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Adipogenic impairment of adipose tissue-derived mesenchymal stem cells in subjects with metabolic syndrome: possible protective role of FGF2

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Context: The decreased expansion capacity of adipose tissue plays a crucial role in the onset of disorders associated with metabolic syndrome.
Objetive: The aim of this study was to examine the state of adipose tissue-derived mesenchymal stem cells (ASCs) from obese subjects with different metabolic profiles.
Design: This was a 2-year study to enroll subjects who underwent bariatric surgery or cholecystectomy.
Setting: University Hospital
Patients and Intervention: Patients who underwent either bariatric surgery (20 morbidly obese) or cholecystectomy (40 subjects) participated in the study.
Main Outcome Measures: ASCs were obtained from both visceral and subcutaneous adipose tissue. Adipogenic, fibrotic genes expression was quantified by qPCR; Smad7 and fibroblast growth factor 2 (FGF2) were quantified by western blotting and ELISA respectively. The susceptibility of ASCs to apoptosis, their population doubling time and clonogenic potential were evaluated
Results The worsening metabolic profile of the subjects was accompanied by a decrease in the intrinsic levels of adipogenic genes expression, reduced proliferation rate, clonogenic potential and exportation of FGF2 to the cell surface of the ASCs derived from both tissues. In addition, the ASCs from NonMS subjects showed differences in susceptibility to apoptosis and expression of TGFß signaling inhibitory protein Smad7 with respect to the ASCs from MS subjects.
Conclusions/Interpretation Our results suggest that the decrease in adipogenic genes mRNA and clonogenic potential as well as the accumulation of fibrotic proteins with metabolic alterations could be a relevant mechanism controlling the number and size of neogenerated adipocytes and involved in adipose tissue expansion alteration.

PRECIS: We studied the state of ASCs from obese subjects with different metabolic profiles, finding that ASCs impairment could contribute to the adipose tissue expandability decrease in metabolic syndrome

INTRODUCTION

It has been suggested that the expansion capacity of the fat depots is not the same for all subjects and, once the storage capacity limit is exceeded, lipids are accumulated ectopically in other organs, inducing the secretion of pro-inflammatory factors leading to metabolic syndrome (1). Alternatively, it has also been proposed that the preservation of the architecture and functionality of adipose tissue could contribute to some obese individuals remaining metabolically healthy and not developing any cardiovascular disorders commonly associated with obesity (2). In either case, adipose tissue-derived mesenchymal stem cells (ASCs) could be involved in the dysfunction of adipose tissue associated with obesity since

they not only ensure the long-term renewal of adipocytes but also the availability of new adipocytes during the expansion of adipose tissue through hyperplasia.

In fact, *in vitro* studies have confirmed the decrease in the adipogenic differentiation ability of the ASCs from obese subjects (3-7). This intrinsic impaired adipogenic differentiation ability in obesity seems to be accompanied by a reduced ability to differentiate into other mesenchymal lineages (6; 8-10), through premature cell senescence signals (6, 11) and through the exhaustion of proangiogenic ASCs (12).

Considering that the expansion of adipose tissue also depends on the state of the ASCs, it is likely that ASCs impairment could contribute to a decrease in adipose tissue expandability and that ASCs functionality may differ between subjects with different metabolic profiles. For this reason, the aims of the present study were to assess the adipogenic genes expression levels *in vitro* of differentiated adipocytes from ASCs from subjects with different metabolic profiles; to measure the expression levels of some of the proteins involved in adipose tissue fibrosis in ASCs; and to describe the susceptibility to apoptosis, the proliferative and clonogenic potential, and the surface retention of fibroblast growth factor 2 (FGF2) in the ASCs.

MATERIALS AND METHODS

Subjects

Paired visceral (specifically greater omental) and subcutaneous adipose (from the abdominal region) tissue biopsies were obtained from 60 subjects who underwent bariatric surgery (20 morbidly obese patients) or cholecystectomy (40 subjects) at the Virgen de la Victoria Clinical Hospital and the Civil Hospital (Malaga, Spain) during 2013-2014. The hospital ethics committee approved the study and informed consent was obtained from the participants. All the subjects were under 58 years of age. None had infectious disease, type 2 diabetes or drug treatment for this condition. Participants were classified according to their BMI as healthy normoweight subjects (Nw) (BMI<25 kg/m^2) or, in subjects with BMI ≥25 kg/m^2, as either without or with metabolic syndrome (NonMS and MS, respectively) 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 (SVF) derived from visceral and subcutaneous adipose tissue.

Paired samples of subcutaneous and visceral adipose tissue were minced and stirred for 70 minutes in a water bath at 37°C in a type I collagenase enzyme solution (0.150% in PBS+1.0% BSA). The resulting tissue suspension was centrifuged, the floating adipocytes were separated by decantation and SVF was filtered, centrifuged and resuspended in erythrocyte lysis buffer for ten minutes at room temperature. After washing, SVF was resuspended and incubated for approximately 16 hours in growth medium consisting of DMEM/F-12 supplemented with FBS (0.1 mL/mL), streptomycin (100 ug/mL), penicillin (100 U/mL) and L-glutamine (2 mM). The number of cells needed to perform the different assays was reached by cell subculturing up to passage two or three under standard culture conditions at 37°C in a humidified atmosphere with 5% $CO_{2(g)}$.

Immunophenotypic characterization by flow cytometry

Immunophenotypic characterization of the visceral ASC (visASCs) and subcutaneous ASC (subASCs) in passage three was performed using flow cytometry based on cell surface markers CD73- Phycoerythrin (PE) (BD Biosciences) and CD105- Allophycocyanin (APC) (Miltenyi Biotec). The ASC were detached with trypsin/EDTA (pH 7.0-7.6), washed with

PBS, resuspended in blocking buffer (PBS supplemented with 3.0% BSA), and incubated for ten minutes on ice. Aliquots of 1 x 10^5 cells each were dispensed into polypropylene tubes to which was added 10 µL or 20 µL of monoclonal mouse antibody solution against the respective cell surface markers, conjugated with its corresponding fluorochrome, according to the manufacturer's instructions. One tube was for labeling the corresponding IgG1-PE and IgG1-APC isotype controls (Miltenyi Biotec). All the tubes were incubated for 30 minutes on ice and protected from light. The cells were washed twice with blocking buffer and resuspended in 1000 µL of PBS to acquire 1 x 10^4 events per tube using a CyAn^{TM} ADP High-speed Analyzer (Beckman Coulter, Barcelona, Spain).

Adipogenic differentiation

The visASCss and subASCss in passage one were seeded at 10,000 cells/cm^2 and cultured under standard culture conditions. When cells reached 80-90% confluence, the expansion medium was replaced during 72 hours with adipogenic medium composed of DMEM/F12 supplemented with FBS (0.1 mL/mL), streptomycin (100 µg/mL), penicillin (100 U/mL), L-glutamine (2 mM), insulin (10 µM), isobutylmethylxanthine (0.5 mM), dexamethasone (1.0 µM), pioglitazone (10 µM), rosiglitazone (0.5 µM), biotin (33 µM), pathenonate (17 µM). After 72h of adipogenic induction, the medium was replaced by the above medium without isobutylmethylxanthine, biotin and pathenonate. Adipogenesis was confirmed after 21 days by Oil Red O staining to visualize the characteristic cytoplasmic lipid droplets.

Gene expression by real-time qPCR (qRT-PCR)

Total RNA was isolated using the RNA-Stat 60 Reagent (Ams Biotechnology, UK) and reverse transcribed to cDNA using the reverse-transcriptase enzyme (Transcriptor Reverse Transcriptase 20U/µL; Roche). qRT-PCR were performed with 20 ng cDNA and carried out using primers and probes specific for TaqMan gene expression assays (Applied Biosystems, Foster City, USA) for the following genes: CCAAT/enhancer binding protein α (CEBPá) (Hs 00269972_s1, RefSeq.NM_004364.3), peroxisome proliferator-activated receptor γ (PPARã) (Hs01115513_m1, RefSeq.NM_005037.5), GLUT4 (Hs00168966_m1, RefSeq NM_001042.2), Fatty acid binding protein 4 (FABP4) (Hs01086177_m1, RefSeq NM_001442.2), Perilipin 1 (PLIN1) (Hs00160173_m1, RefSeq NM_001145311.1), Perilipin 2 (PLIN2) (Hs00765634_m1, RefSeq NM_001122.3), Collagen type I alpha 1 (Col1a1) (Hs 00164004_m1, RefSeq.NM_000088.3), collagen type III alpha 1 (Col3a1) (Hs00943809_m1, Fibronectin 1 (FN1) (Hs 00365052_m1, RefSeq. NM_002026.2), Transforming growth factor β receptor 1 (TGFâ-R1) (Hs00610320_m1, RefSeq. NM_001130916.1), Transforming growth factor β receptor 2 (TGFâ-R2) (Hs00234253_m1, RefSeq. NM_001024847.2), Transforming growth factor β1 (TGFâ1) (Hs00998133_m1, RefSeq. NM_000660.4), Smad7 (Hs 00998193_m1, RefSeq. NM_001190821.1) and as endogenous control for the target gene in each reaction, ribosomal protein L13A (RPL13A) (Hs 04194366-g1, RefSeq. NM_001270491.1) was used. Specific signals were normalized with respect to the endogenous control according to the $2^{-\Delta\Delta Ct}$ formula for mRNA expression of CEBPα, PPARγ, GLUT4, FABP4, PLIN1 and PLIN2 cells cultured in adipogenic medium, taking the cultured cells in expansion medium as calibrator, and $2^{-\Delta Ct}$ formula for mRNA expression of the other genes.

Immunoblot analysis

For the extraction of cytoplasmic proteins from the visASCss and subASCs in passage three, NE-PER buffer+protease/phosphatase inhibitor (Thermo Scientific) was used. Protein extracts (22 µg) mixed in 4X Laemmli loading buffer with 2-mercaptoethanol (Bio-Rad) were loaded on a 7.5% SDS-PAGE gel for electrophoresis then transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in tris-buffered saline Tween-20 (TBST)+5% BSA for one hour at room temperature. Then, they were incubated overnight at

4°C with primary rabbit–antihuman antibody for Vinculin (Abcam), with primary mouse–antihuman antibody for Smad7 (Santa Cruz, USA). The membranes were washed thoroughly for 60 min with TBST before incubation with the secondary Goat Anti-Mouse IgG1 [1:5000] and Goat Anti-Rabbit IgG [1:10 000] horseradish peroxidase–conjugated (Abcam) for one hour at room temperature. Antibody complexes were visualized by chemiluminescence solution using the Universal Hood II-ChemiDoc XRS. Signals were quantified by densitometry using the ImageJ image processing program (National Institute of Health, USA) and expressed as relative quantities with respect to the optical density values of the respective loading control.

Apoptosis susceptibility assay

The visASCs and subASCs in passage two were suspended in maintenance medium and seeded into six-plates at 1 x 10^4/cm^2. After eight days under standard culture conditions, apoptosis was induced for 24 hours by replacing the maintenance medium with Eagle's Minimum Essential Medium, FBS-free, or supplementing this medium with 100 ng/mL of TNFα. The apoptotic effects of both conditions were quantified by flow cytometry labeling of phosphatidylserine with Fluorescein Isothiocyanate (FITC)-conjugated annexin V (Miltenyi Biotec).

Determination of population doubling time (PDT)

The visASCs and subASCs in passage two were seeded in duplicate at 3000 cells/cm^2 in twelve-well plates. The cells were detached with trypsin at two, four, six and eight days of culture, and counted using the trypan blue exclusion assay with neubauer chamber. The 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 day six and eight, respectively, after seeding.

ELISA

The visASCs and subASCs in passage two were resuspended in maintenance medium and seeded in six-well plates at a density of 1.5 x 10^4/cm^2 for eight days. After 72 hours from the last change of medium, the cells were washed with 2 M NaCl (20 mM HEPES, pH 7.4) for 5 seconds to remove cell surface-bound FGF2 according to Zaragosis *et al.* (13). Quantikine® ELISA kit (R&D Systems, Inc) was used to detect FGF2 according to manufacturer's instructions. Data are expressed as mean ± standard error picograms of the secreted FGF2 per 10^6 cells at the time of harvest.

Colony forming unit assay

The visASCs and subASCs in passage two were seeded in triplicate at 50 cells/cm^2 and incubated for 14 days under standard culture conditions. Cells were fixed and stained with crystal violet solution (0.5% in methanol) for ten minutes. After washing with water and air drying, the colonies formed by more than 50 cells were counted.

Statistical Analysis

The results were expressed as mean values ± SEM. The Shapiro-Wilk test was used to test for normality. 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, Chicago, IL). P values < 0.05 were considered statistically significant.

RESULTS

Anthropometric characterization and metabolic profile of the subjects

MS and NonMS subjects had significantly higher BMI, waist circumference, waist-hip ratio and uric acid compared to Nw subjects. The MS subjects showed further deterioration in their

metabolic profile since most of the metabolic variables evaluated in these patients showed statistically significant differences compared to the other two groups, with the exception of systolic blood pressure, creatinine, urea and apoliprotein A (**Supplemental Table 1**).

Decreased expression levels of adipogenic genes in the visASCs and subASCs from the MS subjects

To verify the possible contribution of ASCs to the generation of adipocytes with lower expression levels of adipogenic genes, we isolated the adipose-SVF and we induced *in vitro* adipogenic differentiation of the ASCs from subjects with different metabolic profiles.

Immunophenotypic characterization of visASCs and subASCs in passage three confirmed that the percentage of cells that coexpressed the multipotent mesenchymal cell markers CD73 and CD105 exceeded 94% (**Supplemental Figure 1**).

Oil Red staining confirmed cytoplasmatic lipid accumulation in the ASCs cultured for 21 days in adipogenic medium (**Supplemental Figure 2A**). We did not observe significant differences in the mRNA levels of the transcription factors PPARγ and CEBPα between groups (**Supplemental Figure 2B**). However, gene expressions of GLUT4 and FABP4 in subASCs from the MS subjects were significantly lower than those seen in subASCs from the other patient groups (**Figure 1A**). VisASCs from the MS subjects also showed lower gene expression levels of GLUT4 and PLIN2 compared to Nw and NonSM subjects, repectively (**Figure 1A**). Interestingly, mRNA levels of these genes were negatively and significantly correlated with BMI (**Figure 1B**), HOMA-IR (**Figure 1C**), LDL-cholesterol and Total-cholesterol (**Figure 1D**).

Decreased GLUT4 and PLIN1 mRNA levels in the visASCs and the subASCs is correlated with increased expression levels of fibrotic proteins.

In vitro experiments have shown that alterations in the expression pattern of extracellular matrix proteins can affect the expression of genes of the adipogenic lineage (14-15). Thus we subsequently examined mRNA levels of some of the proteins involved in the increased tissue fibrosis associated with obesity.

Expression levels of FN1 were negatively correlated with GLUT4 transcriptional levels in visASCs (R = -0.402; P = 0.079) and subASCs (R= -0.430; P = 0.058), but only PLIN1 mRNA had a significant negative correlation with expression levels of FN1 in subASCs (**Figure 2A**). Gene expression analysis also revealed statiscally significant negative correlations between Col3a1 and PLIN1 mRNA levels in subASCs (**Figure 2B**) and between Col1a1 and GLUT4 expression levels both visASCs and subASCs (**Figure 2C**). In adittion, Col1a1 mRNA levels in subASCs were positively associated with BMI and HOMA-IR (**Figure 2D**).

Moreover, although no significant differences were seen in the expression levels of TGFß1 and TGFß receptors, we detected significantly elevated Smad7 mRNA levels in the subASCs from NonMS subjects (**Figure 3A**). Furthermore, western blot analysis enabled us to confirm a significant increase in Smad7 protein levels in the cytoplasmic fraction of the subASCs from the NonMS subjects compared to the MS subjects (**Figure 3B**).

Lower susceptibility to apoptosis, but longer PDT in the ASCs from the MS subjects

Flow cytometry assessment (**Figure 4A**) revealed that the visASCs and the subASCs from the NonMS subjects had a higher percentage of apoptotic cells when stimulated with TNFα than those from the MS patients, significantly so in the visASCs (**Figure 4B**).

While the PDT of the subASCs from the MS subjects was significantly higher than in the Nw and NonMS subjects, the decreasing proliferative potential shown by the visASCs from the MS subjects did not reach statistical significance (**Figure 5**). **Figure 5** also shows that the PDT of the ASCs, mainly in the subASCs, was positively correlated with HOMA-IR as well as with plasma glucose and triglyceride levels.

Increased clonogenic potential and concentration of FGF2 retained on the surface of the subASCs from the NonMS subjects.

Given that *in vitro* experiments have shown that autocrine signaling associated with FGF2 increases the rate of proliferation and the clonogenic potential of ASCs (13; 16), we analyzed whether amounts of FGF2 retained on the cell surface of the ASCs from the MS and NonMS subjects could be different.

Application of ELISA in the collected wash solution revealed a significantly decreased concentration of FGF2 retained by the visASCs and subASCs with increased HOMA-IR (**Figure 6A**) and plasma glucose levels respectively (**Figure 6B**). In addition, we detected a significant increase in the FGF2 concentration retained by the subASCs derived from the NonMS subjects compared to those derived from the other two groups of patients (**Figure 6C**).

Crystal violet staining facilitated counting of the colonies generated over 14 days by the low density culture of the ASCs (**Figure 6D**). **Figure 6E** shows that the number of colonies generated by the visASCs and subASCs from MS subjects was significantly lower than in the Nw subjects. While the number of colonies generated by the subASCs had a significant negative correlation with the BMI (**Figure 6F**) and the HOMA-IR in the patients (**Figure 6G**), the clonogenic potential of the subASCs from the NonMS subjects was significantly higher than in the MS subjects and did not differ significantly from that of the Nw subjects (**Figure 6E**).

DISCUSSION

Previous studies have documented the decrease in the adipogenic differentiation capacity of ASCs from obese patients (3-7). Here we describe, for the first time, that with the increase in BMI, HOMA-IR and cholesterol, the adipocytes generated from visASCs and subASCs showed a decrease in the intrinsic expression levels of adipogenic genes in parallel with an increase in fibrotic protein expression. We also observed that a worsening metabolic profile in the subjects was accompanied by a decrease in the proliferative rate, clonogenic potential and export of FGF2 to the cell surface of the ASCs derived from both tissues.

Previous gene expression analyses have shown that subcutaneous adipose tissue FABP4 mRNA expression levels from obese patients with insulin resistance were lower than those lean subjects (17) and the decrease in GLUT4 mRNA levels of subcutaneous adipose tissue is related more to insulin resistance and type 2 diabetes than to obesity itself (18). In line with these results, we found that the adipocytes generated from subASCs of MS subjects had lower FABP4 and GLUT4 expression levels than those generated from ASCs of Nw subjects. Since FABP4 is involved in uptake of fatty acids (19) and GLUT4-mediated glucose uptake induces expression of the lipogenic transcription factor carbohydrate-responsive element-binding protein (20), it is likely that the decreased FABP4 and GLUT4 expression in adipocytes affects the cytoplasmic lipid accumulation and the consequent expansion by hypertrophy of the adipose tissue in MS subjects. It has been confirmed that decreased GLUT4 in the adipose tissue of insulin-resistant obese subjects is accompanied by a decrease in lipogenic enzymes (21). When comparing the two obese groups, only the adipocytes generated from the subASCs showed significant differences in GLUT4 and FABP4 expression levels between the NonMS and MS groups, suggesting a possible protective role of subcutaneous adipose tissue on the metabolic profile of NonMS subjects.

We also observed that the decrease in PLIN1 and GLUT4 expression levels was not accompanied by variations in the amounts of mRNA of the adipogenic transcription factors C/EBPα and PPARγ but by increased expression of FN1, Col3a1 and Col1a1. Therefore, we did not rule out the possibility that the transcription factors associated with TGFß signaling, and involved in the increased expression of fibrotic proteins, have interfered with the

transcription of adipogenic genes, as it is now recognized that TGFß signaling can influence gene expression in precursor cell differentiation (22-23).

The significant increase in Col1a1 expression in the subASCs, which is associated with the increase in BMI and HOMA-IR, suggests a contribution of the subASCs to the increase in pericellular tissue fibrosis seen in adipocytes from obese subjects (24). Because increased tissue fibrosis has been negatively correlated with the average size of small and medium adipocyte fractions (24-26), it is likely that the adipocytes generated from the subASCs with high levels of Col1a1 expression also have restricted hypertrophic capacity. In fact, it has been confirmed that the level of Col6a3 mRNA expression in small adipocytes is significantly higher than in large adipocytes (27).

The adipose tissue remodeling that occurs during obesity is not only accompanied by a disproportionate synthesis of extracellular matrix components (28) but also by a decreasing number of adipocytes (3; 29). Interestingly, the subASCs of the NonMS subjects expressed significantly elevated levels of Smad7, a TGFß signaling inhibitory protein, suggesting the possibility that they are protected from the increased fibrosis associated with TGFß signaling. Consistent with this interpretation, *in vivo* experiments have shown that blocking TGFß signaling protects mice from diabetes and hepatic steatosis (30-31), whereas overexpression of Smad7 decreases accumulation of fibrotic proteins in the kidney of diabetic rats (32). Increased Smad7 expression levels may also be responsible for the increased susceptibility to apoptosis seen in the ASCs from NonMS subjects, as *in vitro* experiments have confirmed that Smad7 can sensitize cells to TNFα-induced apoptosis (33) probably through their inhibitory effects on nuclear factor-kappa b signaling (34).

The increase in PDT in the subASCs may decrease the availability of precursor cells and thereby contribute to the decrease in the number of adipocytes observed in the adipose tissue of obese insulin resistant subjects (3; 29; 35). Thus, the higher proliferation rate observed in the subASCs of the NonMS subjects, compared to the MS subjects, suggests that their subcutaneous adipose tissue might be better able to expand to caloric intake, as *in vivo* experiments have shown that subASCs proliferate in response to a high-fat diet (36). Consistent with the decrease in proliferative potential of the ASCs associated with a worsening metabolic profile in the patients, the number of colonies generated by the visASCs and the subASCs of the MS subjects was significantly lower than in the Nw subjects.

Since FGF2 is currently known as one of the intrinsic regulators of ASCs proliferation and differentiation (37), it might be responsible for maintaining the proliferative and clonogenic potential as well as GLUT4 and FABP4 expression levels observed mainly in the subASCs of the NonMS subjects. Concordantly, the concentration of FGF2 retained on the surface of these cells was significantly higher in NonMS subjects than in the other two groups of subjects. In fact, it has been shown that ASCs express an FGF2 autocrine signaling loop that promotes the maintenance of clonogenic potential and prevents the loss of differentiation ability (13; 16). Furthermore, previous experiments have also confirmed that the treatment of ASCs with FGF2 can not only reduce the expression levels of fibrotic proteins such as fibronectin and type I collagen, but also reverse the TGFß-induced myofibroblastic phenotype (38). In line with this study, we have observed that an increased concentration of FGF2 retained on the surface of the subASCs from the NonMS subjects is accompanied by decreased expression levels of Col1a1. Thus, our results suggest that FGF2, together with Smad 7, could exert a protective function in the activation of TGFß signaling according to recent evidence that suggests a higher vulnerability of subASCs from obese subjects (39).

Altogether, our results indicate that visASCs and subASCs may be involved in the variations in adipose cellularity that occur during the development of insulin resistance through a decrease in their clonogenic and proliferative potential, generation of adipocytes

with lower levels of GLUT4, FABP4 and PLIN2 expression, and high expression of fibrotic proteins. Moreover, ASCs may contribute to the protective effects of subcutaneous adipose tissue expansion in the NonMS obese subjects by stimulating the autocrine signaling associated with the surface retention of FGF2. Thus, our results suggest that the decrease in adipogenic genes mRNA and clonogenic potential as well as the accumulation of fibrotic proteins with metabolic alterations could be a relevant mechanism controlling the number and size of neogenerated adipocytes and involved in adipose tissue expansion alteration.

Abbreviations

APC Allophycocyanin

ASC adipose tissue-derived mesenchymal stem cells

CEBPα CCAAT/enhancer binding protein α

COL1A1 Collagen type I alpha 1

COL3A1 Collagen type III alpha 1

FABP4 Fatty acid binding protein 4

FGF2 fibroblast growth factor 2

FITC Fluorescein Isothiocyanate

FN1 Fibronectin 1

MS subjects with metabolic syndrome

NonMS subjects without metabolic syndrome

Nw normoweight subjects

PDT population doubling time

PE Phycoerythrin

PLIN Perilipin

PPARγ peroxisome proliferator activated receptor γ

PVDF polyvinylidene fluoride

qRT-PCR Quantitative real-time PCR

RPL13A Ribosomal protein L13A

subASCs subcutaneous ASC

SVF stromal vascular fraction

TGFβ-R1 Transforming growth factor β receptor 1

TGFβ-R2 Transforming growth factor β receptor 2

visASCs visceral ASC

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Authors' contributions

W.O.O. designed the experiment, researched data, and wrote the manuscript. L.C.A., S.L., M.C.P. researched data and contributed to the discussion. J.A.T. researched data and edited the manuscript. M.R.B.L. selected the control patients, scientifically revised the manuscript and contributed to the discussion. R.E.B and F.J.T. designed the experiment, reviewed, edited the manuscript and supervised the study.

F.J.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure 1. Expression levels of adipogenic genes in the visASCs and subASCs after 21 days of adipogenic differentiation. A: GLUT4, FABP4, PLIN1 and PLIN2 mRNA expression levels in the ASCs of subjects grouped according to their metabolic profile. **B-D:** Correlations between expression levels of adipogenic genes and BMI, HOMA-IR and cholesterol. GLUT4, FABP4, PLIN1 and PLIN2 mRNA values were determined by qPCR and normalized to RPL13A mRNA, taking the cultured cells in non-adipogenic medium as calibrator. (Nw, n = 5; NonMS, n = 6; MS, n = 9). *, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of the Nw subjects; †, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of the NonMS subjects.

Figure 2. Expression levels of adipogenic genes and mRNA levels of proteins associated with adipose tissue fibrosis. A-B: Correlation between the expression levels of PLIN1 with FN1and Col3a1 mRNA in subASCs. **C:** Correlation between the expression levels of Glut4 and Col1a1 mRNA in the ASCs. **D:** Correlation between the transcriptional levels of Col1a1 in the ASCs with the BMI and HOMA-IR in the patients. Values are reported as mean ± standard error (Nw, n = 5; NonMS, n = 6; MS, n = 9).

Figure 3. Expression of proteins involved in TGFß signaling. A: mRNA expression levels of TGFß signaling components in the ASCs from the subjects grouped by metabolic profile. Values are reported as mean ± standard error (Nw, n = 5; NonMS, n = 6; MS, n = 9). **B:** Smad7 expression detected by western blot. 22 µg of proteins from the cytoplasmic fraction from the ASCs from subjects with different metabolic profiles were loaded on the respective lanes of polyacrylamide gels (7.5%), undergoing electrophoresis under denaturing conditions and transferred to PVDF membranes. The graph represents the relative optical density values revealed by specific labeling against Smad7 compared to those from the Vinculin loading control. Values are reported as mean ± standard error (NonMS, n = 4; MS, n = 4). *, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of the Nw subjects; †, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of the NonMS subjects.

Figure 4. Induction of cell apoptosis in visceral and subASCs from obese subjects with different metabolic profiles cultured for 24 hours in serum-free medium with and without 100 ng/mL TNFα. A: Detection of apoptotic cells by flow cytometry based on negative staining for propidium iodide, but positive staining for annexin V conjugated to FITC. **B:** Quantification by flow cytometry of the percentage of ASCs showing early apoptosis in the obese subjects grouped according to their metabolic profile (NonMS, n = 4; MS, n = 6). †, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of the NonMS subjects.

Figure 5. Population doubling time (PDT) of the visASCs and subASCs from the Nw and obese subjects with different metabolic profiles. The ASCs in passage two were seeded in duplicate at a density of 3000 cells/cm^2 in twelve-well plates and the PDT was calculated between the sixth and eighth day of culture. (Nw, n = 7; NonMS, n = 7; MS, n = 10). *, Significantly different results (Mann-Whitney U test, $P < 0.05$) compared to those of Nw subjects; †, Significantly different results (Mann-Whitney U test, $P < 0.05$) to those of NonMS subjects. R, Spearman's rho.

Figure 6. Clonogenic potential and FGF2 retention on the surface of visASCs and subASCs. A-C: Correlation between concentrations of FGF2 exported towards the surface of the ASCs with HOMA-IR **(A)** and plasma glucose concentrations in the patients **(B)**. FGF2 concentration retained on the surface of the ASCs from subjects grouped by metabolic profile **(C)**. (Nw, n = 4; NonMS, n = 6; MS, n = 9). **(D)**: Crystal violet solution staining of colonies generated by ASCs seeded at low density and grown under standard conditions for 14 days in six-well plates. **E-G**:Number of colonies observed in the ASCs of the different patient groups **(E)**, correlation between number of colonies with BMI **(F)** and HOMA-IR in the patients **(G)**.(Nw, n = 7; NonMS, n = 10; MS, n = 10). * Significantly different results (Mann-Whitney test, $P<0.05$) compared to those of Nw subjects; † significantly different results (Mann-Whitney test, $P<0.05$) compared to those of NonMS subjects.