miR-519d Overexpression Is Associated With Human Obesity

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Obesity is a consequence of imbalance of food intake and energy expenditure that results in storage of energy as fat, primarily in adipose tissue. MicroRNAs are non-coding RNAs that regulate gene expression in metabolic pathways and they are also involved in fat-cell development. The aim of this study was to evaluate whether microRNA dysfunction contributes to obesity. We analyzed, by microarray, the expression profile of 1,458 microRNAs in subcutaneous adipose tissue (SAT) from nondiabetic severely obese (*n* = 20) and nonobese adults (*n* = 8). Among 42 differently expressed microRNAs, we confirmed by reverse-transcription PCR (RT-PCR) that miR-519d was overexpressed whereas the protein levels of peroxisome proliferator-activated receptor-α (PPARA) (a predicted miR-519d target) were lower, at western analysis, in severely obese vs. nonobese subjects. We also show that miR-519d specifically and dose-dependently suppressed translation of the PPARA protein, and increased lipid accumulation during preadipocyte differentiation. Because PPARA plays a central role in fatty acid homeostasis, and in the transcriptional regulation of genes that are necessary for maintenance of the redox balance during the oxidative catabolism of fatty acids, we suggest that PPARA loss and miR-519d overexpression could be associated with metabolic imbalance and subsequent adipocyte hypertrophy in SAT during obesity.

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INTRODUCTION

A multitude of polymorphisms located in genes and candidate regions concur to determine an individual's susceptibility to weight gain (1). The potential role of these susceptibility genes in obesity insurgence may be influenced by various epigenetic and environmental factors (2). Over 50% of all gene expression traits in adipose tissue were recently shown to be closely correlated with obesity-related clinical traits (3). Transcription factors and microRNAs are the most numerous gene regulatory factors in multicellular genomes (4). It has been estimated that the human genome contains 1,000 microRNAs and that they could regulate nearly 30% of human genes (5,6). MicroRNAs are non-coding RNAs (19–25 nucleotide long) capable of regulating gene expression at transcriptional and translational level (7). The role of microRNAs in fat-cell development and in lipid metabolism has been investigated in cultured human preadipocytes and in mouse hepatocytes (8–10), but data on this issue in the subcutaneous adipose tissue (SAT) of obese patients are limited (11). We have explored the role of microRNAs in the regulation of gene expression in severe obesity using a microarray analysis of 1,458 microRNAs in SAT from 20 severely obese patients vs. pools of RNA from 8 nonobese subjects.

We found that one microRNA, namely miR-519d, was higher and that protein levels of peroxisome proliferator-activated-receptor α (PPARA), a predicted miR-519d target, were lower in SAT from obese vs. nonobese subjects. We found that miR-519d suppressed translation of the PPARA protein and increased lipid accumulation during adipocyte differentiation. Our findings suggest that miR-519d overexpression and alteration of PPARA protein expression could be associated with obesity.

METHODS AND PROCEDURES

Subjects

Twenty-eight subjects were enrolled in this study. Subjects were divided into two groups based on BMI: nonobese subjects (BMI <30 kg/m^2, *n* = 8, 3/8 men) and severely obese subjects (BMI >40 kg/m^2, *n* = 20, 50% men). The severely obese subjects (mean age ± s.e.m.: 41.1 ± 3.3; BMI (mean kg/m^2 ± s.e.m.): 42.7 ± 1.2) underwent laparoscopic surgery for severe obesity (gastric banding). They were investigated at their

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maximum weight. The nonobese subjects (mean age ± s.e.m.: 42.2 ± 7.5; BMI (mean kg/m^2 ± s.e.m.): 24.7 ± 1.6) underwent abdominal surgery (laparoscopic cholecystectomy) because of benign diseases. Neoplasia, diabetes, and alcohol abuse were excluded in all but two subjects: one obese subject was affected by type 2 diabetes and another was addicted to alcohol. All subjects provided written informed consent to the study and the research protocol was approved by the Ethics Committee of our Faculty of Medicine.

Samples—SAT samples

Samples of SAT (about 1 cm^3 of fat, average 100–600 mg) were excised from skin in the lower abdomen area during surgery. All biopsies were snap-frozen and stored in liquid nitrogen until RNA isolation. SAT samples were used for total RNA and for protein purifications. A blood sample was collected from all fasted subjects the day before surgery for adipokine analysis and other biochemical measurements (see **Supplementary Methods and Procedures** online).

RNA extraction and microRNA expression profile

Total RNA was extracted from frozen SAT samples and hybridized to microRNA microarray chips (miRCURY LNA miRNA Array V8.1 (208002V8.1-Lot n. 20393.03); Exiqon, Copenhagen, Denmark)) that contain complementary probes for the mature forms of all microRNAs registered in miRBase 8.1 (12). The microarray consists of 1,458 capture probes, perfectly matched to microRNAs annotated in miRBase 8.2 of the miRBase microRNA Registry (http://microrna.sanger.ac.uk/), control probes, and mismatch probes. The reference RNAs, consisting of a pool of total RNA isolated from three control men (CM) and five control women (CW), served as controls for obese males and obese women (OBW), respectively.

Microarrays were scanned and data were analyzed, normalized, and filtered (see **Supplementary Methods and Procedures** online). All microarray data reported herein are in accordance with Minimum Information About a Microarray Experiment (MIAME) guidelines and have been deposited in the NCBI GEO database (Accession number GSE12726–GPL 7162).

Bioinformatic approach

Biological targets of microRNAs were predicted using the TargetScan Release 4.2 algorithm that searches for conserved 8mer and 7mer sites (in the untranslated regions (UTRs)) that match the seed region of each microRNA (13). The data obtained were combined and analyzed using the KEGG database (http://www.genome.ad.jp/kegg/) (14) to identify the biological processes that involve the target genes of microRNAs. Among the various KEGG pathways, we selected those with at least two genes up- or downregulated at the level of $P < 0.01$, and that could be targets of microRNAs. In particular, in the case of the insulin-signaling pathway, the P value was 1.75e-3 and 4.40e-8 for genes targeted by down- and upregulated microRNAs, respectively. In the case of the adipocytokine signaling pathway, the P value was 9.39e-4 for target genes of upregulated microRNAs.

Real-timePCR quantification of microRNAs and mRNA

We used the TaqMan miRNA Assay Protocol to assay by real-time quantitative PCR the subset of microRNAs constituted by miR-519d, miR-150, and miR-659 (normalized to U6 RNA). **Supplementary Table S1** online shows the primers and reverse-transcription PCR (RT-PCR) conditions, we used to assay mRNA of the selected microRNA target genes, namely, insulin receptor substrate 2 (*IRS2*), solute carrier family two-facilitated glucose transporter-member 4 (*SLC2A4*), *PPARA*, carnitine palmitoyltransferase 1A (*CPT1A*), 18S, and mRNA of the adipocyte protein homologous to myelin P2 (*aP2*), a mature adipocyte-specific marker.

Protein extraction and western analysis

Total proteins were extracted, quantified, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot analyses of PPARA, SLC2A4, peroxisome proliferator–activated receptor gamma, coactivator 1α (PPARGC1A), CPT1A, and α-actinin proteins were performed using antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) (see **Supplementary Methods and Procedures** online).

Transfection and inhibition experiments

The oligonucleotides, plasmids (pGL3-control and pRL-PPARA encoding firefly luciferase and *Renilla* luciferase, respectively) and cell lines used for cell transfection experiments are detailed in **Supplementary Methods and Procedures** online. Human embryonic kidney-293 cells were seeded in 24-well plates with 500 µl of antibiotic-free medium the day before transfection to allow adherence and to reach 70–90% confluence at the time of transfection. The experimental conditions used for cotransfection are detailed in **Supplementary Methods and Procedures** online. Twenty-four hours after transfection, we measured firefly and *Renilla* luciferase activities using a dual luciferase assay according to the manufacturer's instructions (Promega, Napoli, Italy).

Preadipocyte culture and differentiation

We purchased Poietics primary human visceral preadipocytes from Cambrex BioScience (Walkersville, MD) and grew them according to the manufacturer's protocol (see **Supplementary Methods and Procedures** online). The cells were passaged twice before differentiation (see **Supplementary Methods and Procedures** online).

Adipocyte staining and quantification

After 14 days of differentiation, we stained the adipocyte culture to evaluate the presence of lipid droplets, which are indicators of differentiation. We washed the cells in phosphate-buffered saline and fixed them in 10% formalin for 1 h. We then washed the cells in water and stained the lipids for 15 min with Oil-Red-O prepared by mixing vigorously three parts of a stock solution (0.5% Oil-Red-O in 98% isopropanol) with two parts of water and then eliminating undissolved particles with a 0.4-µm filter. We then washed cells with water and counted the number of adipocytes under a light microscope. Relative lipid levels were assessed by redissolving the Oil-Red-O present in stained cells in 98% isopropanol and then determining absorbance at 550 nm. We next evaluated differentiation by measuring the levels of the mature adipocyte-specific marker aP2 by RT-PCR as reported in **Supplementary Methods and Procedures** online.

RESULTS

The general and biochemical characteristics of the severely obese and nonobese individuals are listed in **Table 1**. Mean levels of serum adipsin ($P = 0.002$), ghrelin ($P = 0.001$), leptin ($P = 0.001$), resistin ($P = 0.03$), and glucose ($P = 0.03$) were significantly higher in obese than in nonobese subjects. Fasting insulin levels in obese patients were 12.48 ± 5.12 mU/ml (mean ± s.d.). The clinical history of obese patients did not reveal any abnormality at the level of hormones T3, T4, FT3, FT4, TSH, Ab-peroxidase, Ab-thyroglobulin, PRL, FSH, LH, E2, progesterone, and testosterone.

Microarray analysis

Expression profiling revealed that a large set of microRNAs was expressed in SAT (**Figure 1a**). Forty-two microRNAs differed by at least ±1.5-fold in 17/20 obese subjects vs. the nonobese control pools (see **Supplementary Table S2** online). In detail, 21/42 were upregulated (**Figure 1b,c**) and 21/42 were downregulated (see **Supplementary Figure S1** online). A relevant sex difference in microRNA expression emerged from microarray data (**Figure 1a**). Among the differentially

Table 1 General and biochemical characteristics of the study subjects

Parameter[a]	Severely obese subjects (n = 20)	Nonobese subjects (n = 8)	P[b]
Age (years)	41.1 ± 3.3	42.2 ± 7.5	
BMI (kg/m^2)	42.7 ± 1.2	24.7 ± 1.6	<0.001
Total cholesterol (mmol/l)	5.1 ± 0.3	5.0 ± 0.3	
Triacylglycerol (mmol/l)	1.4 ± 0.1	1.1 ± 0.2	
Adiponectin (μg/ml)	20.1 ± 2.0	25.9 ± 5.3	
Adipsin (ng/ml)	240.5 ± 18.9	113.4 ± 36.7	0.002
Ghrelin (pg/ml)	160.7 ± 6.3	112.8 ± 10.0	0.001
Glucose (mmol/l)	5.2 ± 0.2	4.5 ± 0.2	0.03
Leptin (ng/ml)	29.6 ± 4.1	8.4 ± 4.0	0.001
Resistin (ng/ml)	3.5 ± 0.5	2.2 ± 0.4	0.03
TNF-α (pg/ml)	7.5 ± 0.2	8.4 ± 1.3	
IL-6 (pg/ml)	7.9 ± 0.8	5.5 ± 0.9	

IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.
[a]Parameters are reported as mean ± s.e.m. [b]Differences among means were tested by Mann–Whitney U-test and considered statistically significant at $P < 0.05$.

expressed microRNAs, miR-519d, miR-498, and miR-150 were upregulated, whereas miR-659 and miR-371-3p_MM2 were downregulated in 20/20 obese subjects.

Evaluation of microRNA expression by microRNA-specific real-time RT-PCR

RT-PCR (15) assays were available for three of the five microRNAs that were differentially expressed in all subjects: miR-519d and miR-150 (upregulated) and miR-659 (downregulated) (see **Supplementary Figure S2** online). The mean expression levels of miR-519d were 1.88 ± 1.39 (RQ ± s.d.) in OBW and 6.72 ± 4.88 in obese men (OBM) vs. CW and CM, respectively. The mean expression levels of miR-150 were 0.86 ± 0.33 in OBW and 1.43 ± 0.38 in OBM vs. CW and CM, respectively. Lastly, the mean expression levels of miR-659 were 1.36 ± 0.59 in OBW and 0.42 ± 0.14 in OBM vs. CW and CM, respectively (data not shown).

Bioinformatic prediction of microRNA targets

The functional role of microRNA is strictly related to interaction with target genes. We used TargetScan (release 4.2) as target prediction algorithm (13) because it evaluates various features of microRNA/mRNA interactions, and targets are ranked by "total context score," which is based on site type, site number, and site context.

All target genes for miR-150/519d/498 predicted by the TargetScan web tool were combined, and analyzed using the KEGG database (http://www.genome.ad.jp/kegg/) (14) (data not shown). Among the deregulated pathways were the insulin and adipocytokine signaling pathways, which are often involved in obesity insurgence and in obesity-associated diseases (16). From these two pathways, we selected the *PPARA* and *SLC2A4* genes (both of which are targets of miR-519d), the *PPARGC1A* gene (which is a target of miR-150), and the *IRS2* gene (which is a target of miR-659) because they had good microRNA accessibility (ΔG analysis) as evaluated with the mFold program (http://mfold.bioinfo.rpi.edu/) and/or a favorable context score (data not shown).

Another interesting target gene of miR-659 is *CPT1A*; it had a low context score but, based on its prominent role in lipid metabolism and because *CPT1A* is induced by peroxisome proliferators in the liver, we included it in the mRNA and protein evaluations to investigate its possible role, in association with *PPARA*, in obesity.

mRNA and protein expression

The mRNA expression of selected putative target genes in SAT from obese and nonobese subjects was verified by RT-PCR (see **Supplementary Figure S3** online). The relevant sex difference in microRNA expression, which first emerged from microarray data and RT-PCR results (**Figure 1a** and **Supplementary Figure S2** online), was also confirmed at mRNA level. All genes were expressed in SAT from all subjects. The levels of PPARA were particularly high in OBM (mean RQ ± s.d.: 6.5 ± 3.0), as were CPT1A levels in both OBM (mean RQ ± s.d.: 2.3 ± 1.0) and OBW (mean RQ ± s.d.: 3.4 ± 1.2) vs. CM and CW, respectively (data not shown).

We evaluated the selected target proteins by western blot in 4/20 obese subjects, 2 OBW (OBW2 and OBW8) and 2 OBM (OBM5 and OBM10) who had different levels of the tested microRNAs (see **Supplementary Figure S2a,b** online), and in nonobese control pools. Protein concentrations of IRS2, SLC2A4, PPARGC1, and CPT1A were similar in the obese and nonobese subjects, whereas the PPARA protein was detected in the controls but not in the obese subjects (**Figure 2**).

Validation of the interaction between miR-519d and the PPARA 3′ UTR

PPARA is involved in the regulation of a number of genes relevant for metabolic pathways (17,18). Therefore, functional interactions between miR-519d and its putative binding sites on the 3′ UTR of the PPARA mRNA could be associated with obesity. To determine whether the miR-519d binding sites on the PPARA mRNA are functional, we cotransfected a plasmid that expresses the *Renilla* luciferase gene under the control of the cytomegalovirus promoter with the 3′ UTR region of the PPARA mRNA that contains the putative miR-519d binding sites and pre-miR-519d. As transfection control, we used a plasmid that expresses firefly luciferase. The ratio between the activities of the two luciferase enzymes represented the expression level.

As shown in **Figure 3**, a pre-miR-519d concentration as low as 100 nmol/l significantly reduced *Renilla* luciferase expression, and inhibition was dose-dependent. To rule out a nonspecific interaction between pre-miR-519d and the *Renilla* luciferase-expressing construct, we cotransfected the above-indicated plasmids with 200 nmol/l of pre-miR-519d and

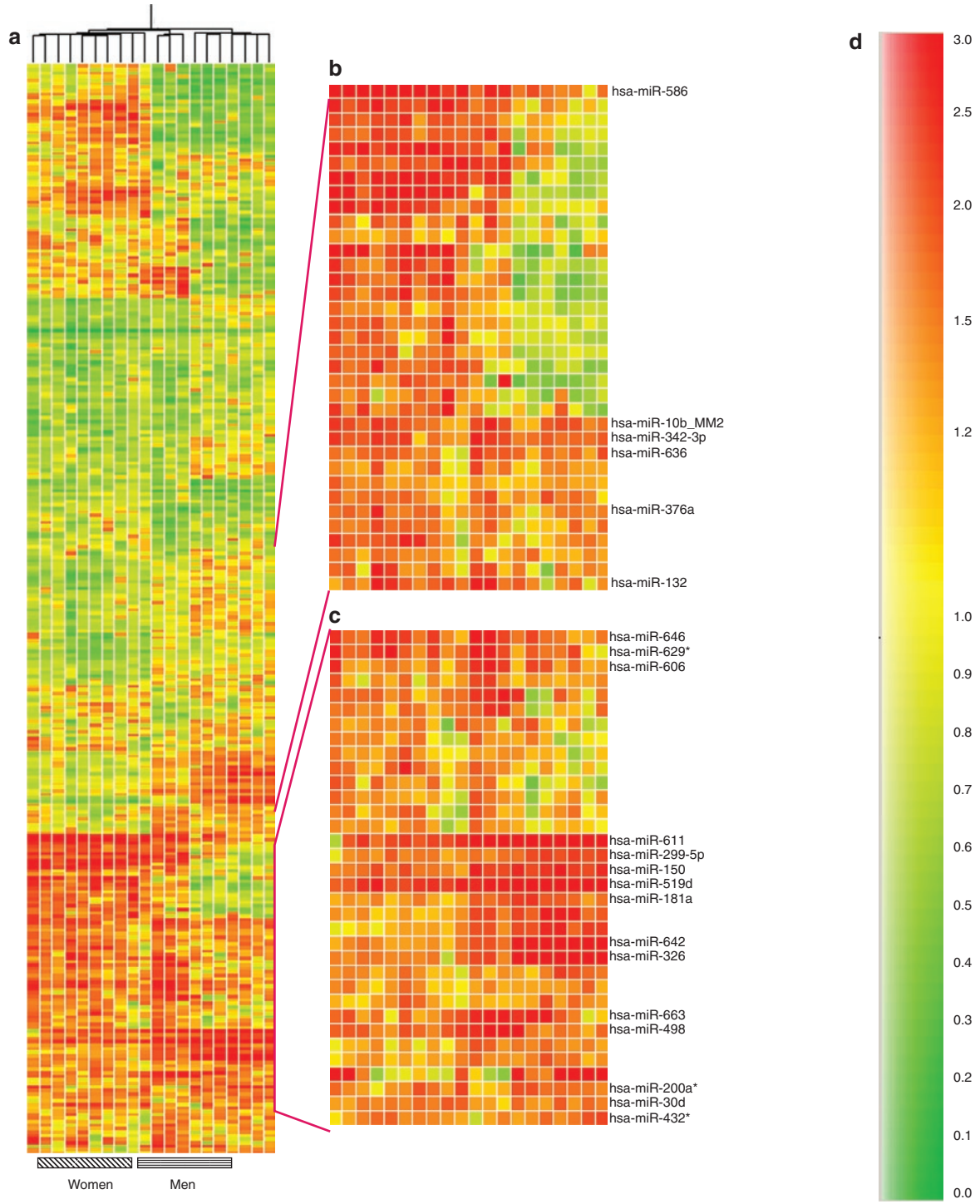

Figure 1 Unsupervised hierarchical clustering of upregulated microRNAs in severely obese subjects. (**a**) Unsupervised hierarchical clustering of microRNA expression profiles of subcutaneous adipose tissue from obese men and women obtained with Exiqon LNA microRNA Arrays V 8.1. The rows represent different microRNA probes on the microarray, and the columns represent individual RNA samples. The analysis reveals a common signature relative to a few microRNAs. Expansion of selected regions (**b** and **c**) shows microRNA upregulated in at least 17/20 subjects. The scale (**d**) indicates relative microRNA expression changes in $\log_2$ units, where 1 represents the mean expression level of a given microRNA across samples.

with an anti-miR-519d that blocks miR-519d. Anti-miR-519d restored *Renilla* luciferase expression in a dose-dependent manner. Moreover, at an anti-miR-519d concentration of 200 nmol/l, the expression levels of *Renilla* luciferase were comparable to those obtained with transfection of the plasmid alone. These data are compatible with a functional interaction between miR-519d and the 3′ UTR of the PPARA mRNA, thereby suggesting that changes in miR-519d levels may modulate the expression of genes regulated by this transcription factor.

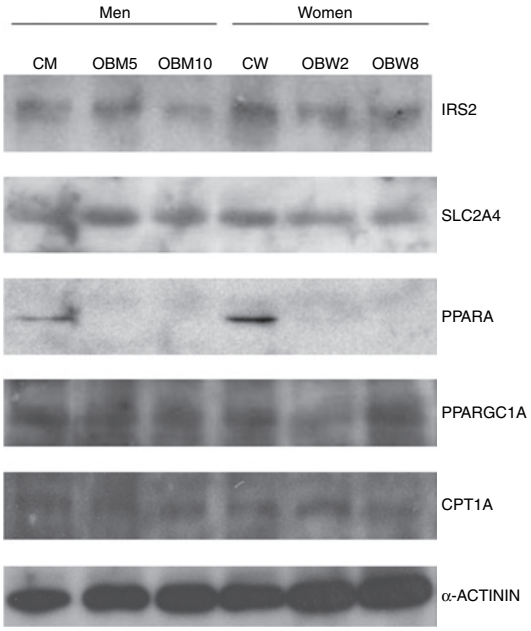

Figure 2 PPARA is downregulated in subcutaneous adipose tissue from severely obese subjects. Western blot analyses of selected predicted targets of deregulated microRNAs in obese subjects. Total proteins from subcutaneous adipose tissue of obese subjects (OBM and OBW) and controls (CM and CW) were used to analyze the protein levels of IRS2, SLC2A4, PPARA, PPARGC1A, and CPT1A. PPARA was not detected in obese subjects (OBM5, OBM10, OBW2, and OBW8). Equal amounts of protein were loaded in each lane as assessed by protein staining with Ponceau S and hybridization with α-actinin. CM, control men; CW, control women; CPT1A, carnitine palmitoyltransferase 1A; IRS2, insulin receptor substrate 2; PPARA, peroxisome proliferator–activated receptor-α; OBM, obese men; OBW, obese women; PPARGC1A, peroxisome proliferator–activated receptor gamma, coactivator 1α; SLC2A4, solute carrier family two-facilitated glucose transporter-member 4.

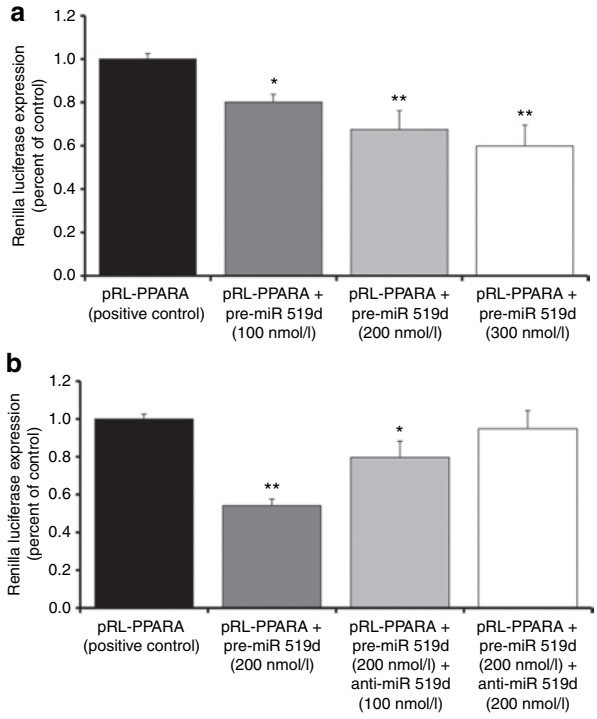

Figure 3 miR-519d binds and functionally interacts with PPARA 3′ UTR. (**a**) pre-miR-519d dose-dependently inhibited the expression of a *Renilla* luciferase-expressing construct that contains the 3′ UTR region of the *PPARA* gene harboring miR-519d putative binding sites; pRL-PPARA = positive control. (**b**) Anti-miR-519d dose-dependently blocked the pre-miR-519d-induced inhibition of the expression of *Renilla* luciferase containing the PPARA 3′ UTR region. Each sample, except the positive control, was transfected with a 200 nmol/l final concentration of pre-miR-519d and the indicated concentration of the anti-miR-519d. **P* < 0.05; ***P* < 0.01. PPARA, peroxisome proliferator–activated receptor-α; UTR, untranslated region.

Analysis of the effect of miR-519d on preadipocyte differentiation

To evaluate whether the miR-519d has a functional role in adipocyte differentiation, we transfected 200 nmol/l of pre-miR-519d and anti-miR-519d in primary human visceral preadipocytes. As control we transfected anti-miR-143 that blocks adipocyte differentiation (8). During adipocyte differentiation, high concentrations of miR-519d increased lipid levels, and low concentrations decreased lipid levels (Figure 4a). As expected, low levels of miR-143 also reduced adipocyte differentiation. This result implicates miR-519d in the adipocyte differentiation process. To verify that reduction of miR-519d levels reduced the overall process and not only lipid content within the cells, we also quantified, by RT-PCR, the differentiation marker aP2 (adipocyte protein homologous to myelin P2), which is normally expressed only in mature adipocytes. Levels of the aP2 transcript were decreased in cells treated with anti-miR-519d and anti-miR-143, and increased in cells treated with pre-miR-519d thereby confirming that miR-519d exerts a proadipogenic effect (Figure 4b).

DISCUSSION

We used microRNA microarray analysis of SAT to investigate the role of microRNAs in the regulation of gene expression in human severe obesity. Intriguingly, microRNA expression profiling revealed some gender-related patterns in our patients. This may be linked to the different prevalence of obesity between men and women. However further studies are required to verify and understand this observation. In our study, we focused on a microRNA that was deregulated in both sexes. In fact, miR-519d was overexpressed and protein levels of PPARA, one of the predicted miR-519d targets, were reduced in SAT from all obese vs. nonobese subjects. We found that miR-519d suppressed translation of the PPARA protein and increased lipid accumulation during adipocyte differentiation. *In vitro*, the effect of miR-519d on adipogenesis was similar to that of miR-143, a well-known marker of adipogenesis (8). Our findings suggest that miR-519d overexpression and alteration of PPARA protein expression could be associated with obesity.

Our study has two main strengths. Firstly, by studying intact SAT, we avoided artefacts arising from manipulations used to separate the various cell populations. Moreover, we were able

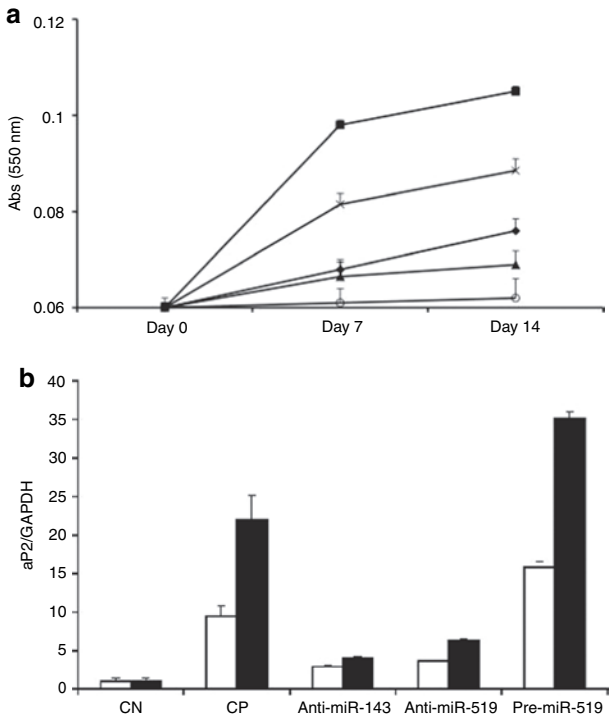

Figure 4 miR-519d enhances adipogenic differentiation. (**a**) pre-miR-519d (rectangles) increases adipogenic differentiation of primary human visceral preadipocytes measured as amount of cell-bound lipid specific dye evaluated spectrophotometrically; cells differentiated with differentiation media show accumulation of cell-bound dye (positive differentiation control: ×), whereas cells treated with anti-miR-519d (diamonds), anti-miR-143 (triangles), and with regular media without differentiation factors (circles) show scarce accumulation of cell-bound lipid specific dye. (**b**) Differentiation in adipocytes is accompanied with an increase of the adipocyte protein homologous to myelin P2 (*aP2*) gene expression, which is a mature adipocyte-specific marker in cells treated with differentiation media; cells treated with pre-miR-519d show a further increase in aP2 levels, whereas cells treated with anti-miR-143, anti-miR-519d, and with regular media (CN) show only a modest increase of this marker compared to the positive control (CP). White and black bars represent respectively aP2 expression levels after 7 and 14 days in culture. Data represent the mean (±s.d.) of at least three independent experiments and are expressed as 2^{-C_t} ratio between the gene of interest and GAPDH, which was used for normalization of expression.

to evaluate gene interactions in a specific pathophysiological microenvironment, namely, SAT. Secondly, because individuals enrolled in the study were not affected by diabetes, except for one case (noninsulin-treated), we were able to evaluate changes in microRNA expression and microRNA-mediated regulation of proteins without confounding signals from diabetes, which is frequently associated with obesity.

PPARA is highly expressed in tissues that rely on fatty acid oxidation as their primary energy substrate, namely, heart, liver, and skeletal muscle (19). PPARA appears to mediate the balance between cellular fatty acid metabolism and glucose homeostasis, particularly under stress conditions (20). In the presence of ligands, i.e., fatty acids, PPARs form heterodimers with the retinoid X receptor-α, and bind to the peroxisome proliferator–response elements in the promoter regions of target genes thereby increasing their transcriptional activation (18,21). The target genes of PPARA are primarily those involved in energy metabolism and substrate utilization, namely, genes involved in fatty acid uptake, fatty acid esterification, fatty acid β-oxidation, glucose oxidation, mitochondrial transport, and energy uncoupling (21).

Evidence obtained in various mouse models supports a link between PPARA and obesity. In fact, in obese diabetic KKAy mice, activation of PPARA by its agonist Wy-14,643 prevented adipocyte hypertrophy, and reduced inflammation and the expression of macrophage-specific genes in white adipose tissue (22). In addition, Wy-14,643 decreased the weight of both white adipose tissue and brown adipose tissue in KKAy mice compared with pair-fed mice, which suggests that PPARA activation prevents obesity (22).

The insulin-sensitizing, glycemia-lowering, and lipidemia-lowering effects of the novel PPARA/G dual activator GCP-02 have been investigated in insulin-resistant obese mice (23). GCP-02 was more effective than rosiglitazone, a PPARG activator, in improving insulin sensitivity, ameliorating glucose tolerance, suppressing L-alanine-induced gluconeogenesis, and decreasing plasma levels of cholesterol, triglycerides, and free-fatty acids. But, whereas rosiglitazone caused weight gain, GCP-02 reduced body weight in this animal model, but did not affect food intake (23). It is noteworthy that rosiglitazone causes weight gain and edema also in humans.

PPARA gene function has been studied also in *PPARA* knockdown mice and in mice treated with the PPARA agonist fenofibrate (24). The comparison of liver transcript profiles revealed that genes of the oxidative phosphorylation and β-oxidation metabolic pathways were upregulated in fenofibrate-treated mice and downregulated in *PPARA* knockdown mice (24). Lastly, using a mouse model, Li *et al.* (25) reported that during white adipose tissue remodeling induced by the β3-adrenergic receptor agonist CL-316, 243, *PPARA* and its downstream targets increased fatty acid catabolism and suppressed proinflammatory signaling. Based on the data showing that PPARA plays a relevant role in adipose tissue function by increasing adiposity in animal models, it is conceivable that its decrease observed in SAT of our obese population could decrease fatty acid oxidation. In our obese population, the observed decrease in PPARA protein levels could thus represent a novel marker associated with human obesity.

In our obese patients, the decrease of PPARA protein in SAT occurred even in the presence of high levels of mRNA, which could indicate post-transcriptional regulation. In addition, the presence in PPARA of an extremely long 3′ UTR with several putative microRNA binding sites also reinforces post-transcriptional regulation in this gene. Our data demonstrate that miR-519d binds to the PPARA 3′ UTR thereby reducing the levels of the protein in human embryonic kidney-293 cells. We also found that during adipogenesis miR-519d stimulated adipocyte differentiation in dose-dependent manner. Furthermore, miR-519d was recently reported (26) to be a part of a microRNA signature of pluripotency in human embryonic stem cell cultures. In fact, miR-519d was higher in undifferentiated human

embryonic stem cell than in differentiated human embryonic stem cell (26). Also based on the latter report, the higher miR-519d expression in SAT from obese vs. nonobese subjects and the miR-519d's role in preadipocyte differentiation are in line with an altered microRNA-based adipocyte differentiation mechanism in obesity.

In conclusion, we show that the PPARA protein is lost and that the expression of miR-519d is increased in SAT from severely obese patients. We demonstrate that miR-519d functionally binds to the PPARA 3′ UTR and that its expression and inhibition are related to an increase and decrease, respectively, of adipogenesis. Because PPARA plays a central role in fatty acid homeostasis, as well as in the transcriptional regulation of genes that are necessary for maintenance of the redox balance during the oxidative catabolism of fatty acids (18,21,27), we suggest that PPARA loss and miR-519d expression could be associated with metabolic imbalance and subsequent adipocyte hypertrophy in SAT from severely obese subjects.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

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DISCLOSURE

The authors declared no conflict of interest.

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