The subcutaneous adipose tissue reservoir of functionally active stem cells is reduced in obese patients

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ABSTRACT **It has been demonstrated that the adipose tissue, a highly functional metabolic tissue, is a reservoir of mesenchymal stem cells. The potential use of adipose-derived stem cells (ADSCs) from white adipose tissue (WAT) for organ repair and regeneration has been considered because of their obvious benefits in terms of accessibility and quantity of available sample. However, the functional capability of ADSCs from subjects with different adiposity has not been investigated. It has been our hypothesis that ADSCs from adipose tissue of patients with metabolic syndrome and high adiposity may be functionally impaired. We report that subcutaneous WAT stromal vascular fraction (SVF) from nonobese individuals had a significantly higher number of $CD90^+$ cells than SVF from obese patients. The isolated ADSCs from WAT of obese patients had reduced differentiation potential and were less proangiogenic. Therefore, ADSCs in adipose tissue of obese patients have lower capacity for spontaneous or therapeutic repair than ADSCs from nonobese metabolically normal individuals.—Oñate, B., Vilahur, G., Ferrer-Lorente, R., Ybarra, J., Díez-Caballero, A., Ballesta-López, C., Moscatiello, F., Herrero, J., Badimon, L. The subcutaneous adipose tissue reservoir of functionally active stem cells is reduced in obese patients. *FASEB J.* 26, 4327–4336 (2012). www.fasebj.org**

Key Words: thrombospondin 1 · cardiovascular risk factors · hypoxia · proliferation · angiogenesis · differentiation

Adipose tissue, traditionally regarded as an energy storage organ, is now considered an endocrine tissue and a source of adult stem cells, adipose-derived stem cells (ADSCs; ref. 1). ADSCs share many properties with the well-described bone marrow mesenchymal stem cells (BM-MSCs), including *ex vivo* expansion, differentiation capacity, and mesenchymal characteristic lineage markers (2). However, in contrast to BM-MSCs, ADSCs can be easily isolated from human subcutaneous adipose tissue and in great quantities (3), which makes them an attractive alternative for cell therapy purposes. Among the most interesting characteristics of ADSCs is their potential to stimulate angiogenesis, reduce apoptosis, and exert anti-inflammatory properties, which suggests an active role of ADSCs in revascularization of ischemic damaged tissues (4–8). Most of these effects are believed to be mediated *via* paracrine activity.

At present, age, adipose tissue depot site, and gender have been shown to modify the number and the proliferation, differentiation, and angiogenic capacity of ADSCs (9–13). However, the effect of cardiovascular risk factors on ADSC potential has not been previously addressed. Indeed, several human studies have demonstrated that hypercholesterolemia, diabetes, and hypertension impair the number and function of bone

Abbreviations: ADSC, adipose-derived stem cell; ADSCmo, adipose-derived stem cell from morbidly obese patient; ADSCn, adipose-derived stem cell from nonobese individual; AU, arbitrary unit; BMI, body mass index; BM-MSC, bone marrow mesenchymal stem cell; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cell; FABP4, fatty acid binding protein 4; FBS, fetal bovine serum; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cell; LDL, low-density lipoprotein; LPL, lipoprotein lipase; mAb, monoclonal antibody; MSC, mesenchymal stem cell; PCR, polymerase chain reaction; PDT, population doubling time; PECAM-1, platelet endothelial cell adhesion molecule 1; P/S, penicillin/streptomycin; SVF, stromal vascular fraction; TBP, TATA-binding protein; TSP-1, thrombospondin 1; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor; WAT, white adipose tissue

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doi: 10.1096/fj.12-207217

marrow-derived circulating progenitor cells (14–21). Yet, the effect of different degree of adiposity and/or metabolic syndrome on the functional capability of adult stem cells—particularly in ADSCs—has not been investigated. We hypothesized that human ADSCs from obese patients with metabolic syndrome may have impaired capabilities for spontaneous or therapeutic tissue repair. Here we report that ADSCs from white adipose tissue (WAT) of patients with obesity and metabolic syndrome have lower proliferative and angiogenic potential than ADSCs from WAT of nonobese metabolically normal individuals.

MATERIALS AND METHODS

Subjects

Subcutaneous WAT was obtained from morbidly obese patients [body mass index (BMI)>40 kg/m^2; n=16] that underwent bypass gastric surgery and from nonobese patients (BMI<25 kg/m^2; n=8) who underwent liposuction surgery. During the procedures, bypass gastric surgery patients were kept with isofluorane (2%), whereas liposuction surgery was performed under propofol/ramifentanil. Tissues were obtained with informed consent from patients, and the study protocol was approved by the Centro Medico Teknon Ethical Committee, consistent with the principles of the Declaration of Helsinki. Blood samples from all subjects were obtained at the time of intervention in order to evaluate the following biochemical parameters: glucose, triglyceride, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, urea, total proteins, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and creatinine levels. Patients used regular medication as recommended in the guidelines if it was necessary.

Cell isolation and culture

Isolation of ADSCs was performed with a modified method of a previously described technique (1). Briefly, adipose tissue was washed, minced, and digested with 1 mg/ml collagenase I-A (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco; Life Technologies, Inc., Grand Island, NY, USA) for 1 h at 37°C with gentle agitation. The digested tissue was sequentially filtered through a 100-μm mesh and centrifuged for 10 min at 1200 rpm at room temperature. The supernatant, containing mature adipocytes, was aspirated, and the pellet was identified as the stromal vascular fraction (SVF). SVF was resuspended in culture medium [DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (P/S); Gibco] and seeded. Finally, cells were incubated overnight at 37°C with 5% CO_2 under two different oxygen conditions: normoxic (21% O_2) and hypoxic (1% O_2). At 24 h after incubation, the medium was changed to remove nonadherent cells. Adherent cells were referred as ADSCs. For ADSC expansion, medium was changed every 2–3 d. Cells were always maintained under the original oxygen condition in all passages.

Flow cytometry characterization

Surface marker analysis of the SVF, ADSCs from nonobese individuals (ADSCns), and ADSCs from morbidly obese patients (ADSCmos), cultured under normoxic and hypoxic conditions (passage 3), was performed by using the fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies (mAbs) against CD105, CD90, CD29, CD44, CD45, and CD34 and their respective isotype control mAbs (BD Biosciences, San Diego, CA, USA). Briefly, the cell suspension was washed and resuspended with 1× PBS supplemented with 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% sodium azide. Cells (10^6) were incubated with the specific mAbs at 4°C for 30 min, fixed with 1× PBS supplemented with 3% BSA, 0.1% sodium azide, and 0.1% paraformaldehyde and analyzed by flow cytometry. Before acquiring SVF cells, erythrocytes were lysed with 1 ml of quick lysis solution (Cytognos, Salamanca, Spain). At least 3×10^4 events were acquired from each sample.

Growth kinetics of ADSCs

To determine the growth kinetics of cultured ADSCs, wells were seeded with 10^4 cells/well (passage 3) and cultured under both normoxic and hypoxic conditions, as described previously (22). Cells from 2 duplicate wells were harvested and counted every other day. ADSC numbers were plotted against the number of days cultured. The exponential-growth phase was determined, and population doubling time (PDT) was calculated using the formula $PDT = T_e/[(\log N_2 - \log N_1)/\log_2]$, where T_e is time (d) in the exponential-growth phase, N_1 is the number of cells at the beginning of the exponential-growth phase, and N_2 is the number of cells at the end of the exponential-growth phase.

Relative real-time polymerase chain reaction (PCR) and gene expression

Total RNA was extracted from undifferentiated ADSCns and ADSCmos, and from adipocyte- and endothelial cell (EC)-differentiated ADSCns and ADSCmos by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Life Technologies, Foster City, CA, USA) was used for reverse transcription of RNA. The expression of adipocyte [lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4)] and EC markers [platelet EC adhesion molecule 1 (PECAM-1), vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR1, and von Willebrand factor (vWF)] and thrombospondin 1 (TSP-1) were evaluated at the mRNA level by PCR assays (Applied Biosystems, Life Technologies), conducted according to the manufacturer's instructions. Relative gene expression values were calculated by the $\Delta\Delta C_t$ method. The raw gene expression values were normalized according to the expression of TATA-binding protein (TBP) gene.

Western blotting

Whole-cell extracts of ADSCns and ADSCmos cultured under hypoxic conditions were made in RIPA buffer. Total protein in cell extracts was determined, and 25 μg cell lysate was subjected to electrophoresis on 8% gels and then transferred to nitrocellulose membrane. After blocking in 5% nonfat milk 1 h at room temperature, blots were incubated with TSP-1 antibody (1:1000; mouse mAb; Abcam, Cambridge, UK) at 4°C overnight. Secondary antibody consisted of horseradish peroxidase-conjugated antibodies (Dako, Glostrup, Denmark) and was detected using the SuperSignal chemiluminescence system (Pierce; Rockford, IL, USA). Protein expression was determined using Image Lab software (Bio-

Rad, Richmond, CA, USA), and β-tubulin (Abcam) was used for protein loading control.

TSP-1 ELISA

Conditioned medium of ADSCns and ADSCmos cultured under hypoxic conditions was collected, and TSP-1 was quantified using a Quantikine kit (R&D Systems; Abingdon, UK), according to the manufacturer's protocol. All values were normalized to total medium protein.

In vitro tube formation assay

Conditioned medium was collected from ADSCns and ADSCmos cultured under hypoxic conditions. Human umbilical vein ECs (HUVECs) at passage 5 were seeded in 96-well plates coated with growth factor-reduced Matrigel (BD Biosciences) at a density of 2×10^4 cells/well. HUVEC growth medium was supplemented with conditioned medium (1:1). Al least 3 wells were used for each sample of conditioned medium. HUVEC serum-free growth medium and nonconditioned ADSC culture medium were used as a negative control; HUVEC growth medium (20% FBS) served as positive control. Plates were incubated at 37°C with 5% CO_2 and 21% O_2 for 6 h. Total length of tubular structures was counted with ImageJ 1.43u software (U.S. National Institutes of Health, Bethesda, MD, USA).

In vitro cell differentiation

For differentiation studies, ADSCs at passage 3 and cultured under hypoxic conditions were plated at a density of 2×10^3 cells/cm^2 on 6-well culture plates and were allowed to grow to confluence. The culture medium was then replaced with the specific differentiation medium, which was changed every 2–3 d for the full induction period.

Adipogenic differentiation

Confluent cells were cultured in adipogenic medium with DMEM supplemented with 10% FBS, 1% P/S, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), 200 μM indomethacin (Sigma-Aldrich) and 1.7 μM insulin (Sigma-Aldrich) (23). Culture medium was changed every 2–3 d for 21 d. Differentiated cells were detected with Herxheimer staining for identifying intracellular lipid accumulation and by examining the expression of the adipocyte cell markers LPL and FABP4 using real-time PCR.

EC differentiation

Cells were cultured with EC differentiated medium [M-199 supplemented with 3% FBS, 1% P/S (Life Technologies), 10 ng/μl fibroblast growth factor (BD Biosciences), and 50 ng/μl VEGF (Sigma-Aldrich)] for 7 d. Subsequent differentiation was evaluated by cord formation on plating on Matrigel (BD Biosciences; ref. 24) and by examining expression of the EC markers PECAM-1, VEGFR2, VEGFR1, and vWF by PCR.

Statistical analyses

Statistical analyses were performed using StatView software. Data are expressed as means ± SE. Statistical significance was assessed by unpaired Student's *t* test. Values of $P < 0.05$ were considered statistically significant. Correlation significances were determined as linear correlations.

RESULTS

Patient data

The clinical characteristics of the subjects are detailed in **Table 1**. For analytical purposes, subjects were grouped by BMI. As such, morbidly obese patients (n=16) presented a BMI > 40 kg/m^2 (44.44±1.29 kg/m^2) whereas nonobese individuals (n=8) presented a BMI < 25 kg/m^2 (22.26±0.88 kg/m^2). Morbidly obese patients had hyperglycemia (142.13±11.95 mg/dl glucose; P<0.05) and lower levels of HDL cholesterol and total cholesterol/HDL ratio than nono-

TABLE 1. *Clinical characteristics of the subjects who participate in the study*

Characteristic	Morbidly obese patients	Nonobese individuals	*P*
n	16	8	
Age	41.56 ± 3.07	38.28 ± 2.48	NS
BMI (kg/m^2)	44.44 ± 1.29	22.26 ± 0.88	<0.0001
Biochemical parameter			
Glucose (mg/dl)	142.13 ± 11.95	61.54 ± 7.35	0.0003
Triglyceride (mg/dl)	135.00 ± 18.41	131.14 ± 40.72	NS
Cholesterol (mg/dl)	184.19 ± 10.58	186.71 ± 9.41	NS
HDL (mg/dl)	33.20 ± 3.05	48.32 ± 2.82	0.0045
LDL (mg/dl)	133.33 ± 12.97	157.43 ± 11.33	NS
Urea (mg/dl)	23.61 ± 1.90	29.03 ± 2.71	NS
Total protein (g/dl)	7.16 ± 0.31	7.03 ± 0.35	NS
GOT (U/L)	17.78 ± 2.98	32.50 ± 14.58	NS
GPT (U/L)	19.32 ± 4.67	33.72 ± 16.54	NS
GPT/GOT	1.15 ± 0.11	0.94 ± 0.14	NS
Creatinine (mg/dl)	0.83 ± 0.07	0.55 ± 0.10	0.036
Cholesterol/HDL	5.92 ± 0.48	3.98 ± 0.38	0.01
HDL/LDL	0.25 ± 0.03	0.31 ± 0.02	NS

Data are presented as the means ± SE. GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; NS, not significant.

TABLE 2. *Phenotypic characterization of human subcutaneous stromal vascular fraction*

Antigen	Nonobese individuals	Morbidly obese patients	*P*
$CD90^+$ (%)	35.31 ± 7.70	19.11 ± 3.07	<0.05
$CD29^+$ (%)	32.16 ± 8.94	34.82 ± 6.99	NS
$CD44^+$ (%)	9.05 ± 5.91	6.49 ± 1.32	NS
$CD45^+$ (%)	14.40 ± 5.30	7.47 ± 1.81	NS

Percentages of ADSC surface markers were analyzed in the subcutaneous stromal vascular fraction from nonobese individuals and morbidly obese patients. Data are presented as means ± SE; NS, not significant.

bese individuals ($P<0.05$), supporting their metabolic syndrome condition.

SVF and ADSC phenotypic characterization

We analyzed the presence of ADSC surface markers (CD90, CD29, and CD44) in the subcutaneous SVF from WAT of morbidly obese and nonobese subjects. CD45 was used as a marker of hematopoietic cells (**Table 2**). Flow cytometry results revealed the presence of higher numbers of $CD90^+$ cells in WAT from nonobese individuals (35.31±7.7%) than in WAT of morbidly obese patients (19.11±3.07%). No differences between groups were seen when we analyzed $CD29^+$ (nonobese: 32.16±8.94%; morbidly obese: 34.82±6.99%) and $CD44^+$ (nonobese: 9.05±5.91%; morbidly obese: 6.49±1.32%) cells.

ADSCns and ADSCmos were harvested and cultured in two different oxygen conditions: normoxia (21% O_2) and hypoxia (1% O_2). After *in vitro* culture (passage 3) we measured the presence of CD90, CD29, CD44, CD105, CD34, and CD45 in the cultured cells. The percentages of $CD90^+$, $CD29^+$, $CD44^+$, and $CD105^+$ cells achieved 90–100% in both groups of subjects regardless of oxygen culture conditions (**Table 3**). No $CD45^+$ or $CD34^+$ cells were observed in the cultured cells.

Growth kinetics of cultured ADSCs under normoxic and hypoxic conditions

To evaluate the effects of oxygen concentration on ADSCn and ADSCmo growth, we analyzed the effect of both normoxic and hypoxic conditions (**Fig. 1*A***) in growth kinetics up to 10 d.

When ADSC numbers were monitored over time, an exponential cell growth curve was obtained (Fig. 1*B*, *C*). When comparing ADSC growth curves in both oxygen conditions, ADSCs cultured in hypoxic conditions showed faster growth kinetics than ADSCs cultured in normoxic conditions ($P<0.05$). This significant effect was shown in both ADSCns (Fig. 1*B*) and ADSCmos (Fig. 1*C*). The lag phase before cell growth was longer in ADSCns than in ADSCmos in both oxygen conditions, but the differences did not reach statistical significance (Fig. 1*D*).

PDT was calculated from the exponential-growth phase of the growth curves. PDT was longer in ADSCmos than in ADSCns cultured in normoxic conditions (ADSCmos: 3.20±0.06 d, ADSCns: 2.38±0.21 d; $P<0.05$; Fig. 1*E*) and in hypoxic conditions (ADSCmos: 2.66±0.085 d, ADSCns: 1.88±0.055 d; $P<0.0005$; Fig. 1*F*). Indeed, ADSCs from subcutaneous WAT of obese patients were significantly less proliferative than ADSCs from subcutaneous WAT of nonobese individuals.

Angiogenic potential

To study the angiogenic potential of ADSCns and ADSCmos, we measured the gene and protein expression levels of the antiangiogenic molecule TSP-1. ADSCmos presented higher expression levels of TSP-1 as both mRNA [ADSCns: 556.15±284.16 arbitrary units (AU), ADSCmos: 2017.96±324.88 AU; $P=0.016$; **Fig. 2*A***] and protein (ADSCns: 0.13±0.05 AU, ADSCmos: 0.98±0.22 AU; $P=0.0024$; Fig. 2*B*). These values correlate with BMI (mRNA: $R^2=0.44$, $P=0.0037$; protein: $R^2=0.49$, $P=0.0025$). TSP-1 secreted levels (Fig. 2*C*) were higher in ADSCmo than ADSCn conditioned medium (ADSCn: 71.78±12.56 ng TSP-1/mg total protein, ADSCmo: 121.06±12.2 ng TSP-1/mg total protein). When we analyzed the effect of the conditioned medium from ADSCmos and ADSCns on the capillary-like tube formation capacity of HUVECs in Matrigel (Fig. 2*D*), we found that conditioned medium from ADSCmos induced a significantly reduced formation of capillary-like structures (ADSCns: 9813.31±1086.65 AU, ADSCmos: 7387.78±518.34 AU; $P=0.035$; Fig. 2*D*).

TABLE 3. *Phenotypic characterization of human ADSCs*

	Normoxic conditions		Hypoxic conditions		
Antigen	ADSCn	ADSCmo	ADSCn	ADSCmo	*P*
$CD90^+$ (%)	96.79 ± 1.86	98.7 ± 0.67	98.29 ± 1.45	99.80 ± 0.11	NS
$CD29^+$ (%)	99.33 ± 1.34	99.60 ± 0.50	96.60 ± 1.90	93.05 ± 5.36	NS
$CD44^+$ (%)	95.73 ± 1.49	98.40 ± 1.30	95.97 ± 7.50	91.00 ± 7.13	NS
$CD105^+$ (%)	92.6 ± 1.5	93.7 ± 0.7	91.2 ± 2.1	94 ± 0.9	NS
$CD34^+$ (%)	0.3 ± 0.1	0.1 ± 0.1	0.6 ± 0.3	0.1 ± 0.1	NS
$CD45^+$ (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NS

ADSC surface markers were analyzed in ADSCns and ADSCmos cultured after 3 passages under normoxia (21% O_2) or hypoxia (1% O_2) conditions. Data are presented as means ± SE. NS, not significant.

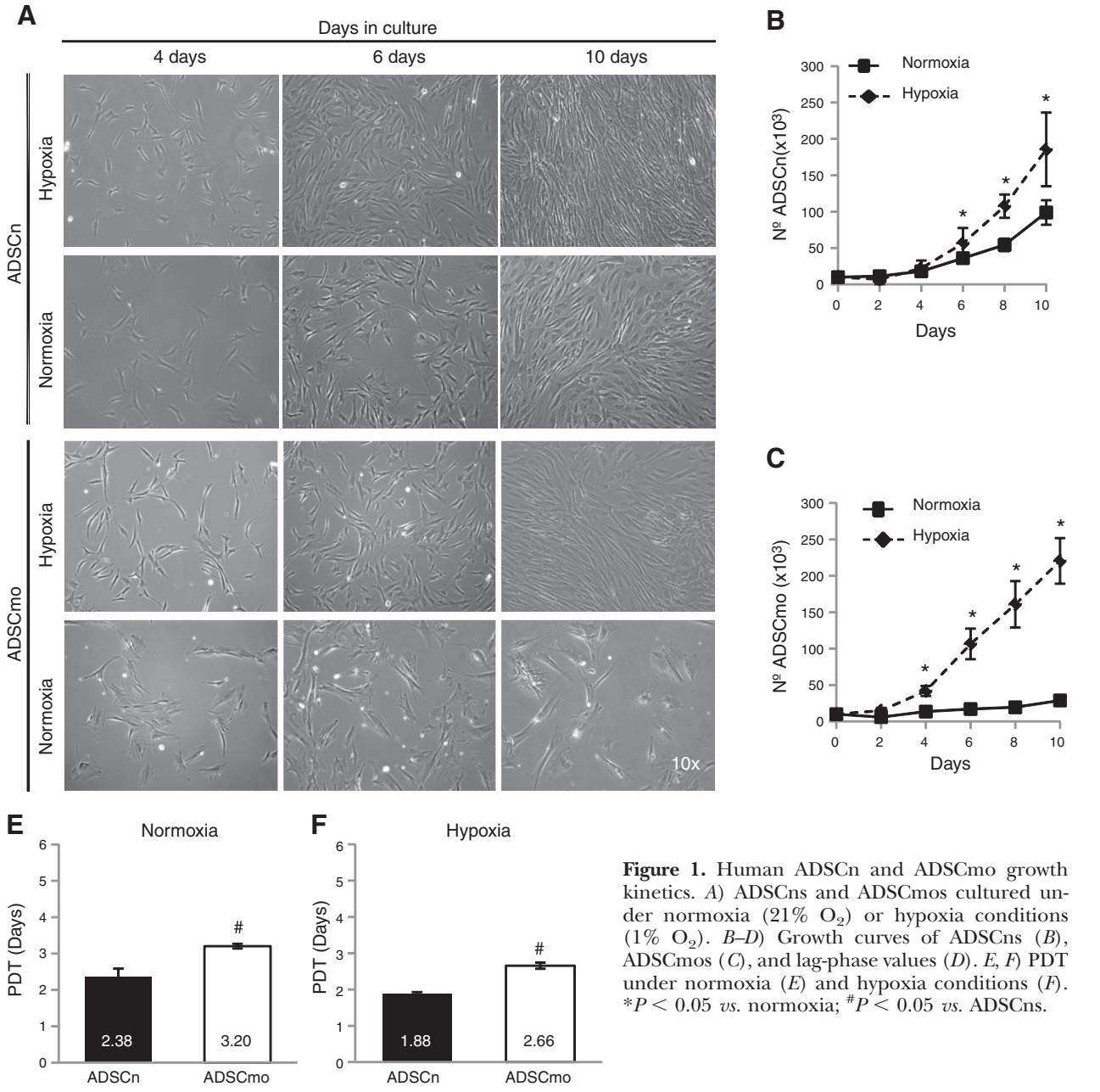

Figure 1. Human ADSCn and ADSCmo growth kinetics. *A*) ADSCns and ADSCmos cultured under normoxia (21% O_2) or hypoxia conditions (1% O_2). *B–D*) Growth curves of ADSCns (*B*), ADSCmos (*C*), and lag-phase values (*D*). *E, F*) PDT under normoxia (*E*) and hypoxia conditions (*F*). $^*P < 0.05$ *vs.* normoxia; $^{\#}P < 0.05$ *vs.* ADSCns.

Adipogenic differentiation capacity

To evaluate the potential of ADSCns and ADSCmos to differentiate, cells cultured in hypoxic conditions were induced to differentiate to adipocytes (**Fig. 3**). Both cell types showed adipocyte differentiation; however, ADSCns showed larger-size lipid vesicles positive for Herxheimer's staining (Fig. 3*A*). Moreover, differentiated ADSCns showed a significantly higher expression of the adipocyte cell markers FABP4 (ADSCns: 22,400-fold, ADSCmos: 4300-fold) and LPL (ADSCns: 30,000-fold, ADSCmos: 1700-fold; Fig. 3*B*).

EC differentiation capacity

ADSCns and ADSCmos showed EC differentiation (**Fig. 4**). While undifferentiated ADSCns and ADSCmos did not form capillary-like ring structures in Matrigel (Fig. 4*A*), EC-differentiated ADSCs formed capillary-like networks. These networks were of similar lengths for both EC-differentiated ADSCs (ADSCns: 6264.81±1454.23 AU, ADSCmos: 7040.72±1234.94 AU; Fig. 4*B*).

The expression of EC molecular markers in differentiated and undifferentiated ADSCns and ADSCmos (**Fig. 5**) showed some differences. ADSCns and ADSCmos significantly increased the expression of PECAM-1 after EC differentiation (ADSCns: 17-fold, $P=0.03$; ADSCmos: 12-fold, $P=0.0028$). Expression levels of VEGFR1 were not modified after EC differentiation, although a nonsignificantly higher expression was seen in ADSCmos before and after differentiation. VEGFR2, CD34, and vWF had very low expression levels regardless of cell type and the differentiation process in these cells.

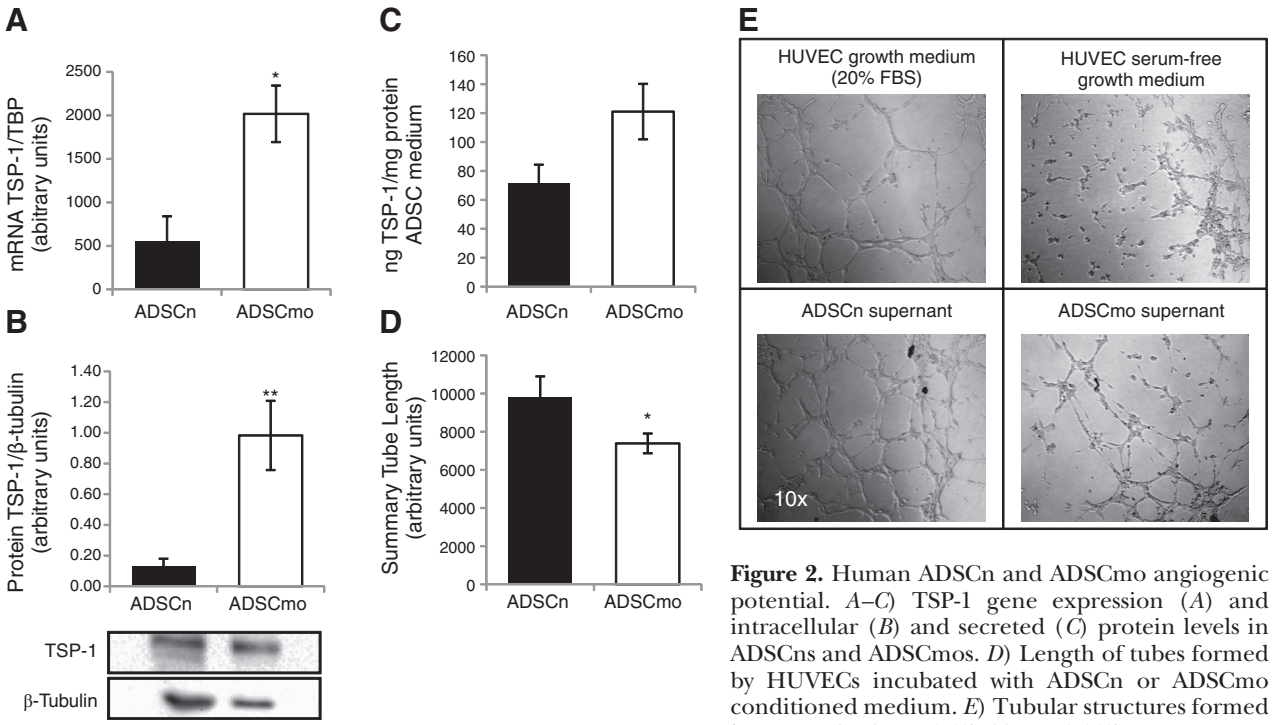

Figure 2. Human ADSCn and ADSCmo angiogenic potential. *A–C*) TSP-1 gene expression (*A*) and intracellular (*B*) and secreted (*C*) protein levels in ADSCns and ADSCmos. *D*) Length of tubes formed by HUVECs incubated with ADSCn or ADSCmo conditioned medium. *E*) Tubular structures formed by HUVECs. $*P < 0.05$, $**P < 0.005$.

DISCUSSION

ADSCs have recently become an alternative source of pluripotent stem cells for cell therapy. Certainly, ADSCs have been used in various preclinical models and clinical trials. However, previous studies using circulating progenitor cells from patients with cardiovascular risk factors have shown impaired properties. As such, evidence suggests that smoking, hypertension, coronary artery disease, diabetes, and hypercholesterolemia reduce the number and functional activities of isolated endothelial progenitor cells. Endothelial progenitor cell proliferation, migration, adhesion, and *in vitro* vasculogenesis are impaired in patients with cardiovascular risk factors (14, 17–21, 25, 26). As to ADSCs, age, adipose tissue depot site, and gender have the potential to modify the functionality and quality of ADSCs (9–13). However, little is known about the effect of obesity in endogenous ADSCs. In the present study, we compared subcutaneous ADSCs from morbidly obese patients and from nonobese individuals, analyzing growth behavior, differentiation capacity, and angiogenic potential.

We observed that WAT from morbidly obese patients presented a lower percentage of $CD90^+$ cells within the SVF. This result is in agreement with a study reporting reduced committed preadipocyte numbers in obese patients (27). However, the percentages of $CD29^+$ and $CD44^+$ cells were similar in SVFs from both morbidly obese and nonobese subjects.

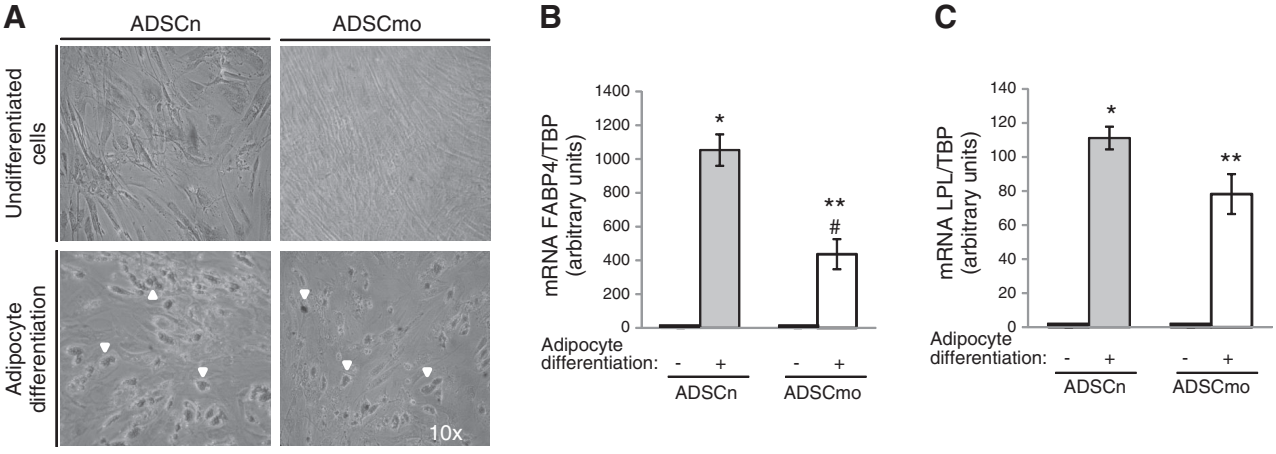

Figure 3. Differentiation of human ADSCns and ADSCmos toward adipogenic phenotype. Adipogenic differentiation of ADSCns and ADSCmos was detected by Herxheimer staining of lipids (*A*, arrowheads) and the expression of adipocyte molecular markers FABP4 (*B*) and LPL (*C*). $*P < 0.005$, $**P < 0.0005$ *vs.* undifferentiated cells; $^{\#}P < 0.005$ *vs.* ADSCns.

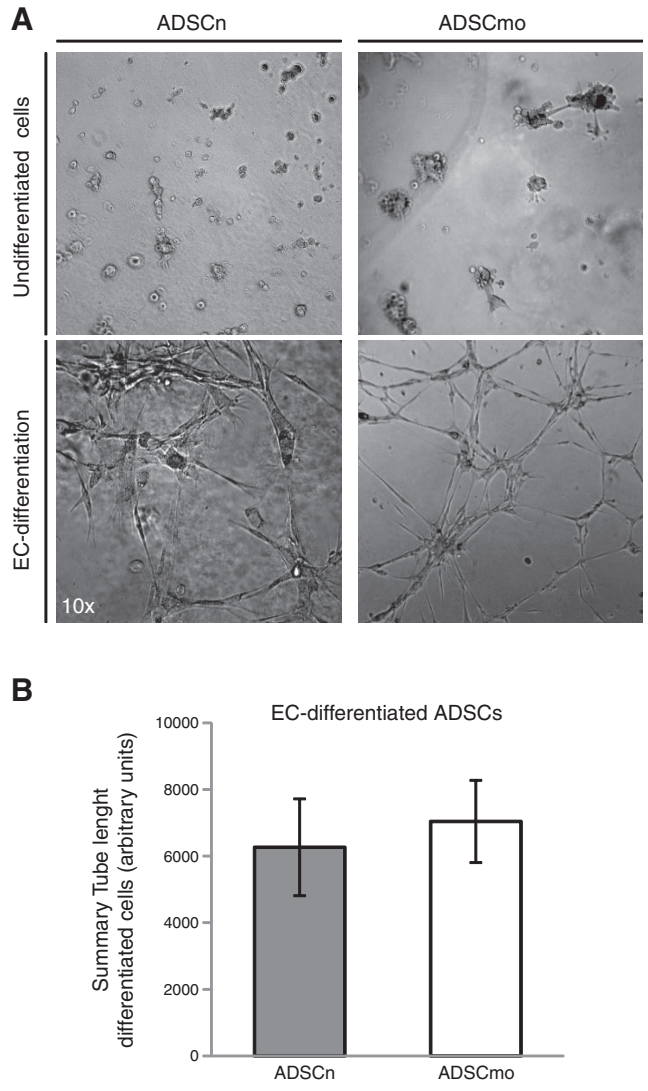

Figure 4. Differentiation of human ADSCns and ADSCmos toward ECs. *A*) Acquisition of EC phenotype of differentiated ADSCns and ADSCmos was evaluated by formation of capillary-like ring-structures when plating cells onto Matrigel. *B*) Total length of the formed tubes of EC-differentiated ADSCs was quantified.

For clinical cell therapy purposes, it would be beneficial to obtain the most ADSCs in the shortest period of time. Hypoxia is an important microenviromental factor in major aspects of stem cell biology, including survival, proliferation viability, pluripotency maintenance, differentiation, and migration (28–32). Despite the fact that the anatomical sites of MSC niches in the body are relatively oxygen deficient, MSCs are usually cultured under normoxic conditions. Although hypoxia, concomitant with serum deprivation, has been demonstrated to induce apoptosis in MSCs (33, 34), culturing MSCs under physiologically relevant low-oxygen-tension conditions may uniquely benefit the proliferation, differentiation, growth factor secretion, and migration/homing potential of transplanted cells. Previous studies have demonstrated that culturing BM-MSCs and human ADSCs under hypoxic conditions increased their proliferation capacity (30, 35, 36), although those cells presented an extended lag phase in order to acclimatize to culture conditions (29). Moreover, it has been shown that hypoxia increases angiogenic potential and the release of paracrine factors in murine and human BM-MSCs and ADSCs (35, 37–39). Indeed, the tissue-regenerative potential of BM-MSCs in the repair of murine infarcted myocardium and hindlimb ischemia (32, 37) and MSC wound-healing effects (36, 40) has been improved by hypoxic preconditioning. Therefore, we cultured ADSCns and ADSCmos under two different oxygen conditions (normoxia and hypoxia) in order to compare growth kinetics and function between ADSCmo and ADSCn. Firstly, when we cultured ADSCs under normoxic conditions, both ADSCns and ADSCmos were 90–100% positive for CD90, CD29, CD105, and CD44. ADSCs cultured under hypoxic conditions elicited similar results in surface marker expression of pluripotency. These results showed that neither hypoxia nor obesity modifies the molecular phenotype of expanded ADSCs.

However, we tested the effects of cell culture oxygen levels on cellular function. Hypoxia seems to modify ADSC proliferation. We observed an improvement in growth kinetics of ADSCns and ADSCmos cultured under hypoxic conditions. Interestingly, ADSCs from obese patients showed decreased proliferation capacity. ADSCmos presented slower PDTs than ADSCns cultured under both normoxic and hypoxic conditions.

In previous experimental animal studies with mice, rats, and rabbits, ADSCs were shown to have angiogenic properties through the release of paracrine factors (4–6). In fact, ADSCs are known to secrete a large number of angiogenic factors, including VEGF, hepatocyte growth factor, insulin-like growth factor 1, transforming growth factor-β, and monocyte chemotactic protein 1, among many others, which suggests an active role of ADSCs in promoting revascularization of the ischemic tissue (41–44).

To this end, we studied whether ADSCmos had a different angiogenic potential. We measured levels of TSP-1 at the gene expression and intracellular and secreted-protein levels. TSP-1 is an adipokine with antiangiogenic effects, the expression and secretion of which have been shown to be strongly modulated by insulin and glucose levels in adipocytes from rats with diet-induced obesity (45) and the degree of obesity in human adipose tissue from extremely obese and insulin-resistant subjects (46, 47). In all cases, TSP-1 levels were higher in ADSCmos than in ADSCns. Conditioned medium from ADSCmos induced a decrease in HUVEC capillary-like ring formation with respect to ADSCn conditioned medium, indicating a much lower proangiogenic capacity of ADSCmos.

To study how obesity may affect ADSC differentiation capacity, we induced differentiation of ADSCns and ADSCmos toward both adipocytes and ECs (24, 48). Several studies have shown an inverse correlation between BMI and preadipocyte differentiation capacity. In women, obesity reduces the differentiation capacity

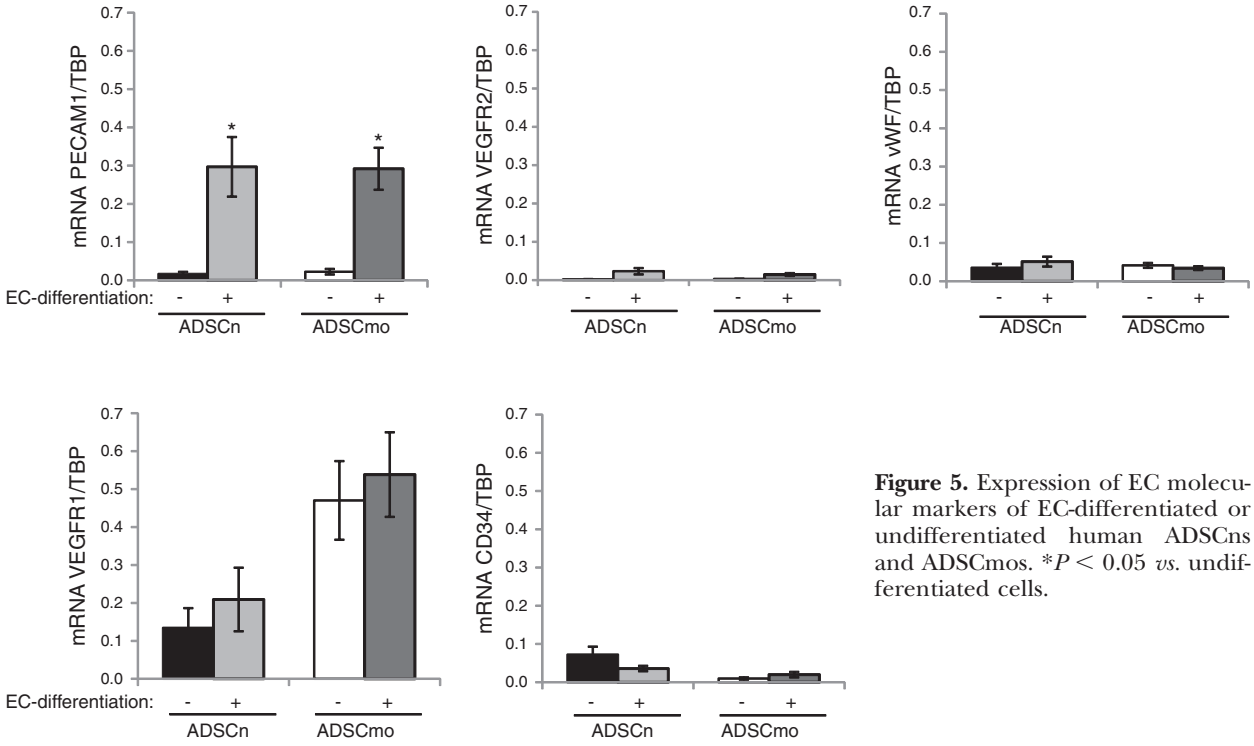

Figure 5. Expression of EC molecular markers of EC-differentiated or undifferentiated human ADSCns and ADSCmos. $*P < 0.05$ *vs.* undifferentiated cells.

of the adipose SVF (10, 27), and in Pima Indians, preadipocyte differentiation correlated negatively with the degree of central obesity (49). Impaired adipocyte differentiation of preadipocytes in obesity has also been reported (50). Here we found that ADSCmos showed lower differentiation capacity to adipocytes than ADSCns. Although the expression of the adipocyte-specific genes LPL and FABP4 increased after the differentiation process, ADSCmos presented lower expression levels compared to ADSCns.

ADSC differentiation to ECs also showed differences between both cells. Indeed, the expression of EC-specific genes did not follow the same pattern in ADSCmos and ADSCns. ADSCmos expressed higher levels of VEGFR1 than ADSCns before and after EC-differentiation. Only *in vitro* differentiated ADSCs presented the ability to form capillary-like ring structures. EC differentiation similarly modified the expression of PECAM-1, while VEGFR2, CD34, and vWF expression levels were almost undetectable in both cell types and did not change their expression after the differentiation process.

In summary, ADSCs from morbidly obese patients with metabolic syndrome show impairment in proliferation, angiogenic capacity, and differentiation potential. These effects may negatively influence their regenerative potential when used in cell therapy and also in spontaneous repair of minor organ damage. Indeed, ADSCs have already been tested in several clinical trials, from repair of heart ischemic injury to Crohn's disease and multiple sclerosis (51–55). However, our observations indicate that the therapeutic strategies based on autologous ADSC implantation would be impaired in patients with obesity and metabolic syndrome. FJ

This work was supported by grants SAF2010/16549, CIBERobn-CB06/03, and Retic-TERCEL RD06/0010/0017 from Instituto Salud Carlos III (Madrid, Spain) and Fundación Jesús Serra (Barcelona, Spain). L.B. is the Cardiovascular Research Chair, Universitat Autònoma de Barcelona (Barcelona, Spain). B.O. was the recipient of a predoctoral fellowship from Instituto Salud Carlos III. G.V. is a recipient of a contract from the Spanish Ministry of Science and Innovation (MICINN; RyC-2009-5495). R.F.-L. is a recipient of a Sara Borrell contract from Instituto Salud Carlos III, MICINN. The authors thank Marta Sánchez and Vanesa Rodríguez for their technical support. The authors declare no conflicts of interest.

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Received for publication March 5, 2012.
Accepted for publication June 20, 2012.