-related hypoxic environment may have latent effects on human adipose tissue-derived mesenchymal stem cells (hASCs) with potential consequences in mature cells. This study explores the immunophenotypic profile of hASCs obtained from lean and obese individuals and its potential relationship with the altered plasticity of hASCs observed in obesity. In this context, an altered pattern of cell surface marker expression in obese-derived hASCs in both undifferentiated and differentiated stages is demonstrated. Differences in proliferation, migration, and differentiation capacity of hASCs from obese adipose tissue correlated with alterations in cell surface expression. Remarkably, altered plasticity observed in obese-derived hASCs was maintained in the absence of hypoxia, suggesting that these cells might be obesity conditioned.

### INTRODUCTION

White adipose tissue (WAT) is conceivably the most plastic organ in the body, capable of considerable expansion and retraction in response to chronic deregulation in the energy balance. Adipose tissue expansion leads to obesity and contributes to obesity-related insulin resistance and its

consequent morbidities. Human adipose tissue-derived mesenchymal stem cells (hASCs) are progenitor cells that are part of a structural component of the stroma surrounding mature adipocytes and maintain tissue homeostasis by regulating the number of mature adipose cells [1, 2].

Increasing evidence indicates that the homeostatic environment as well as the specific

tissue source can influence the characteristics of hASCs, chiefly with regard to proliferation, migration, apoptotic susceptibility, and lipolytic capacity. In addition, the differentiation potential of isolated progenitor cells to adipogenic and osteogenic lineages has been found to be dependent on anatomical origin (subcutaneous, omental pericardic, or thymic) [3, 4]. There is no clear explanation for these distinctions, but local and systemic factors may help explain some of them. For example, low oxygen tension has dramatic effects on hASC physiology and impacts adipose tissue functionality. Accordingly, adipogenic differentiation of hASCs appears to be promoted by “high” in vitro oxygen tension (21%), whereas proliferation is enhanced at lower oxygen levels (5%) [5]. Analogously, low oxygen tension alters the physiological characteristics of embryonic, hematopoietic, and neuronal stem cells [6, 7]. Reduced adipose tissue oxygen partial pressure is a hallmark of obesity, presumably because adipose tissue vascularization is insufficient to maintain oxygen levels in expanding tissue, with potential implications for tissue function. Indeed, previous work showed that obese-derived hASCs are precommitted toward adipocyte differentiation with an upregulated inflammatory gene expression associated with their loss of stem cellness [8].

We hypothesized that this predisposition combined with a hostile environment mediated by hypoxia could have latent effects on hASCs with potential consequences in mature cells. Because the immunophenotype features of hASCs have been shown to be dependent on the adipose tissue source [9] and distinct stem cell immunophenotypes might impact adipogenic differentiation [10], changes in the immunophenotype profile in the context of an obese phenotype may result in dysfunctional behavior.

We show here that the antigen surface profile in undifferentiated hASCs and during adipogenic differentiation differs according to body mass index (BMI). Hypoxia has an equivalent effect on the plasticity of hASCs from healthy and obese donors; however, these effects do not correlate with the different immunophenotypic profiles detected in lean and obese hASCs. Overall, our results demonstrate that obesity determines the stem cell population in adipose tissue, and persistent hypoxia is necessary but not sufficient to orchestrate significant functional changes in mature adipocytes.

## MATERIALS AND METHODS

### Reagents

Dulbecco's modified Eagle's medium (DMEM) high-glucose/Ham's F12 medium (F12; 1:1), phosphate-buffered saline (PBS), trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, <https://www.thermofisher.com>). Hanks' Balanced Salt Solution (HBSS) and penicillin/streptomycin were purchased from PAA Laboratories (Pasching, Austria, <http://www3.gehealthcare.com>). Collagenase, type 1, was purchased from Worthington Biochemical Corp. (Lakewood, NJ, <http://www.worthington-biochem.com>). Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit and Vybrant CFDA SE Cell Tracer Kit for flow cytometry were purchased from Invitrogen (Thermo Fisher Scientific). Bovine serum albumin (BSA), mitomycin C, sodium azide, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), biotin, human insulin solution, pantothenate acid, Oil Red, paraformaldehyde, isopropanol, and Nile Red were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI, <https://www.caymanchem.com>).

Monoclonal antibodies for flow cytometry were purchased from BD (Franklin Lakes, NJ, <http://www.bd.com>), Beckman Coulter (Brea, CA, <https://www.beckmancoulter.com>), and e-Bioscience (San Diego, CA, <http://www.ebioscience.com>).

### Study Subjects

Subcutaneous adipose tissue from healthy female donors undergoing abdominal plastic surgery ( $n = 8$  lean, BMI  $23 \pm 1$  kg/m<sup>2</sup>;  $n = 8$  obese, BMI  $35 \pm 5$  kg/m<sup>2</sup>) was used in the study. Donors were classified as lean or obese based on BMI following World Health Organization criteria [11]. All participants gave their informed consent and the study was reviewed and approved by the Ethics and Research Committee of University Hospital Joan XXIII from Tarragona, Spain. All samples were part of an adipose-tissue biobank collection from our institution, ensuring the traceability and storage conditions of all samples.

### hASC Isolation and Cell Culture

hASCs were isolated following published protocols [2, 12]. Briefly, washed lipoaspirate tissues were digested with collagenase in PBS-0.2% BSA at 37°C for 1 hour with agitation, followed by centrifugation for 5 minutes at 300g at room temperature (RT). Collected stromal vascular cells were cultured in stromal culture medium (SCM) consisting of DMEM/F12, 10% FBS, and 1% antibiotics (penicillin and streptomycin) at 37°C, 5% CO<sub>2</sub> overnight. hASCs primary cultures at passage 0 were grown to 90% confluence and then harvested with trypsin-EDTA, and aliquots of  $1 \times 10^6$  cells were cryopreserved in liquid nitrogen until required [10]. To thaw for use, each vial of hASCs was removed from liquid nitrogen and rapidly thawed at 37°C in a water bath. Thawed cells were transferred to a 15-ml disposable centrifuge tube containing 10 volumes of the required growth medium, followed by centrifugation at 300g at RT for 5 minutes. The cell pellet was resuspended in fresh SCM and transferred to a culture vessel at  $5 \times 10^3$  cells/cm<sup>2</sup>. Cells were maintained at 37°C in a humidified tissue culture incubator containing 5% CO<sub>2</sub> under high (21%) or low (5%) oxygen concentration. Medium was changed twice weekly and passaging was done at near confluence (>80%). Cells were washed in PBS and detached with trypsin for immunophenotypic analysis by flow cytometry, migration and proliferation studies, or directly induced for adipogenic differentiation.

### Immunophenotyping

hASCs ( $2 \times 10^5$  cells) were incubated with a panel of primary antibodies, as previously described [11]; the antibodies were specifically chosen based on the known profile of bone marrow (BM)-derived MSCs. Cells were incubated for 20 minutes at RT (protected from light) with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated antibodies to human cluster of differentiation (CD) 36, CD49b, CD147, HLA-abc, and HLA-class II (HLA II); PeCy5-conjugated antibodies to human CD90, CD29, CD106, CD105, CD73, and CD34; PeCy7-conjugated antibodies to human CD45 and CD117; and anti-allophycocyanin (APC)-conjugated antibodies to human CD184 and CD44. Finally, hASCs were suspended in 400  $\mu$ l of HBSS without calcium and magnesium, and the vital dye Hoechst 33342 (Ho342; 40  $\mu$ g/ml final concentration) was added to discriminate nucleated cells. Samples were kept in the dark at 4°C until analysis. Data were collected using a MoFlo analyzer (Beckman Coulter) equipped with three lasers operating at 351, 488, and 633 nm. Nile Red fluorescence

was measured using a 630/30-nm band-pass filter. Surface markers were detected using FITC (530/30-nm band-pass filter), PeCy5 (560/40-nm band-pass filter), PeCy7 (600/30-nm band-pass filter), and APC (650/50-nm band-pass filter) channels. Gating was based on forward scatter and side scatter dot plots. A side scatter versus Ho342 dot-plot was used to discriminate nucleated cells from debris. At least 20,000 events were analyzed in each run.

### Hypoxia Treatment

Although oxygen tension in adipose tissue depends on blood flow, it varies typically between 3% and 11% [13]. An oxygen concentration of 5% was chosen as optimal for inducing changes in gene expression as described in BM-derived MSCs [14, 15]. hASCs from 3 lean and 3 obese donors were thawed in SCM at 5% O<sub>2</sub> and maintained at this oxygen tension throughout the experiment. Adipogenic differentiation potential was analyzed immediately after cells were removed from the hypoxia incubator.

### Cell Proliferation

Proliferation was quantified with the Vybrant carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) Cell Tracer Kit (Thermo Fisher Scientific), which provides a versatile and well-retained cell tracing reagent. Cells were detached from culture plates, washed in cold PBS, and gently resuspended in prewarmed (37°C) PBS containing 2  $\mu$ M CFDA-SE at approximately  $1 \times 10^6$  cells/ml. Following incubation for 15 minutes at 37°C, cells were collected by centrifugation, resuspended in fresh prewarmed medium, and incubated for a further 30 minutes to ensure complete modification of the probe. After a final wash, CFDA-SE fluorescence was measured with the FC 500 Series flow cytometer (Beckman Coulter) using 488-nm excitation. The approximate excitation and emission peaks of the product after hydrolysis were 492 nm and 517 nm, respectively. At least 20,000 events were analyzed in each run.

### Cell Migration

A migration assay was performed using the Transwell system (8- $\mu$ m pore polycarbonate membrane) as previously described [16, 17]. Briefly, the lower chamber wells were filled with DMEM with 0.1% BSA and without FBS. DMEM plus 30% FBS was used as a positive control of migration. hASCs from lean or obese individuals ( $8 \times 10^4$  cells in 0.2 ml) were added to the upper well of the chamber and incubated for 24 hours at 37°C in a humidified atmosphere at 5% or 21% O<sub>2</sub>. Cells that migrated to the lower chamber were collected and counted using an automatic cell counter (Bio-Rad TC10; Bio-Rad Laboratories, Hercules, CA, <http://www.bio-rad.com>). Cells in the lower side of the Transwell membrane were fixed with 4% glutaraldehyde for 2 hours and stained overnight with 2% toluidine blue. Transwells were then rinsed with water and the upper membrane side was cleaned with a cotton swab to eliminate nonmigratory cells. Finally, the membrane was cut and placed onto a slide. We counted the number of cells on the lower side in five different, randomly selected  $\times 10$  fields using a bright-field microscope (Zeiss Axio Vert.A1; Zeiss, Oberkochen, Germany, <http://www.zeiss.com>). At least three independent experiments were performed using hASCs from different lean and obese individuals.

### Adipogenic Differentiation

To derive mature adipocytes, hASCs from lean and obese individuals were induced to differentiate according to published protocols [10]. Briefly, confluent hASCs were cultured for 3 days in adipogenic

induction medium containing DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM dexamethasone, 500 mM IBMX, 33 mM biotin, 5 mM rosiglitazone, 100 nM insulin, and 17 mM pantothenate. Cultures were replenished every 3 days with maintenance medium (supplemented medium without IBMX and rosiglitazone) for 2 weeks, after which differentiation potential was assessed. To do this, cells were washed with PBS, fixed with 2% paraformaldehyde for 30 minutes, and washed again with deionized water. Adipogenic differentiation was assessed by Oil Red O staining (0.3% Oil Red O in isopropanol) for 1 hour at room temperature, after which cells were washed and visualized with a microscope (Zeiss Axio Vert.A1).

### Quantification of Lipid Droplets

Measurement of intracellular triacylglycerol was performed by Nile Red staining essentially as described by Greenspan et al. [18]. A working solution was prepared immediately before use by dilution of the stock solution (100  $\mu$ g/ml Nile Red in dimethyl sulfoxide) in PBS to give a final concentration of 0.05  $\mu$ g/ml. Following trypsin detachment, the cells were washed once with ice-cold PBS and Nile Red was added. The cells were incubated for 15 minutes at RT, centrifuged, and washed once with HBSS without calcium or magnesium. Subsequently, the cells were resuspended in PBS-1% BSA and 5% sodium azide, and fluorescence was measured as described under Immunoblot Analysis. Accumulation of lipid droplets was also visualized by staining the cells with Oil Red O. Cells were fixed in formalin solution 10% (Sigma-Aldrich) for 30 minutes and stained with Oil Red O for 45 minutes. After staining, dishes were washed and photographed (Zeiss Primovert; Zeiss).

### Gene Expression Analysis

Total RNA was isolated from adipose tissue or cells using the RNeasy Lipid Tissue Midi Kit (Qiagen Science, Hilden, Germany, <https://www.qiagen.com>). RNA quantity was measured at 260 nm and purity was assessed by the OD260/OD280 ratio. One microgram of RNA was transcribed to cDNA with random primers using the Reverse Transcription System (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>). Quantitative gene expression was evaluated by real-time PCR (qPCR) on a 7900HT Fast Real-Time PCR System using TaqManR Gene Expression Assay (Applied Biosystems) for quantifying human adiponectin (APM) (Hs 00605917\_m1) and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Hs00234592\_m1). Results were calculated using the comparative threshold cycle (Ct) method and expressed relative to the expression of the housekeeping genes cyclophilin 1A (PPIA) (Hs 04194521\_s1) and 18S (Hs03928985).

### Immunoblot Analysis

Cells were lysed and homogenized in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Sigma-Aldrich), and the protein concentration was determined with the BCA Protein Assay Kit (Thermo Fisher Scientific, Boston, MA). Equal amounts of total protein were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to Immobilon membranes (EMD Millipore, Billerica, MA, <https://www.emdmillipore.com>) and blocked [19]. Immunoreactive bands were visualized with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific) and images were captured using the VersaDoc imaging system and Quantity One software (Bio-Rad).

## Statistical Analysis

Results are expressed as mean  $\pm$  SD. Comparisons between groups were performed with Student's *t* test. Significance was determined as  $p < .05$ . Flow cytometry data were analyzed using FlowJo Software (Tree Star, Ashland, OR, <http://www.flowjo.com>) and statistical significance was tested with the unpaired Student's *t* test.

## RESULTS

### Characterization of hASCs From Lean and Obese Individuals

Isolated hASCs were prepared and stained according to the protocol described by Pachón-Peña et al. [10] and following the reagent manufacturer's recommendations. Flow cytometry analysis of cell marker expression revealed that undifferentiated hASCs showed minimal functional and quantitative criteria as established by the International Society of Cell Therapy and the International Federation for Adipose Therapeutics and Science. hASCs were positive for the expression of typical surface antigens, including CD90 (Thy-1 antigen), CD29 (integrin  $\beta$  1), CD44 (hyaluronic acid receptor), CD106 (vascular cell adhesion molecule 1, VCAM-1), CD73 (ecto-5'-nucleotidase), CD36 (glycoprotein IV, GPIV), CD147 (neurothelin), CD49b (integrin  $\alpha$ 2), human leukocyte antigen (HLA) class I -abc and HLA-major histocompatibility complex class II antigen (HLA-II), and negative for the expression of CD34 (single-chain transmembrane glycoprotein), CD45 (leukocyte common antigen), and CD117 (transmembrane tyrosine-kinase receptor) (Table 1). This expression profile has been described in previous studies and recognizes a specific hASC population [20–22]. All experiments were performed with these cells at passages 3–7.

### Obesity Disturbs hASCs Plasticity and Immunophenotypic Profile

Previous results demonstrated that the metabolic phenotype of CD34<sup>+</sup> MSCs isolated from obese adipose tissue significantly differed from equivalent cells isolated from lean adipose tissue [23]. To question whether an obese environment might similarly affect the plasticity of isolated hASCs, we evaluated proliferation, migration, and differentiation capacity of hASCs obtained from lean and obese individuals. To study the proliferative capacity, we used the Vybrant CFDA-SE Kit to evaluate the number of labeled cells during 72 hours. We found that proliferation of obese-isolated hASCs was significantly greater than hASCs isolated from lean individuals (total mean fluorescence intensity [MFI]  $230 \pm 16$  and  $192 \pm 28$  [arbitrary units], respectively;  $p < .05$ ) (Fig. 1A, 1B).

Next, we evaluated migration of hASCs using Transwell assays. Obese hASCs showed significantly greater basal migration capacity than lean hASCs ( $p < .05$ ) (Fig. 1C, 1D). We then induced adipogenic differentiation by culturing hASCs in defined medium. After 10 days in culture, mature adipocytes derived from hASCs from lean and obese individuals (mAd-hASCs) showed a similar gene upregulation of adipogenic markers such as adiponectin (APM) and PPAR $\gamma$ , as compared with equivalent noninduced cultures. However, a decrease in the expression of genes related to lipid metabolism, such as fatty acid binding protein 4 and lipoprotein lipase, was detected in mAd-hASCs derived from obese donors as compared with those from lean donors ( $p < .05$ ) (Fig. 2A). Protein expression analysis confirmed that although a comparable level of PPAR $\gamma$  protein was detected between obese and

**Table 1.** Immunophenotypic profile of undifferentiated human adipose-derived stem cells isolated from lean and obese individuals<sup>a</sup>

Surface markers	Lean		Obese	
	%	MFI	%	MFI
CD44	97 $\pm$ 4	488 $\pm$ 219	99 $\pm$ 1	172 $\pm$ 30
CD90	93 $\pm$ 4	217 $\pm$ 100	90 $\pm$ 14	275 $\pm$ 80
CD105	97 $\pm$ 2	58 $\pm$ 24	90 $\pm$ 14	124 $\pm$ 49
CD73	90 $\pm$ 13	89 $\pm$ 5	97 $\pm$ 3	81 $\pm$ 8
CD147	94 $\pm$ 6	49 $\pm$ 7	88 $\pm$ 10	40 $\pm$ 12
CD29	2 $\pm$ 1	31 $\pm$ 5	6 $\pm$ 4	15 $\pm$ 2 <sup>b</sup>
CD106	9 $\pm$ 7	28 $\pm$ 2	82 $\pm$ 5	315 $\pm$ 35 <sup>b</sup>
HLA II	96 $\pm$ 2	28 $\pm$ 7	53 $\pm$ 4	62 $\pm$ 20 <sup>b</sup>
HLA abc	98 $\pm$ 2	90 $\pm$ 21	95 $\pm$ 5	112 $\pm$ 26
CD49b	99 $\pm$ 1	28 $\pm$ 7	95 $\pm$ 3	13 $\pm$ 5
CD45	3 $\pm$ 2	3 $\pm$ 1	0 $\pm$ 0	5 $\pm$ 2
CD34	16 $\pm$ 8	3 $\pm$ 2	19 $\pm$ 14	19 $\pm$ 5
CD117	1 $\pm$ 1	15 $\pm$ 2	29 $\pm$ 21	22 $\pm$ 2

<sup>a</sup>Human adipose-derived stem cells obtained from five lean and obese donors and cultured under high oxygen levels were stained with the panel of antibodies described and analyzed by flow cytometry using 488-nm excitation on the MoFlo cytometer (Beckman Coulter). Values are reported as the mean  $\pm$  SD and were compared with Student's *t* test.

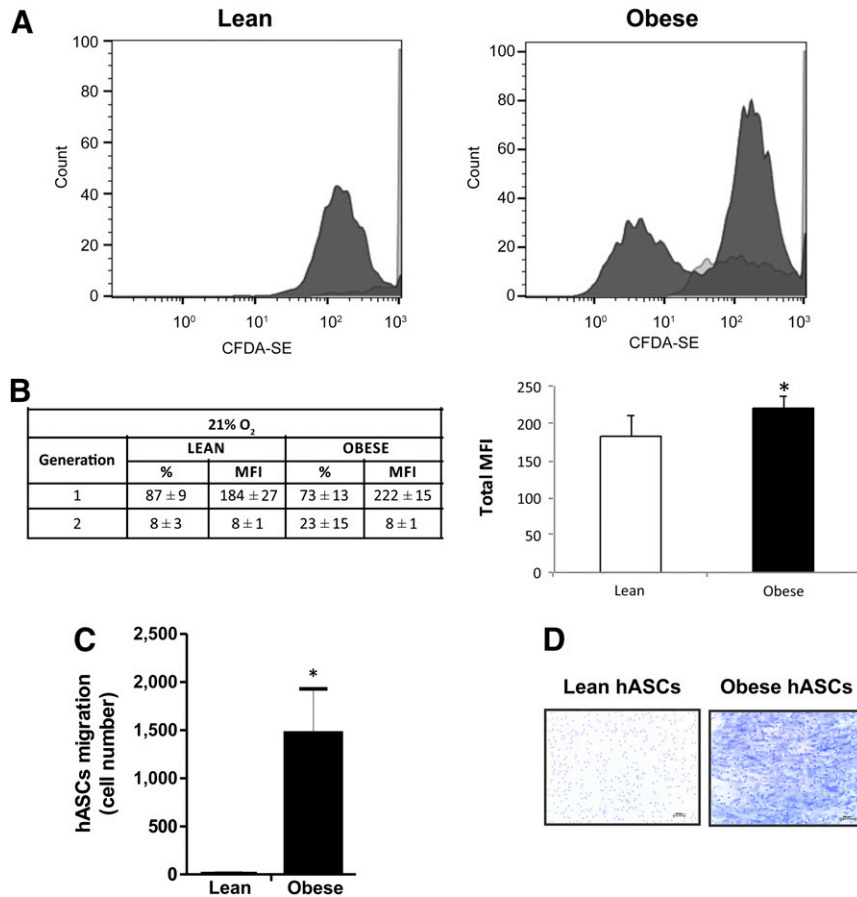
<sup>b</sup> $p < .05$ .

Abbreviation: MFI, mean fluorescence intensity (arbitrary units).

lean mAd-hASCs, obese cells showed a decrease in markers of lipid accumulation including fatty acid synthase (FASN), hormone sensitive lipase (HSL), and PERILIPIN compared with equivalent lean mAd-hASCs (Fig. 2B). Accordingly, lipid droplet formation evaluated by Oil Red O staining (Fig. 2C) and Nile Red staining ( $p < .05$ ) (Fig. 2D) was significantly greater in lean-derived mAd-hASCs compared with obese-derived mAd-hASCs after 12 days of differentiation. Collectively, these results reveal that hASCs isolated from an obese environment have an increased proliferative and migration capacity but an impaired intracellular lipid accumulation.

It is recognized that variations in MSC phenotypes are intimately associated with cell-specific and CD marker profiles [24, 25]. To question whether an obese environment modifies the immunophenotype of hASCs, we evaluated surface antigen expression by flow cytometry in undifferentiated hASCs and mAd-hASCs from lean and obese individuals. We found that marker expression was dependent on the differentiated status of the cells, but also on the clinical phenotype of the source tissue. Accordingly, expression of HLA-II and CD106 was significantly increased in obese-isolated undifferentiated hASCs relative to cells isolated from lean individuals, whereas expression of CD29 was significantly decreased (Fig. 3). Also, we observed decreased CD106 expression during differentiation of lean hASCs, but this was increased in differentiated obese hASCs. Furthermore, CD49b was significantly upregulated in mAd-hASCs from lean but not obese individuals compared with undifferentiated cells, and HLA-ABC expression was downregulated in obese but not lean hASCs during differentiation (Fig. 3).

Remarkably, quantitative flow cytometric analysis revealed two distinct populations of undifferentiated and mAd-hASCs from lean individuals based on expression of CD36 (CD36<sup>dim</sup>/CD36<sup>bright</sup>) (Fig. 4A, 4B). Regardless of this profile, high expression of CD36 in mAd-hASCs from both lean and obese individuals was detected (73%  $\pm$  16% vs. 69%  $\pm$  9% in cell percentage; total MFI: 779  $\pm$  166 vs. 718  $\pm$  2, respectively). Collectively, these data reveal that



**Figure 1.** Proliferation and migration capacity of hASCs under high oxygen. **(A):** hASCs isolated from lean and obese individuals were cultured in growth medium at high (21%) oxygen levels. hASCs were treated with 2  $\mu$ M CFDA-SE for 30 minutes and detected according to the recommended staining protocol. Cell division results in sequential halving of CFDA-SE fluorescence, resulting in a cellular fluorescence histogram in which the peaks represent successive generations. **(B):** Successive generation data expressed in percent and MFI values. **(C):** Quantification of migration capacity of hASCs isolated from lean and obese individuals after 24 hours. **(D):** Representative toluidine blue-stained cells (magnification,  $\times 10$ ). Values are reported as the mean  $\pm$  SD of hASCs isolated from eight independent, lean and eight obese individuals. \*,  $p < .05$ . Abbreviations: CFDA-SE, carboxy-fluorescein diacetate, succinimidyl ester; hASC, human adipose-derived stem cell; MFI, mean fluorescence intensity (arbitrary units).

the altered plasticity observed in hASCs from an obese environment is paralleled by changes in cell surface markers, both in undifferentiated and differentiated cultures.

### Hypoxia-Related Plasticity Dysfunction in hASCs Correlates With an Altered Immunophenotypic Profile

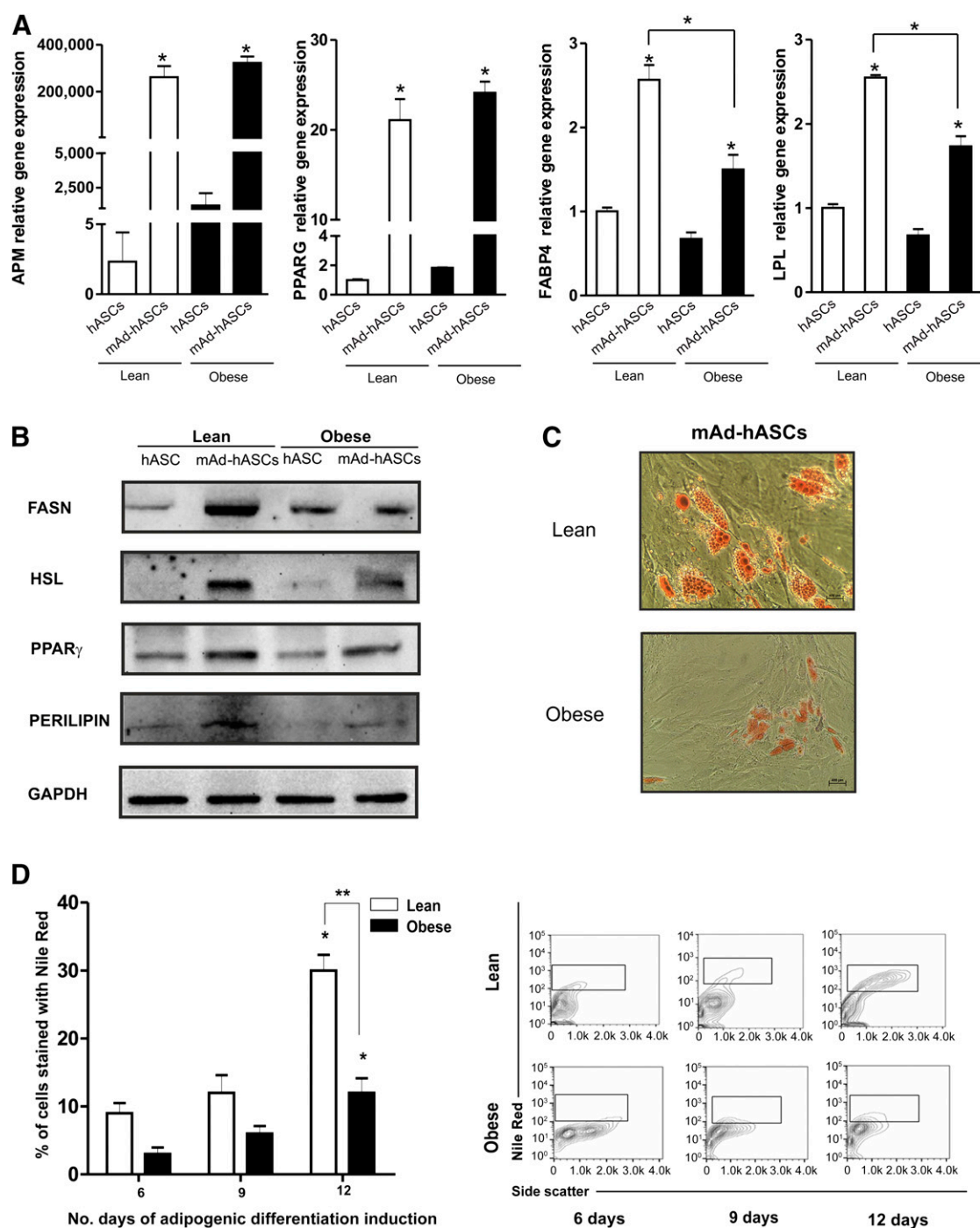
Adipose tissue hypoxia is potentially implicated in adipocyte dysfunction [26, 27]. Accordingly, hASCs isolated from obese patients display a gene expression profile associated with a loss of “stem cellness” and a commitment to adipocyte differentiation [28, 29]. This predisposition, combined with a hypoxic environment, may affect hASCs with consequences for mature adipocytes.

We first evaluated whether low in vitro oxygen culture conditions (5% O<sub>2</sub>), which mimics the physiological oxygen tension in adipose tissue in vivo [30–32], impacted proliferation. hASCs isolated from lean individuals exhibited a higher proliferative capacity at 5% O<sub>2</sub> compared with normal oxygen tension (total MFI: 380  $\pm$  49 vs. 192  $\pm$  28, respectively;  $p < .05$ ) (compare Fig. 1B and Fig. 5B). In contrast, proliferation of hASCs isolated from obese individuals remained unchanged (total MFI: 195  $\pm$  49 vs. 230  $\pm$  16) (compare Fig. 1B and 5B). We also found an increased

migration capacity of lean hASCs after 24 hours of incubation at 5% O<sub>2</sub> (Fig. 5C, 5D) as well as an increase in the quantity of apoptotic cells (data not shown), whereas the migration capacity of obese hASCs was decreased under the same conditions (Fig. 5C, 5D).

Next, we analyzed the effects of low oxygen for intracellular lipid formation. Although culture at 5% O<sub>2</sub> attenuated lipid droplet formation in both lean and obese mAd-hASCs compared with 21% O<sub>2</sub>, accumulation of lipids in lean-derived mAd-hASCs was greater than in obese-derived mAd-hASCs after 12 days of adipogenic differentiation (26%  $\pm$  14% and 6%  $\pm$  3%, respectively).

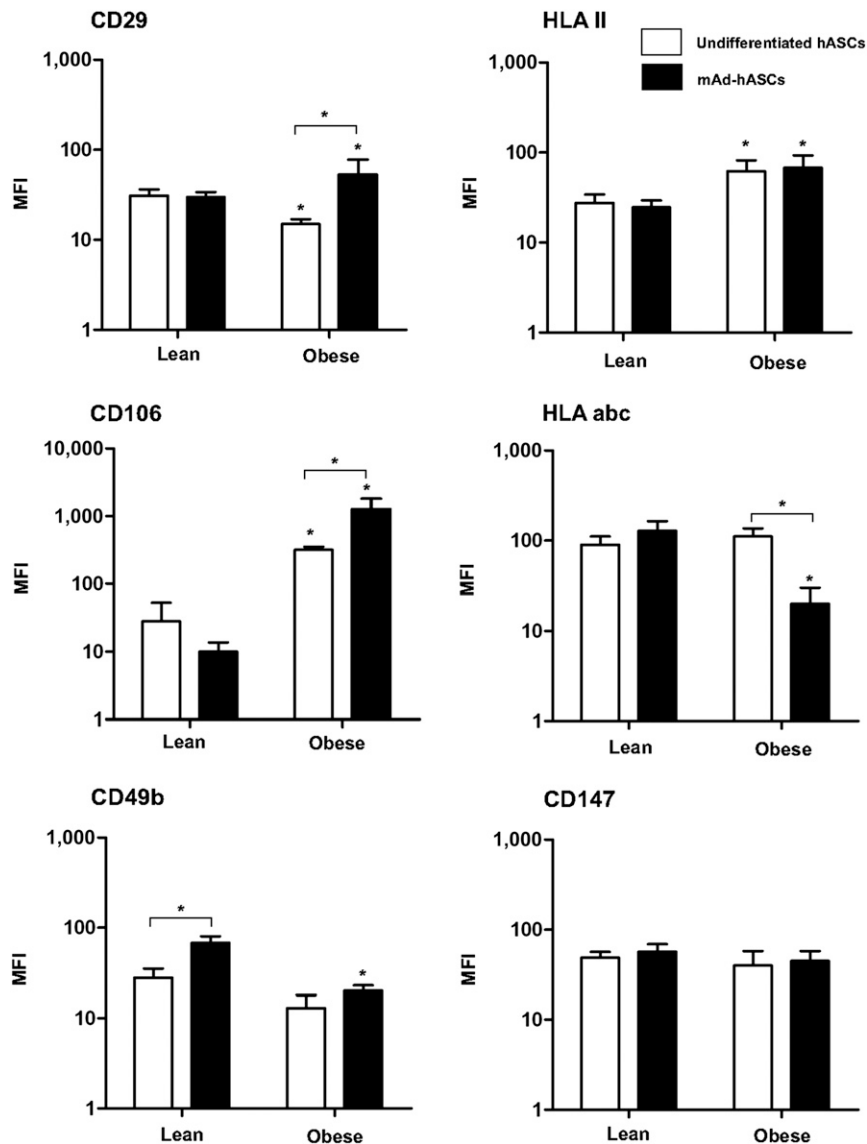
We then measured surface marker expression during differentiation in low oxygen. Because of the low number of adipogenic-differentiated hASCs from obese individuals, global surface marker expression analysis during differentiation of obese hASCs at 5% O<sub>2</sub> was not possible. As described, data from flow cytometry revealed two populations of undifferentiated and mAd-hASCs from lean individuals with distinct expression of CD36<sup>+</sup> (CD36<sup>dim</sup>/CD36<sup>bright</sup>) under normal oxygen levels (Fig. 4). Strikingly, the relative proportion of these subsets varied at low oxygen levels. Accordingly, a decrease in the percentage of undifferentiated hASCs from lean and obese individuals (85% and 70% in the CD36<sup>bright</sup> population at 5% O<sub>2</sub>, respectively) was observed, as well as a decrease in the mean



**Figure 2.** Adipogenic differentiation capacity of hASCs. **(A):** Expression of APM and PPAR $\gamma$  mRNA was analyzed by real-time polymerase chain reaction in hASCs and differentiated mAd-hASCs from the same lean and obese individuals. Results are given as mean  $\pm$  SD from three independent experiments performed in duplicate. \*,  $p < .05$  versus undifferentiated hASCs. **(B):** Protein expression of FASN, HSL, PPAR $\gamma$ , and PERILIPIN was analyzed by Western blotting under the same conditions as in **A**. A representative immunoblot is shown. **(C):** Representative cytochrome images of intracellular lipid enrichment in hASCs from lean and obese individuals cultured at 21% O $_2$  (magnification,  $\times 20$ ) **(D):** Quantification of Nile Red staining of hASCs from lean and obese individuals cultured for 6, 9, and 12 days in adipogenic differentiation medium at 21% O $_2$  and stained with Nile Red (0.05  $\mu$ g/ml). \*,  $p < .01$  for 12 days versus 6 and 9 days; \*\*,  $p < .01$  for hASCs from lean versus obese individuals at 12 days of adipogenic differentiation. Abbreviations: APM, adiponectin; FASN, fatty acid synthase; hASC, human adipose-derived stem cell; HSL, hormone sensitive lipase; mAd-hASCs, mature adipocytes derived from human adipose-derived stem cells from lean and obese individuals; PPAR, peroxisome proliferator-activated receptor; PERILIPIN, lipid droplet-associated protein.

fluorescence intensity (2-fold and 1.4-fold in lean and obese subjects, respectively) (Fig. 6A, 6C). Interestingly, among CD49 $^+$  cells, two populations with different phenotypes (CD49b $^{dim}$ /CD49b $^{bright}$ ) were also identified at low oxygen

levels both in lean and obese populations (Fig. 6B, 6D). Collectively, our data uncover a distinguishing immunophenotypic profile in hASCs in response to low oxygen levels, which also associates with the donor BMI.



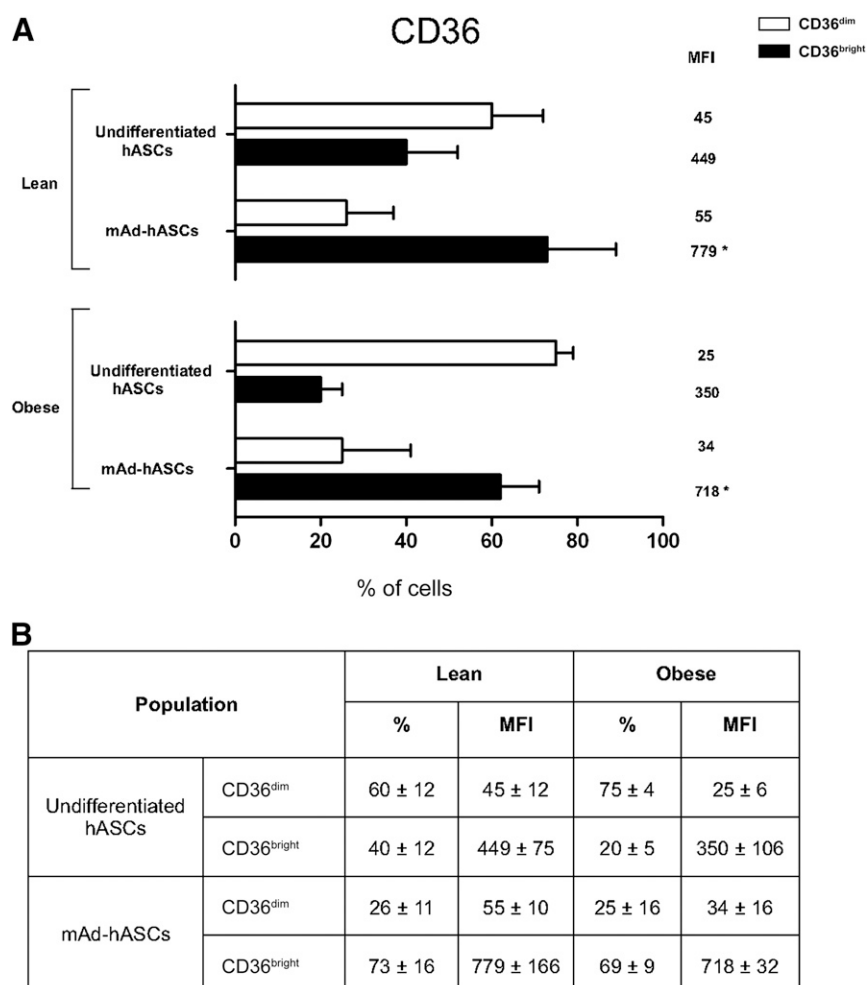
**Figure 3.** Immunophenotypic profile of undifferentiated and differentiated hASCs isolated from lean and obese individuals cultured at 21% O<sub>2</sub>. Undifferentiated hASCs (white bars) and mAd-hASCs (black bars) were stained with the panel of antibodies described and analyzed by flow cytometry using a MoFlo analyzer (Beckman Coulter) equipped with 3 lasers operating at 351, 488, and 633 nm. Surface markers were detected using fluorescein isothiocyanate (530/30-nm band-pass filter), PeCy5 (560/40-nm band-pass filter), PeCy7 (600/30-nm band-pass filter), and APC (650/50-nm band-pass filter) channels. Gating was based on forward scatter and side scatter dot plots. A side-scatter versus Ho342 dot plot was used to discriminate nucleated cells from debris. Values are expressed in MFI and reported as the mean  $\pm$  SD of hASCs from eight independent lean and eight obese individuals. \*,  $p < .05$ . Abbreviations: hASC, human adipose-derived stem cell; mAd-hASC, mature adipocytes derived from human adipose-derived stem cells from lean and obese individuals; MFI, mean fluorescence intensity (arbitrary units).

## DISCUSSION

Adipose tissue is a major source of MSCs, which possess an assortment of features that make them suitable for regenerative and immunomodulatory applications [33]. hASCs are affected by changing environmental conditions that result in an adaptation of their properties to a particular function. Here, we demonstrate that compared with a lean environment, adipose tissue from obese individuals contains a dysfunctional population of hASCs that can be identified by a unique immunophenotypic profile and aberrant differentiation.

Our results reveal that obese hASCs have a high proliferation capacity, significantly higher basal migration, and reduced lipid

droplet formation relative to lean hASCs. This altered plasticity of obese hASCs is paralleled by changes in cell surface marker expression, under basal conditions and also during differentiation. Hypoxia disturbs the plasticity of lean hASCs in a manner similar to the behavior observed in obese hASCs; however, notwithstanding the changes in surface marker expression detected under low oxygen levels, the immunophenotypic profile of obese hASCs is not mimicked in lean hASCs by hypoxia. These results point to the presence of a different population of hASCs as a consequence of the obese state and not only of environmental hypoxia. Consistent with previous studies [34–36], our data indicate that low oxygen levels maintain hASCs in their undifferentiated state and decrease their differentiation potential to adipocytes. In accordance



**Figure 4.** Membrane CD36 expression on undifferentiated and differentiated hASCs cultured at 21% O<sub>2</sub>. **(A):** Quantification of CD36 expression in hASCs and mAd-hASCs from lean and obese individuals cultured at 21% O<sub>2</sub>. **(B):** Data expressed in percentage of cells and MFI indicating the presence of two populations of CD36<sup>+</sup> with CD36<sup>dim</sup>/CD36<sup>bright</sup>. Values are reported as the mean ± SD of hASCs from eight independent lean and eight obese individuals. \*, *p* < .05. Abbreviations: hASC, human adipose-derived stem cell; mAd-hASC, mature adipocytes derived from human adipose-derived stem cells from lean and obese individuals; MFI, mean fluorescence intensity (arbitrary units).

with these findings, other studies have shown that high donor BMI results in a potentially compromised osteogenic capacity in vitro [37]. Low oxygen levels, as observed in adipose tissue from obese subjects, may account for the immunophenotypic changes in these cells, triggering functional differences.

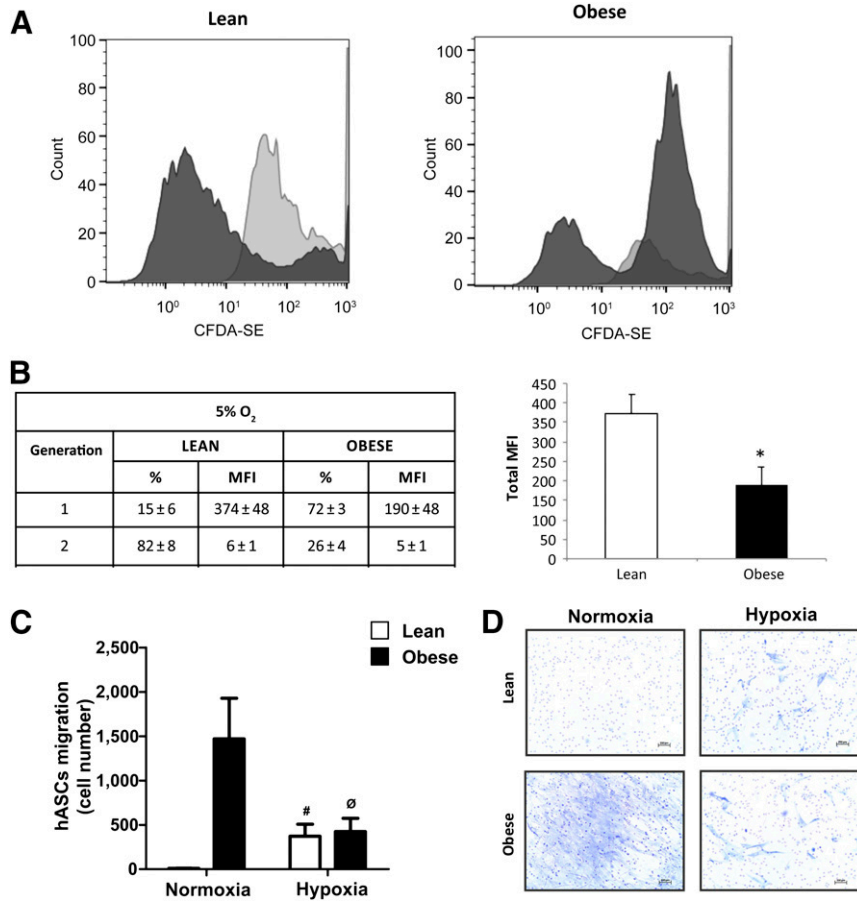
Despite the relevance of cell surface marker expression as a tool to identify and track stem cells, including hASCs, no studies have been published that comprehensively examine surface marker expression in hASCs from lean and obese individuals. This is particularly relevant when considering that these cells are proposed as a promising tool for regenerative therapies, and differences in surface antigen expression may define a specific profile in both the undifferentiated and in the mature states. These observations may allow an understanding of how these cells might function in regenerative medicine applications.

Surface marker expression is routinely determined by flow cytometry to classify cell type, but it should be noted that an MSC cannot be classified based solely on a single marker protein. Moreover, consecutive single-parameter measurements for determination of an expression pattern of various stem cell markers may suffer from artifacts due to heterogeneous cell populations.

Consequently, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have proposed three minimal criteria to define human MSCs [19]. We have defined hASCs according to these criteria [20–22]. Other methods have been frequently used to identify, isolate, and characterize human and murine ASCs from adipose tissue [23, 38]. Thus, previous studies have reported that human and murine WAT is a rich reservoir of CD45<sup>+</sup> CD34<sup>+</sup> ASCs, with a high basal migration capacity in lean-derived, but not obese-derived, ASCs [23, 39]. The discrepancies between our data and those of other authors regarding migratory capacity may be due to differences in the type of hASCs isolated, because CD34<sup>+</sup> cells used in these earlier reports represent the classical phenotype of endothelial progenitor cells (EPCs). These EPCs differentiate into endothelial cells and might contribute to vessel formation. Indeed, they express typical surface markers (CD34+/KDR+/CD45–) attributed to true circulating EPCs derived from bone marrow [40] but do not represent the niche of hASCs that generate mature adipocytes.

A striking finding of our work was the increased migratory capacity of hASCs from obese individuals. Interestingly, these cells show an upregulation of CD106 and HLA II surface cell markers in



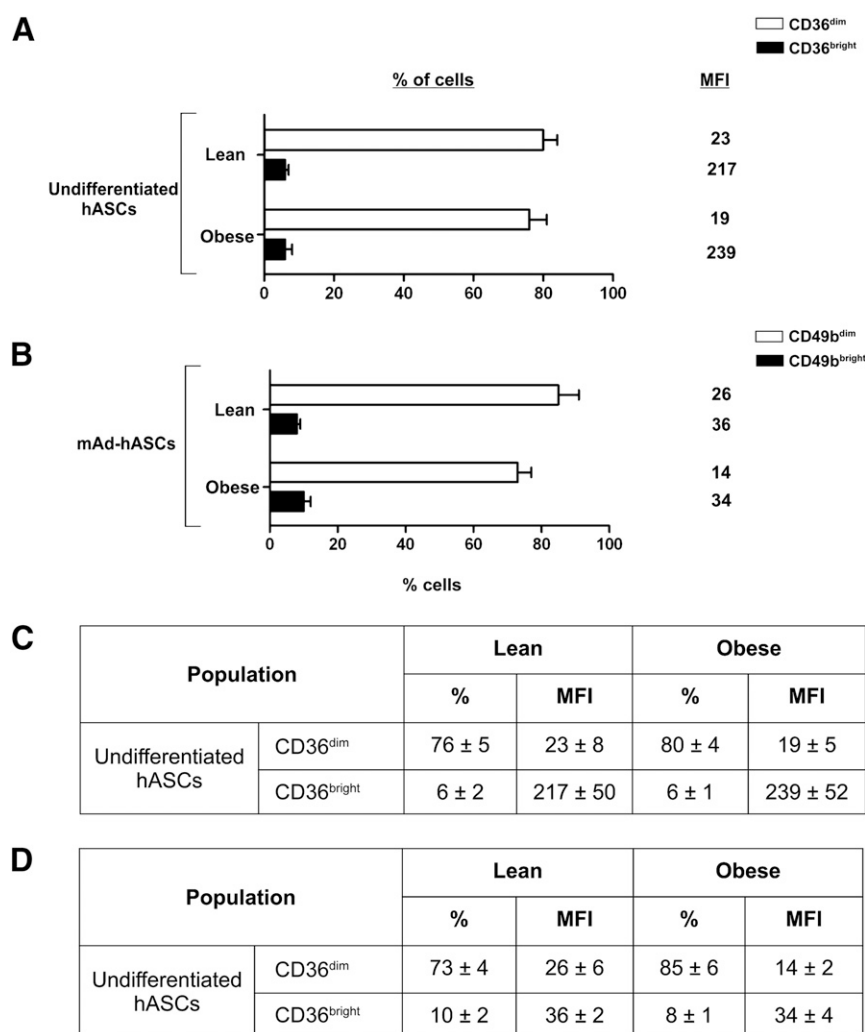


**Figure 5.** Proliferation and migration capacity of hASCs under low oxygen. **(A):** hASCs isolated from lean and obese individuals were cultured in growth medium at 5% O<sub>2</sub>. hASCs were treated with 2  $\mu$ M CFDA-SE as described in Figure 1 legend. **(B):** Successive generation data are expressed in percent and MFI values. Values are reported as the mean  $\pm$  SD of hASCs from three independent lean and three obese individuals. \*,  $p < .05$  versus lean. **(C):** Migration capacity of hASCs isolated from lean and obese individuals at 21% O<sub>2</sub> and after 24-hour exposure to 5% O<sub>2</sub>. Values are reported as the mean  $\pm$  SD of hASCs from three independent lean and three obese individuals. #,  $p < .01$  versus lean under normoxic conditions;  $\emptyset$ ,  $p < .01$  versus obese under normoxic conditions. **(D):** Representative toluidine blue-stained cells (magnification,  $\times 10$ ). Abbreviations: CFDA-SE, carboxy-fluorescein diacetate, succinimidyl ester; hASC, human adipose-derived stem cell; MFI, mean fluorescence intensity (arbitrary units).

undifferentiated cells and, although we acknowledge that no mechanistic conclusions can be reached from these observations, both proteins are involved in migration and immunomodulatory events. We show that CD49b, a transmembrane glycoprotein that noncovalently associates with CD29 (integrin  $\beta 1$ ) to form the integrin  $\alpha 2\beta 1$  complex known as very late activation antigen 2 (VLA-2), was upregulated in normal oxygen levels in mAd-hASCs. CD49b is recognized as a collagen receptor that plays a critical role in the establishment of resting memory CD4 T cells of the BM and the transmigration of effector cells into the BM through the sinusoidal endothelium [41, 42]. Although CD49b has been described to be involved in generation of T-helper cell memory, where and how CD49b works during the immune response remains unclear. Additionally, several studies have reported that MSCs exert their immunomodulatory activities through direct cell-cell contact and/or secretion of soluble factors [43]. Cell-cell adhesion mediated by CD106 is known to be critical for T-cell activation and leukocyte recruitment to the site of inflammation, and, therefore, plays an important role in evoking effective immune responses. Moreover, CD106 is critical for MSC-mediated immunosuppression [44] and for the binding

of hematopoietic progenitor cells [45]. Our data lead us to speculate that increased expression of HLA II/CD106 in a subpopulation of undifferentiated and differentiated hASCs, together with upregulated expression of CD49b in differentiated hASCs from obese individuals, could be related to the powerful immunosuppressive activity of hASCs. Further experiments are required to elucidate the downstream mechanisms and potential applications of these findings.

The significance of the two populations of cells with distinct CD36 expression remains to be investigated. CD36 has been described to regulate the expression of genes involved in lipid metabolism and has been also shown to be essential in the regulation of adipocyte differentiation and cellular lipid uptake [46]. Our data showing modulation of CD49b/CD36 expression at low oxygen levels and high expression of CD36 on differentiated hASCs are consistent with previous studies demonstrating a key role for this receptor in modulation of adipocyte transcriptional regulators, such as PPAR $\gamma$ , and adipogenic differentiation [46–49]. Further experiments will be required to determine the significance of these distinct populations, especially in relation to CD49b in the context of



**Figure 6.** Membrane CD36 and CD49b expression in undifferentiated hASCs cultured under low oxygen. **(A, B):** Quantification of CD36 **(A)** and **(B)** CD49b expression in undifferentiated hASCs from lean and obese individuals at 5% O<sub>2</sub>. **(C, D):** Data expressed in percentage of cells and MFI values indicating the presence of two populations of CD36<sup>+</sup> and **(D)** CD49b<sup>+</sup> cells, with dim/bright MFI with respect to percentage of cells at 21% O<sub>2</sub>. Values are reported as the mean ± SD of hASCs from three independent lean and three obese individuals. \*, *p* < .05. Abbreviations: hASC, human adipose-derived stem cell; mAd-hASC, mature adipocytes derived from human adipose-derived stem cells from lean and obese individuals; MFI, mean fluorescence intensity (arbitrary units).

low oxygen tension. Other  $\alpha 2$  integrins are regulated during osteogenic stimulation [50] and have also been frequently associated with enhanced osteogenic differentiation capacity at low oxygen levels [51]. We show here that hypoxia regulates CD49b expression, and the modest percentage of undifferentiated hASCs cells with high levels of surface CD49b might predict a higher osteogenic differentiation capacity of these cells in low oxygen. Additional studies to evaluate the potential relationship between cell surface markers and the osteogenic versus adipogenic potential of hASCs should be considered.

## CONCLUSION

Our data demonstrate that proliferation, migration, and differentiation capacities, as well as the immunophenotypic profile of hASCs, are modulated by the microenvironment within adipose tissue. In the obese context, low oxygen levels and

chronic low-grade inflammation are considered key microenvironmental factors that might determine commitment and plasticity of progenitor cells within adipose tissue. We have observed that hypoxia disturbs cell plasticity in undifferentiated and also in adipogenic-differentiated hASCs isolated from obese individuals, and these changes are paralleled by modulation of the immunophenotypic profile. The altered behavior observed in obese hASCs independently of oxygen levels, however, points to additional environmental factors as a potential origin of the characteristic dysfunction of hASCs observed in obesity.

Our results highlight the importance of examining stem cells within their physiologic environment to better understand their functional properties, which may be invaluable for developing novel cytotherapeutic strategies with possible clinical impact. Additional studies will be required to further assess the characteristics of these cells and to elucidate their true physiological nature.

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## AUTHOR CONTRIBUTIONS

G.P.-P., J.P., S.F.-V., and J.V.: conception and design, collection and/or assembly of data, manuscript writing; C.S., M.E., and X.D.: collection and/or assembly of data, manuscript writing; W.O.-O., R.S., and F.J.T.: collection and/or assembly of data.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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