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FNDC4, a novel adipokine that reduces lipogenesis and promotes fat browning in human visceral adipocytes

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ABSTRACT

Background: Fibronectin type IIIdomain-containing protein 4 (FNDC4) constitutes a secreted factor showing a high homology in the fibronectin type III and transmembrane domains with the exercise-associated myokine irisin (FNDC5). We sought to evaluate whether FNDC4 mimics the anti-obesity effects of FNDC5/irisin in human adipose tissue.
Methods: Plasma and adipose tissue samples of 78 patients with morbid obesity undergoing bariatric surgery and 26 normal-weight individuals were used in the present study.
Results: Plasma FNDC4 was decreased in patients with morbid obesity, related to obesity-associated systemic inflammation and remained unchanged six months after bariatric surgery. Visceral adipose tissue from patients with morbid obesity showed higher expression of FNDC4 and its putative receptor GPR116 regardless of the degree of insulin resistance. FNDC4 content was regulated by lipogenic, lipolytic and proinflammatory stimuli in human visceral adipocytes. FNDC4 reduced intracytosolic lipid accumulation and stimulated a brown-like pattern in human adipocytes, as evidenced by an upregulated expression of UCP-1 and the brown/beige adipocyte markers *PRDM16, TMEM26* and *CD137*. Moreover, FNDC4 treatment upregulated mitochondrial DNA content and factors involved in mitochondrial biogenesis (*TFAM, NRF1* and *NRF2*). Human *FNDC4*-knockdown adipocytes exhibited an increase in lipogenesis and a reduction of brown/beige-specific fat markers as well as factors involved in mitochondrial biogenesis.
Conclusions: Taken together, the novel adipokine FNDC4 reduces lipogenesis and increases fat browning in human visceral adipocytes. The upregulation of FNDC4 in human visceral fat might constitute an attempt to attenuate the adipocyte hypertrophy, inflammation and impaired beige adipogenesis in the obese state.
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1. Introduction

Regular physical activity and exercise induce major and depot-specific adaptations in adipose tissue, such as an increase in mitochondrial activity, a decrease in adipocyte hypertrophy and the regulation of adipokines, that mediate in part the improvement in metabolic health [1]. Exercise also promotes the transdifferentiation of a cell population within the white adipose tissue to brown-like cells, in a process known as fat browning [2,3]. Active brown-like or beige adipocytes exhibit multi-locular lipid droplets, a high thermogenic activity due to abundant UCP-1-expressing mitochondria as well as a unique molecular signature, including the expression of TBX1, TMEM26, and CD137,

Abbreviations: ACTB, actin beta; ADIPOQ, adiponectin, C1Q and collagen domain containing; CD137, TNF receptor superfamily member 9; CYTB, cytochrome B; FABP4, fatty acid binding protein 4, adipocyte; FNDC4, fibronectin type III domain containing 4; FNDC5, fibronectin type III domain containing 5; GPR116, adhesion Gprotein-coupled receptor F5; IGT, impaired glucose tolerance; ITGA5, integrin subunit α 5; NG, normoglycemia; NRF1, nuclear respiratory factor 1; NRF2, nuclear factor, erythroid 2 like 2, transcript variant 2; PPARG2, peroxisome proliferator-activated receptor (PPAR) γ, transcript variant 2; PPARGC1A, PPARγ coactivator 1 γ; RYGB, Roux-en-Y gastric bypass; RT, room temperature; SVFC, stromal vascular fraction cells; T2D, type 2 diabetes; TFAM, transcription factor A, mitochondrial, transcript variant 2; TMEM26, transmembrane protein 26, transcript variant 1; UCP1, uncoupling protein 1.

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markers not expressed in mature brown or white adipocytes [2,4,5]. The identification of the skeletal muscle as an endocrine organ has provided a mechanistic explanation for the beneficial effects of exercise on the prevention of metabolic diseases [6,7]. In this regard, several muscle-secreted factors or myokines act on adipocytes as positive (IL-6, irisin, BAIBA and meteorin-like) and negative (myostatin) regulators of fat browning. Thus, the crosstalk between skeletal muscle and adipose tissue is of considerable interest, since myokine and adipokine dysregulation might contribute to the onset of obesity and associated metabolic alterations [6].

FNDC5 (also known as FRCP2) is a myokine that belongs to the fibronectin type III domain family (FNDC1–5) [2,8]. The *FNDC5* gene encodes a protein with a sequence that includes a signal peptide, a fibronectin type III domain, a hydrophobic transmembrane domain and a cytosolic C-terminal domain. Exercise and/or peroxisome proliferator-activated receptor (PPAR) γ coactivator 1-α (PGC-1-α) activation induce the proteolytic cleavage of FNDC5 to form the active form, irisin, that is secreted from skeletal muscle [2]. Besides being a myokine, FNDC5/irisin is also an adipokine that can modulate adipocyte function in an autocrine, paracrine and endocrine manner [9–11]. On binding its putative receptor in adipocytes, the αV class of integrin, as a preformed dimer [12,13], FNDC5/irisin inhibits adipogenesis and promotes fat browning *via*ERK, p38 MAPK and Wnt signaling. Moreover, FNDC5/irisin treatment promotes mitochondrial biogenesis through the upregulation of *PPARGC1A, TFAM, NRF1* and *NRF2* in human subcutaneous adipose tissue explants [14] and adipocytes [15]. FNDC5 treatment or overexpression in mice also attenuates adipose tissue inflammation by inhibiting M1 macrophage polarization and proinflammatory cytokine production [16–18]. These results point to irisin as an appealing therapeutic target for metabolic diseases. However, inconsistencies in published data regarding the circulating irisin levels in the context of human obesity and insulin resistance highlight the need for accurate methods for irisin measurement and/or for improved study designs [19–21]. In addition, discrepancies have been also found in the impact of *Fndc5* deletion on adiposity and insulin sensitivity in mice [16,22]. These results suggest that other family members might compensate for the *Fndc5*loss-of-function*invivo*.

The fibronectin type III domain family member FNDC4 (also known as FRCP1) displays a high homology with FNDC5 (60% identity on fibronectin type III domain and 86% identity in the transmembrane domain) [8]. FNDC4 is cleaved and the N-terminal portion is secreted from cells in accordance with the release mechanism of FNDC5/irisin [23]. FNDC4 is highly expressed in liver and brain, and to a lesser extent, in heart, skeletal muscle and adipose tissue [8,24]. Despite their high homology, FNDC4 and FNDC5 exhibit different expression profiles in response to exercise and inflammation [24]. FNDC4 transcript levels are robustly upregulated in three experimental models of inflammation and in human inflammatory conditions, but not by exercise [24]. FNDC4 is upregulated in human activated neutrophils and macrophages as well as in inflamed sites of the intestine of patients with inflammatory bowel disease, suggesting its role as an anti-inflammatory factor. Interestingly, FNDC4 improves inflammation, endoplasmic reticulum stress and insulin resistance *via* the AMPK/HMOX1 pathway in palmitate-treated murine adipocytes and adipose tissue [25]. Furthermore, adipose tissue deletion of the seven transmembrane G-protein coupled receptor 116 (GPR116), the putative receptor of FNDC4, results in impaired glucose tolerance and insulin resistance in mice [26]. However, the role of FNDC4 in human obesity has not been yet explored. Thus, we hypothesize that FNDC4 mimics the anti-obesity effects of FNDC5/irisin in human adipose tissue based on their high homology. We therefore sought to: 1)explore the potential differences in circulating FNDC4 in obesity and insulin resistance before and after weight loss achieved by bariatric surgery; 2)characterize *exvivo* the expression and regulation of FNDC4 in human adipose tissue in relation to obesity and obesity-associated type 2 diabetes; 3)analyze *invitro* the effect of FNDC4 treatment and gene silencing on lipogenesis, fat browning and mitochondrial biogenesis in human visceral adipocytes.

2. Materials and methods

2.1. Patient selection and study design

Plasma FNDC4 and FNDC5 were evaluated in a cross-sectional study with 104 samples from 26 normal-weight subjects (healthy volunteers or patients undergoing a Nissen funduplication) and 78 patients with morbid obesity undergoing bariatric surgery recruited at the Clínica Universidad de Navarra. Obesity was defined as a BMI ≥ 30 kg/m^2, and normal weight as a BMI < 25 kg/m^2. Patients with obesity were subclassified into two groups [normoglycemia (NG) and impaired glucose tolerance (IGT) or type 2 diabetes (T2D)] following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes [27]. An interventional study was also performed to investigate the variations of circulating FNDC4 and FNDC5 after bariatric surgery, using plasma samples from 64 patients with morbid obesity before and 6 months after sleeve gastrectomy ($n = 18$) or Roux-en-Y gastric bypass (RYGB) ($n = 42$). An intraoperative liver biopsy was obtained during bariatric surgery to establish a histological diagnosis of nonalcoholic fatty liver disease (NAFLD) [28]. The study protocol was conducted according to the principles of the Declaration of Helsinki, and approved by the Ethical Committee responsible for the research (028/2009).

2.2. Clinical assessments and analytical measurements

Body composition was measured by air-displacement plethysmography (Bod-Pod®, COSMED, Rome, Italy) and visceral adiposity was assessed by bioelectrical impedance (Tanita AB-140 ViScan®, Tanita Corp., Tokyo, Japan). Resting energy expenditure (REE) was determined by indirect calorimetry (Vmax29; SensorMedics Corporation, Yorba Linda, CA). Biochemical assays were performed as previously described [29]. The HOMA (homeostasis model assessment) score of insulin resistance was calculated with the formula: fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5. An indirect measure of insulin sensitivity was calculated by using the quantitative insulin sensitivity check index (QUICKI; 1/[log(fasting insulin in μU/mL) + log(fasting glucose in mg/dL)]. The adipocyte insulin resistance (Adipo-IR) index, as a surrogate of adipocyte dysfunction, was calculated as fasting NEFA (mmol/L) x fasting insulin (pmol/L). FNDC4 (MBS9332722, MyBiosource, San Diego, CA) and FNDC5 (SEN576Hu, USCN Life Science Inc., Wuhan, China) were measured by ELISA, with intra- and inter-assay coefficients of variation being < 15% and < 15%, respectively, for the former, and < 10% and < 12%, respectively, for the latter.

2.3. Adipose tissue handling

Paired samples of omental and subcutaneous fat ($n = 59$) were collected during elective surgical procedures for lean (Nissen funduplication) and obese (sleeve gastrectomy or RYGB) patients. Fat samples were immediately stored at −80 °C for gene and protein expression analyses. A portion of omental and subcutaneous adipose tissue was fixed in 4% formaldehyde for histological analyses. Another portion of omental fat was used for the isolation of adipocytes and stromal vascular fraction cells (SVFC) by 2% collagenase digestion, as previously described [29]. Total RNA isolation and purification was performed using QIAzol® Reagent (Qiagen, Hilden, Germany) and RNeasy Lipid Tissue Mini Kit (Qiagen) for adipose tissue and adipocytes, and TRIzol® Reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen) for SVFC, according to the manufacturer's instructions.

2.4. Real-time PCR

Transcript levels of the *ADIPOQ, CD68, FABP4, FNDC4, FNDC5, ITGA5, GPR116, PPARG2, PPARGC1A, TFAM, NRF1, NRF2, PRDM16, CD137, TMEM26* and *UCP1* genes were quantified by real-timePCR (7300 Real-TimePCR System, Applied Biosystems, Foster City, CA). Primers and

probes were designed using the software Primer Express 2.0 (Applied Biosystems) (**Supplemental Table1**). The cDNA was amplified at the following conditions: 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 59 °C, using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The endogenous housekeeping gene *18S* rRNA (Applied Biosystems) was used as loading control and relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ formula [30]. Relative mRNA expression was expressed as fold expression over the calibrator sample (average of gene expression corresponding to the lean group) as previously described [30]. All samples were run in triplicate and the average values were calculated.

2.5. Western-blot studies

Protein samples (30 μg) were separated by SDS-PAGE (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad Laboratories, Inc., Hercules, CA) under denaturing conditions and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblotting. Blots were incubated overnight at 4 °C with rabbit polyclonal anti-human FNDC4 (SAB1401807-100G, Sigma, St Louis, MO), polyclonal anti-human FNDC5 (ab93373, Abcam Ltd., Cambridge, UK), polyclonal anti-human GPR116 (ab136262, Abcam) and monoclonal anti-human integrin αV (ab179475, Abcam) (dilution 1:1000) or mouse monoclonal anti-actin (A5441, Sigma) antibody (dilution 1:5000). The antigen-antibody complexes were visualized using horseradish peroxidase-conjugatedanti-rabbit or anti-mouse antibodies (1:5000) and the enhanced chemiluminescence ECL Plus detection system (Amersham Biosciences). The intensity of the bands was determined by densitometric analysis with the Gel Doc™ gel documentation system and Quantity One 4.5.0 software (Bio-Rad) and normalized with β-actin density values.

2.6. Immunohistochemistry of FNDC4 and FNDC5

The immunodetection of FNDC4 and FNDC5 in sections (6 μm) of omental and subcutaneous adipose tissue was carried out by the indirect immunoperoxidase method [11]. Sections were incubated overnight at 4 °C with rabbit polyclonal IgG recognizing FNDC4 protein (SAB1401807-100G, Sigma) or the carboxy-terminal end of FNDC5 (ab93373, Abcam) (dilution 1:100). As negative controls, primary antibodies were omitted or pre-adsorbed with their respective immunogens to assess non-specific staining and specific binding, respectively. Briefly, for pre-adsorption negative controls, primary antibodies were preincubated with recombinant FNDC4 or FNDC5 (100 ng/mL) (AG-40B-0124-C010 and AG-40B-0102-C010, Adipogen, Liestal, Switzerland) overnight at 4 °C and the pre-adsorbed antibodies were then incubated in the histological sections instead of the primary antibody alone.

2.7. Cell culture and treatment

Human SVFC were grown to confluence in six-well plates and differentiated to mature adipocytes, as previously described [29]. SVFC and differentiated adipocytes (10^{th} day of differentiation) were serum-starved for 24 h and then treated with increasing concentrations of FNDC4 (Adipogen), FNDC5 (Adipogen), insulin (Sigma), acylated ghrelin (Tocris, Ellisville, MO), desacyl ghrelin (Tocris), leptin (PeproTech EC, Inc., Rocky Hill, NJ), isoproterenol (Sigma), atrial natriuretic peptide (Bachem, Bubendorf, Switzerland), TNF-α (PeproTech), lipopolysaccharide (LPS) (Sigma) and TGF-β (PeproTech) for 24 h. One sample per experiment was used to obtain control responses in the presence of the solvent.

2.8. FNDC4 knockdown by siRNA transfection

MISSION® esiRNA targeting human *FNDC4* mRNA (EHU045461) and MISSION® siRNA Universal Negative Control number 1 (SIC001) were purchased from Sigma-Aldrich. MISSION® esiRNA are a heterogeneous mixture of siRNAs that all target the same mRNA sequence, which conducts highly specific and effective gene silencing. Control and *FNDC4* siRNAs (100 pmol/L, final concentration) were complexed with 5 μL of Lipofectamine® 2000 reagent (Invitrogen) in serum-freeOpti-MEM®I (Invitrogen), according to the manufacturer's protocol. After 20-min incubation at room temperature (RT), the mix was added to cells and incubated at 37 °C for 4 h. The transfection mixes were then completely removed and fresh adipocyte culture media were added. Knockdown effectiveness was determined after 24 h by real-time PCR. MISSION® esiRNA *FNDC4* treatment resulted in an average knockdown of 86% and 87% of *FNDC4* transcripts in adipocytes and SVFC, respectively (**Supplemental Fig.1**).

2.9. Measurement of intracellular triacylglycerol accumulation

Intracellular triacyglycerol (TG) content was measured by enzymatic methods as well as by Oil Red O staining, in accordance with previously published procedures [29].

2.10. Analysis of mitochondrial DNA content

The amount of mitochondrial DNA (mtDNA), extracted and purified using DNeasy Blood and Tissue Kit (Qiagen), was determined by real-timePCR of the mitochondrial cytochrome B(*CYTB*) gene normalized to the nuclear β-actin(*ACTB*) gene (**Supplemental Table1**), as previously described [31]. The real-timePCR was performed with 25 ng of total DNA using the TaqMan® Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions.

2.11. Immunocytochemistry of UCP1

Differentiated human omental adipocytes were grown on glass coverslips and fixed in 4% formaldehyde as described earlier [11]. Cells were incubated overnight at 4 °C with rabbit polyclonal anti-UCP1 antibody (ab10983, Abcam) (1:500). After washing three times, cells were incubated with Dako Real™ EnVision™ HRP-conjugatedanti-rabbit/mouse (Dako) for 1 h at RT. After washing in PBS, the peroxidase reaction was visualized with a 3,3'-DAB/H_2O_2 solution (Dako), as chromogen and Harris hematoxylin solution (Sigma) as counterstaining.

2.12. Statistical analysis

Statistical analyses were performed using the SPSS 15.0 software. Data are expressed as mean ± S.E.M. The normality of the variables of the study was assessed by the Kolmogorov-Smirnov's and Shapiro-Wilk's test. Parameters that did not fulfil normal distribution criteria [*i.e.* circulating triacyglycerol or C-reactive protein (CRP)] were logarithmically transformed (log10) to improve symmetry for statistical analyses. Differences between mean values were determined using Student's *t*-test or onewayANOVA followed by Scheffé's or Dunnett's tests, where appropriate, for quantitative variables, and assessment of chi-square (χ^2) distributions for categorical variables. Comparisons between pre- and post-surgical values were made by paired two-tailed Student's *t*-tests. Pearson's correlation coefficients (r)and stepwise multiple linear regression analysis were used to analyze the association between variables. A P value <.05 was considered statistically significant.

3. Results

3.1. Low plasma FNDC4 concentrations in patients with obesity were related to systemic inflammation and remained unchanged after bariatric surgery

The clinical characteristics of our cohort are shown in Table1. Twenty-five patients (24.0%) were on antihypertensive treatment, 11 subjects (10.5%) were on oral antidiabetic drugs and 12 individuals (11.5%) were on lipid-lowering medications. Plasma FNDC4

Table1
Clinical characteristics of the subjects included in the study.

	Lean	Obese NG	Obese IGT/T2D	*P*
n	26	37	41	–
Sex (male/female)	13/13	14/23	18/23	0.628
Age (years)	45 ± 3	40 ± 2	45 ± 1	0.053
Height (m)	1.67 ± 0.02	1.66 ± 0.02	1.66 ± 0.01	0.814
Weight (kg)	65 ± 3	124 ± 4[a]	126 ± 4[a]	<**0.00001**
BMI (kg/m^2)	22.9 ± 0.6	45.3 ± 1.7[a]	46.4 ± 1.4[a]	<**0.00001**
Fat free mass (%)	79.7 ± 2.0	50.0 ± 1.4[a]	48.5 ± 1.2[a]	<**0.00001**
Body fat (%)	20.5 ± 1.8	50.1 ± 1.4[a]	51.4 ± 1.1[a]	<**0.00001**
Visceral fat (%)	14.7 ± 2.3	50.9 ± 1.3[a]	48.9 ± 1.4[a]	<**0.00001**
Waist circumference (cm)	76 ± 4	124 ± 3[a]	129 ± 3[a]	<**0.00001**
REE (kcal)	–	2266 ± 88	2445 ± 103	0.203
Glucose (mg/dL)	90 ± 3	91 ± 2	123 ± 7[b]	<**0.00001**
Glucose 2-h OGTT (mg/dL)	–	118 ± 4	199 ± 16[b]	<**0.00001**
Insulin (μU/mL)	5.7 ± 0.8	20.2 ± 2.1	27.5 ± 4.9[a,b]	**0.007**
Insulin 2-h OGTT (μU/mL)	–	109.4 ± 12.5	107.4 ± 13.9	0.915
HOMA	1.3 ± 0.2	4.7 ± 0.5	8.9 ± 1.8[a]	**0.006**
QUICKI	0.38 ± 0.01	0.32 ± 0.01[a]	0.31 ± 0.01[a]	<**0.00001**
FFA (mg/dL)	15.0 ± 1.3	17.6 ± 1.1	18.6 ± 1.9	0.226
Glycerol (mg/dL)	18.7 ± 2.5	35.1 ± 4.0[a]	44.0 ± 5.1[a]	<**0.0001**
Adipo-IR index	20 ± 2	91 ± 13[a]	109 ± 20[a]	**0.006**
Triacylglycerol (mg/dL)	60 ± 5	125 ± 14	156 ± 24[a]	**0.028**
Total cholesterol (mg/dL)	191 ± 6	194 ± 6	190 ± 6	0.888
LDL-cholesterol (mg/dL)	108 ± 5	121 ± 6	118 ± 5	0.422
HDL-cholesterol (mg/dL)	67 ± 6	47 ± 3[a]	44 ± 2[a]	<**0.0001**
AST (IU/L)	21 ± 3	15 ± 1	16 ± 1	0.330
ALT (IU/L)	24 ± 6	26 ± 3	27 ± 2	0.803
Alkaline phosphatase (IU/L)	85 ± 10	103 ± 6	101 ± 6	0.276
γ-GT (IU/L)	16 ± 5	20 ± 2	29 ± 4	0.051
CRP (mg/L)	2.1 ± 0.6	8.6 ± 1.6[a]	9.8 ± 2.0[a]	**0.002**
Uric acid (mg/dL)	4.4 ± 0.2	7.9 ± 1.8	6.2 ± 0.2	0.310
Leptin (ng/mL)	6.6 ± 1.0	44.5 ± 4.5[a]	52.0 ± 5.5[a]	**0.001**
Antihypertensive therapy, n (%)	0 (0%)	12 (5.4%)	13 (32.5%)	0.102
Antidiabetic therapy, n (%)	0 (0%)	2 (4.5%)	9 (22.5%)	**0.035**
Lipid-lowering therapy, n (%)	0 (0%)	6 (16.2%)	6 (15.0%)	0.400

NG, normoglycaemia; IGT, impaired glucose tolerance; T2D, type 2 diabetes; BMI, body mass index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; REE, resting energy expenditure; OGTT, oral glucose tolerance test; FFA, free fatty acids; CRP, high-sensitivity C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, -glutamyltransferase. Bold values denote statistically significant *P* values. Differences between groups were analyzed by one-way ANOVA followed by Scheffé's test or χ^2 test, where appropriate. [a]$P < .05$ *vs* lean NG subjects; [b]$P < .05$ *vs* obese NG patients.

concentrations ranged from 6.2 to 761.6 ng/mL, and the mean was 117.4 ng/mL, while plasma levels of FNDC5 ranged from 0.17 to 9.69 ng/mL, and the mean was 3.05 ng/mL. No drug effects were found to influence circulating concentrations of FNDC4 and FNDC5 (all $P > .05$). No sexual dimorphism was found in plasma FNDC4 of our cohort (females 118.1 ± 16.0, males 116.5 ± 16.5 ng/mL, $P = .944$). However, lower plasma FNDC5 concentrations were observed in women compared with those in men (females 2.77 ± 0.20, males 3.42 ± 0.25 ng/mL, $P = .038$). Obesity was associated with a reduction in circulating concentrations of FNDC4 (obese 99.2 ± 7.5, lean 162.2 ± 34.2 ng/mL, $P = .012$) and FNDC5 (obese 2.84 ± 0.15, lean 3.62 ± 0.41 ng/mL, $P = .027$) compared with normal-weight individuals (Fig.1). Accordingly, univariate analyses showed a negative correlation between both secreted factors and markers of adiposity (FNDC4 and body weight, $r = -0.21$, $P < .05$; FNDC5 and BMI, $r = -0.20$, $P < .05$). Noteworthy, plasma FNDC4, but not FNDC5, was positively correlated with C-reactive protein, a marker of obesity-associated systemic inflammation (**Supplemental Table2**). Even if the cohort is split according to obesity status, the positive association of plasma FNDC4 with CRP ($r = 0.37$, $P = .004$) was maintained in patients with morbid obesity, while it was lost in normal-weight individuals ($r = 0.06$, $P = .938$). No effect of glucose intolerance or T2D was observed in plasma FNDC4 and FNDC5 (both $P > .05$) (Fig.1). Multiple linear regression analysis revealed that insulin sensitivity, as evidenced by the QUICKI index, was not a major determinant for circulating concentrations of FNDC4 and FNDC5 (Table2). Sex ($P < .05$) and body composition ($P < .05$) accounted for 12.5% ($P < .05$) of plasma FNDC5 variance, but not to FNDC4 variance. Otherwise, C-reactive protein ($P < .01$) contributed independently to 18.4% ($P < .05$) of plasma FNDC4 levels after controlling for the effects of age, sex and body composition.

Since one of the best-known hepatic alterations associated with obesity is NAFLD, we also aimed to investigate the regulation of FNDC4 and FNDC5 in this condition. The presence of biopsy-provenNAFLD was associated with increased circulating concentrations of FNDC4 (NAFLD 99.68 ± 11.69, non-NAFLD53.07 ± 18.53 ng/mL, $P = .047$) and FNDC5 (NAFLD 2.74 ± 0.25, non-NAFLD1.42 ± 0.33 ng/mL, $P = .038$) in patients with morbid obesity. A previous study found that circulating FNDC5/irisin was positively associated with portal inflammation in patients with biopsy-provenNAFLD [32]. In line with this observation, univariate analyses revealed a positive correlation of circulating FNDC4 and FNDC5 with AST, a marker of liver injury (**Supplemental Table3**). We, therefore, speculate that both FNDC4 and FNDC5 levels may increase with the worsening of NAFLD, acting, at least in part, as compensatory mechanisms to limit liver inflammation.

To gain further insight into the relationship of FNDC4 with obesity-associated metabolic derangements and inflammation, we next investigated the impact of weight loss achieved by bariatric surgery on the circulating levels of the myokine. Sleeve gastrectomy and RYGB constitute the most widely used bariatric surgical procedures with proven effects on metabolic improvement and obesity resolution [33]. As expected, after an average period of 6 months, patients with morbid obesity undergoing bariatric surgery experienced a profound decrease in body weight, whole-body and visceral adiposity, an improved lipid profile and an increase in insulin sensitivity (Table3). These anthropometric and metabolic improvements were more evident for RYGB than for sleeve gastrectomy. No significant changes were observed in inflammatory markers, such as C-reactive protein or uric acid, 6 months after both surgical procedures. Noteworthy, no changes were observed in plasma FNDC4 after bariatric surgery (Fig.1C and E), whereas FNDC5 levels

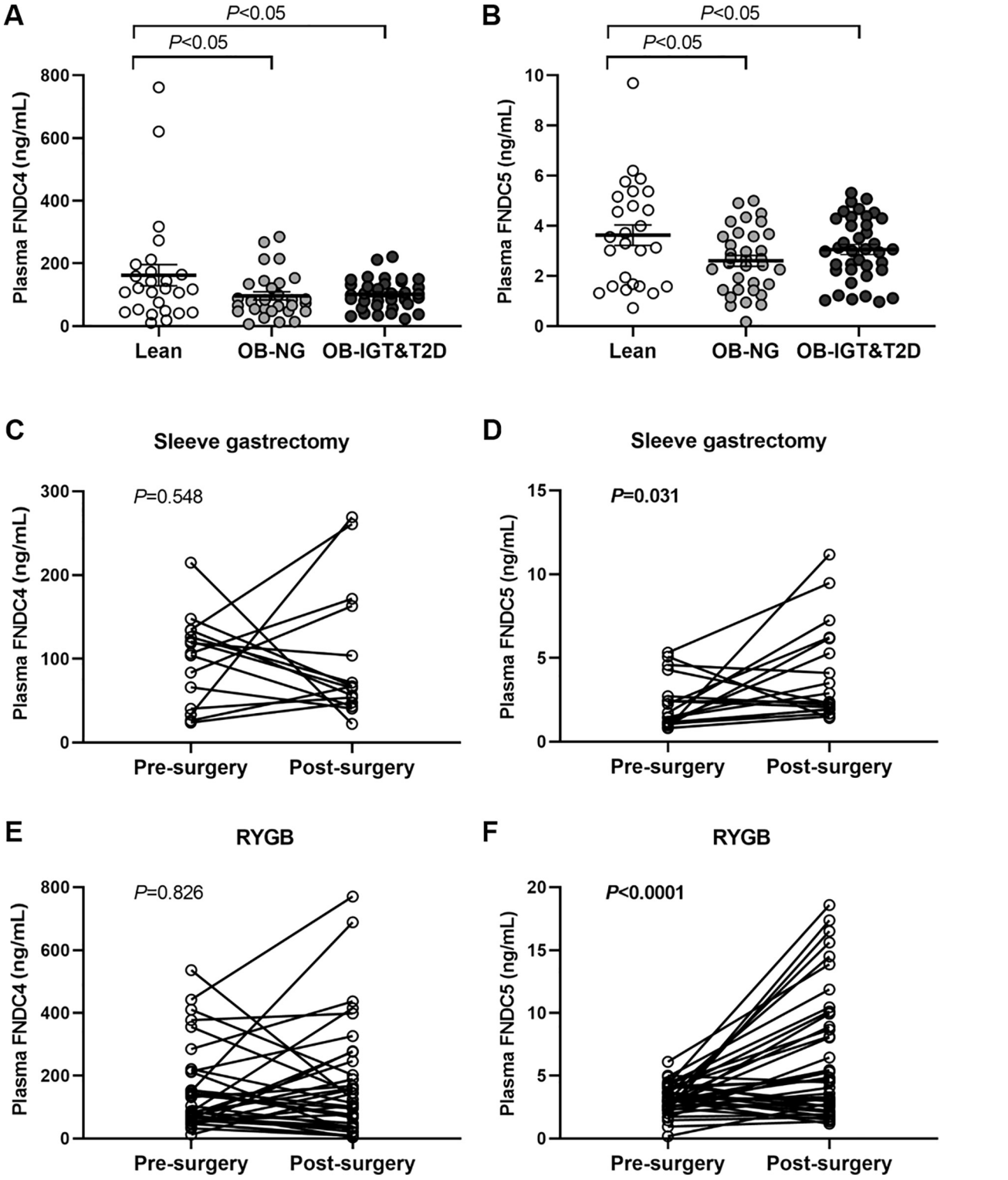

Fig.1. Effect of obesity, insulin resistance and weight loss achieved by bariatric surgery on plasma FNDC4 concentrations. Fasting plasma FNDC4 (A)and FNDC5 (B)in lean individuals and patients with obesity and NG or IGT&T2D. Comparison of plasma FNDC4 and FNDC5 concentrations before and after weight loss achieved by sleeve gastrectomy (C, D)or RYGB (E, F). Statistical differences were assessed by one-way ANOVA followed by Scheffé's test or by two-tailed paired Student's *t*-test, where appropriate.

were significantly increased after sleeve gastrectomy (baseline 2.27 ± 0.36, 6 months 4.08 ± 0.69 ng/mL, $P = .031$) and RYGB (baseline 3.14 ± 0.18, 6 months 6.54 ± 0.77 ng/mL, $P < .001$) (Fig.1D and F). Interestingly, the correlations of FNDC4 with markers of adiposity (body weight $r = -0.11$, $P = .422$; BMI,$r = -0.18$, $P = .191$) and CRP($r = 0.17$, $P = .468$) were not observed 6 months after bariatric surgery, which might reflect the post-surgical improvement of obesity-associated inflammation.

3.2. Characterization of FNDC4 in human adipose tissue

The human adipose tissue constitutes a production site of FNDC4 (Fig.2). When compared to the transcriptional levels of *FNDC5*, FNDC4 was the most abundantly expressed adipokine in both omental (*FNDC4*1.00 ± 0.00 *vs* *FNDC5*0.49 ± 0.12, $P < .0001$) and subcutaneous (*FNDC4*1.00 ± 0.00 *vs* *FNDC5*0.42 ± 0.08, $P < .0001$) fat depots. To

Table2
Multiple linear regression analyses with plasma FNDC4 and FNDC5 as dependent variables for all subjects in the cross-sectional study.

	Plasma FNDC4		Plasma FNDC5	
Model I	*β*	*P*	*β*	*P*
Age	−1.493	**0.030**	0.011	0.444
Sex	−4.338	0.812	−1.077	**0.014**
Fat mass (%)	2.848	0.785	−0.477	**0.047**
Fat free mass (%)	1.723	0.873	−0.490	**0.048**
QUICKI	218.272	0.255	−1.449	0.717
CRP	21.594	**0.002**	0.149	0.326
Adjusted R^2	0.184	**0.015**	0.125	**0.038**
Model II	*β*	*P*	*β*	*P*
Age	−1.335	0.052	0.009	0.527
Sex	0.739	0.966	−0.755	0.053
Fat mass (%)	5.732	0.584	−0.386	0.111
Fat free mass (%)	5.498	0.610	−0.379	0.127
CRP	19.945	**0.004**	0.137	0.373
Adjusted R^2	0.140	**0.025**	0.094	0.056
Model III	*β*	*P*	*β*	*P*
Age	−1.031	0.343	0.012	0.382
Sex	−4.143	0.876	−0.892	**0.030**
Fat mass (%)	16.261	0.312	−0.494	**0.025**
Fat free mass (%)	17.200	0.289	−0.508	**0.023**
QUICKI	243.079	0.393	−2.438	0.498
Adjusted R^2	0.013	0.336	0.133	**0.012**

BMI, body mass index; CRP, C-reactive protein; WBC, white blood cells; QUICKI, quantitative insulin sensitive check index. *β* is the regression coefficient, which allows evaluating the relative significance of each independent variable in multiple linear regression analyses. Adjusted R^2 expresses the percentage of the variance explained by the independent variables in the different models (*i.e.* 0.133 is 13.3%). Statistical significant values are inbold.

identify the cell type responsible for FNDC4 and FNDC5 production in human adipose tissue, the presence of both factors was evaluated by immunohistochemistry and real-time PCR. A positive brown immunostaining for FNDC4 and FNDC5 was observed in fully mature adipocytes, as well as in the SVFC of omental and subcutaneous fat depots from obese patients (Fig.2A-B). Adipose tissue fraction analysis revealed an increase ($P < .05$) in *FNDC4* mRNA levels in adipocytes in comparison to SVFC, which, otherwise, was the main source of *FNDC5* in omental adipose tissue (Fig.2C). We next examined potential fat depot- and sex-differences in *FNDC4* and *FNDC5* mRNA expression. *FNDC4* was expressed to a higher ($P < .0001$) extent in the subcutaneous adipose tissue (Fig.2D), while no fat depot-related differences were found for *FNDC5* transcript levels. No sexual dimorphism was detected for the gene expression of *FNDC4* and *FNDC5* in omental and subcutaneous adipose tissue (all $P > .05$) (Fig.2E-F).

3.3. Obesity was associated with an altered expression of FNDC4 and its receptor GPR116 in human adipose tissue

We next sought to investigate the impact of obesity and insulin resistance on the expression of FNDC4 and FNDC5 and their recently identified receptors G-protein-coupled receptor 116 (GPR116) [26,34] and integrin αV (ITGA5) [12], respectively, in human adipose tissue. Patients with obesity exhibited an upregulation ($P < .05$) of mRNA and protein expression of FNDC4 in visceral adipose tissue, whereas FNDC5 mRNA and protein were downregulated ($P < .05$) in visceral and subcutaneous fat depots (Fig.3A-F). Higher GPR116 and ITGA5 mRNA and protein expression was found in visceral adipose tissue in association with obesity, without changes in the expression of these receptors in the subcutaneous fat depot (Fig.3A-F). Interestingly, FNDC4 protein levels in omental adipose tissue were positively associated with body weight, BMI, body fat and leptin, while those of FNDC5 were negatively correlated with these markers of adiposity (**Supplemental Table3**). In addition, the protein expression of both FNDC4 and FNDC5 were negatively correlated with serum transaminases AST and ALT, respectively, which are indicators of liver injury. A negative association of omental adipose tissue FNDC5 with markers of systemic inflammation, including C-reactive protein, was also detected. Insulin resistance did not modify the gene and protein expression of both adipokines in omental and subcutaneous adipose tissue (Fig.3A-F). Local adipocyte insulin resistance, as evidenced by Adipo-IR, was not a major determinant for FNDC4 and FNDC5 expression in adipose tissue or their circulating levels (**Supplemental Tables2 and 3**). The influence of local adipose tissue inflammation on FNDC4 and FNDC5 was also investigated in visceral adipose tissue, a depot with high macrophage accumulation in the obese state. We measured the transcript levels of *CD68*, a pan-marker of macrophages, in SVFC from omental adipose tissue from patients with morbid obesity. The gene expression of *CD68* was increased approximately 2-fold in SVFC from insulin-

Table3
Effect of weight loss achieved by sleeve gastrectomy or RYGB on clinical characteristics of obese patients.

	Sleeve gastrectomy (n = 18)			RYGB (n = 42)		
	Pre-surgery	Post-surgery	*P*	Pre-surgery	Post-surgery	*P*
Sex (male/female)	6	12	0.347	18	24	0.347
Age (years)	47 ± 3	48 ± 3	**0.002**	41 ± 2	42 ± 2	<**0.0001**
Weight (kg)	103 ± 4	76 ± 4	<**0.0001**	132 ± 4	101 ± 3	<**0.0001**
BMI (kg/m^2)	41.3 ± 2.8	29.0 ± 1.4	<**0.0001**	48.1 ± 2.5	37.1 ± 1.7	<**0.0001**
Fat free mass (%)	50.2 ± 1.9	63.6 ± 2.5	<**0.0001**	49.4 ± 1.4	58.2 ± 1.8	<**0.0001**
Body fat (%)	49.8 ± 1.8	36.5 ± 3.0	<**0.0001**	50.6 ± 1.4	42.1 ± 1.8	<**0.0001**
Waist circumference (cm)	119 ± 3	95 ± 3	<**0.0001**	131 ± 4	112 ± 3	<**0.0001**
Glucose (mg/dL)	115 ± 12	94 ± 5	**0.013**	106 ± 6	91 ± 2	**0.001**
Insulin (mg/dL)	33.7 ± 8.0	10.2 ± 2.1	**0.024**	25.6 ± 3.9	17.8 ± 3.0	**0.029**
HOMA	8.8 ± 2.8	2.2 ± 0.4	**0.030**	9.1 ± 1.5	4.3 ± 0.7	**0.022**
QUICKI	0.30 ± 0.04	0.37 ± 0.06	<**0.0001**	0.30 ± 0.01	0.34 ± 0.01	<**0.0001**
Triacylglycerol (mg/dL)	117 ± 20	92 ± 12	0.166	161 ± 29	99 ± 7	**0.028**
Total cholesterol (mg/dL)	170 ± 7	181 ± 9	0.329	196 ± 6	156 ± 5	<**0.0001**
LDL-cholesterol (mg/dL)	98 ± 6	103 ± 8	0.654	124 ± 6	97 ± 4	<**0.0001**
HDL-cholesterol (mg/dL)	48 ± 5	59 ± 4	<**0.00001**	46 ± 4	41 ± 2	0.104
AST (IU/L)	15 ± 2	15 ± 1	0.872	16 ± 1	18 ± 1	0.175
ALT (IU/L)	23 ± 3	17 ± 2	0.062	27 ± 3	26 ± 2	0.796
Alkaline phosphatase (IU/L)	72 ± 8	63 ± 5	0.416	109 ± 5	112 ± 4	0.535
γ-GT (IU/L)	25 ± 4	22 ± 4	0.189	26 ± 4	15 ± 1	**0.005**
CRP (mg/L)	5.2 ± 1.5	1.5 ± 0.6	0.080	7.6 ± 1.5	4.2 ± 0.1	0.080
Uric acid (μmol/L)	5.2 ± 0.4	4.8 ± 0.4	0.329	8.2 ± 1.7	5.3 ± 0.2	0.103
Leptin (ng/mL)	60.3 ± 12.2	16.6 ± 3.9	**0.015**	48.6 ± 5.6	26.4 ± 3.5	**0.003**

BMI, body mass index; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; NEFA, non-esterified fatty acids; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, γ-glutamyltransferase; CRP, high-sensitivity C-reactive protein; TNF-α, tumor necrosis factor γ. Bold values denote statistically significant *P* values. Differences between pre- and post-surgical values were analyzed by paired two-tailed Wilcoxon *t*-test.

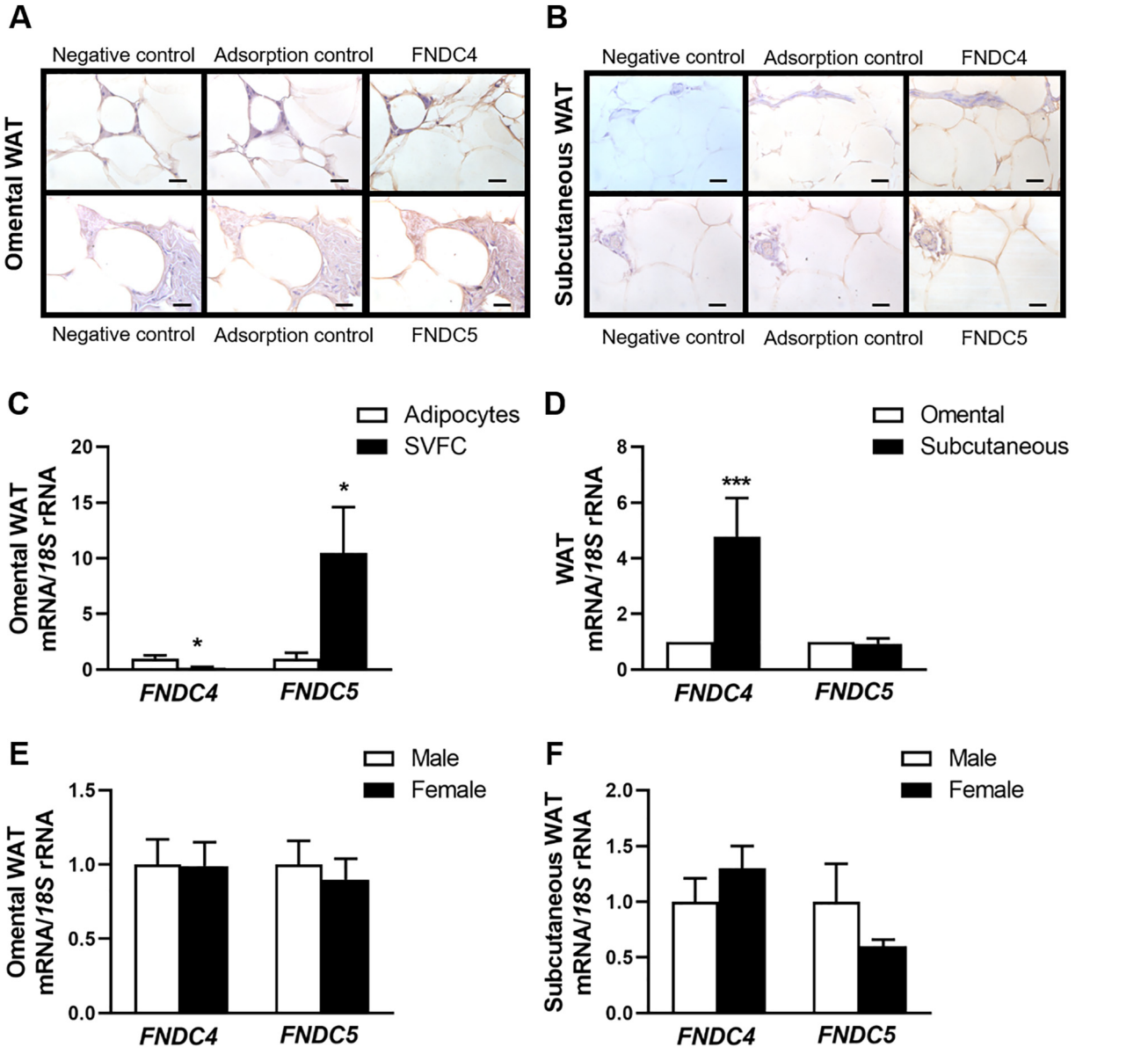

Fig.2. Characterization of FNDC4 in human adipose tissue. Immunohistochemical staining for FNDC4 and FNDC5 in paired omental (A)and subcutaneous (B)WAT ($n = 5$ per group) (magnification ×200, scale bar = 50 μm). (C)Transcript levels of *FNDC4* and *FNDC5* in subfractions of adipocytes and stroma-vascular fraction cells (SVFC) of omental adipose tissue obtained from patients with obesity. (D)Fat-depot- and (E, F)sex-dependent differences in mRNA levels of *FNDC4* and *FNDC5* in human adipose tissue. The gene expression in the subfractions of adipocytes, in omental fat depots or in males was considered 1. Statistical differences were assessed by Student's *t*-test. $^{*}P < .05$, $^{***}P < .001$ *vs.* subfractions of adipocytes, omental adipose tissue or male subjects.

resistant patients compared to insulin-sensitive individuals, but without statistical changes (IGT/T2D 3.10 ± 1.26 *vs*NG1.50 ± 0.57 A.U., $P > .05$). Interestingly, a strong negative correlation was found between the transcripts of *FNDC4*($r = -0.87$, $P < .01$) and *FNDC5*($r = -0.90$, $P < .001$) in adipocytes and *CD68* mRNA in SVFC obtained from paired fat samples, suggesting an anti-inflammatory role of FNDC4 and FNDC5 in visceral fat.

3.4. FNDC4 expression was regulated by lipogenic, lipolytic and proinflammatory factors in human adipocytes

Given the metabolic relevance of the visceral adipose tissue, the next experiments were focused on this fat depot. Since our results point to a strong association of FNDC4 with the control of human adipose tissue expansion and inflammation, the effect of lipogenic (insulin, acylated and desacyl ghrelin), lipolytic (leptin, isoproterenol and ANP), and pro-inflammatory (TNF-α, TGF-β and LPS) factors on the regulation of FNDC4 and FNDC5 was assessed in human omental adipocytes. The stimulation with acylated and desacyl ghrelin markedly up-regulated*FNDC4* and *FNDC5* mRNA (Figs.4A-B) in adipocytes, while insulin did not affect their transcription levels. Leptin (Figs.4C-D) and TNF-α(Figs.4E-F) treatment down-regulated*FNDC4* and *FNDC5* gene expression, while neither isoproterenol nor ANP modified them. *FNDC5* transcript levels, but not *FNDC4* mRNA, were up-regulated in response to TGF-β and LPS(Figs.4E-F).

The potential self- and cross-regulation between FNDC4 and FNDC5 and their receptors was also determined. FNDC4 treatment upregulated its own expression at the physiological concentration of 10 ng/mL, and downregulated ITGA5 only at the highest concentration (100 ng/mL) (**Supplemental Fig.2A**). By contrast, FNDC5 downregulated the gene expression of *FNDC4* and the receptors *GPR116* and *ITGA5* at all concentrations tested, and only the pharmacological concentration of 100 ng/mL reduced its own mRNA expression (**Supplemental Fig.2B**).

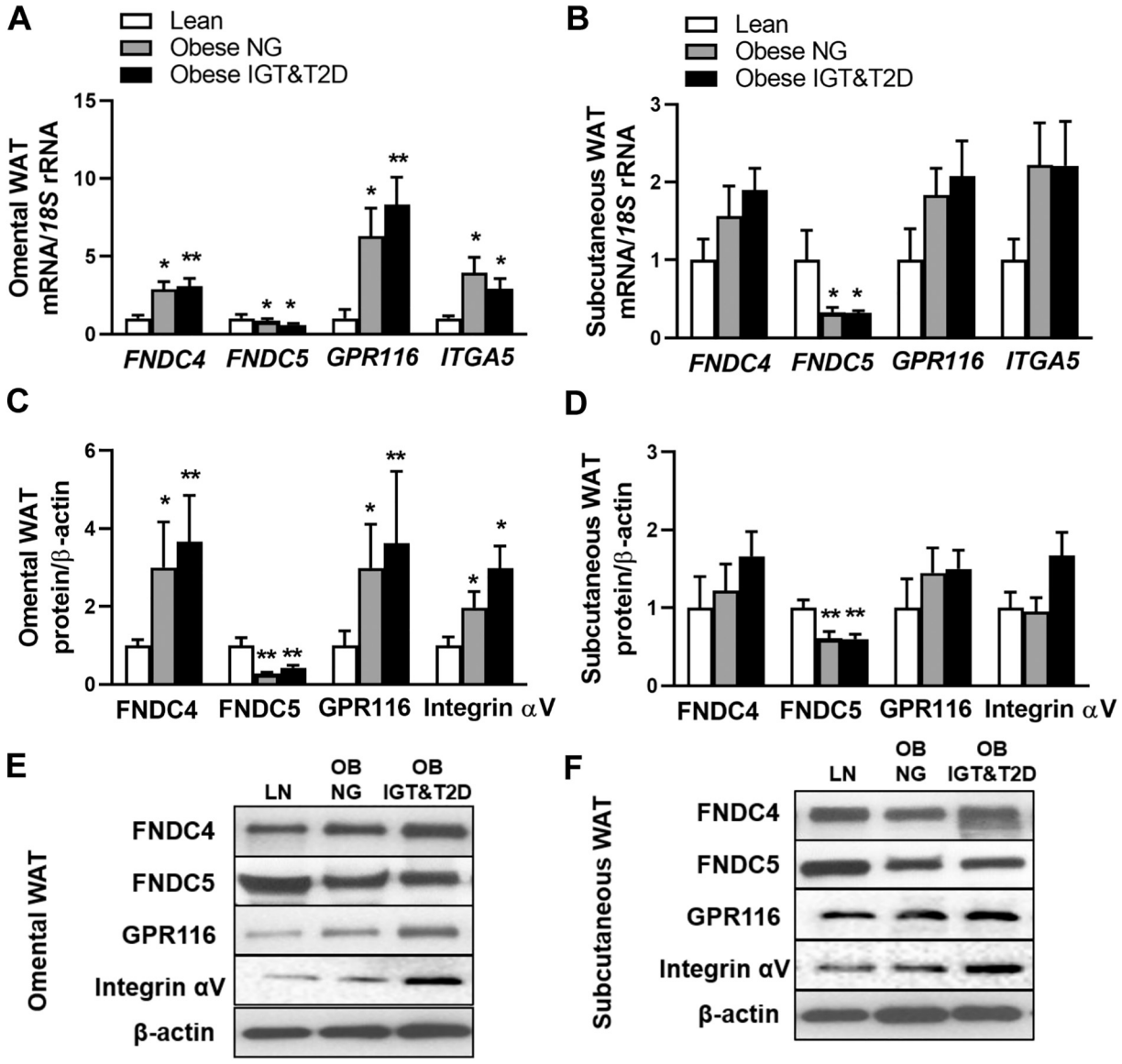

Fig.3. Impact of obesity and insulin resistance on the expression of FNDC4 and its receptor GPR116 in human adipose tissue. Gene (A, B)and protein (C, D)expression of FNDC4, FNDC5, GPR116 and integrin αV in paired omental and subcutaneous WAT samples obtained from lean individuals and patients with obesity and NG or IGT&T2D. The gene and protein expression in lean patients was considered 1. Representative blots of omental (E)and subcutaneous (F)adipose tissue are shown. Statistical differences were assessed by a one-way ANOVA followed by Tukey's test. $^{*}P < .05$, $^{**}P < .01$ *vs.* lean individuals.

3.5. FNDC4 inhibited lipogenesis in human adipocytes

To explore the direct effect of FNDC4 on human adipocyte metabolism, both SVFC and differentiated adipocytes were exposed to increasing concentrations of FNDC4 (1, 10 and 100 ng/mL) for 24 h. The effect on lipogenesis was assessed by examining the mRNA expression of the master transcription factor of adipogenesis *PPARG2* and its downstream target genes, fatty acid binding protein 4 (*FABP4*, also known as aP2) and adiponectin (*ADIPOQ*), as well as the lipid accumulation accompanying this biological process by enzymatic methods and Oil Red O staining (Fig.5). The size of lipid droplets was markedly decreased in the presence of physiological concentrations of both FNDC4 and FNDC5, compared with unstimulated cells (Fig.5A). The addition of FNDC4 induced a significant decrease ($P < .05$) in *PPARG2*, *FABP4* and *ADIPOQ* transcript levels in both differentiated adipocytes and SVFC(Fig.5B). Similarly, FNDC5 stimulation diminished ($P < .05$) *PPARG2* and *FABP4* gene expression in both adipocytes and SVFC, but the downregulation ($P < .05$) of *ADIPOQ* mRNA was only evident in adipocytes (Fig.5D). In agreement with these results, both FNDC4 and FNDC5 significantly reduced ($P < .05$) lipid accumulation in differentiated adipocytes and SVFC(Fig.5C and E). To gain further insight to the anti-lipogenic action of FNDC4, we reduced the constitutive expression levels of *FNDC4* in human visceral adipocytes using a pool of siRNAs targeting *FNDC4* mRNA. Differentiated adipocytes with *FNDC4* gene silencing exhibited an upregulation of *PPARG2, FABP4* and *ADIPOQ* (both $P < .05$) transcript levels (Fig.5F). Notably, intracellular triacylglycerol accumulation was increased by 87% in *FNDC4*-silenced adipocytes (Fig.5G). *FNDC4*-knockdown SVFCs did not change the expression of *PPARG2, FABP4* and *ADIPOQ* (both $P > .05$).

3.6. FNDC4 increased mitochondrial biogenesis and fat browning in human adipocytes

Subsequently, we sought to elucidate the potential role of FNDC4 as a driver of fat browning in human adipocytes. The 10 ng/mL concentrations of FNDC4 and FNDC5 stimulated a brown-like pattern after 24-h treatment in human adipocytes, as evidenced by an upregulated expression of UCP-1(Fig.6A). In parallel, an upregulation of brown- and beige

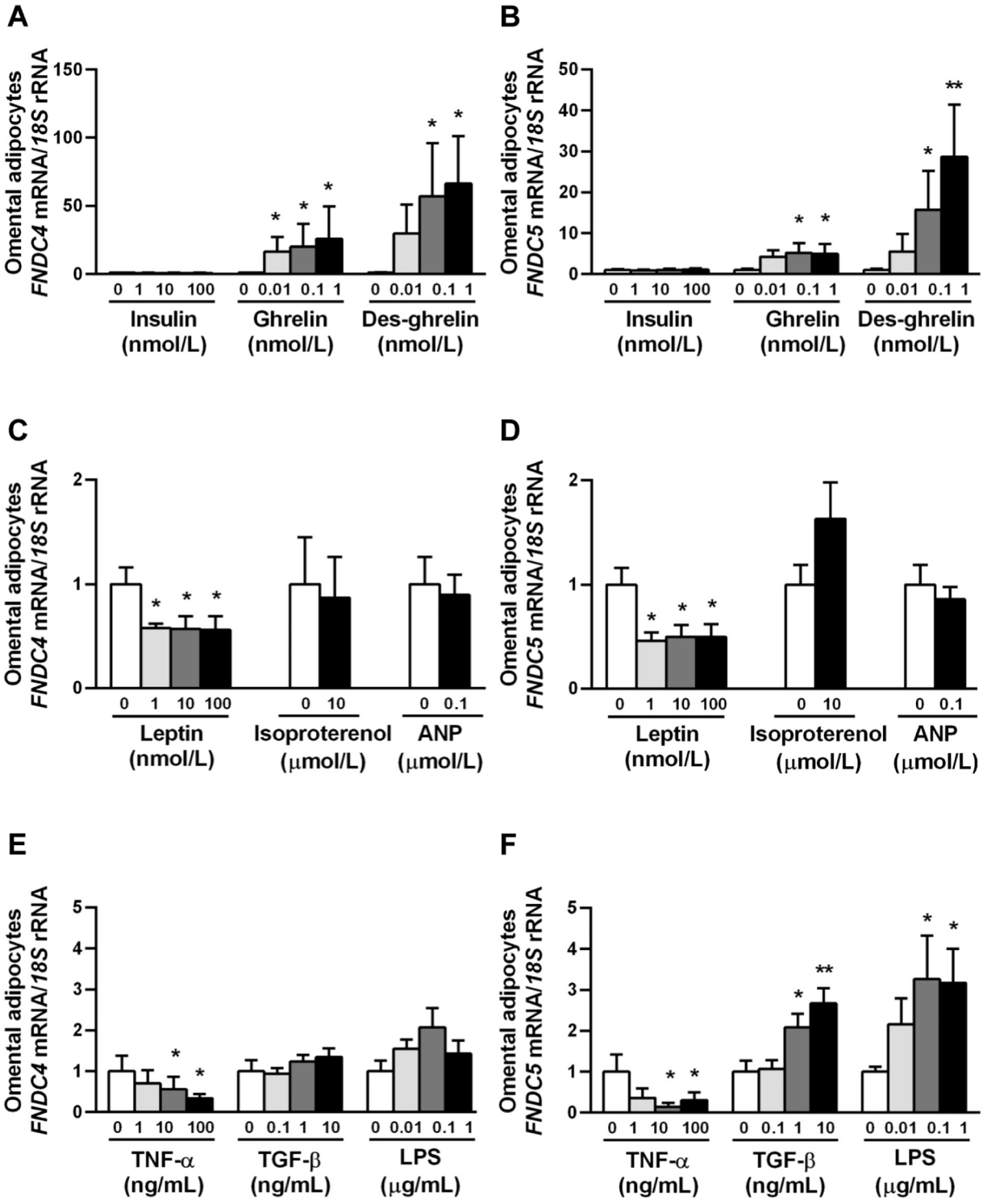

Fig.4. Regulation of FNDC4 in human visceral adipocytes. Effect of lipogenic (A, B), lipolytic (C, D), and pro-inflammatory (E, F)stimuli on the transcription of *FNDC4* and *FNDC5* in human differentiated omental adipocytes. The mRNA levels of unstimulated cells were considered 1. Statistical differences were assessed by one-way ANOVA followed by Dunnett's test. $^{*}P < .05$, $^{**}P < .01$ *vs.* unstimulated adipocytes.

adipocyte markers, such as *PRDM16, TMEM26, CD137* and *UCP1*, was observed after a 24-h exposure to FNDC4 or FNDC5 in differentiated adipocytes, but not in SVFCs (Fig.6B and C). Noteworthy, *FNDC4* gene silencing in adipocytes downregulated the expression of the beige-adipocyte marker *CD137*(Fig.6D).

The impact of FNDC4 on mitochondrial biogenesis, a hallmark of fat browning, was also evaluated. To this end, we analyzed the mitochondrial copy number, evidenced by mtDNA content, and the expression of mitochondrial biogenesis-related factors in FNDC4-treated adipocytes and SVFCs. The highest concentrations of FNDC4 and FNDC5 induced a ~ 2-fold($P < .05$) and ~ 4-fold ($P < .05$) increase in mtDNA content in differentiated adipocytes and SVFCs (Fig. 7A and B). Although the transcript levels of PGC-1α(*PPARGC1A*) remained unchanged after FNDC4 and FNDC5 treatment, the gene expression of *NRF1* and *TFAM*, which are PGC-1-α downstream molecules and controllers of mitochondrial genome replication, was elevated following FNDC4 and

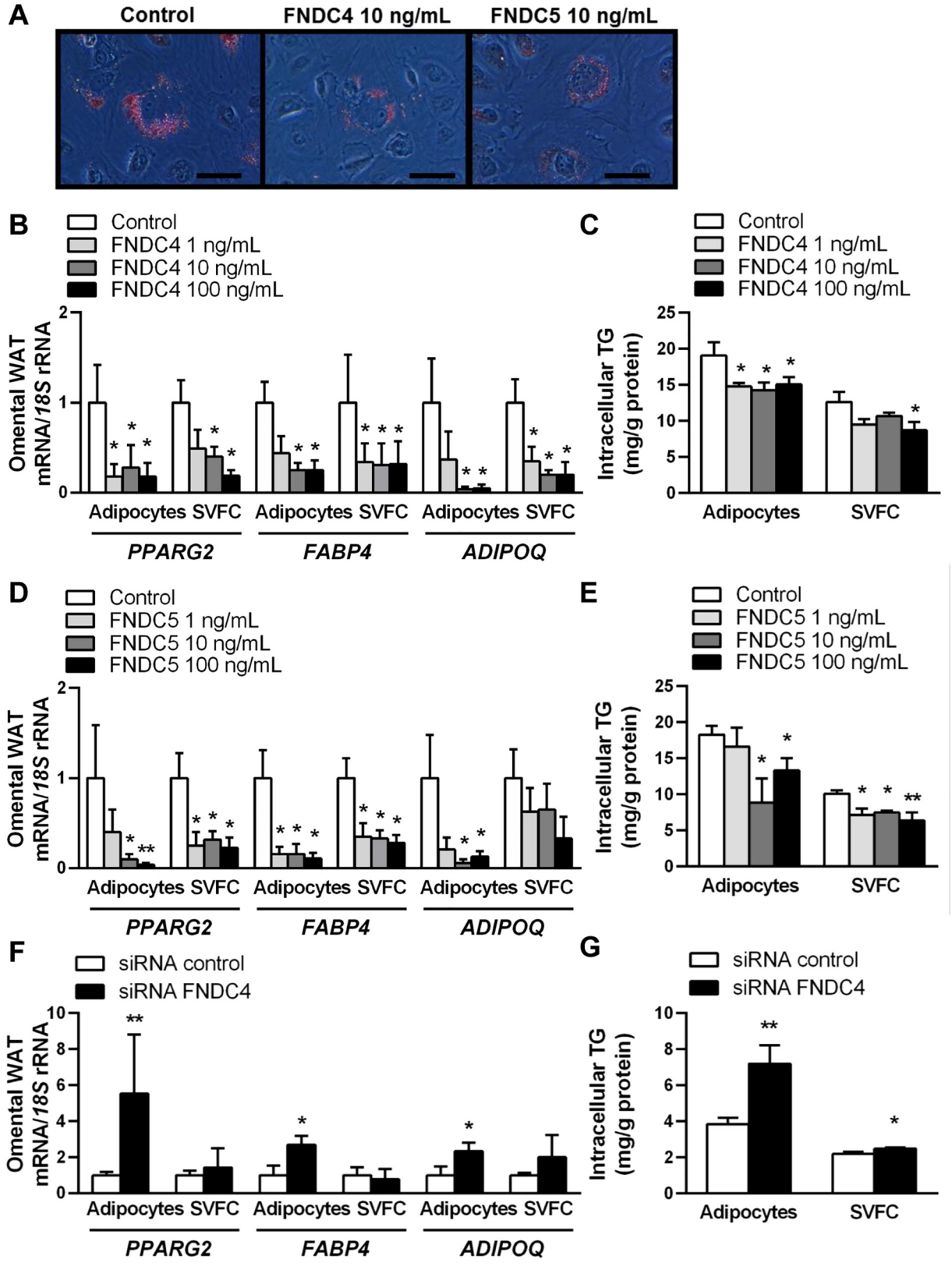

Fig.5. FNDC4 treatment inhibited human lipogenesis. (A)Representative images showing Oil Red O-stained differentiated adipocytes in the absence (*left panel*) and presence of FNDC4 (10 ng/mL) (*central panel*) or FNDC5 (10 ng/mL) (*right panel*) for 24 h (magnification ×400, scale bar = 50 μm). Bar graphs illustrate the mRNA levels of *PPARG, FABP4* and *ADIPOQ* as well as intracellular triacylglycerol content in human omental differentiated adipocytes and SVFC stimulated with increasing concentrations of FNDC4 (B, C)and FNDC5 (D, E)for 24 h as well as in *FNDC4*-knockdown adipocytes and SVFCs (F, G). The gene expression of unstimulated cells or scramble siRNA-treated cells was assumed to be 1. Statistical differences were assessed by Student's *t*-test or a one-way ANOVA followed by Dunnett's test, where appropriate. $^{*}P < .05$, $^{**}P < .01$ *vs.* unstimulated or siRNA control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

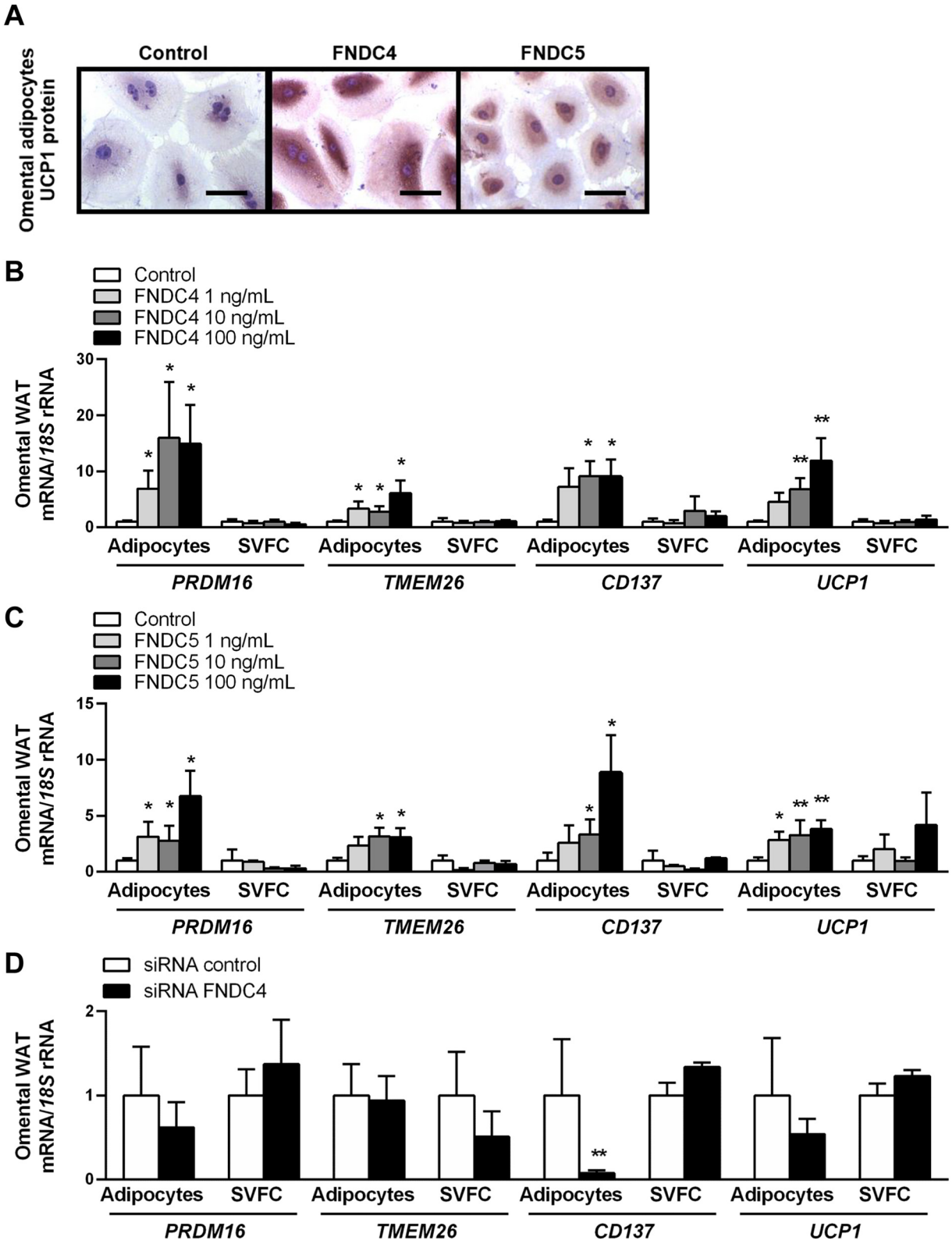

Fig.6. FNDC4 promoted fat browning in human visceral adipocytes. (A)UCP-1 staining, as evidenced by immunocytochemistry, in differentiated adipocytes in the absence (*left panel*) and presence of FNDC4 (10 ng/mL) (*central panel*) or FNDC5 (10 ng/mL) (*right panel*) for 24 h (magnification ×400, scale bar = 50 μm). Bar graphs illustrate the transcript levels of brown and beige adipocyte-specific genes in human omental differentiated adipocytes and SVFC stimulated with increasing concentrations of FNDC4 (B)and FNDC5 (C)for 24 h as well as in *FNDC4*-knockdown adipocytes and SVFC (D). The gene expression of unstimulated cells or scramble siRNA-treated cells was assumed to be 1. Statistical differences were assessed by Student's *t*-test or one-way ANOVA followed by Dunnett's test. $^{*}P < .05$, $^{**}P < .01$ *vs.* unstimulated or siRNA control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

FNDC5 treatment (10 and 100 ng/mL) in adipocytes for 24 h (Fig. 7C and D), but not in SVFCs. Interestingly, *FNDC4*-silenced adipocytes exhibited a downregulation of *NRF1* and *TFAM*(Figs.7E), confirming its role in mitochondrial biogenesis.

4. Discussion

FNDC4 is the closest homolog to FNDC5/irisin [8], a hormone that improves adipose tissue function by stimulating fat browning [9,10]

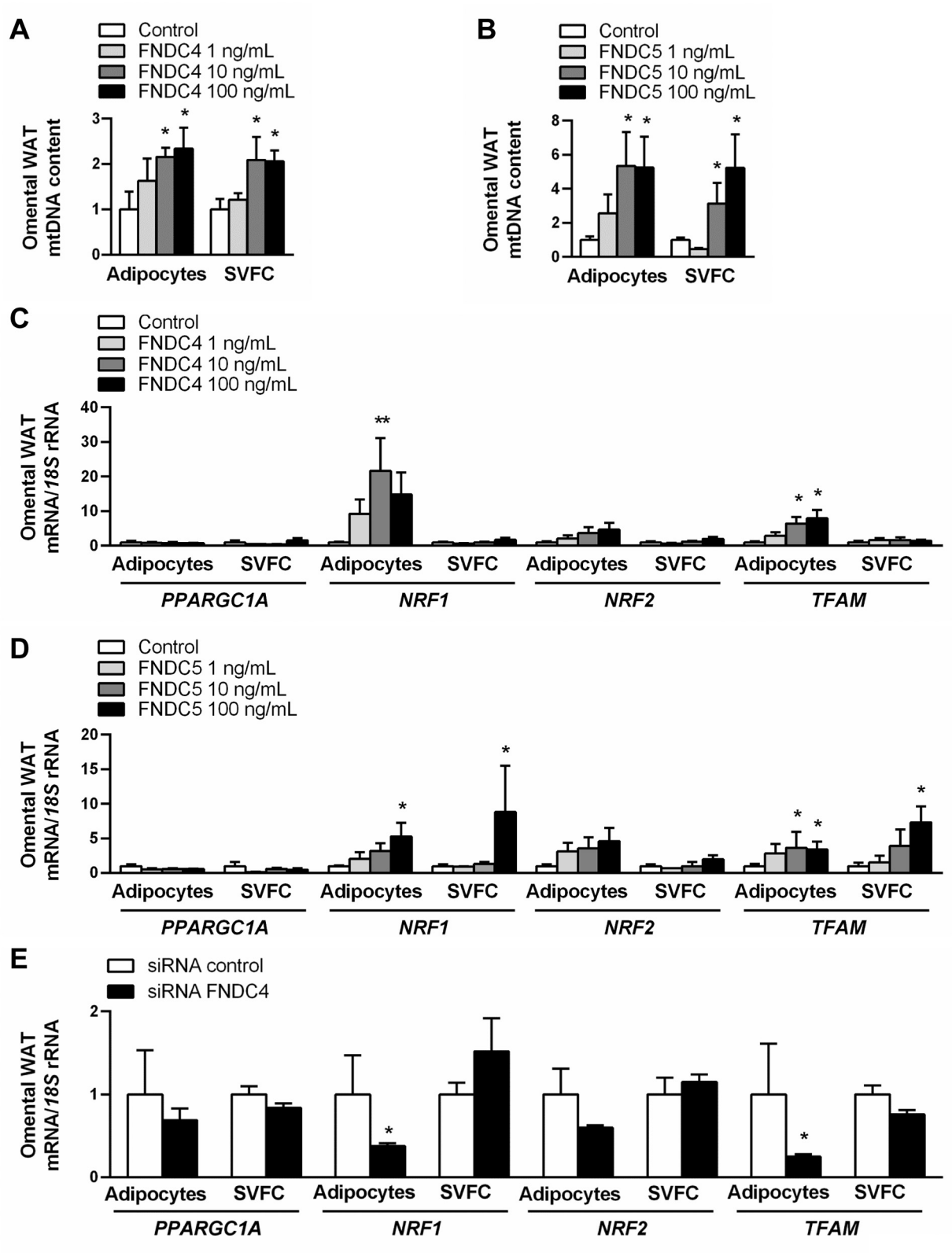

Fig.7. FNDC4 induced mitochondrial biogenesis in human visceral adipocytes. Bar graphs illustrate the mitochondrial DNA amount and the expression of factors involved in mitochondrial biogenesis in human omental differentiated adipocytes and SVFC stimulated with increasing concentrations of FNDC4 (A, C)and FNDC5 (B, D)for 24 h as well as in *FNDC4*-knockdown adipocytes and SVFC (E). The gene expression of unstimulated cells or scramble siRNA-treated cells was assumed to be 1. Statistical differences were assessed by Student's *t*-test or a one-way ANOVA followed by Dunnett's test. $^{*}P < .05$, $^{**}P < .01$ *vs.* unstimulated or siRNA control cells.

and inhibiting adipocyte differentiation [35,36] and inflammation [18,37]. Earlier studies suggest that the adipose tissue constitutes an important target for the anti-inflammatory actions of FNDC4 in mice [23,26]. This study has explored whether FNDC4 directly acts on human adipose tissue for regulating adipocyte metabolism. The main findings are that: i)plasma FNDC4 is decreased in human obesity and related to obesity-associated inflammation; ii) the visceral adipose tissue expression of FNDC4 and its receptor GPR116 are increased in obesity and obesity-associated T2D; iii) FNDC4 is regulated by lipogenic, lipolytic and proinflammatory factors in human visceral adipocytes; and iv) FNDC4 inhibits lipogenesis and stimulates fat browning and mitochondrial biogenesis in human visceral adipocytes, mimicking the anti-obesity actions of FNDC5.

In the present study, we found that plasma FNDC4 was diminished in patients with obesity and associated with systemic inflammation. Consistent with our finding, *Fndc4*, but not *Fndc5*, is robustly upregulated in three models of inflammation: i)dextran sodium sulfate-mediated colitis, ii) autoimmune glomerulonephritis; and iii) ischemic kidney [24]. In this regard, FNDC4 strongly binds to murine and human macrophages in order to induce an anti-inflammatory state *via* the reduction of macrophage phagocytosis and proinflammatory factors, such as TNF-α, MCP-1 or the chemokines CCL2, CCL4, CXCL9 and CXCL10 [24,38]. Accordingly, our data show that TNF-α treatment downregulated both *FNDC4* and *FNDC5* transcription in human visceral adipocytes. Interestingly, FNDC4 is barely expressed in macrophages [24]. In our hands, adipocytes constituted the major cellular source of FNDC4 in the adipose tissue, suggesting that FNDC4 is synthesized and released from adipocytes acting on adipose tissue-recruited macrophages in an autocrine-paracrine manner potentially counteracting adipose tissue-inflammation. In line with this observation, patients with morbid obesity exhibited higher expression of FNDC4 and its putative receptor GPR116 in the visceral fat, a depot characterized by higher adipocyte hypertrophy and recruitment of macrophages during the pathological expansion of the adipose tissue in the obese state. Interestingly, a strong negative association was found between *FNDC4* and *FNDC5* in adipocytes and the macrophage pan-marker*CD68* in SVFC obtained from paired visceral fat samples. Furthermore, FNDC4 is positively regulated by itself, contributing to a self-maintained loop to overcome adipose tissue inflammation.

Systemic and local adipocyte insulin resistance were not major determinants of FNDC4 in visceral adipose tissue. Accordingly, in our *invitro* studies, insulin treatment did not modify the expression of these factors in human visceral adipocytes. Moreover, plasma concentrations of FNDC4 were decreased in patients with obesity, but remained similar among patients with NG,IGT or T2D. To our knowledge, the present study shows, for the first time, that obesity-associated inflammation rather than insulin resistance increases the abundance of FNDC4 in human visceral adipose tissue.

Bariatric surgery attenuates obesity-associated inflammation, specifically by decreasing several proinflammatory factors, such as CRP,IL-6 and leptin, and increasing anti-inflammatory markers, including adiponectin [30,39]. Moreover, surgical strategies for weight loss induce changes in circulating levels of several myokines, such as IL-6, IL-8 or FNDC5/irisin, that are related to changes in body composition, especially in fat-free mass (FFM) [40,41]. Our results show that weight loss achieved by sleeve gastrectomy and RYGB was associated with an increase in FNDC5/irisin concentrations, an adipo-myokine with well-knownanti-inflammatory properties in the adipose tissue [18,37]. By contrast, circulating concentrations of the anti-inflammatory FNDC4 remained unchanged after bariatric surgery. A plausible explanation for the reduced plasma FNDC4 in obesity and the inability of bariatric surgery to modulate FNDC4 is that adipose tissue is not the main source for plasma FNDC4. In this sense, other FNDC4-producing sites, such as skeletal muscle, liver or brain, are major determinants of circulating FNDC4. Interestingly, the skeletal muscle is a source [8] and target [42] of FNDC4, inducing muscle cell differentiation and repair *via*Wnt/β-catenin pathway. Therefore, loss of muscle mass due to a sedentary lifestyle and obesity-associated metabolic diseases, might contribute to the observed low FNDC4 levels in obesity. In addition, a threshold level for adiposity before any effect on circulating FNDC4 is observed might condition the expression of pro- or anti-inflammatory factors 6 months after surgery. In line with this observation, despite CRP was markedly reduced 6 months after sleeve gastrectomy or RYGB,CRP levels were not completely normalized. Moreover, some proinflammatory factors, such as TNF-α or MCP-1, are unaffected by bariatric surgery [30,39]. Further investigations are needed to unravel whether lack of changes of FNDC4 after surgery are cause or consequence of weight loss as well as the relevance of other FNDC4-producing tissues in the obesity status.

Myokines induce profound changes in adipose tissue, such as a decrease in adipocyte cell size and TG content, together with an increase in mitochondrial activity and regulation of adipokines [6,7]. We report, for the first time, that the functional FNDC4 and FNDC5 receptors, namely GPR116 and integrin αV (ITGA5), are upregulated in the visceral adipose tissue of patients with obesity. ITGA5 is highly expressed in human adipose tissue stem cells, and repressed during adipogenesis [43]. Moreover, loss of ITGA5 increases the expression of *PPARG* and the number of lipid droplets during the differentiation of human adipose tissue stem cells [43]. Our results corroborate previous findings about the role of FNDC5/irisin as a negative regulator of adipogenesis [2,14,15,35,36,44,45]. Moreover, in the present study, we unravel a novel role of FNDC4 in human adipose tissue, mimicking the inhibitory effect of FNDC5/irisin on human lipogenesis. In this regard, FNDC4 treatment reduced triacylglycerol content and repressed the expression of *PPARG2* gene, a key transcriptional factor known to be involved in adipocyte differentiation as well as the mRNA levels of *FABP4* and *ADIPOQ*, late markers of mature adipocytes. Interestingly, *FNDC4* gene silencing resulted in an increase in *PPARG2, FABP4* and *ADIPOQ* in undifferentiated SVFCs and differentiated adipocytes. Moreover, *FNDC4* expression was oppositely regulated by both lipogenic (acylated and desacyl ghrelin) and lipolytic (leptin) factors, supporting its role in control of adipocyte size. Unexpectedly, a previous study found that the putative receptor of FNDC4, GPR116, is highly expressed in murine mature adipocytes and *Gpr116* knockdown impairs murine 3 T3-L1 preadipocyte differentiation [26]. Discrepancies with this study of Nie and colleagues might be ascribed to species-specific differences (mouse *vs.* human) and/or the potential binding of FNDC4 to other receptors in the adipose tissue, including integrin αV. Further investigations are warranted to address this point. Together, owing to the herein found anti-lipogenic properties of FNDC4 and FNDC5, the upregulation of their receptors GPR116 and ITGA5 in visceral fat found in the present study seems to be a compensatory mechanism to overcome adipocyte hypertrophy in the obese state.

The impact of myokines on fat browning has provided another novel mechanism to explain the benefits of physical activity on weight loss and metabolic disease prevention [2,6,7]. In this regard, FNDC5/irisin and other myokines (IL-6, irisin, BAIBA,meteorin-like or myostatin) are well-known regulators of fat browning [2,6,7]. As stated in previous studies [8–10,24], we have corroborated that FNDC4 and FNDC5 are produced by the human adipose tissue. An interesting finding of the present study is the ability of FNDC4 to promote fat browning in a similar manner to FNDC5/irisin. Specifically, the stimulation of adipocytes with FNDC4 augmented the protein expression of UCP-1, a hallmark of adaptive thermogenesis, and upregulated the expression of several brown and beige adipocyte markers (*PRDM16, TMEM26, CD137* and *UCP1*) in human differentiated adipocytes. Moreover, FNDC4 promoted mitochondrial biogenesis, another feature of fat browning, by increasing the adipocyte expression of *NRF1* and its downstream target *TFAM*, a mitochondrial transcription factor that controls the process of mitochondrial biogenesis, as well as mtDNA content in adipocytes and SVFCs. In human and experimental obesity, beige adipogenesis [46–49] and mitochondrial biogenesis [50,51] become dysfunctional. Interestingly, proinflammatory factors secreted by adipose tissue-embedded immune cells repress the expression of brown-specific genes in brown and beige

adipocytes [47,52]. This fact suggests that inflammation is responsible, at least in part, for the obesity-related loss of brown-like characteristics of adipose tissue.

5. Conclusion

FNDC4 was initially described as an anti-inflammatory factor [24], also in murine adipose tissue [25]. In the present study, we have characterized the anti-lipogenic and fat browning-inducing properties of FNDC4 in human adipose tissue, of which to our current knowledge are novel and seminal findings. Taken together, the upregulation of FNDC4 in visceral adipose tissue embodies a mechanism to attenuate adipocyte hypertrophy, inflammation and impaired beige adipogenesis in the obese state, thereby suggesting its potential as an anti-obesity therapy.

Contributions of authors

AR designed the study. BF-Q, MP,AWH-P, VV, RM, VC, SB, PP,JG-A, GF and AR collected and analyzed data. CS,JS and GF enrolled the patients. AR wrote and edited the manuscript. VC, SB, JG-A, GF and AR revised the manuscript critically for important intellectual content. All the authors participated in final approval of the version to be published. AR and GF are the guarantors of this work and contribute equally to it, had full access to all the data, and take full responsibility for the integrity of data and the accuracy of data analysis.

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Declaration of competing interest

No potential conflicts of interest relevant to this article were reported.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2020.154261.

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