Differences in the Osteogenic Differentiation Capacity of Omental Adipose-Derived Stem Cells in Obese Patients With and Without Metabolic Syndrome

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Multiple studies have suggested that the reduced differentiation capacity of multipotent adipose tissue-derived mesenchymal stem cells (ASCs) in obese subjects could compromise their use in cell therapy. Our aim was to assess the osteogenic potential of omental ASCs and to examine the status of the isolated $CD34^{negative}$-enriched fraction of omental-derived ASCs from subjects with different metabolic profiles. Omental ASCs from normal-weight subjects and subjects with or without metabolic syndrome were isolated, and the osteogenic potential of omental ASCs was evaluated. Additionally, osteogenic and clonogenic potential, proliferation rate, mRNA expression levels of proteins involved in redox balance, and fibrotic proteins were examined in the $CD34^{negative}$-enriched fraction of omental-derived ASCs. Both the omental ASCs and the $CD34^{negative}$-enriched fraction of omental ASCs from subjects without metabolic syndrome have a greater osteogenic potential than those from subjects with metabolic syndrome. The alkaline phosphatase and osteonectin mRNA were negatively correlated with nicotinamide adenine dinucleotide phosphate oxidase-2 mRNA and the mRNA expression levels of the fibrotic proteins correlated positively with nicotinamide adenine dinucleotide phosphate oxidase-5 mRNA and the homeostasis model assessment. Although the population doubling time was significantly higher in subjects with a body mass index of 25 kg/m^2 or greater, only the $CD34^{negative}$-enriched omental ASC fraction in the subjects with metabolic syndrome had a higher population doubling time than the normal-weight subjects. The osteogenic, clonogenic, fibrotic potential, and proliferation rate observed in vitro suggest that omental ASCs from subjects without metabolic syndrome are more suitable for therapeutic osteogenic applications than those from subjects with metabolic syndrome. **(*Endocrinology* 156: 4492–4501, 2015)**

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Abbreviations: AP, alkaline phosphatase; ASC, multipotent adipose tissue-derived mesenchymal stem cell; BMI, body mass index; Col1a1, collagen type I alpha 1; Col3a1, collagen type III α1; Col5a1, collagen type V α1; Col6a3, collagen type VI α3; Ct, cycle threshold; HOMA-IR, homeostasis model assessment of insulin resistance; MS, subjects with metabolic syndrome; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; non-MS, subjects without metabolic syndrome; NOX2, cytochrome b-245, beta polypeptide; NOX5, EF-hand calcium binding domain 5; Nw, normal-weight subjects; Sp7, transcription factor osterix; PDT, population doubling time; RefSeq, reference sequence; ROS, reactive oxygen species; RPL13A, ribosomal protein L13a; RT-qPCR, real-time quantitative PCR; Runx2, Runt-related transcription factor 2; SOD1, superoxide dismutase 1 cytoplasmic; SOD3, superoxide dismutase 3 extracellular.

The use of multipotent adipose tissue-derived mesenchymal stem cells (ASCs) for therapeutic approaches in regenerative medicine has grown substantially in the last decade (1). Given their accessibility, abundance, and ease of use, they have been used as a cell source for skeletal muscle regeneration (2). They have been used in combination with osteoinductive biomaterials and osteogenic molecules in various animal models and have also been used in several clinical trials on bone reconstruction (3). In developed countries, obesity has reached approximately 30% of the population (1), and it is associated with both degenerative musculoskeletal disease (4) and bone fracture risk (5). It is increasingly evident that these obese subjects could be candidates for regenerative therapies. In vitro experiments outside the adipose tissue environment have demonstrated the reduced capacity for adipogenic differentiation (6–11) and premature senescence (10, 12) in ASCs derived from obese subjects. The deterioration of ASCs associated with obesity appears to compromise their therapeutic potential for musculoskeletal diseases because in vitro experiments have also confirmed a diminished capacity for osteogenic differentiation (10, 13, 14). However, it is well described that 3%–57% of obese subjects, although having a high body mass index (BMI), do not develop the metabolic abnormalities that are generally associated with obesity (15, 16).

Numerous findings suggest that white adipose tissue dysfunction plays a crucial role in the origin, progression, and development of the metabolic impairment associated with obesity (17–19), and the capacity for expansion of this tissue seems to be one of the most important determinants contributing to metabolic complications (20). Considering that adipose tissue expansion also depends on the state of its precursor cells, it seems likely that ASC functionality differs among obese subjects with different metabolic profiles. The ASCs of obese subjects without metabolic syndrome may be more suitable for therapeutic osteogenic applications than those of obese subjects with metabolic syndrome. Therefore, in this study we first evaluated the osteogenic potential of omental ASCs in subjects, taking into account not only the BMI but also the metabolic profile. We also examined the status of the $CD34^{negative}$-enriched fraction isolated from omental adipose tissue, evaluating its osteogenic and clonogenic potential and proliferation rate, as well as the mRNA expression levels of proteins involved in both the redox balance and the adipose tissue fibrosis associated with obesity.

Research Design and Methods

Subjects

A total of 47 subjects were recruited at the Virgen de la Victoria Clinical Hospital (Malaga, Spain). All subjects were younger than 58 years of age and none had an infectious disease or type 2 diabetes (or were receiving medical therapy for this condition). Omental adipose tissue biopsies were obtained from subjects who underwent bariatric surgery (morbidly obese) or cholecystectomy (normal weight, overweight, and obese). The hospital ethics committee approved the study and the informed consent of all participants was obtained. Patients with a BMI of 25 kg/m^2 or greater were diagnosed as subjects with and without metabolic syndrome according to the International Diabetes Federation criteria. Due to the limited availability of tissue, we were unable to conduct the experiments on all biopsies provided by all the subjects recruited. The number of experiments with cells from different donors (n) is specified in the respective assays.

Isolation and expansion of the stromal vascular fraction derived from omental adipose tissue

The samples of omental adipose tissue were subjected to enzymatic digestion in a type I collagenase solution at 0.150% supplemented with 1.0% bovine serum albumin in a shaking water bath at 37°C for 70 minutes. The tissue suspension obtained after enzymatic digestion was centrifuged at $500 \times g$ for 10 minutes, and floating adipocytes were separated from precipitated stromal vascular fraction, which was passed through 100-μm pore filters and centrifuged at $500 \times g$ for 5 minutes. The resulting cell pellet was resuspended in erythrocyte lysis buffer for 10 minutes at room temperature and after being centrifuged at $400 \times g$ for 5 minutes was resuspended in growth medium consisting of DMEM/F12 supplemented with fetal bovine serum (0.1 mL/mL), streptomycin (100 μg/mL), penicillin (100 U/mL), and L-glutamine (2 mM). Approximately 16 hours after seeding, the first medium change was carried out, and the cells were allowed to proliferate under standard culture conditions at 37°C in a humid atmosphere with 5% CO_2, with two or three medium changes per week, until they reached 90% confluence.

Isolation of the $CD34^{negative}$ fraction from the omental ASCs at passage zero and immunophenotypic characterization

The passage zero omental ASCs were detached with trypsin and resuspended in 300 μL PBS buffer supplemented with 0.5% BSA and 2 mM EDTA. One hundred microliters of FcR blocking reagent and 100 μL of specific antihuman CD34 antibody conjugated with magnetic beads were added and incubated at 4°C for 30 minutes to then separate the $CD34^{negative}$ fraction using a column and magnetic separator, according to the manufacturer's instructions (Miltenyi Biotec). During the immunophenotypic characterization, passage zero $CD34^{negative}$-enriched omental ASCs were resuspended in PBS buffer supplemented with 3.0% BSA and incubated for 30 minutes on ice and protected from light, with monoclonal antibodies specific for phenotypic and fluorochrome-conjugated markers (CD34-fluorescein isothiocyanate; eBioscience; CD45-PE-Cy7BD; Pharmingen; CD31-APC, CD90-APC; Miltenyi Biotech) or the respective isotype control. After washing with the buffer solution, the cells were resuspended in 1000 μL PBS and finally 1×10^4 events per tube were acquired using a CyAn ADP high-speed analyzer (Beckman Coulter).

Osteogenic differentiation

The omental ASCs in passage 2 or CD34negative-enriched fraction in passage zero were seeded at a density of 10 000 cells/cm^2 and cultured under standard culture conditions at 37°C in a humid atmosphere with 5% CO_2. When cells reached 80%–90% confluence, the expansion medium was replaced with osteogenic medium composed of DMEM/F12 supplemented with fetal bovine serum (0.1 mL/mL), streptomycin (100 μg/mL), penicillin (100 U/mL), L-glutamine (2 mM), dexamethasone (0.1 μM), ascorbic acid (200 μM), and β-glycerophosphate (20 mM). After the period of differentiation, the osteogenic condition was confirmed by Alizarin Red staining solution (2.0% in deionized water, pH 4.3) and the alkaline phosphatase (AP) and osteonectin gene expression were quantified by real-time quantitative PCR (RT-qPCR).

Gene expression by RT-qPCR

The cultured cells were washed with PBS and lysed immediately using TRIZOL (Invitrogen). RNA from the cells was isolated and purified with the RNeasy lipid kit (QIAGEN) and reverse transcribed to cDNA using the reverse transcriptase enzyme (transcriptor reverse transcriptase 20 U/μL; 03531287001; Roche) in the thermocycler (2720 Thermal Cycler; Applied Biosystems). RT-qPCRs were performed with 20 ng cDNA for AP (Hs01029144_m1, reference sequence [RefSeq] NM_000478.4), osteonectin (Hs00234160_m1, RefSeq NM_003118.3), runt-related transcription factor 2 (Runx2) (Hs00231692_m1, RefSeq NM_001015051.3), Sp7 transcription factor (osterix) (Hs01866874_s1, RefSeq NM_001173467.1), cytochrome b-245 β-polypeptide (nicotinamide adenine dinucleotide phosphate oxidase [NOX]-2) (Hs00166163_m1, RefSeq NM_000397.3), NOX EF-hand calcium binding domain 5 (NOX5) (Hs00225846_m1, RefSeq NM_001184779.1), superoxide dismutase 1 cytoplasmic (SOD1) (Hs00533490_m1, RefSeq NM_000454.4), superoxide dismutase 3 extracellular (SOD3) (Hs00162090_m1, RefSeq NM_003102.2), collagen type Iα1 (Col1a1) (Hs 00164004_m1, RefSeq NM_000088.3), collagen type IIIα1 (Col3a1) (Hs00943809_m1, RefSeq NM_000090.3), collagen type Vα1 (Col5a1) (Hs00609133_m1, RefSeq NM_000093.4), collagen type VIα3 (Col6a3) (Hs00915125_m1, RefSeq NM_004369.3) genes.

The amplifications were performed using a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) on an ABI 7500 real-time PCR system (Applied Biosystems). RT-qPCRs were carried out for all genes using specific TaqMan gene expression assays. Each sample was performed in duplicate, and negative controls were included in all the reactions. The reactions consisted of an initial denaturing of 10 minutes at 95°C and then 40 cycles of 15 seconds denaturing phase at 95°C and 1 minute annealing and extension phase at 60°C. A threshold cycle (Ct) value was obtained for each amplification curve and a DCt value was first calculated by subtracting the Ct value for human ribosomal protein L13a (RPL13A) (Hs 04194366-g1, RefSeq NM_001270491.1) from the Ct value for each sample and transcript. Thus, specific signals were normalized with respect to endogenous control RPL13A according to the $2^{-\delta Ct}$ formula for mRNA expression of NOX2, NOX5, SOD1, SOD3, Col1a1, Col3a1, Col5a1, Col6a3, and $2^{-\delta\delta Ct}$ for mRNA expression of AP, osteonectin, Runx2, and osterixin cells cultured in osteogenic medium, taking the cultured cells in expansion medium as calibrator.

Colony-forming unit assay

The CD34^{negative}-enriched omental ASC fraction in passage zero was seeded in triplicate at a density of 50 cells/cm^2 and incubated for 14 days under standard culture conditions at 37°C in a humid atmosphere with 5% CO_2. Cells were fixed and stained with crystal violet solution (0.5% in methanol) for 10 minutes. After washing with water and air drying, the colonies formed by more than 50 cells were counted.

Determination of population doubling time

The CD34^{negative}-enriched omental ASC fraction in passage zero was seeded in duplicate at a density of 3000 cells/cm^2 in 12-well plates. The cells were detached with trypsin at 2, 4, and

Table 1. Biological Characteristics of Nw Individuals, MS Patients, and Non-MS Patients

	Nw (n = 10)	Non-SM (n = 15)	SM (n = 22)
Age, y	42.10 ± 2.33	40.60 ± 2.21	41.45 ± 2.09
BMI, kg/m^2	23.01 ± 0.66	38.73 ± 2.73[a]	39.95 ± 2.53[a]
Waist circumference, cm	80.60 ± 2.02	110.92 ± 5.89[a]	121.81 ± 5.47[a]
Waist to hip ratio	0.86 ± 0.02	0.91 ± 0.02	0.97 ± 0.02[a,b]
HOMA-IR	1.54 ± 0.24	2.87 ± 0.42[a]	3.66 ± 0.39[a]
Serum insulin, μIU/mL	6.89 ± 0.98	11.95 ± 2.07	14.0 ± 1.42[a]
Serum glucose, mg/dL	88.67 ± 2.45	94.20 ± 2.87	106.0 ± 4.39[a,b]
Triglycerides, mg/dL	92.50 ± 17.08	112.67 ± 7.17	165.95 ± 18.66[a,b]
HDL cholesterol, mg/dL	58.10 ± 3.87	47.36 ± 4.07[a]	40.91 ± 2.75[a]
Systolic blood pressure, mm Hg	120.50 ± 3.21	117.73 ± 6.99	132.89 ± 4.61
Diastolic blood pressure, mm Hg	77.40 ± 2.12	77.0 ± 3.10	83.39 ± 2.06
Serum urea, mg/dL	89.80 ± 2.28	94.20 ± 2.87	102.0 ± 2.24[a,b]
Serum creatinine, mg/dL	0.75 ± 0.06	0.77 ± 0.04	0.94 ± 0.06[a]
Uric acid, mg/dL	3.46 ± 0.19	4.63 ± 0.24[a]	5.74 ± 0.33[a,b]
GGT, U/L	13.80 ± 1.28	31.0 ± 3.78[a]	44.91 ± 10.59[a]
GOT, U/L	13.90 ± 1.47	20.47 ± 4.12	22.45 ± 1.90[a]
GPT, U/L	22.30 ± 1.93	33.40 ± 5.12	51.95 ± 5.43[a,b]

Abbreviations: GGT, γ-glutamil transferase; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvate transaminase; HDL cholesterol, high-density lipoprotein cholesterol. Values are means ± SEM.

[a] Results are significantly different (Mann-Whitney; $P < 0.05$) from Nw individuals.

[b] Results are significantly different (Mann-Whitney; $P < 0.05$) from Non-MS patients.

6 days of culture and were counted using the trypan blue exclusion assay with neubauer chamber. The population doubling time (PDT) was calculated using the formula, $PDT = 48/\log_2(N_2/N_1)$, where N_1 and N_2 are the number of cells on days 4 and 6, respectively, after seeding.

Statistical analysis

The results were expressed as mean values ± SE. Comparisons between more than two groups were performed using the nonparametric Kruskal-Wallis test and between two unpaired groups using the nonparametric Mann-Whitney *U* test. The correlation between variables was calculated with Spearman's Rho. All the statistical analysis was done using SPSS (version 17.0; SPSS Inc). Values of $P < .05$ were considered statistically significant.

Results

Anthropometric characterization and metabolic profile of the subjects

The clinical characteristics of the subjects are shown in Table 1. Individuals having a BMI of 25 kg/m^2 or greater with metabolic syndrome (MS) and without (Non-MS) had significantly higher waist circumference, waist to hip ratio, uric acid, γ-glutamiltransferase values and lower high-density lipoprotein cholesterol values than normal-weight (Nw) subjects. The MS subjects showed greater deterioration in their metabolic profile because, except for blood pressure, all metabolic variables evaluated in these patients showed statistically significant differences compared with the Nw subjects. In addition, the waist to hip ratio, concentrations of glucose, triglycerides, urea, uric acid, and glutamic pyruvate transaminase in the MS patients were significantly higher than in the Non-MS and the Nw subjects.

Association of BMI and the presence of MS with the osteogenic potential of omental ASCs

After 21 days in osteogenic medium, calcium accumulation was demonstrated by staining with Alizarin Red S in cultured omental ASCs from subjects with different BMIs and metabolic profiles (Figure 1A). The RT-qPCR allowed us to confirm a reduction in the mRNA expression levels of AP and osteonectin in subjects with a BMI of 25 kg/m^2 or greater compared with Nw subjects (Figure 1B). Although we found no statistically significant correlation between AP and osteonectin expression levels with BMI ($R = -0.515$; $P = .087$, and $R = -0.510$, $P = .090$, respectively), there was a tendency for decreased expression levels of both osteogenic markers. Grouping subjects according to their metabolic profile revealed a statistically significant decrease in expression levels of AP and osteonectin in the omental ASCs from MS subjects compared with the Non-MS and Nw subjects (Figure 1C).

To verify whether the decreased osteogenic potential of the omental ASCs from the MS subjects was influenced by alterations in the expression of osteogenic transcription factors, we evaluated the Runx2 and osterix mRNA levels by RT-qPCR. Although osterix mRNA tended to be lower in the omental ASCs from both Non-MS and MS subjects, neither of the two osteogenic transcription factors showed significant differences between the groups of subjects (Figure 1D).

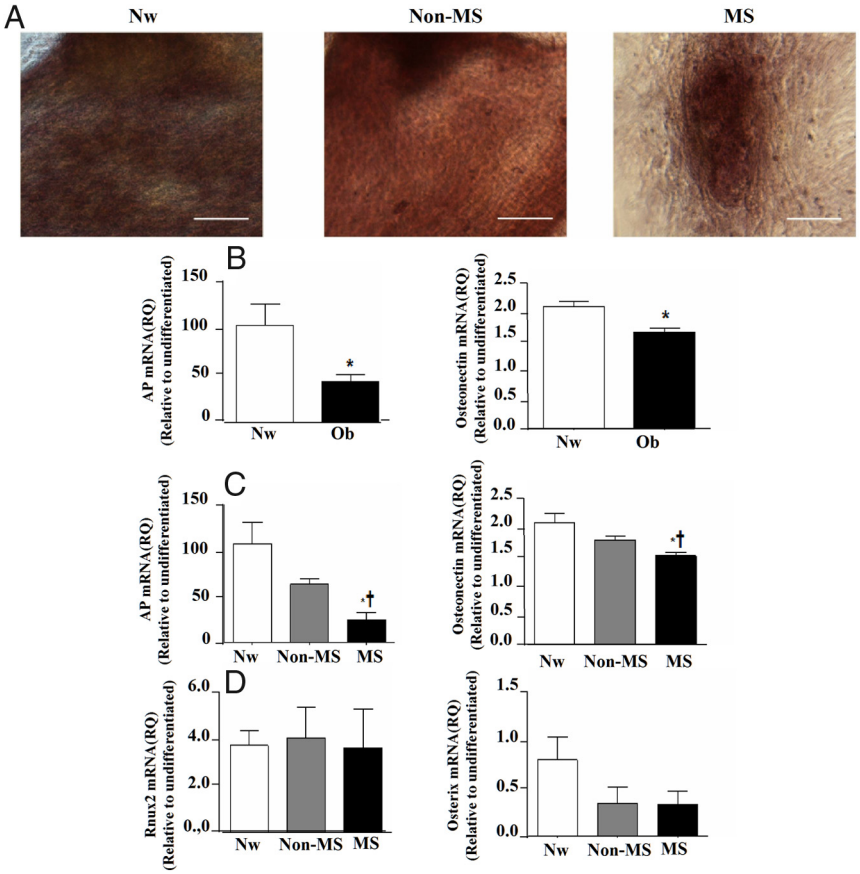

Figure 1. Osteogenic potential of omental-derived ASCs from subjects with different BMI and metabolic profile. A, Alizarin Red staining of omental ASCs from Nw, Non-MS, and MS subjects after 21 days in osteogenic medium. Scale bar, 100 μm. B and C, mRNA expression levels of AP and osteonectin in the omental ASC of subjects grouped by BMI and metabolic profile. D, mRNA expression levels of Runx2 and osterix in the omental ASCs of subjects grouped by metabolic profile. mRNA values of AP, osteonectin, Runx2, and osterix were quantified by RT-qPCR, and mRNA was normalized to RPL13A and expressed relative to the respective cells cultured in nonosteogenic medium. Values are reported as mean ± SE (Nw, n = 4; Ob, n = 8; Non-MS, n = 4; MS, n = 4). *, Results significantly different (Mann-Whitney; $P < .05$) from Nw individuals; †, results significantly different (Mann-Whitney; $P < .05$) from Non-MS subjects. RQ, relative quantification.

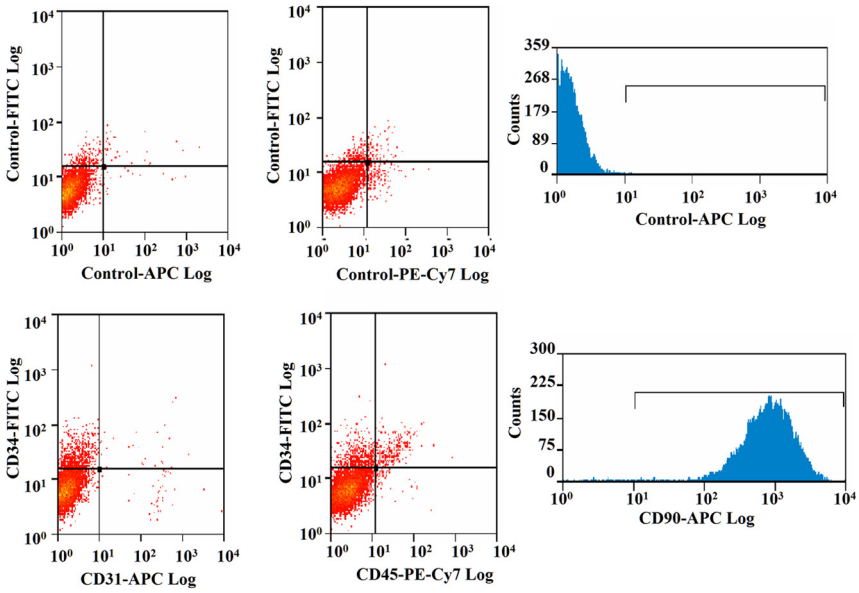

Figure 2. Immunophenotypic characterization of CD34^{negative}-enriched omental ASC fraction in passage zero. The flow cytometry analysis showed a low presence of CD34$^+$/CD31$^+$ endothelial cells and CD34$^-$/CD45$^+$ macrophages in the newly isolated fraction as well as High level expression of CD90, a common marker with other mesenchymal stem cells (n = 10).

Association of BMI and the presence of MS with the osteogenic potential of the CD34^{negative}-enriched omental ASC fraction

In vitro experiments have shown that the CD34$^-$CD31$^-$CD45$^-$ fraction has greater osteogenic differentiation capacity than the CD34$^+$CD31$^-$CD45$^-$ fraction (21). Here we applied magnetic beads coupled with anti-CD34 antibodies to omental ASCs in passage zero to obtain an enriched CD34$^-$CD31$^-$CD45$^-$ fraction of cells and assessed their osteogenic potential. Immunophenotypic characterization confirmed that 84.0% ± 5.9% of these cells isolated by magnetic beads failed to express CD34. Only 1.6% ± 1.9% and 2.1% ± 2.0% were CD34$^+$CD31$^+$ endothelial cells and CD34$^-$CD45$^+$ macrophages, respectively. Furthermore, 96.1% ± 1.2% of the isolated cells expressed the multipotent mesenchymal cell marker CD90 (Figure 2).

When the subjects were grouped according to their BMI, osteogenic stimulation of the CD34^{negative}-enriched fraction revealed no significant differences in the osteogenic potential (Figure 3A). However, osteonectin and AP mRNA showed decreased expression levels in the CD34^{negative}-enriched omental ASC fraction from the MS subjects compared with the Non-MS and Nw subjects (Figure 3B).

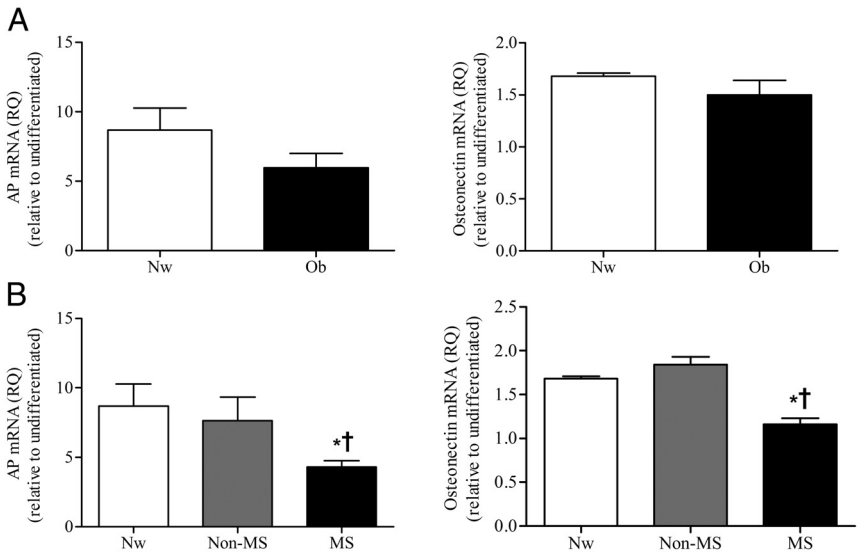

Figure 3. Osteogenic potential of the CD34^{negative}-enriched, omental-derived ASC fraction from subjects with different BMI and metabolic profiles. A and B, mRNA expression levels of AP and Osteonectin in the CD34^{negative}-enriched omental ASC fraction of subjects grouped by BMI and metabolic profile. mRNA values of AP and osteonectin were quantified by RT-qPCR, and mRNA was normalized to RPL13A and expressed relative to the respective cells cultured in nonosteogenic medium. Values are reported as mean ± SE (Nw, n = 3; Ob, n = 8; Non-MS, n = 4; MS, n = 4). *, Results significantly different (Mann-Whitney; $P < .05$) from Nw; †, results significantly different (Mann-Whitney; $P < .05$) from Non-MS patients. RQ, relative quantification.

Expression levels of NOX 2, NOX5, SOD1, and SOD3 mRNA of the CD34^{negative}-enriched omental ASC fraction

Multiple studies have confirmed the influence of reactive oxygen species (ROS) on osteogenesis, suggesting that oxidative stress may suppress osteogenic differentiation (22). Because it has been established that oxidative stress is closely linked to MS (23) and it has been demonstrated that nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase is one of the main sources of ROS production in the adipose tissue of obese subjects (24), we examined the mRNA expression levels of several NADPH oxidase family members (Table 2).

Expression levels of NOX2 and NOX5 were elevated in the CD34^{negative}-enriched fraction from the MS subjects and, in particular,

Table 2. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised (Monoclonal or Polyclonal)	Dilution Used
CD34		CD34 microbead kit	Miltenyi Biotec, 130–046–702	Mouse; monoclonal	1:05
CD34		Antihuman CD34 FITC	eBioscience, 11–0349	Mouse; monoclonal	1:21
CD31		Antihuman CD31 APC	Miltenyi Biotec, 130–092–652	Mouse; monoclonal	1:11
CD45		Antihuman CD45 PE-Cy7	BD Pharmingen, 557 748	Mouse; monoclonal	1:21
CD90		Antihuman CD90 APC	Miltenyi Biotec, 130–095–402	Mouse; monoclonal	1:11

Abbreviation: FITC, fluorescein isothiocyanate.

NOX2 mRNA was significantly higher than in the Nw and Non-MS subjects. Importantly, we also observed a statistically significant negative correlation between NOX2 mRNA and AP and osteonectin mRNA (Figure 4A).

In addition, we measured the expression of antioxidant enzymes involved in catalyzing the dismutation of superoxide, the main product of the enzyme NADPH, into hydrogen peroxide. The SOD1 mRNA expression levels were high in the enriched fraction from CD34^{negative}-MS subjects but were not significantly different from the Nw and Non-MS subjects. Interestingly, SOD1 mRNA expression levels were significantly correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) and plasma insulin of the subjects (Figure 4B).

mRNA expression levels of collagen I, III, V, and VI in the CD34^{negative}-enriched omental ASC fraction

Different in vivo experiments have confirmed the fibrogenic potential of multipotent mesenchymal cells (25–27), indicating the risk of fibrosis associated with cell therapy. Because NADPH-dependent redox signaling has been involved in the fibrotic response in tissue repair (28), we examined the mRNA expression levels of some collagen isotypes.

We found a positive correlation between Col1a1, Col3a1, Col5a1, and Col6a3 mRNA and NOX5 mRNA, which was statistically significant for Col5a1 and Col6a3 (Figure 5A). mRNA of the four collagen isotypes also correlated positively with HOMA-IR; in particular, Col3a1 correlated significantly (Figure 5B). The expression level of these collagens tended to be lower in Nw subjects, but only Col3a1 was significantly increased in MS subjects (Figure 5C).

Association of BMI and the presence of MS with clonogenic potential and proliferative rate of the CD34^{negative}-enriched omental ASC fraction

The relatively high frequency of ASCs, their high yield, and their high proliferation rate during in vitro expansion have contributed to increasing expectations for their biomedical use (29). We therefore determined the clonogenic potential and PDT of the CD34^{negative}-enriched fraction from obese subjects with different metabolic profiles.

No statistically significant differences in the number of colonies, generated over 14 days by culture at low density of the CD34^{negative}-enriched fraction, were found between subjects when they were grouped by BMI (Figure 6A) or when grouped according to their metabolic profile (Figure 6B). However, we observed a significant negative correlation between the number of colonies and BMI (Figure 6C).

The PDT of the CD34^{negative}-enriched fraction from subjects with a BMI of 25 kg/m^2 or greater was sig-

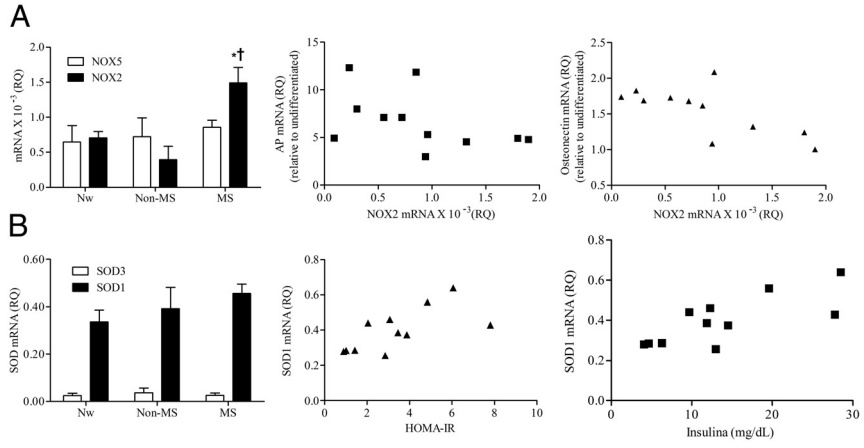

Figure 4. Dysregulated expressions of proteins involved in cellular redox status in the CD34^{negative}-enriched omental ASC fraction. A, Increased expression of NOX2 and NOX5 in Non-MS subjects and correlation of NOX2 mRNA with AP (R = −0.627, P = .039) and osteonectin mRNA (R = −0.682, P = .021). B, mRNA expression levels of SOD1 and SOD3 in the CD34^{negative}-enriched omental ASC fraction of subjects grouped by metabolic profile. Correlation of SOD1 mRNA was performed with HOMA-IR (R = 0.673, P = .023) and serum insulin (R = 0.618, P = .043). NOX2, NOX5, SOD1, and SOD3 mRNA values were quantified by RT-qPCR and normalized to mRNA RPL13A. Values are reported as mean ± SE (Nw, n = 3; Non-MS, n = 4; MS, n = 4). *, Results significantly different (Mann-Whitney; $P < .05$) from Nw individuals; †, results significantly different (Mann-Whitney; $P < .05$) from Non-MS subjects. RQ, relative quantification.

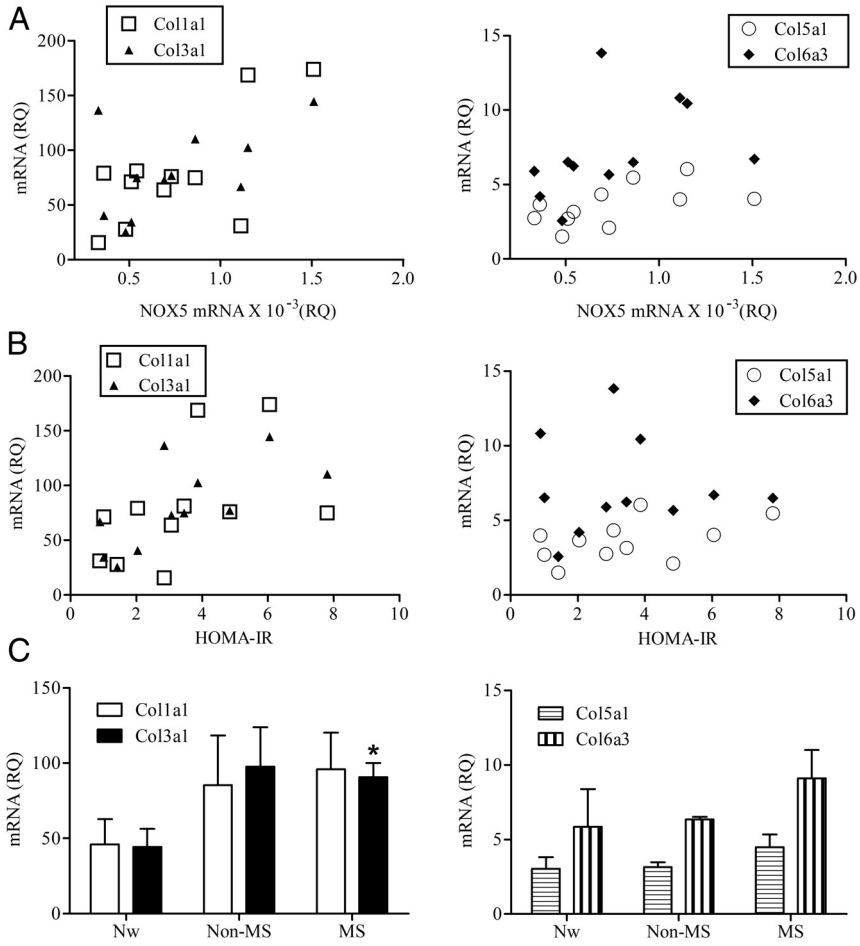

Figure 5. Expression of fibrotic proteins in the CD34^{negative}-enriched omental ASC fraction. A, Correlation between mRNA expression levels of NOX5 and Col1a1 (R = 0.555; P = .077), Col3a1 (R = 0.445; P = .170), Col5a1 (R = 0.627; P = .039), and Col6a3 (R = 0.645; P = .032). B, Correlation between HOMA-IR and Col1a1 (R = 0.600; P = .051), Col3a1 (R = 0.773; P = .005), Col5a1 (R = 0.445; P = .170), and Col6a3 (R = 0.045; P = .894). C, mRNA expression levels of Col1a1, Col3a1, Col5a1, and Col6a3 in the CD34^{negative}-enriched omental ASC fraction of subjects grouped by metabolic profile. Col1a1, Col3a1, Col5a1, and Col6a3 mRNA values were quantified by RT-qPCR and normalized to mRNA RPL13A. Values are reported as mean ± SE (Nw, n = 3; Non-MS, n = 4; MS, n = 4). *, Results significantly different (Mann-Whitney; $P <$ .05) from Nw individuals. RQ, relative quantification.

nificantly greater than that of the Nw subjects (Figure 6D). Interestingly, only the CD34^{negative}-enriched fraction from the MS subjects showed greater PDT than that of the Nw subjects (Figure 6E). In addition, a positive correlation between the HOMA-IR and the PDT of the CD34^{negative}-enriched omental ASC fraction (Figure 6F) was observed.

Discussion

The present study confirmed the decreased osteogenic potential of omental ASCs from subjects with BMI of 25 kg/m^2 or greater confirmed that both the omental ASCs and the CD34^{negative}-enriched omental ASC fraction from the Non-MS subjects have a greater osteogenic potential than that from the MS subjects. The deterioration of the osteogenic potential of the omental ASCs from the MS subjects was not accompanied by decreased expression levels of the osteogenic transcription factors Runx2 and osterix but rather by alterations in the transcriptional pattern of enzymes involved in cellular redox balance. Furthermore, the levels of mRNA expression of the fibrotic proteins Col1a1, Col3a1, Col5a1, and Col6a3 correlated positively with both NOX5 mRNA and HOMA-IR. Whereas the number of colonies generated by the CD34^{negative}-enriched omental ASC fraction correlated negatively with BMI, PDT correlated positively with HOMA-IR. Interestingly, the PDT of the CD34^{negative}-enriched omental ASC fraction was significantly higher in the subjects with a BMI of 25 kg/m^2 or greater, but only the MS subjects had a higher PDT than the Nw subjects.

Although preclinical and clinical studies suggest that ASCs are safe, reliable, and applicable to cell therapy, it is still unclear whether age, gender, and BMI affect their therapeutic potential (30). In this study we emphasize the need to consider not only BMI but also the metabolic profile of subjects during clinical assessments associated with the use of ASCs in regenerative medicine, and we propose that the omental ASCs from Non-MS subjects may be more suitable for therapeutic osteogenic applications than those from MS subjects.

Our results confirmed those of other studies in which the decrease in the osteogenic differentiation capacity of ASCs in obese patients has been documented (10, 13, 14). However, grouping the same subjects according to their metabolic profile showed that only the osteogenic potential of omental ASCs from the MS subjects had a statistically significant decrease with respect to that from the Nw subjects.

Previous studies aimed at the characterization of ASC subpopulations have documented differences in the expression levels of mesenchymal stem cell markers (31) and in their proliferation rate and differentiation potential (32,

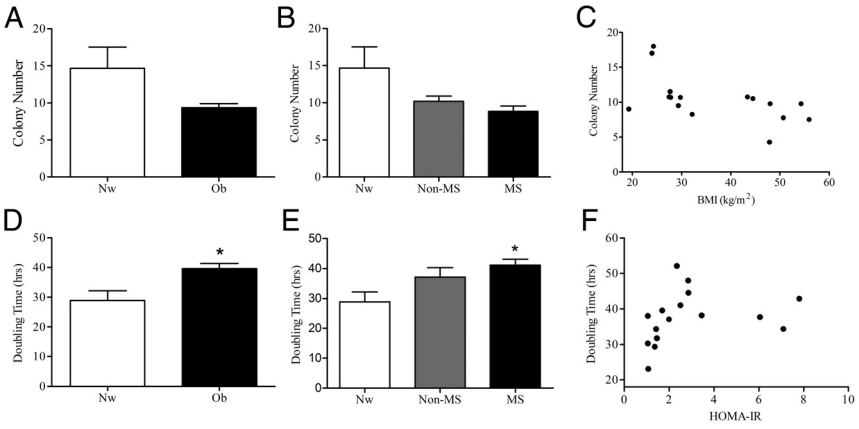

Figure 6. Clonogenic potential and population doubling time of omental-derived CD34^{negative}-enriched ASC fraction from subjects with a different BMI and metabolic profile. A–C, Zero passage cells were seeded in six-well plates at a density of 50 cells/cm^2 and maintained in culture for 14 days. The number of colonies observed after 14 days of culture in the CD34^{negative}-enriched, omental-derived ASC fraction of subjects grouped by BMI and metabolic profile. C, The number of colonies correlated negatively with BMI (R = −0.613; P = .012). D–F, Zero passage cells were seeded in 12-well plates at a density of 3000 cells/cm^2 and the PDT was calculated at 4 and 6 days of culture. PDT of subjects was grouped by BMI and metabolic profile. F, PDT correlated positively with HOMA-IR (R = 0.556; P = .025). Values are reported as mean ± SE (Nw, n = 3; Ob, n = 13; Non-MS, n = 5; MS, n = 8). *, Results significantly different (Mann-Whitney; $P < .05$) from Nw.

33). Cell therapy based on the use of selected populations is increasingly relevant (31). In our study, to decrease the contribution of variations in the abundance of subpopulations with different osteogenic potentials to the differences found between the groups of patients studied, we focused on the CD34^{negative}-enriched omental ASC fraction, isolating it by applying magnetic beads, according to Suga et al (21). This fraction demonstrates greater osteogenic potential than the CD34$^+$ fraction.

The osteogenic differentiation assay carried out on the CD34^{negative}-enriched omental ASC fraction, unlike the results obtained in the whole fraction of omental ASCs, revealed no differences in the expression levels of AP and osteonectin between the Nw subjects and the subjects with a BMI of 25 kg/m^2 or greater. However, it did display a decrease in the osteogenic potential of the cells from the MS subjects compared with those from the Non-MS and the Nw subjects.

Use of the RT-qPCR technique enabled us to confirm that the CD34^{negative}-enriched omental ASC fraction from the MS subjects presented NOX2 mRNA expression levels intrinsically superior to that of the Nw and Non-MS subjects and that the expression levels of NOX2 were negatively correlated with both osteonectin and AP mRNA. These results suggest the involvement NOX2 in the deterioration of the osteogenic potential of the ASCs from the MS subjects because in vitro experiments have demonstrated the inhibitory influence of ROS on osteogenic differentiation (22).

ROS have been implicated not only in the inhibition of osteogenic differentiation (22) but also in reducing colony formation by precursor cells (34), in cell senescence in adipose tissue (35), and in the fibrotic response associated with the activation of the NADPH pathway (28). In line with these studies, we also observed a positive correlation between NOX5 mRNA and the expression levels of collagen associated with adipose tissue fibrosis and a decrease in the clonogenic potential and proliferation rate with increasing BMI and HOMA-IR, respectively.

It is likely that the differences observed in the NOX2 and NOX5 mRNA among the precursor cells from subjects with different metabolic profiles could be attributable in part to the lower toxicity of the omental depot, which probably characterizes individuals who have not yet developed MS. In fact, previous studies have confirmed that omental adipose tissue inflammation is strongly associated with insulin resistance independent of BMI (36–39). Furthermore, in vitro experiments have shown that proinflammatory transcription factors and cytokines are involved in controlling the expression of NOX2 (40, 41) and NOX5 (42). In line with our suggestions, it has been reported that the paracrine effects of macrophages in adipose tissue are associated with both the inhibition of proliferation (43) and the increased expression of genes encoding extracellular matrix proteins in adipose progenitor cells (44, 45).

Given that visceral adipose tissue dysfunction appears to result from the diminished expansion capacity of sc adipose tissue (20, 46), it is unlikely that sc ASCs from MS subjects could be recommended for cell therapy because alterations in the reservoir of stem cells from sc adipose tissue in diabetic rat models have been described (47). However, previous experiments have found that in Nw subjects sc ASCs are more capable of osteogenic differentiation than omental ASCs (48). It has also been suggested that omental ASCs are more vulnerable to the loss of their stemness and multilineage differentiation potential (13). Unfortunately, the small availability of sc adipose tissue biopsies did not allow us to examine the state of the CD34^{negative} fraction. Another limitation of our study was that, also due to the lack of availability of biopsies, experiments performed on the CD34^{negative}-enriched omental ASC fraction did not correspond to the same subjects

who were evaluated for the osteogenic potential of omental ASCs. Therefore, our results are susceptible to the effects of intersubject variability. Furthermore, these results must be validated by in vivo experiments in models that reproduce the clinical stage of bone repair.

The main benefit from our findings is that they suggest taking into account not only the BMI but also the metabolic profile of the subjects during the implementation of cell therapies aimed at bone repair. We also provide evidence that the therapeutic use of ASCs from MS subjects is limited due to the significant decrease in their osteogenic potential and proliferation rate. Because we confirmed that the deterioration in the metabolic profile of the subjects was accompanied by increased expression levels of NADPH enzymes and fibrotic proteins in the ASCs, it is likely that ASC-based therapies are not without potential risks.

In summary, the osteogenic, clonogenic, and fibrotic potential and proliferation rate in vitro suggest that omental ASCs from Non-MS subjects are more suitable for therapeutic osteogenic applications than those from MS subjects. Future studies aimed at identifying and correcting mechanisms involved in the intrinsic deterioration of ASC in obesity would be important for the successful development of autologous cell therapy directed at bone repair.

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