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ORIGINAL ARTICLE

Thyroid hormone responsive Spot 14 increases during differentiation of human adipocytes and its expression is down-regulated in obese subjects

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Context: Very limited information is available regarding the function of human thyroid hormone responsive Spot 14 (human S14, hS14) in adipogenesis and human adiposity.
Objective: To evaluate hS14 levels during differentiation of human pre-adipocytes, in human fat depots and isolated fat cells.
Design: This was a cross-sectional study.
Subjects: A total of 161 omental (OM) and 87 subcutaneous (SC) adipose tissue samples obtained during elective surgical procedures from a population who varied widely in terms of obesity.
Measurements: *hS14* gene expression and protein levels during adipogenesis were assessed by RT–PCR, western blot, and using an automated confocal imaging approach.
Results: *hS14* gene expression levels were decreased in OM adipose tissue from overweight (−42.0%) and obese subjects (−56.5%) compared with lean subjects ($P<0.05$ and $P<0.0001$, respectively). hS14 mRNA (but not hS14-related) was inversely associated with obesity measures such as body mass index ($P=0.001$), percent fat mass ($P=0.001$), waist-to-hip ratio ($P=0.020$), and systolic blood pressure ($P=0.031$). *hS14* gene expression and protein levels were up-regulated at the early stages of differentiation of human pre-adipocytes as well as for 3T3-L1 cells. That observation was most prominent in those individual cells exhibiting the more marked differentiation features. *hS14* gene expression levels increased by ~45 000-fold in mature adipocytes. Increased hS14 levels were also found in stromal-vascular cells/pre-adipocytes (3.8-fold, $P<0.05$) and in adipose tissue samples (1.9-fold, $P<0.0001$) from SC compared with OM fat depots.
Conclusions: These results suggest that hS14 is involved in human adipogenesis, but inversely related to obesity and OM fat accumulation.
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Keywords: adipogenesis; pre-adipocytes; mature adipocytes; adipose tissue; gene expression; thyroid hormone responsive Spot 14

Introduction

The levels of critical factors in fatty acid and triacylglycerol biosynthesis are tightly controlled by different nutritional, hormonal, and developmental conditions. Feeding fasted animals with high-carbohydrate low-fat diets causes a dramatic induction of many genes involved in *de novo* lipogenesis such as glucokinase, pyruvate kinase, acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FASN), and malic enzyme.[1–5] In addition to insulin (Ins) and glucose, thyroid hormone (T_3) is a powerful inducer of lipogenesis.[6–8] Thyroid hormone responsive Spot 14 (THRSP or S14) is another specific factor whose gene expression and protein levels in lipogenic tissues are strongly linked to T_3, Ins, and

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glucose. For this reason, S14, a 17-kDa acidic protein found in the cytoplasm as well as in the nucleus of lipogenic cells,[9] has been postulated to have a function in lipogenesis.[8,10]

In mice, hepatic S14 shows a rapid and robust response to signals that induce lipogenesis, such as glucose[11] and thyroid hormone administration.[12,13] Indeed, both carbohydrate[14] and thyroid hormone response elements[15] have been identified upstream of the start site of transcription for *S14* gene. Moreover, increased liver S14 mRNA is directly correlated with lipogenesis and lipogenic enzymes as well as with decreased β-oxidation,[16] suggesting that hepatic S14 may be involved in the synthesis of fatty acids. In fact, long-chain polyunsaturated fatty acids administration down-regulated S14 mRNA in rat liver and in cultured hepatocytes.[17] In agreement with these observations, *in vitro* knockdown experiments confirmed that S14 is required for lipogenesis.[10,18] Otherwise, according to *S14* null mice data, S14 is necessary for normal lipogenesis in the lactating mammary gland, but not in liver,[19] in which S14-related (S14-R or MIG12) might compensate the lack of S14.[20] Finally, *S14* gene has been recently related to the regulation of diet-induced obesity, as *S14* gene deletion leads to an improvement in age-associated glucose intolerance[21] in mice.

Human *S14* (*hS14*) gene, located at chromosome 11q13.5,[22] has been cloned and shares a 78–81% homology with rat *S14* gene.[23,24] However, very limited information concerning *hS14* gene expression levels is available in human beings. To determine whether *hS14* gene has a function in regulation of lipid storage in human fat depots, Kirschner and Mariash[25] studied the response to a 48-h fast in six obese and in six non-obese subjects. These authors found that *hS14* gene expression levels were strongly down-regulated in the abdominal subcutaneous (SC) adipose tissue of non-obese subjects in response to fasting, but only minimally down-regulated in obese individuals.[25]

Given this paucity of information, we aimed to evaluate hS14 during differentiation of human pre-adipocytes to mature adipocytes (MAs) using an automated confocal imaging approach. We also studied the expression levels of hS14 and hS14-R (MIG12) in isolated stromal-vascular cells (SVCs) and MAs isolated from human fat biopsies and during adipogenesis in human commercially available pre-adipocytes. Finally, we analyzed *hS14* gene expression levels in omental (OM) and SC fat from a cohort of subjects who varied widely in terms of obesity. The findings reported here suggest that hS14 (but not hS14-R) is directly associated with adipogenesis in human adipocytes, but inversely related with obesity and OM fat accumulation.

Materials and methods

Experimental procedures

Cell isolation. Approximately 5 g of SC and OM fat samples obtained during the surgical procedures practiced in four subjects were aseptically isolated and all visible connective tissues were removed. Tissues were finely minced and subjected to a 1 h digestion at 37 °C in a shaking water bath. The digestion buffer included 100 mM HEPES (Sigma Aldrich, St Louis, MO, USA) buffer containing 120 mM NaCl, 50 mM KCl, 5 mM D-glucose, 1 mM CaCl2, 1.5% type-V BSA, 2% penicillin/streptomycin, and 0.15% collagenase type I solution (Sigma Aldrich). The collagenase type I solution used to isolate SVCs and MAs from fat samples contained ~1.5 mg collagenase type I per ml (CLS type 1, Worthing Biochemical Corp., Lakewood, NJ, USA). The remaining procedure was similar to the earlier described method for isolating SVCs from adipose tissue.[26] Briefly, on disintegration of the adipose tissue aggregates, digested tissue was centrifuged and two cellular fractions, a pellet of SVCs and a supernatant of MAs, were placed in 20 ml of phosphate-buffered saline (PBS) 2% penicillin/streptomycin and passed through sterile nylon mesh filters (autoclaved metal screen, Becton Dickinson Biosciences, Erembodegem, Belgium) to isolate digested cells. Finally, both SVCs and MAs ($n=8$ obese (body mass index, BMI $>30\,kg\,m^{-2}$) women) fractions were washed and centrifuged for 5 min at 400 *g* before being stored at −80 °C.

Cell culture. Commercially available cryo-preserved human SC pre-adipocytes from two non-diabetic male subjects with age > 40 and BMI < 25 or BMI > 30 kg m^{-2} (SP-F-1 or SP-F-3, respectively; Zen-Bio, Inc., Research Triangle Park, NC, USA) were plated on T-75 cell culture flasks and cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/nutrient mix F-12 medium (1:1, v/v) supplemented with fetal bovine serum (FBS) 10%, HEPES 1%, glutamine 1%, and penicillin/streptomycin at 10 U ml^{-1} (all from GIBCO, BRL; Grand Island, NY, USA). One week later, human SC pre-adipocytes were resuspended and cultured (~40.000cells cm^{-2}, third passage) in 12- or 96-well plates with pre-adipocyte medium (Zen-Bio, Inc.) composed of DMEM/nutrient mix F-12 medium (1:1, v/v), FBS 10%, HEPES 1%, glutamine 1%, and penicillin/streptomycin 1% in a humidified 37 °C incubator with 5% CO_2. Twenty-four hours after plating, cells were checked for complete confluence (day 0) and differentiation was induced using differentiation medium (DM; Zen-Bio, Inc.), composed of pre-adipocyte medium with human Ins, dexamethasone, isobutylmethyl-xanthine, and PPARγ agonists (rosiglitazone). After 7 days (day 7), DM was replaced with fresh adipocyte medium (Zen-Bio Inc.), composed of DMEM/nutrient mix F-12 medium (1:1, v/v), FBS, HEPES, biotin, panthothenate, human Ins, dexamethasone, penicillin, streptomycin, and amphotericin, according to manufacturers' guidelines. Two weeks after the initiation of differentiation (day 14), cells appeared rounded with large lipid droplets in the cytoplasm. Cells were then considered MAs, harvested, and stored at −80 °C for RNA extraction to study gene expression levels, or fixed and immunostained (96-well plates). For gene expression analyses, three biological replicates ($n=3$) of fat cells from both lean and obese subjects were performed. Undifferentiated control cultures (not subjected to hormonal treatment, but processed at the

same time as the differentiating cultures) were also performed for each experiment.

Murine 3T3-L1 fibroblasts (CCL 92.1, American type culture collection) were grown to confluence at 37 °C in six-well plates in DMEM with no added biotin or pantothenate, containing 10% calf serum in incubators equilibrated with 10% CO_2. Two days post-confluence (day 0), differentiation was induced with isobutylmethyl-xanthine (0.5 mM), dexamethasone (0.25 μM), and Ins (1 pg ml^{-1}) in DMEM containing 10% FBS. After 2 days, the isobutylmethyl-xanthine and dexamethasone were removed and Ins was maintained for 2 additional days. On day 4, and thereafter, DMEM (without Ins supplementation) plus 10% FBS was replaced every 2 days. On days 0, 7, and 14 before starting differentiation protocol, three replications of cells were collected separately for total protein extraction. Cell samples were washed in ice-cold PBS followed by homogenization assay using RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) supplemented with a protease inhibitor cocktail (Sigma Aldrich) at 4 °C for 30 min. Cellular debris were eliminated by centrifugation of the diluted samples at 10 000 *g* for 10 min (4 °C). Protein concentration was then determined using *Lowry* assay.

Cell fixation. Culture media was removed from human adipocytes and freshly prepared 3.7% formaldehyde (Sigma Aldrich) diluted in 1 × PBS and pre-warmed to 37 °C was added (100 μl per well). After incubation at room temperature (RT) for 10 min, the fixation solution was removed and the residual liquid was removed by inverting the plate briefly onto absorbent paper. Plates were washed twice by adding 100 μl per well of 1 × PBS.

Triton X-100 permeabilization and blocking. A total of 1 × PBS was removed and 100 μl per well of a 0.1% Triton X-100 (Sigma Aldrich) solution diluted in 1 × PBS was added. After incubation at RT for 5 min, the permeabilization solution was removed and the residual liquid was drained. Plates were washed twice by adding 100 μl per well of 1 × PBS. A measure of 1 × PBS was removed and 100 μl per well of 1 × PBS supplemented with 5% FBS were added. After incubation at RT for 30 min, we proceeded to the primary antibody staining step.

Primary antibody staining. Once the blocking solution was removed, 50 μl per well of primary antibodies, 2.5 μg ml^{-1} of the anti-THRSP or S14 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 2.5 μg ml^{-1} of the anti-FASN mouse monoclonal antibody (Becton Dickinson Biosciences) were added. After incubation at RT for 1 h, primary antibody solution was removed and plates were washed three times with 1 × PBS before proceeding with the secondary antibody staining step.

Secondary antibody staining. A total of 1 × PBS was removed and 50 μl per well of diluted secondary antibody was added. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Molecular Probes; Eugene, OR, USA) antibody were diluted in blocking solution to 0.05 mg ml^{-1}. After incubation at RT for 1 h in the dark, secondary antibody solution was removed and plates were washed three times with 1 × PBS. A total of 100 μl per well of 1 × PBS containing 2 μg ml^{-1} Hoechst 33342 (Invitrogen) were added, and the plate was covered and let stand protected from light for 15 min. Plates were immediately imaged or stored at 4 °C in the dark before imaging.

Imaging. The images were captured on a BD Pathway 855 Bioimager System (Becton Dickinson Biosciences) with a × 20 objective (NA 075 Olympus, Madrid, Spain) according to the Recommended Assay Procedure and merged using BD Attovision software. Images of hS14 or hS14 and FASN expression in whole human adipocytes cell cultures undergoing the differentiation process were captured as a 4 × 4 or a 2 × 2 montage.

Gene expression analyses

RNA was prepared from both fat biopsies and cellular debris using RNeasy Lipid Tissue Mini Kit (QIAgen; Gaithersburg, MD, USA). The integrity of each RNA sample was checked by either agarose gel electrophoresis or with an Agilent Bioanalyzer (Agilent Technologies; Palo Alto, CA, USA). Total RNA was quantified by means of spectrophotometer (GeneQuant, GE Health Care; Piscataway, NJ, USA) or with the bioanalyzer and 3 μg of RNA was then reverse transcribed to cDNA using High Capacity cDNA Archive kit (Applied Biosystems; Darmstadt, Germany) according to manufacturer's protocol.

Gene expression was assessed by real-time PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using TaqMan technology suitable for relative gene expression quantification. The reaction was performed following manufacturers' protocol in a final volume of 25 μl. The cycle program consisted of an initial denaturing of 10 min at 95 °C then 40 cycles of 15 s denaturizing phase at 92 °C and 1 min annealing and extension phase at 60 °C. Positive and negative controls were included in all the reactions.

The commercially available and pre-validated TaqMan primer/probe sets used were as follows: Cyclophilin A (*PPIA; 4333763, RefSeq. NM_002046.3,*) was used such as endogenous control for all target genes in each reaction and Spot 14 homolog rat (*THRSP; Hs00930058_m1, RefSeq. NM_003251.2*), FASN (*Hs00188012_m1, RefSeq. NM_004104.4*), acetyl-coenzyme A carboxylase α (*Hs00167385_m1, RefSeqs. NM_198834.1, NM_198836.1, NM_19883 7.1, NM_198838.1 and NM_198839.1*), fatty acid binding protein 4 (*FABP4 or aP2; Hs00609791_m1, RefSeq. NM_001442.2*), peroxisome proliferator-activated receptor γ *(PPARg; Hs01115513_m1, RefSeqs. NM_138711.3, NM_138712.3, NM_005037.5 and NM_015869.4)*, and MID1 interacting protein 1 (*MIG1IP1* or

MIG12; Hs00221999_m1, RefSeqs. NM_001098790.1 and NM_021242.4) were the target genes. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human cyclophilin A (PPIA) cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$ so gene expression results are expressed in all cases as expression ratio relative to PPIA gene expression according to manufacturers' guidelines.

Western blot analysis

A total of 100 μg of RIPA protein extracts were separated by SDS–PAGE and transferred to nitrocellulose membranes by conventional procedures. Membranes were immunoblotted with anti-FASN and anti-S14 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc.). Anti-rabbit immunoglobulin G coupled to horseradish peroxidase was used as secondary antibody. Horseradish peroxidase activity was detected by chemiluminescence and quantification of protein expression was carried out using Scion image software.

In vivo *studies*

Subjects and samples. A total of 161 OM and 87 SC adipose tissue samples (within them, 80 paired fat samples) were obtained from both human fat depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric by-pass surgery), washed, fragmented, and immediately flash frozen in liquid nitrogen before being stored at −80 °C. These fat samples were provided from a group of 161 subjects (65 men and 96 women) with a BMI between 18 and 70 kg m^{-2} who were invited to participate at the Endocrinology Service of the Hospital Universitari de Girona Dr Josep Trueta (Girona, Spain), at the Clinica Universitaria de Navarra (Navarra, Spain) and at the Hospital Carlos Haya de Málaga (Málaga, Spain). All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. They had no systemic disease other than type 2 diabetes and obesity and all were free of any infections within the earlier month before the study. Liver disease and thyroid dysfunction were specifically excluded by biochemical work up. Other exclusion criteria for those patients included the following: (1) clinically significant hepatic, neurological, or other major systemic disease, including malignancy; (2) history of drug or alcohol abuse, defined as >80 g per day, or serum transaminase activity more than twice the upper limit of normal; (3) elevated serum creatinine concentration; (4) acute major cardiovascular event in the earlier 6 months; (5) acute illnesses and current evidence of chronic inflammatory or infectious diseases; and (6) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study. All subjects gave written informed consent after the purpose of the study was explained to them. The institutional review board of the participant institutions approved the protocol.

Anthropometric measurements. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. The subjects' waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteal region. The waist-to-hip ratio was then calculated. *Deurenberg's* formula was used to estimate body fat composition in those subjects.[27] Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals.

Analytical determinations. The serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Brea, CA, USA). The coefficient of variation was 1.9%. Total serum cholesterol was measured through the reaction of cholesterol esterase/oxidase/peroxidase using a BM/Hitachi 747. HDL cholesterol was quantified after precipitation with polyethylene glycol at RT. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase, as described earlier.[28] Free T_4, free T_3, and TSH were measured by electro-chemiluminescence (Roche Diagnostics, Basel, Switzerland) with intra- and inter-assay coefficients of variation <5%.

Statistical analyses

Descriptive results of continuous variables are expressed as mean ± s.d. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test. One-way ANOVA, for multiple comparisons, using *post hoc* by Bonferroni's test (when equal variances could be assumed) was used to compare groups. Relation between quantitative variables was tested using Pearson's correlation coefficient. All data from human samples are expressed as mean ± s.d. The statistical analyses and graphics were performed using the program SPSS (v13.0; Chicago, IL, USA).

Results

hS14 expression during adipogenic maturation of pre-adipocytes

The monitoring of hS14 expression during adipogenic maturation of SC human pre-adipocytes to MAs showed a pronounced increasing of nuclear and cytoplasmic hS14 levels (Figure 1). On differentiation, human pre-adipocytes developed microscopically visible lipid droplets starting at

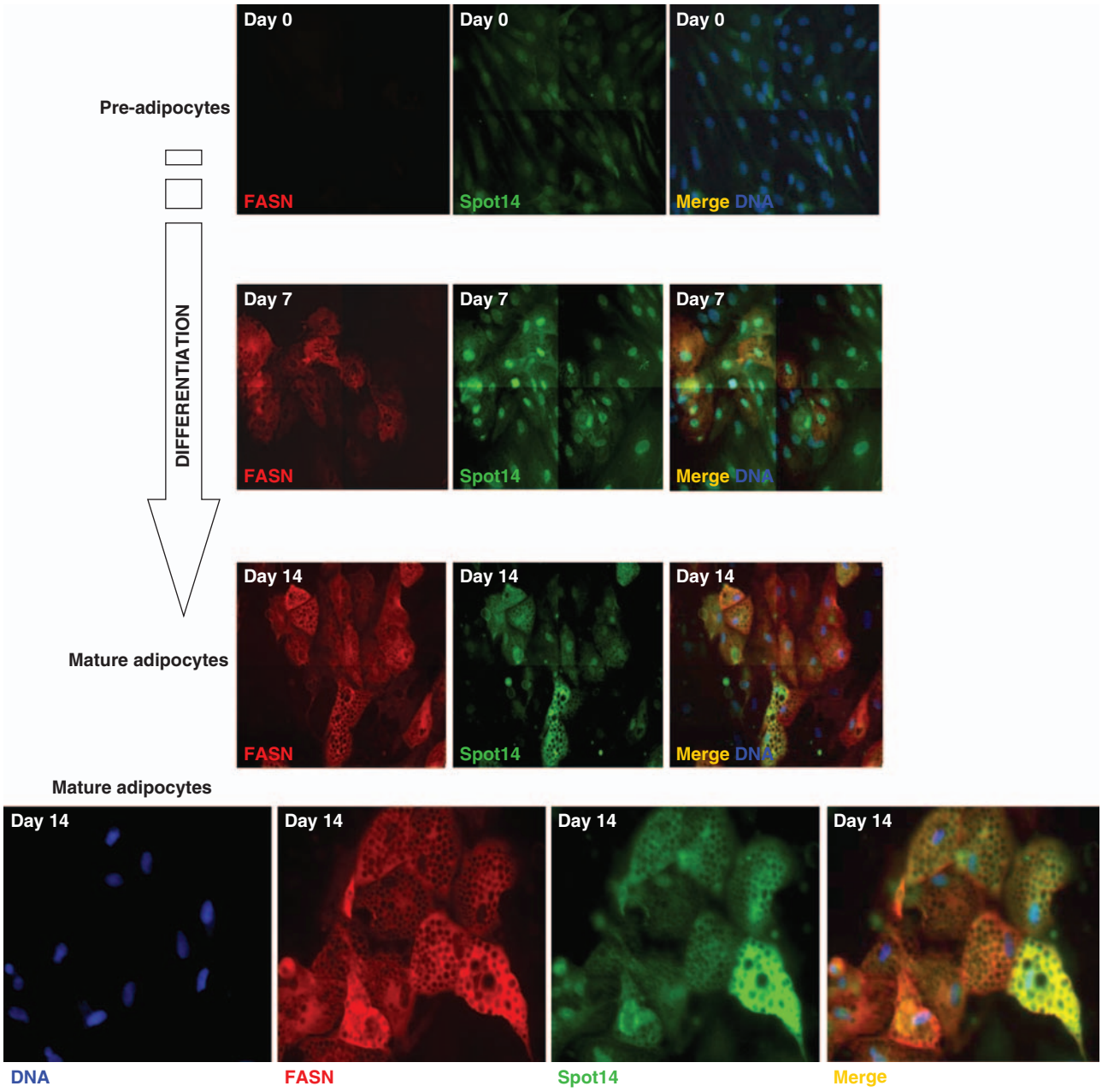
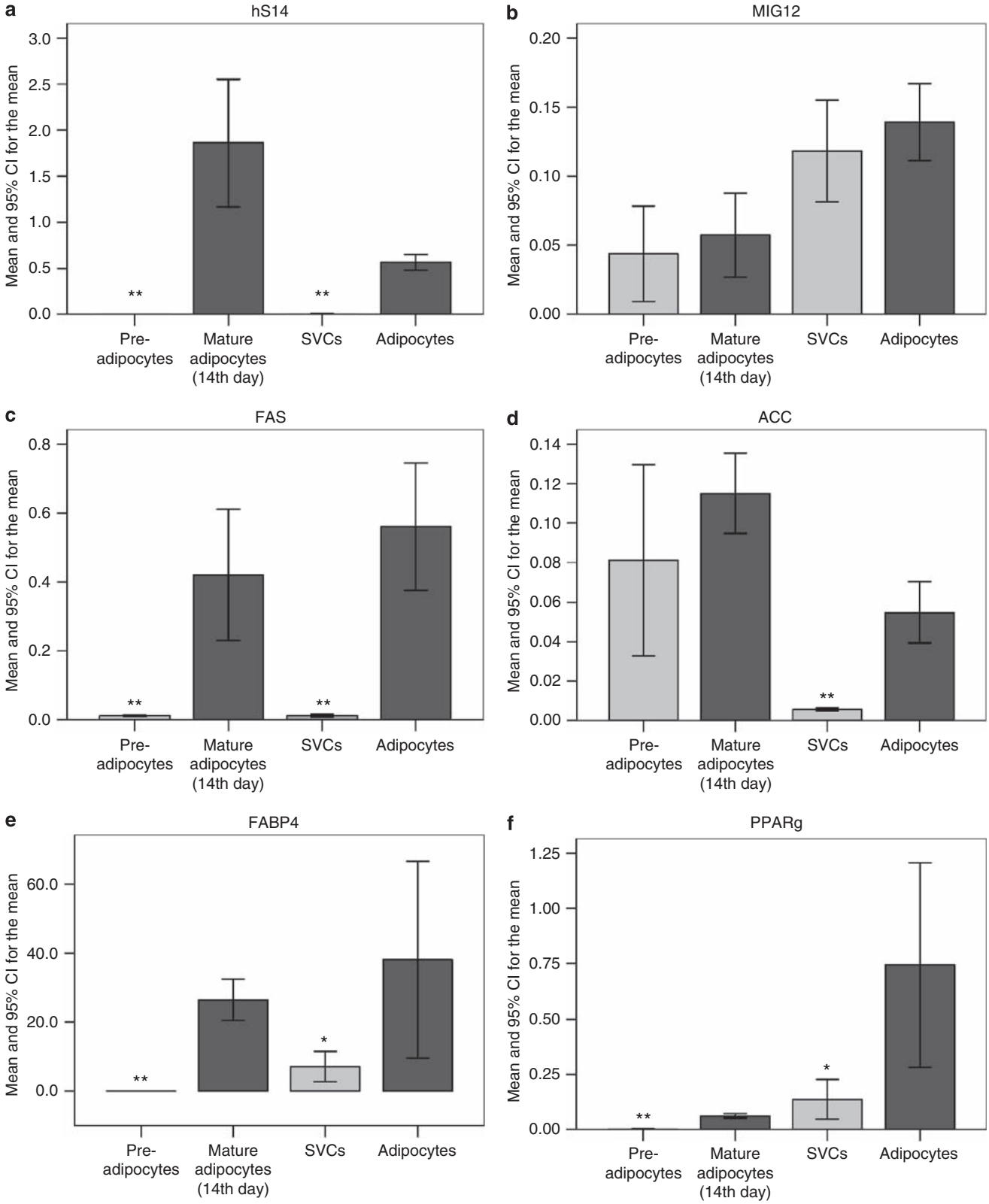


Figure 1 Monitoring of hS14 and FASN expression during adipogenic maturation of human adipocytes. Human adipocytes stained with an anti-hS14 rabbit polyclonal antibody and an anti-FASN rabbit monoclonal antibody on 0, 7, and 14th day. The images were captured in the three different channels for Alexa Fluor 594 (pseudo-colored red, FASN), Alexa Fluor 488 (pseudo-colored green, hS14) and Hoeschst 33,341 (pseudo-colored blue, nuclei-DNA) on a BD Pathway 855 Bioimager System (Becton Dickinson Biosciences) with a $\times$ 20 objective (NA 075 Olympus) as described under Experimental procedures. (The color reproduction of this figure is available on the html full text version of the manuscript.)

7th day. Concomitantly, there was a significant increase in the content of cytoplamic hS14 protein in human adipocytes (Figure 1). The majority of hS14-positive cells were lipid droplets-positive differentiated cells. Importantly, hS14 up-regulation as an early marker of pre-adipocyte differentiation was concurrently accompanied by cytoplasmic accumulation of FASN protein (Figure 1). Negative staining of nuclear and, especially, cytoplasmic S14 and FASN was observed in undifferentiated control cultures.

MAs ($n=6$) differentiated *in vitro* showed highly increased *hS14* gene expression when compared with hS14 levels in pre-adipocytes ($\sim$45 000-fold, $P<0.0001$; Figure 2a). As expected, mRNA levels of other lipogenic factors, namely FASN (Figure 2c), FABP4 (Figure 2e), and PPARg (Figure 2f),


a
hS14
Mean and 95% CI for the mean
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes
b
MIG12
Mean and 95% CI for the mean
0.20
0.15
0.10
0.05
0.00
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes
c
FAS
Mean and 95% CI for the mean
0.8
0.6
0.4
0.2
0.0
**
**
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes
d
ACC
Mean and 95% CI for the mean
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
**
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes
e
FABP4
Mean and 95% CI for the mean
60.0
40.0
20.0
0.0
**
*
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes
f
PPARg
Mean and 95% CI for the mean
1.25
1.00
0.75
0.50
0.25
0.00
**
*
Pre-adipocytes
Mature adipocytes (14th day)
SVCs
Adipocytes

were also significantly up-regulated in differentiated adipocytes (~38-, ~37 900-, and ~26-fold, respectively, $P<0.0001$). ACC (Figure 2d) and MIG12 (Figure 2b) gene expression levels did not change during adipogenesis. No significant differences were observed between cells from obese or lean individuals.

To further confirm our data, we performed western blotting analyses for FASN and S14 with protein samples from 3T3-L1 during adipogenesis (Figure 3). The anti-S14 polyclonal rabbit antibody revealed two bands. The behavior for both the '*upper*' (~1300% higher, $P=0.034$) and the '*lower*' (~15 000% higher in MAs at the 14th day than in pre-adipocytes, $P=0.037$) bands was similar, increasing during differentiation, in parallel to FASN expression (166% higher in MAs, $P=0.001$; Figure 3).

hS14 gene expression in isolated fat cells

Accordingly with our *in vitro* results, human Spot 14 mRNA was highly increased (~130-fold, $P<0.0001$) in MAs compared with SVCs in both SC and OM fat depots (Figure 2a). The gene expression for other lipogenic key-factors, namely FASN (~48-fold, $P<0.0001$; Figure 2c), ACC (~ninefold, $P<0.0001$; Figure 2d), FABP4 (~fivefold, $P<0.05$; Figure 2e), and PPARg (~fivefold, $P<0.05$; Figure 2f) was also higher in MAs than in SVCs, even though not as much as hS14 mRNA. MIG12 (Figure 2b) gene expression levels did not differ between isolated SVCs and MAs.

hS14 gene expression levels in human fat samples

The anthropometric and metabolic characteristics of the study subjects and the relative hS14 gene expression levels in fat depots are summarized in Table 1. hS14 gene expression was significantly decreased in OM adipose tissue samples from obese with or without DM-2 individuals, being in these groups 59.1 and 53.8% lower (respectively, both $P<0.0001$) than hS14 expression in OM fat from lean subjects (Figure 4a). Down-regulated gene expression for hS14 (−42.0%, $P<0.05$) was also found in OM fat depots from overweight subjects. Standard deviation of hS14 mRNA expression values in lean subjects was between 1.5- and 3.0-fold higher than within overweight and obese subjects with or without DM-2 (Table 1a). Although S14 gene expression was consistently decreased in obese groups of both men and women, these differences did not reach the level of significance in SC fat depots (Figure 4b).

Differences for both OM and SC hS14 mRNA levels between groups were easily noticeable in both men and women (Table 1a and b). Indeed, no significant differences for hS14 gene expression between men and women were

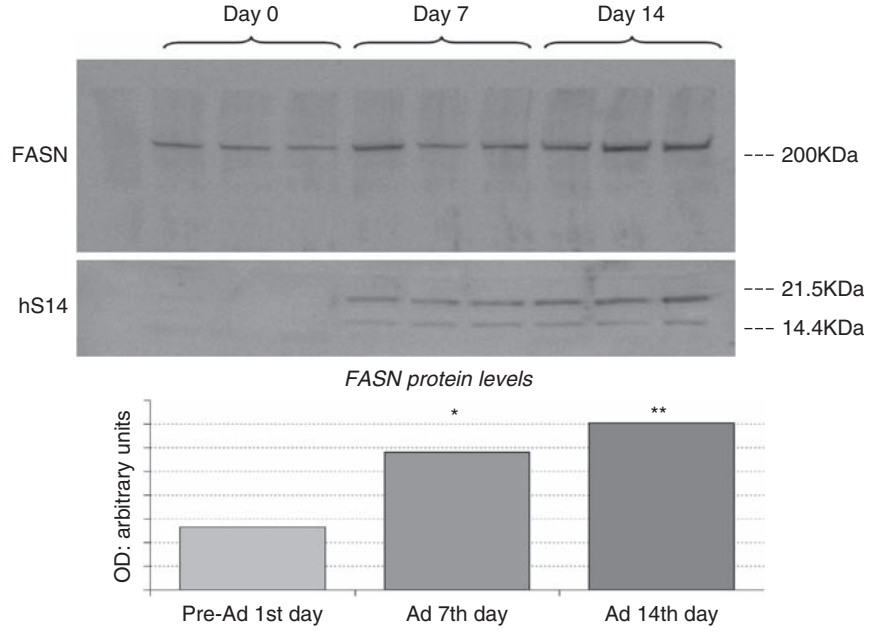


Figure 3 hS14 and FASN protein levels assessed by WB during adipogenic maturation of 3T3-L1. WB results showing two bands between 21.5 and 14.4 kDa. FASN levels (*plot*) increased, as expected, during adipocyte differentiation ($n=3$). **$P<0.001$ and *$P<0.05$ for comparisons between FASN protein levels at 7th or 14th day vs day 0.

Figure 2 RT–PCR: comparisons between immature and mature human fat cells. Mean and 95% confidence interval for the mean of gene expression levels for hS14 (**a**), MIG12 (**b**), FASN (**c**), ACC (**d**), FABP4 (**e**), and PPARg (**f**) in commercially available pre-adipocytes ($n=6$, day 0) *in vitro* differentiated to mature adipocytes ($n=6$, day 14) and in SVCs ($n=8$) and MAs ($n=8$) isolated from both SC ($n=4$) and OM ($n=4$) fat biopsies. **$P<0.001$ and *$P<0.05$ for comparisons between groups of cells (mature vs immature cells) in both *in vitro* and *in vivo* data.

Table 1 Anthropometrical and clinical characteristics of study subjects

	Normoweight	*Overweight*	*Obesity*	*Obesity and DM2*	*P (ANOVA)*
a					
n (women; OM/SC)	15/2	16/14	45/30	20/15	
Age (years)	46 ± 14	51 ± 13	44 ± 13	48 ± 14	0.410
BMI (kg m^{-2})	22.0 ± 2.9	27.0 ± 1.5	42.9 ± 7.9	42.7 ± 6.5	***< 0.0001***
% fat	31.6 ± 4.9	38.6 ± 3.4	56.3 ± 8.8	57.0 ± 6.7	***< 0.0001***
Waist (cm)	76.0 ± 10.5	87.3 ± 6.1	104.3 ± 10.6	119.1 ± 13.8	***< 0.0001***
Hip (cm)	89.5 ± 8.4	102.6 ± 5.5	123.6 ± 18.8	127.0 ± 17.7	***< 0.0001***
Waist-to-hip ratio	0.85 ± 0.07	0.85 ± 0.05	0.88 ± 0.09	0.94 ± 0.06	0.115
Blood glucose (mg per 100 ml)	81.2 ± 9.0	103.6 ± 49.5	96.8 ± 14.5	143.0 ± 58.7	***< 0.0001***
Fasting insulin (μIU ml^{-1})	9.2 ± 5.1	9.3 ± 3.4	14.7 ± 8.5	19.9 ± 10.6	***0.007***
HOMA-IR	1.9 ± 1.2	2.1 ± 0.9	3.3 ± 1.8	7.7 ± 5.2	***< 0.0001***
HbA_{1c}	5.7 ± 0.4	5.6 ± 1.5	5.2 ± 0.7	6.5 ± 2.0	0.067
Total cholesterol (mg per 100 ml)	200.4 ± 35.5	215.5 ± 36.8	193.6 ± 37.0	194.4 ± 40.0	0.270
HDL-cholesterol (mg per 100 ml)	58.7 ± 12.7	67.2 ± 17.0	61.3 ± 52.4	51.4 ± 12.7	0.735
LDL-cholesterol (mg per 100 ml)	121.7 ± 33.4	124.9 ± 41.9	118.6 ± 31.7	120.3 ± 31.5	0.948
Fasting triglycerides (mg per 100 ml)	88.5 ± 38.0	105.3 ± 42.1	117.8 ± 62.2	140.2 ± 89.2	0.147
Systolic blood pressure (mm Hg)	124.2 ± 20.6	126.0 ± 18.2	134.8 ± 21.2	143.9 ± 27.6	0.126
Diastolic blood pressure (mm Hg)	75.7 ± 13.2	77.7 ± 11.1	77.5 ± 11.1	80.8 ± 12.0	0.794
OM hS14 mRNA	**0.607 ± 0.342**	**0.338 ± 0.258**	**0.311 ± 0.160**	**0.269 ± 0.162**	**< 0.0001**
SC hS14 mRNA ($n = 61$)	**0.843 ± 0.472**	**0.565 ± 0.287**	**0.555 ± 0.221**	**0.440 ± 0.145**	**0.088**
b					
n (men; OM/SC)	11/2	16/4	29/16	9/4	
Age (years)	47 ± 17	49 ± 14	42 ± 9	46 ± 6	0.198
BMI (kg m^{-2})	23.1 ± 1.8	27.3 ± 1.8	42.1 ± 7.6	44.8 ± 6.5	***< 0.0001***
% fat	21.5 ± 3.5	27.8 ± 2.6	43.9 ± 8.5	48.1 ± 7.9	***< 0.0001***
Waist (cm)	83.1 ± 8.1	95.1 ± 4.5	120.3 ± 20.7	140.0 ± 4.2	***< 0.0001***
Hip (cm)	91.4 ± 11.1	102.7 ± 4.4	118.0 ± 25.4	134.5 ± 9.2	***0.001***
Waist-to-hip ratio	0.92 ± 0.10	0.93 ± 0.03	0.98 ± 0.03	1.04 ± 0.04	***0.038***
Blood glucose (mg per 100 ml)	79.9 ± 8.8	88.3 ± 9.9	97.0 ± 18.6	139.4 ± 80.6	***0.001***
Fasting insulin (μIU ml^{-1})	9.5 ± 2.8	13.4 ± 7.1	15.2 ± 10.4	22.0 ± 14.2	0.067
HOMA-IR	1.9 ± 0.7	3.0 ± 1.6	4.2 ± 3.3	6.3 ± 4.9	***0.025***
HbA_{1c}	5.4 ± 0.3	5.5 ± 0.4	4.7 ± 0.6	5.1 ± 0.4	***0.002***
Total cholesterol (mg per 100 ml)	169.4 ± 44.1	184.5 ± 37.2	182.3 ± 24.8	205.7 ± 39.0	0.203
HDL-cholesterol (mg per 100 ml)	50.8 ± 11.4	46.8 ± 13.2	44.8 ± 17.1	42.2 ± 11.4	0.629
LDL-cholesterol (mg per 100 ml)	110.7 ± 24.7	116.2 ± 31.5	109.7 ± 29.7	116.0 ± 44.9	0.920
Fasting triglycerides (mg per 100 ml)	73.7 ± 20.5	114.8 ± 42.5	135.5 ± 94.0	236.0 ± 184.2	0.027
Systolic blood pressure (mm Hg)	125.2 ± 16.7	137.8 ± 16.7	138.2 ± 14.8	139.8 ± 23.3	0.268
Diastolic blood pressure (mm Hg)	76.7 ± 10.9	78.8 ± 10.2	86.7 ± 10.8	79.0 ± 19.3	0.210
OM hS14 mRNA	**0.649 ± 0.534**	**0.392 ± 0.382**	**0.248 ± 0.135**	**0.211 ± 0.101**	***0.002***
SC hS14 mRNA ($n = 26$)	**0.536 ± 0.038**	**0.616 ± 0.286**	**0.384 ± 0.135**	**0.435 ± 0.107**	***0.058***

	Non-obese (BMI < 30 kg m^{-2})	*Obese (non-DM-2)*	*P (t-Student)*
n (women+men)	8	28	
BMI (kg m^{-2})	24.9 ± 3.1	42.6 ± 8.0	***< 0.0001***
OM hS14 mRNA	**0.322 ± 0.151**	**0.221 ± 0.085**	***0.019***
OM FAS mRNA	0.197 ± 0.123	0.083 ± 0.073	***0.002***
OM ACC mRNA	0.051 ± 0.056	0.019 ± 0.011	***0.007***
OM MIG12 mRNA	0.066 ± 0.047	0.052 ± 0.029	0.304

Abbreviations: % fat, percent body fat-mass estimated by Deurenberg's formula; ACC, acetyl-CoA carboxilase; ANOVA, analysis of variance; BMI, body mass index; DM-2, type-2 diabetes mellitus; FASN, fatty acid synthase; hA1c, glycosylated hemoglobin; HOMA-IR, homeostasis model assessments of insulin-resistance; hS14, human thyroid hormone responsive Spot 14; MIG12, MID1 interacting protein 1 or Spot 14-related; OM, omental adipose tissue; SC, subcutaneous adipose tissue. Human thyroid hormone responsive Spot 14 (hS14) values are written in bold.

found. Thyroid function was not significantly associated with *hS14* gene expression in these subjects.

Analysis of 80 paired fat samples of OM and SC fat depots revealed that *hS14* gene expression was, as reported earlier,[29] significantly increased (Figure 5a) by 1.93-fold in SC vs OM adipose tissue ($P < 0.0001$). SC and OM *hS14* gene expression levels were significantly correlated ($r = 0.448$, $P < 0.0001$). Interesting, higher hS14 levels (3.8-fold, $P < 0.05$) in SVCs/pre-adipocytes (Figure 5b), but not in isolated MAs (Figure 5c), were also found in cells isolated from SC when compared with those obtained from OM fat depots.

OM hS14 mRNA (but not SC hS14) correlated with parameters such as BMI ($r = -0.281$, $P = 0.001$), percent fat mass ($r = -0.278$, $P = 0.001$; Figure 4c), waist-to-hip ratio ($r = -0.293$, $P = 0.027$), systolic blood pressure ($r = -0.230$, $P = 0.031$), and circulating serum glucose (Figure 4d), but not

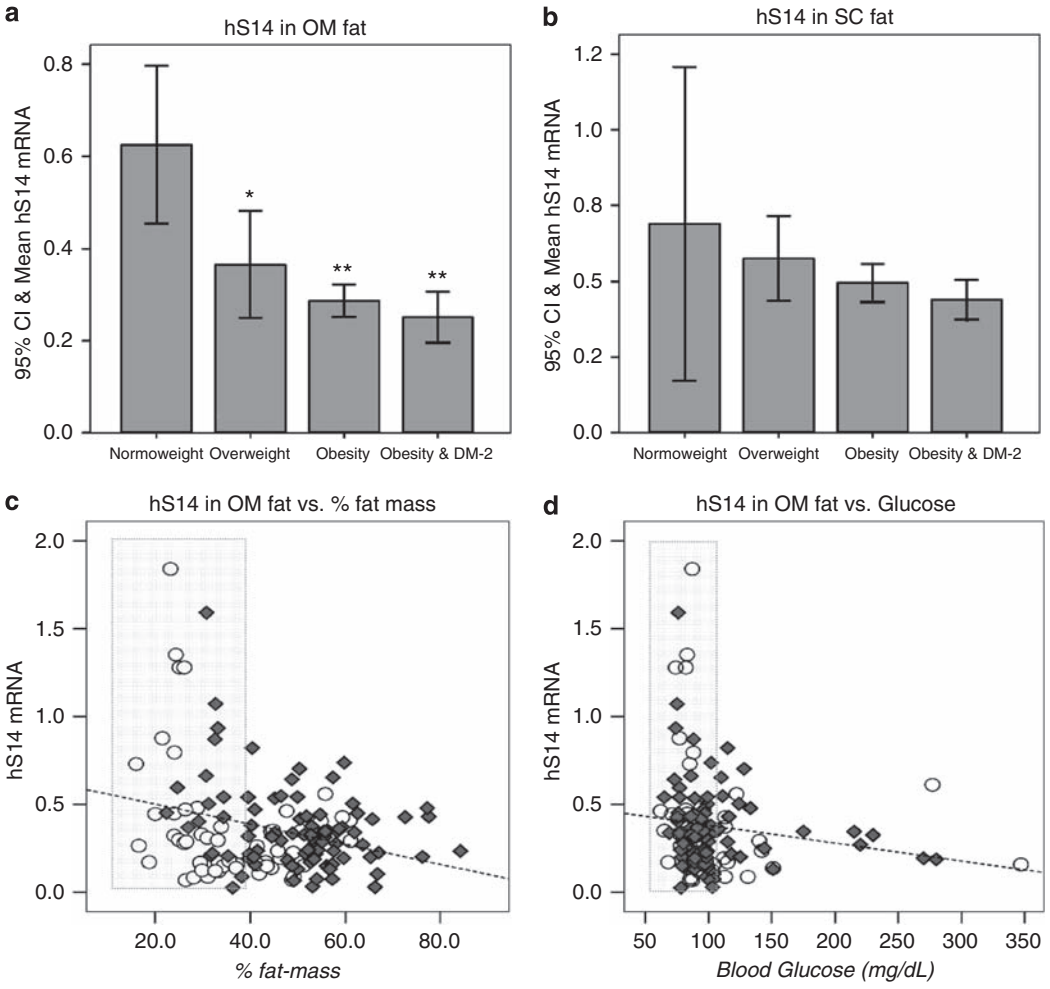


Figure 4 RT–PCR: hS14 gene expression levels vs adiposity. Mean and 95% confidence interval for the mean of gene expression levels for hS14 in omental ($n=161$, **a**) and subcutaneous ($n=87$, **b**) fat from normoweight (BMI $<25\,kg\,m^{-2}$), overweight ($25\leqslant BMI<30\,kg\,m^{-2}$), and obese (BMI $\geqslant 30\,kg\,m^{-2}$) with or without type 2 diabetes mellitus (DM-2) subjects. Linear relationship between hS14 expression level and percent fat mass (**c**; $r=-0.264$, $P=0.001$). hS14 mRNA was inversely correlated, in men (○) as well as among women (◆), also with blood glucose levels (**d**; $r=-0.190$, $P=020$). $^{**}P<0.001$ and $^{*}P<0.05$ for comparisons between normoweight and the rest of the groups.

with fasting triglycerides. Noteworthy, the negative correlation between hS14 and BMI remained significant ($r=-0.341$, $P=0.001$; $n=85$) even when the metabolically compromised subjects (DM-2 and morbidly obese patients) were excluded from the analysis.

Finally, *FASN* and *ACC* gene expression values, assessed in a subset of the patients ($n=36$), were, as well as hS14 levels (−31.4%, $P<0.05$), significantly decreased (−57.9 and −62.7%, respectively, both $P<0.05$) in obese subjects ($n=28$) compared with non-obese individuals ($n=8$), as reported earlier.[30] *S14-R* (MIG12) gene expression levels were not significantly different among groups (Table 1b).

Discussion

Spot 14 was originally identified as an mRNA from rat liver that responded rapidly to thyroid hormone,[13,31] and has been shown to have an important function in the tissue-specific regulation of lipid metabolism.[16,18] In addition to its responsiveness to thyroid hormone, S14 gene transcription is controlled by dietary substrates, such as glucose[11] and polyunsaturated fatty acids,[17] and by fuel-related hormones including Ins and glucagon.[32,33] However, most of this knowledge about S14 mRNA behavior has been described in hepatic tissue from animals and *in vitro* models. The function of hS14 in adipogenic processes, as well as the relationship between hS14 gene expression in adipose tissue and the development of obesity in human beings, has been only evaluated by Kirschner and Mariash[25] in a very small study. We here report hS14 gene expression levels and cellular protein location in human preadipocytes and MAs, and hS14 mRNA in human adipose tissue from a large population that varied widely in terms of obesity and body fat distribution.

hS14 mRNA and protein levels were, as well as *FASN*, *ACC*, *FABP4*, and *PPARg* gene expression levels, highly

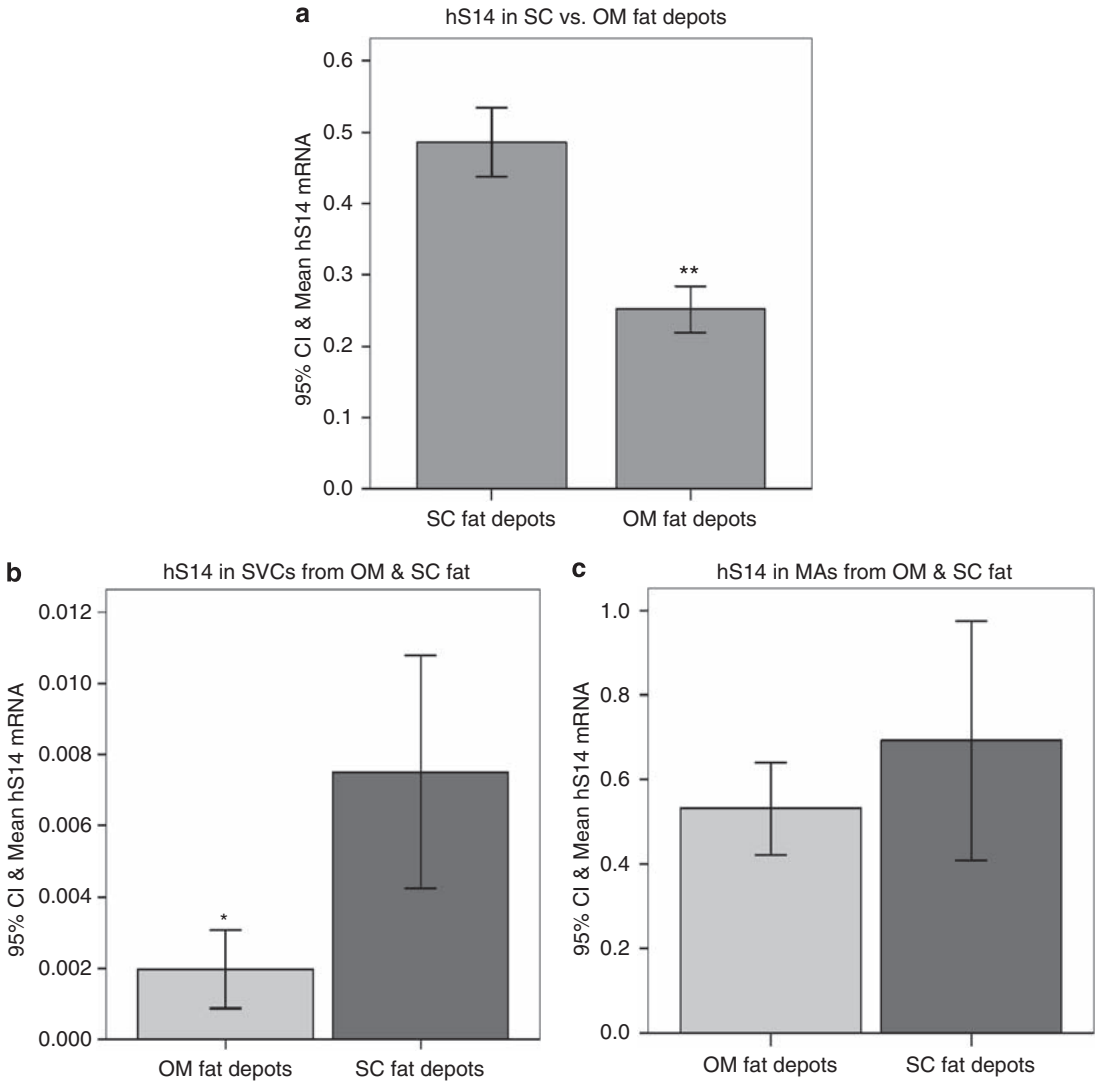


Figure 5 RT–PCR: comparisons between human fat depots. Mean and 95% confidence interval for the mean of gene expression levels for hS14 in subcutaneous (SC) vs omental (OM) fat depots ($n=80$ paired samples; **a**), in stromal-vascular cells (SVCs, **b**) and mature adipocytes (MAs, **c**) isolated from SC and OM fat biopsies from a subset of four individuals ($n=8$ paired samples). $^{**}P<0.001$ and $^{*}P<0.05$ for comparisons between groups.

up-regulated in MAs isolated from fat biopsies and, accordingly, during *in vitro* differentiation of human pre-adipocytes into MAs. In fact, dramatic differences between pre-adipocytes and MAs were detected for hS14 levels, suggesting that hS14 could be a mediator of adipogenesis in human fat depots.

S14 protein was found to be located primarily in the cell nucleus of hepatocytes,[9,34] specifically in the perivenous portion of the hepatic lobule, one of the main sites of fatty acid synthesis.[35] S14 has been earlier suggested to regulate expression of genes encoding key metabolic enzymes, including those required for long-chain fatty acid synthesis.[18,35] Otherwise, S14 might be a cofactor for thyroid receptor-dependent transcriptional activation for lipogenic key factors such as malic enzyme through the physical interaction between S14 and thyroid hormone receptors.[36,37] In agreement with these studies, the expression status of the adipogenic marker FASN[38] and of the hS14 in individual human pre-adipocytes and MAs showed a remarkable nuclear location of hS14 protein. During adipocyte differentiation, and concomitantly with FASN levels, the cytoplasmic accumulation of hS14 protein was more prominent in individual cells exhibiting differentiation features (that is visible lipid droplets). Moreover, as observed for the adipogenic marker FASN, cytoplasmic and nuclear hS14 protein accumulation in differentiated adipocytes reached a maximum at 14th day (Figure 1). These findings suggest that hS14 could not only regulate expression of genes encoding enzymes required for fatty acid synthesis in human adipocytes, but also be engaged in active lipid synthesis, as far as

hS14 co-localizes with FASN in the cytoplasm of differentiated fat cells.

Otherwise, hS14 expression in breast tumors is highly concordant with overabundance of lipogenic enzymes.[39,40] Interestingly, Sanchez-Rodriguez *et al*.[39] found that hS14 overexpression causes inhibition of cell proliferation, induction of cell differentiation, and accumulation of lipid droplets in MCF-7 human breast cancer cells. As earlier observed with FASN and HER2,[38,41] hS14 up-regulation was more evident in human adipocytes developing visible lipid droplets. Finally, Donnelly *et al*.[42] have recently showed that conjugated linoleic acid could reduce tumor lipogenesis and growth through *S14* and *FASN* gene expression suppression in human breast cancer and liposarcoma cells.

On the other hand, and consistent with earlier studies (decreased hepatic S14 mRNA in diabetic rats[32]), we found decreased hS14 levels in OM adipose tissue from obese and/or DM-2 subjects. *hS14* gene expression levels were inversely associated with obesity parameters such as BMI, percent fat mass, and waist circumference. The most striking aspect of increased adiposity and abdominal obesity is its association with Ins resistance and increased circulating free fatty acids.[43] Interestingly, SC hS14 mRNA was significantly down-regulated after fasting in lean, but not in obese volunteers.[25] In our study, fasting conditions were similar in obese and non-obese subjects, suggesting that the differences in *hS14* gene expression were even higher if down-regulation experienced in non-obese subjects would be accounted for.

There is an apparent paradox between the developmental data and the negative correlation between the mRNA levels for hS14 and measures of obesity. However, a remarkable reduction in the expression of genes coding for lipogenic factors such as *SREBP-1c*, *FASN*, *ACC*, *PEPCK*, *ATP Citrate-lyase*, or *Pyruvate Carboxylase*[30,44,45] or involved in adipocyte differentiation[46] has been found in obesity. This suggest that, at least in obese subjects with a large and long-lasting fat excess, the decreased expression of lipogenic genes[47] could be a late and adaptive process, aimed at limiting adipocyte hypertrophy and further development of fat mass.

Finally, higher hS14 mRNA levels in SC pre-adipocytes and/or fat SVCs than in OM ones were found (Figure 5b). Pre-adipocytes from SC fat biopsies show *'higher and faster'* differentiation rate than pre-adipocytes from OM fat depots.[48] In fact, hS14 mRNA levels were invariably higher in SC than in OM fat depots for all studied paired samples. These findings suggest that hS14 could be not only strongly related to human adipogenesis, but also a gene responsible for differences between human fat depots at lipo- and adipogenic capacities.

In summary, the findings reported here suggest that hS14 levels are directly associated with adipogenesis in human adipocytes. Differences at hS14 levels between fat depots could reflect that capacity, slightly higher in SC than in OM fat depots. Interestingly, *hS14* gene expression levels in OM adipose tissue were inversely related to obesity parameters. These findings are compatible with the notion that Ins resistance and DM-2 down-regulate hS14 mRNA levels in human adipose tissue as well as in rat hepatic tissue. Accordingly, a remarkable reduction in the expression of other genes controlling lipogenic factors has been shown in obesity. However, further investigations will be required to evaluate the functional consequences of these findings and the biochemical function of S14, which remains elusive.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

All authors of this manuscript have directly participated in the execution and analysis of the study. All authors are aware of and agree to the content of the manuscript, and all authors have approved the final version submitted and their being listed as an author on the manuscript.

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