CMLS, Cell. Mol. Life Sci. 59 (2002) 1960–1971
1420-682X/02/111960-12

CMLS Cellular and Molecular Life Sciences

Research Article

Carboxypeptidase E and thrombospondin-1 are differently expressed in subcutaneous and visceral fat of obese subjects

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Received 7 August 2002; received after revision 19 Septemer 2002; accepted 24 September 2002

Abstract. The aim of this study was to identify candidate genes for visceral obesity by screening for genes strongly differentially expressed between human subcutaneous and visceral adipose depots. A cDNA microarray with human adipose-derived cDNAs was used as an initial screening to identify genes that are potentially differentially expressed between human subcutaneous and visceral abdominal fat tissues. For the two best candidates, carboxypeptidase E (CPE) and thrombospondin-1 (THBS1) (EST N72406), real-time RT-PCR was performed to confirm their depot specific expression in extremely obese individuals. Both genes appeared to be strongly differentially expressed, having a higher expression in the visceral depot than in the subcutaneous one. For THBS1, the difference in expression between the depots was greater in women than in men. The involvement of CPE and THBS1 in obesity allows us to suggest that the physiological processes controlled by these genes contribute to depot and gender-related differences in the metabolic complications of obesity.

Key words. Visceral adiposity; real-time RT-PCR; CPE; THBS1; differential gene expression.

The existence of different phenotypes of obesity was established over 50 years ago [1]. Vague [2] described how body fat was differently distributed among people, and that upper-body fat (abdominal obesity), in particular, was associated with an increased risk of obesity-related health problems such as insulin resistance, non-insulin-dependent diabetes mellitus (NIDDM), dyslipidaemia, hypertension and atherosclerosis. This has been confirmed more recently in several studies that measured the amount of subcutaneous and visceral fat by computed tomography and showed that the detrimental influence of abdominal obesity on metabolic processes is mediated by the visceral fat depot [reviewed in ref. 3].

Visceral adipocytes are more metabolically active, more sensitive to catecholamine-induced lipolysis and less sensitive to anti-lipolytic effects of insulin, than subcutaneous adipocytes [4, 5]. This observation together with the anatomical location of the visceral fat depot resulted in the 'portal theory' [6], in which the differential health effects of the different fat depots are explained by the higher metabolic activity of the visceral one, which results in increased delivery of non-esterified fatty acids (NEFAs) into the portal vein. NEFAs have direct effects on hepatic metabolism, such as the stimulation of glucose production, very low density lipoprotein secretion and interference with hepatic insulin clearance, and would ultimately lead to glucose intolerance, hypertriacylglycerolaemia and hyperinsulinaemia, respectively [7]. A more recent hypothesis, in line with the adipose organ concept [8], is based on the fact that adipose tissue has important secretion functions [9]. Systemic metabolic disturbance would be caused by bioactive factors secreted differently

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by the two fat depots. Examples are leptin [10, 11], plasminogen activator inhibitor-1 (PAI-1) [12] and interleukin (IL)-6 [13].

Despite physiological differences between visceral and subcutaneous adipocytes, the molecular basis for these differences is still largely unknown, and identification of genes whose expression is strongly site related may contribute to identifying candidate genes for visceral obesity [14]. Up to date, a limited number of known genes have been studied. Of these, leptin [10, 11, 15–18] expression was consistently higher in the subcutaneous as compared to the visceral depot, while angiotensinogen expression was higher in the visceral fat [19]. Contradictory results were published for hormone-sensitive lipase, lipoprotein lipase, tumor necrosis factor-alpha and peroxisome proliferator-activated receptor gamma [15, 16, 18, 20, 21]. Uncoupling protein 2 (UCP2) [22] and glucose transporter 4 (GLUT4) [16] were only analysed in a single study. UCP2 was found to be highly expressed in the visceral depot compared to the subcutaneous one, while GLUT4 showed the inverse pattern. 11-Beta hydroxysteroid dehydrogenase type 1 has recently been identified as a gene involved in visceral obesity [23], and its shown expression was greater in visceral fat than in subcutaneous fat of obese patients [24].

Here, we used a two-step procedure to identify new genes that are differentially expressed by different fat depots. An initial screening to find good candidate genes with a putative depot-specific expression was performed by hybridisation of a cDNA microarray with human adipose tissue-derived cDNA clones with two visceral and two subcutaneous adipose tissue samples. The differential expression of the two best candidate genes was subsequently confirmed in a larger number of visceral and subcutaneous adipose depots from morbidly obese men and women using real-time RT-PCR.

Materials and methods

Subjects and adipose tissue biopsies

The study group consisted of 9 men and 14 women, all morbidly obese (age 23–58 years, BMI 39–81 kg/m^2), who were undergoing weight reduction surgery by biliopancreatic diversion [25]. The study was approved by the Ethics Committee of the 'Hospital Universitari Son Dureta' (Palma de Mallorca, Spain) and all subjects gave written consent for biopsies. Fat biopsies were removed from both the abdominal subcutaneous and visceral (omental) adipose tissue of each subject obtained within 20 min after the start of general anaesthesia. Part of the tissue was immediately frozen in liquid nitrogen and stored at –80 °C for subsequent mRNA analysis. Fresh samples of adipose tissue from 9 subjects (5 men and 4 women) were taken in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4), containing 10 mM HEPES and 6 mM glucose for adipocyte isolation.

Adipocyte isolation

Adipocytes were isolated using the method described by Robdell [26] with some minor modifications. Briefly, tissue samples were minced at room temperature and incubated for 35 min with 1.5 g/l of collagenase (Sigma, Barcelona, Spain) in 10 ml KRB buffer (pregassed with 95% O_2–5% CO_2, pH 7.4), containing 10 mM HEPES, 6 mM glucose and 30 g/l bovine serum albumin (Sigma), at 37 °C in a shaking bath. Cells were filtered though a nylon mesh (250 µm) and subsequently washed three times with a collagenase-free buffer. Three hundred microliters of isolated adipocytes were stored at –80 °C for subsequent mRNA analysis.

Culture of PAZ-6 cell line

PAZ-6 preadipocytes (human immortalised brown adipocytes) kindly provided by Prof. D. Strossberg [27] were cultured in a medium consisting of DMEM/F12/Glutamax-I (Invitrogen, Breda, The Netherlands), supplemented with glucose (4.5 g/l), 15 mM HEPES, penicillin (100 µg/ml), streptomycin (0.1 µg/ml) and 8% fetal calf serum (Invitrogen). Cells were cultured at 37 °C in a 95% air-5% CO_2 atmosphere. The culture medium was replaced every other day. Cells were harvested at 90% confluence.

mRNA isolation

Total RNA was isolated from both fat biopsies and isolated adipocytes using Tripure (Roche, Barcelona, Spain) and also from the PAZ-6 cell line using Trizol (Invitrogen), both according to instructions of the manufacturers. Isolated RNA was quantified by spectrophotometry and its integrity was checked by electrophoresis on a 1% agarose/TBE gel containing ethidium bromide. mRNA was isolated using the quickprep micro mRNA purification kit according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

cDNA-microarray construction

A cDNA microarray was constructed using 154 named human cDNAs, 286 human adipose cDNAs, a set of luciferase cDNA clones as positive controls and a *Salmonella* cDNA as a negative control. All clones were printed in duplicate onto the slides. Of the 286 adipose cDNAs, 244 clones were from the WATM1 library. This lambda gt11 library was made from poly(A) mRNA isolated from subcutaneous white adipose tissue from an adult female using random oligonucleotides and ligation adaptors *Eco*RI/*Not*I. Five clones were from the BATM1 library (lambda gt10), similarly constructed from perirenal brown adipose tissue from children. The same tissue sam-

ple was used for construction of the BATM2 library, but in this case, the poly(A) mRNA was primed with oligo-dT primers and the cDNA was cloned in the *Pst*I and *Bam*HI sites of pTZ18 after dGdC tailing. Thirty-three BATM2 clones were on the array. Finally, 4 cDNA clones were from the Clontech adult human fat cell library HL1108A. All cDNA clone sequences have been deposited at EMBL/GenBank by Bouillaud. The full list of named genes is given in Appendix 1.

The 286 adipose cDNAs were reamplified from 3 µl original PCR product using their original PCR oligonucleotide primers 5′-TGG CGA CGA CTC CTG GAG CCC G and 5′-TGA CAC CAG ACC AAC TGG TAA TGG for WATM1 and Clontech (lambda gt11); 5′-GAG CAA GTT CAG CCT GGT TAA GTC C and 5′-ATG AGT ATT TCT TCC AGG G for BATM1 (lambda gt10) and 5′-GTT GTA AAA CGA CGG CCA GTG CC and 5′-CAC ACA GGA AAC AGC TAT GAC CAT G for BATM2 (PTZ-18) in a total reaction volume of 50 µl containing 20 pmol forward and 20 pmol reverse primer, 1.5 mM $MgCl_2$, 1 × PCR buffer (Invitrogen, Breda, The Netherlands), 0.2 mM dNTPs and 2.5 U Taq polymerase (Invitrogen). The reaction was denatured for 2 min at 94 °C, and amplified during 35 cycles of 40 s 94 °C, 1 min 65 °C (lambda gt11) or 58 °C (lambda gt10 and pTZ18) and 1.5 min 72 °C, with a final incubation of 10 min 72 °C. The resulting PCR products were diluted 1000 times and used as template in a nested PCR. One microlitre was amplified using the same PCR conditions and the following 5′-amine-C6 linked oligonucleotide primers 5′-TCC TGG AGC CCG TCA GTA TC and 5′-ACC GGC GCT CAG CTG GAA TT for WAT1 and Clontech (lambda gt11); 5′-GCC TGG TTA AGT CCA AGC TG and 5′-TCC AGG GTA AAA AGC AAA AG for BATM1 (lambda gt10) and 5′-AAC GAC GGC CAG TGC CAA and 5′-TTA ATA CGA CTC ACT ATA GGG for BATM2 (pTZ18) in a total volume of 100 µl containing 40 pmol forward and 40 pmol reverse primer, 1.5 mM $MgCl_2$, 1 × PCR buffer (Invitrogen), 0.2 mM dNTPs, 5 U Taq polymerase (Invitrogen).

The 154 named human cDNA fragments and the positive and negative controls were amplified using the 5′ Amine-C6 AGG CGA TTA AGT TGG GTA AC and 5′ Amine-C6 AGC GGA TAA CAA TTT CAC AC oligonucleotide primers in a 100-µl reaction volume containing 40 pmol forward and 40 pmol reverse 5′-C6-amine-linked primers, 1.5 mM $MgCl_2$, 1 × PCR buffer (Invitrogen), 0.2 mM dNTPs, 5 U Taq polymerase and 1 µl bacterial glycerol stock. The PCR conditions were as follows: 2 min 94 °C followed by 35 cycles of 40 s 94 °C, 1 min 55 °C, 2.5 min 72 °C and subsequently a final incubation of 10 min 72 °C. To check the quality of the PCR reactions, 1 µl of each PCR product was run on a 1% agarose/TBE gel. Subsequently, amplification products were purified with the Qiaquick purification kit (Qiagen-Westburg, Leusden, The Netherlands) using Millipore filtered water as an elution buffer; eluates were evaporated using vacuum and dissolved in 10 µl spotting buffer (5 × SSC). Microarray slides were produced as described elsewhere [28].

Sample preparation, labelling and microarray hybridisations

Four mRNA samples, two from subcutaneous and two from visceral abdominal adipose tissues from morbidly obese women, were used for the cDNA microarray screening. mRNA from PAZ-6 cells was used as a reference to allow the comparison of sample hybridsed on different slides. Labelled cDNA was obtained from 1 µg mRNA by incorporation of either Cy5-dCTP (fat biopsy sample mRNA) or Cy3-dCTP (reference PAZ-6) during a reverse transcription as previously described [28]. Before labelling, 0.5 ng luciferase mRNA (Promega, Leiden, The Netherlands) was spiked into each sample mRNA, to check the efficiency of the labelling reaction based on the hybridisation signals of the luciferase spots present on the array. Each slide was prehybridised for 5 h and subsequently hybridised overnight at 42 °C simultaneously with a 1:1 (v:v) mixture of the sample cDNA (labelled with Cy5) and the reference cDNA (labelled with Cy3) dissolved in hybridisation buffer. Prehybridisation and hybridisation protocols and subsequent washing steps are been described by Boeuf et al. [28].

Microarray scanning

Slides were scanned as previously described [28]. Two image files were obtained for every slide, corresponding to the Cy3 (λex 550 nm, λem 570 nm) and the Cy5 (λex 649 nm, λem 670 nm) measurements. The image files generated by the scanner were analysed using the software package ArrayVision (Imaging Research, St Catharines, Canada), obtaining the fluorescence intensity for every spot.

CDNA microarray data analysis

Data analysis was performed using the average intensity of duplicate spots. For data normalisation, two corrections were performed. The first correction was performed to normalise for (i) the amount of spotted PCR product, (ii) local hybridisation conditions within a slide and (iii) hybridisation conditions between slides. For every two slides that were compared, corrected Cy5 values were calculated according to the following equation: $Cy5^{corr\ 1}_{spot\ i,\ slide\ 2} = Cy5_{spot\ i,\ slide\ 2} \times (Cy3_{spot\ i,\ slide\ 1}/Cy3_{spot\ i,\ slide\ 2})$. The first correction was followed by a second correction step to normalise for the amount of mRNA labelled and the difference in labelling efficiency between the various samples. For this purpose, corrected Cy5 values were calculated for every two slides that were compared according to the following equation: $Cy5^{corr2}_{spot\ i,\ slide\ 2} = Cy5^{corr1}_{spot\ i,\ slide\ 2} \times$ (me-

dian Cy5 signals$_{slide\ 1}$/median Cy5 signals$_{slide\ 2}$). Genes with a ratio of the normalised fluorescence intensity, obtained from the second equation, between both depots equal to higher or than 2 were considered to be differentially expressed. All genes with a ratio ≥ 2 but with signal intensities lower than the threshold (a signal intensity ≥ 1 in at least one of the tissues being compared) were excluded from further analysis.

Real-time RT-PCR

To validate differential expression, real-time RT-PCR was used. RNA from subcutaneous and visceral fat depots from both adipose tissue (9 men and 14 women) and isolated adipocytes (5 men and 4 women) was used. Five micrograms of total RNA from each sample was incubated for 15 min at 37 °C with 2 U RNase-free DNase (Promega) and 80 U RNase OUT (Invitrogen) in a final volume of 100 µl. The reaction was stopped by adding 10 µl of stopping buffer (10 mM TRIS-HCl pH 7.4, 1 mM EDTA and 3 mM NaCl) and the DNase treated RNA was ethanol precipitated. cDNA was synthesised from 1 µg of DNase-treated total RNA with 150 ng random hexamers using Super Script First Strand Synthesis (Invitrogen) according to the protocol of the supplier. Each cDNA was diluted 1/25, 1/250 and 1/2500 and aliquots were frozen at –20 °C for subsequent PCR reactions. Real-time PCR was done for three housekeeping genes: beta-2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hexokinase 1 (HK) and two target genes: carboxypeptidase E (CPE) and thrombospondin-1 (THBS1/EST N73406) using oligonucleotide primers as given in table 1. PCRs were performed using the LightCycler (Roche Diagnotics Nederland, Almere, The Netherlands). Each reaction contained 0.5 µM of the relevant oligonucleotide primers (table 1), 4 mM $MgCl_2$, 2 µl LightCycler-FastStart DNA Master SYBR Green I (containing FastStart enzyme, dNTPs, $MgCl_2$, and SYBR Green I dye; Roche) and 5 µl of the cDNA dilution buffer (Roche Diagnostics Nederland) in a final volume of 20 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (10 min at 95 °C), followed by 45 cycles consisting of a denaturation step [95 °C for 0 s (B2M, HK, GAPDH, THBS1) or 2 s (CPE)], an annealing step [5 s at 58 °C (B2M, HK, GAPDH) or at 55 °C (CPE and THBS1)] and an extension step (18 s at 72 °C for all). After each cycle, fluorescence was measured at 72 °C (λex 470 nm, λem 530 nm). PCR products were subjected to a melting curve analysis on the Light Cycler and subsequently 1% agarose/TBE gel electrophoresis to confirm amplification specificity (T_m and amplicon size). Real-time RT-PCR was performed in duplicate and a negative control without cDNA template was run in every assay. To allow relative quantification of a target gene between the two tissues, a relative standard curve was constructed for every subject as previously described [29] with some minor modifications. To perform the relative standard curve, arbitrary template concentration values (0.1, 0.01 and 0.001) were given to the three cDNA dilutions (mentioned above) from the visceral tissue, which, according to the microarray results, showed higher expression levels. The 1/25 dilution from the subcutaneous tissue (with a lower expression) was analysed as unknown. Data analyses were performed with the LightCycler analysis software version 3.5 (Roche). To correct for the quality and amount of starting RNA and reverse transcription efficiency, three housekeeping genes were used (B2M, HK and GAPDH). These were analysed for every tissue sample and dilution. The mean was used to normalise the target genes that were tested from a different aliquot of the same cDNA dilution. Normalised target concentrations from the 1/25 dilution were used to compare the expression levels in subcutaneous and visceral fat samples.

Table 1. Real-time RT-PCR primers.

Gene	Primers sequence (5′–3′)	Amplicon size (bp)	T_m amplicon (°C)
B2M	forward: 5′-CCTGAATTGCTATGTGTCTGGGTTTC reverse: 5′-CTCCATGATGCTGCTTACATGTCTCG	249	84
HK	forward: 5′-GAGGAACCAATTTCCGTGTGCTGCT reverse: 5′ CCTTTGATCCCCATGTAGTCCAAGA	177	85
GAPDH	forward: 5′-ATGGGGAAGGTGAAGGTCGGAG reverse: 5′- TCGCCCCACTTGATTTTGGAGG	265	87
CPE1	forward: 5′-TGTCTGACCCCAATCG reverse: 5′- ACTCCTCGGTGTATCT	253	87.5
TSP-1	forward: 5′-CCCTTCAAAACAAATAGGAGTTCA reverse: 5′-ATCCTGTGATTCCAAATGCCAG	118	84

Oligonuclotide primer sequences used for real-time RT-PCR of housekeeping and target genes and the specificity parameters (length and T_m) of the resulting amplicons. B2M, beta-2 microglobulin; HK, hexokinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CPE, carboxypeptidase E; THBS1, thrombospondin-1.

Statistical analysis

The paired t test was used to compare levels of the normalised target concentrations in paired subcutaneous and visceral adipose tissues. Student's t test was used to compare the visceral versus subcutaneous ratios between men and women. Analyses were performed using Windows SPSS version 10.0 software.

Results

We constructed a cDNA microarray containing 154 selected named genes and 286 human adipose tissue-derived cDNA clones. The named genes encompass cDNAs that are involved in basic cellular processes such as apoptosis, the cell cycle, lipid metabolism and adipocyte development. The use of cDNA microarray technology potentially allowed us to simultaneously screen among 440 genes for those that are differentially expressed by the two fat depots. Four slides, two with subcutaneous and two with visceral adipose tissue-derived Cy5-labelled cDNA were hybridised. On all four slides, cDNA from the human adipose PAZ-6 cell line, labelled with the Cy3 dye, was hybridised as a reference; therefore, four comparisons were allowed (two samples from subcutaneous fat were compared with two samples from visceral fat). Differential expression levels between depots could be analysed for approximately 110 cDNAs due to signal limitations in either the sample or the reference. The relatively large number of genes that could not be interpreted was caused by a combination of a stringent threshold that was set at twice the background, to the absence of expression of a significant number of genes in the undifferentiated PAZ-6 cells and, especially, to the small amounts of mRNA (1 μg) that were used for labelling.

The genes that showed a differential expression higher or equal to twofold between depots in three or four of the comparisons performed are given in table 2. Three genes were identified with a consistently higher expression level in the visceral than in the subcutaneous depot: carboxypeptidase E (CPE), expressed sequence tag (EST) N73406 and glucagon receptor (GCGR). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) showed that EST N73406, originating from human perirenal brown adipose tissue, is 100% homologue to thrombospondin-1 (THBS1) and will be considered identical and here referred to as THBS1. Three of four comparisons showed a differential expression in muscle glycogen phosphorylase, transcobalamin 2 and prostaglandin-endoperoxide synthase 1, which were also all higher in the visceral depot.

Of the genes from which differential expression has previously been described, lipoprotein lipase and UCP-2

Table 2. Microarray hybridisation results.

Gene	Accession numbe	Comparison	Normalised fluorescence intensity		Vs/Sc ratio
			subcutaneous (Sc)	visceral (Vs)	
Carboxypeptidase-1 (CPE)	NM_001873	1	1.1	10	8.8
		2	1.3	13	10
		3	1.1	6.2	5.5
		4	1.3	20	15
Glucagon receptor (GCGR)	NM_000160	1	0.53	2.7	5.0
		2	0.78	4.6	5.9
		3	0.53	1.8	3.4
		4	0.78	6.2	7.9
Thrombospondin-1 (THBS1)	XM_031617 N73406	1	0.16	1.1	7.1
		2	1.2	3.2	2.7
		3	0.16	1.1	7.3
		4	1.2	2.9	2.4
Muscle glycogen phosphorylase (PYGM)	NM_005609	2	0.18	2.3	13
		3	0.17	1.4	8.5
		4	0.18	1.1	5.9
Transcobalamin 2 (TCN2)	NM_000355	1	0.53	1.5	2.8
		3	0.53	1.3	2.4
		4	1.2	2.4	2.1
Prostaglandin-endoperoxide synthase 1 (PTGS1)	NM_000962	1	0.59	1.4	2.3
		2	1.3	2.8	2.2
		4	1.3	3.2	2.6

Normalised fluorescence intensities for subcutaneous (Sc) and visceral (Vs) fat, and Vs/Sc ratios are presented. Comparisons: 1, VsA/ScA; 2, VsB/ScB; 3, VsB/ScA; 4, VsA/ScB. Only those with a ratio ≥ 2 in three or four of the comparisons are presented.

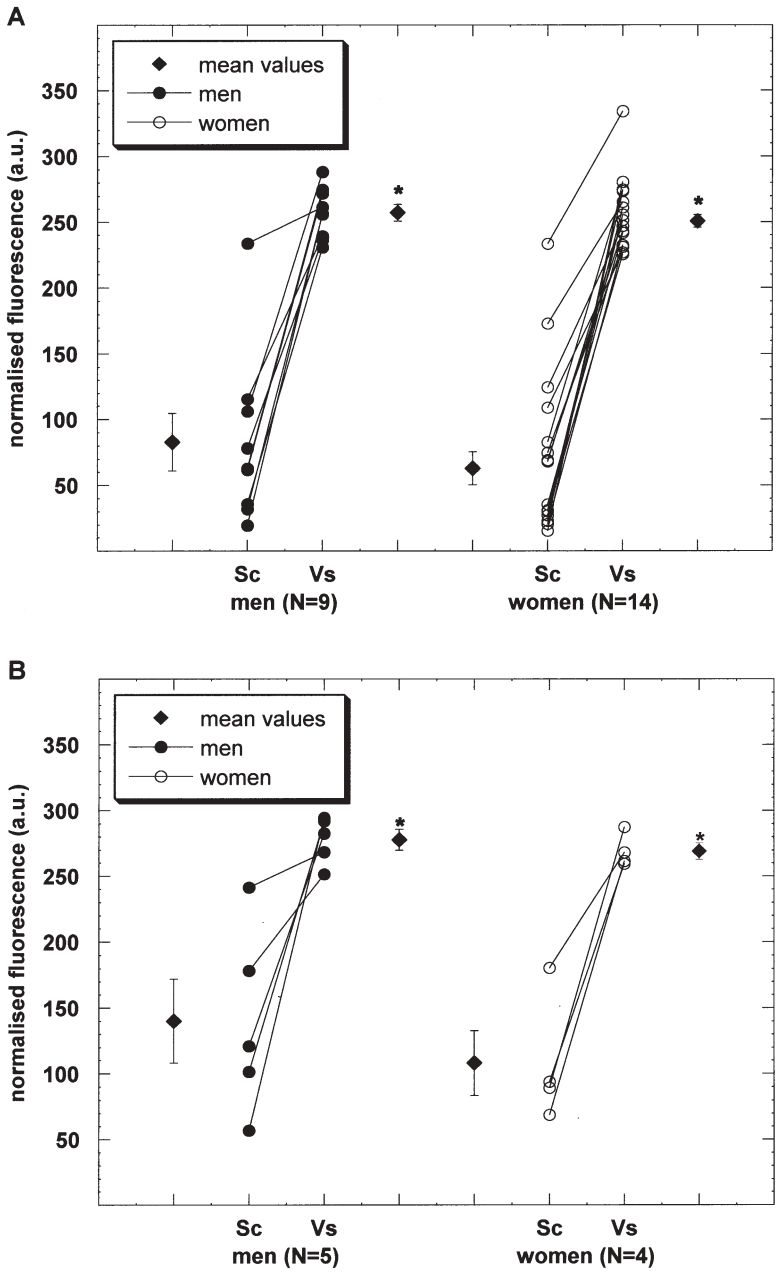

Figure 1. CPE mRNA expression. Normalised mRNA expression levels as determined by real-time RT-PCR for carboxypeptidase E (CPE) in paired samples of subcutaneous and visceral fat depots from adipose tissue (*A*) and isolated adipocytes (*B*) from morbidly obese men and women. Both individual data and means ± SE are presented. Results were statistically compared by paired t test (*$p < 0.03$).

were above threshold, but neither showed a clear consistent differential expression level (ratio ≥ 2) between subcutaneous and visceral fat depots. Since leptin did not meet the stringent requirements set for analysis, and because it constitutes a gene with a wide documented differential expression between depots [10, 11, 15–18], we conducted individual examination. This further microarray data analysis pointed to a higher expression in the subcutaneous than in the visceral depot. Moreover, leptin mRNA levels from the samples used for the microarray hybridisations were also checked by semiquantitative RT-PCR (data not shown) and the differential expression was confirmed.

To validate depot-differential expression and to obtain information on the interindividual differences in more subjects, real-time RT-PCR was performed for two selected genes, CPE and THBS1 (EST N73406). These genes were chosen because consistent and strong signals were obtained in the microarray analysis and a role for these genes in obesity could be envisaged. The expression of these genes was analysed in adipose tissue samples from 14 women (11 for THBS1) and 9 men, all morbidly obese

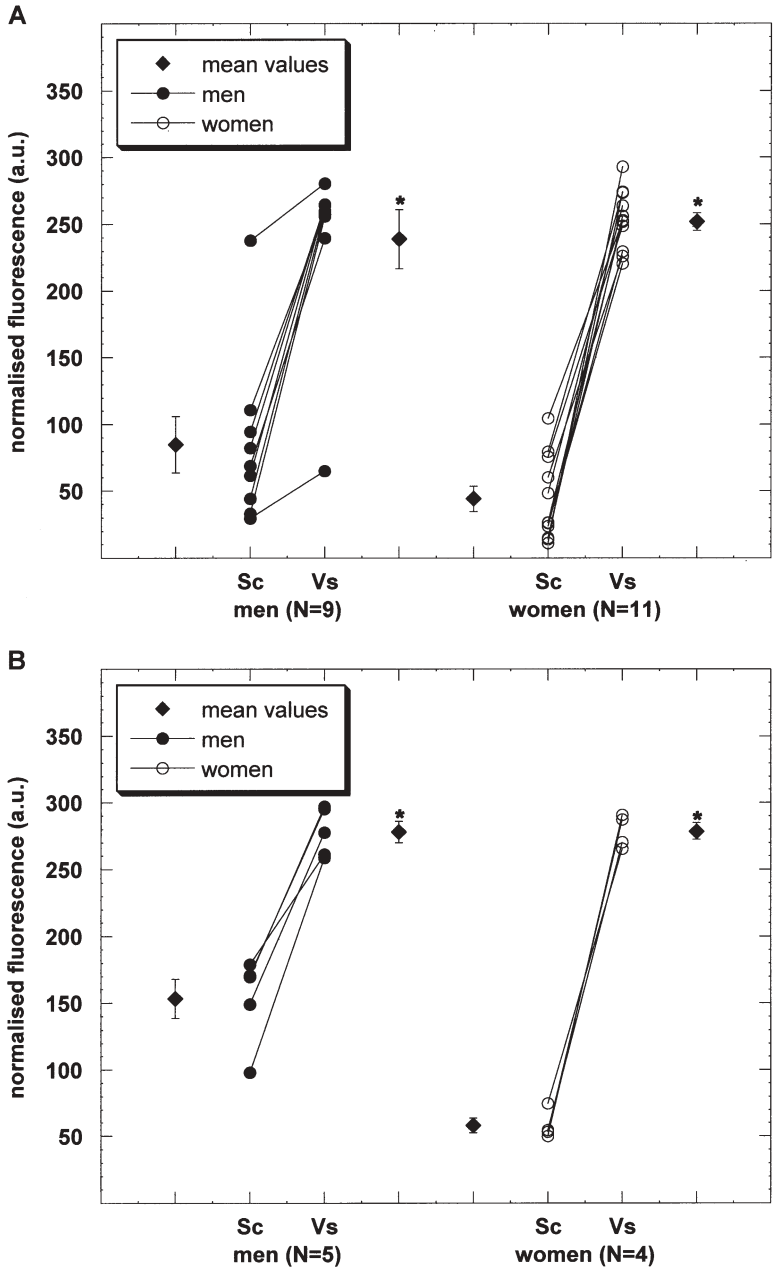

Figure 2. THBS-1mRNA expression. Normalised mRNA expression levels as determined by real-time RT-PCR for thrombospondin-1 (THBS1) in paired samples of subcutaneous and visceral fat depots from adipose tissue (*A*) and isolated adipocytes (*B*) from morbidly obese men and women. Both individual data and means ± SE are presented. Results were statistically compared by paired t test (*$p \leq 0.01$).

subjects. The normalised expression levels obtained by real-time PCR for the CPE and the THBS1 genes in paired tissue samples are shown in figures 1A and 2A. Expression levels were confirmed as being significantly higher in the visceral than in the subcutaneous adipose tissue depot (paired t test $p < 0.03$). Although there were some interindividual variations, the tendency for higher expression in the visceral depot was maintained in all the subjects studied. When the visceral (Vs) subcutaneaous (Sc) ratio between men (BMI 58 ± 2) and women (BMI 53 ± 3) was compared (fig. 3A), a significant difference was found for THBS1, which was greater in women (Student's t test $p < 0.03$), mainly due to a significantly lower expression of this gene in the subcutaneous depot.

To confirm that the difference in mRNA expression levels of CPE and THBS1 in the different fat depots was mainly due to the adipocytes themselves, real-time RT-PCR was also performed in adipocytes isolated from subcutaneous and visceral adipose tissues. The expression levels of both genes in paired fat cell samples (figs. 1B,

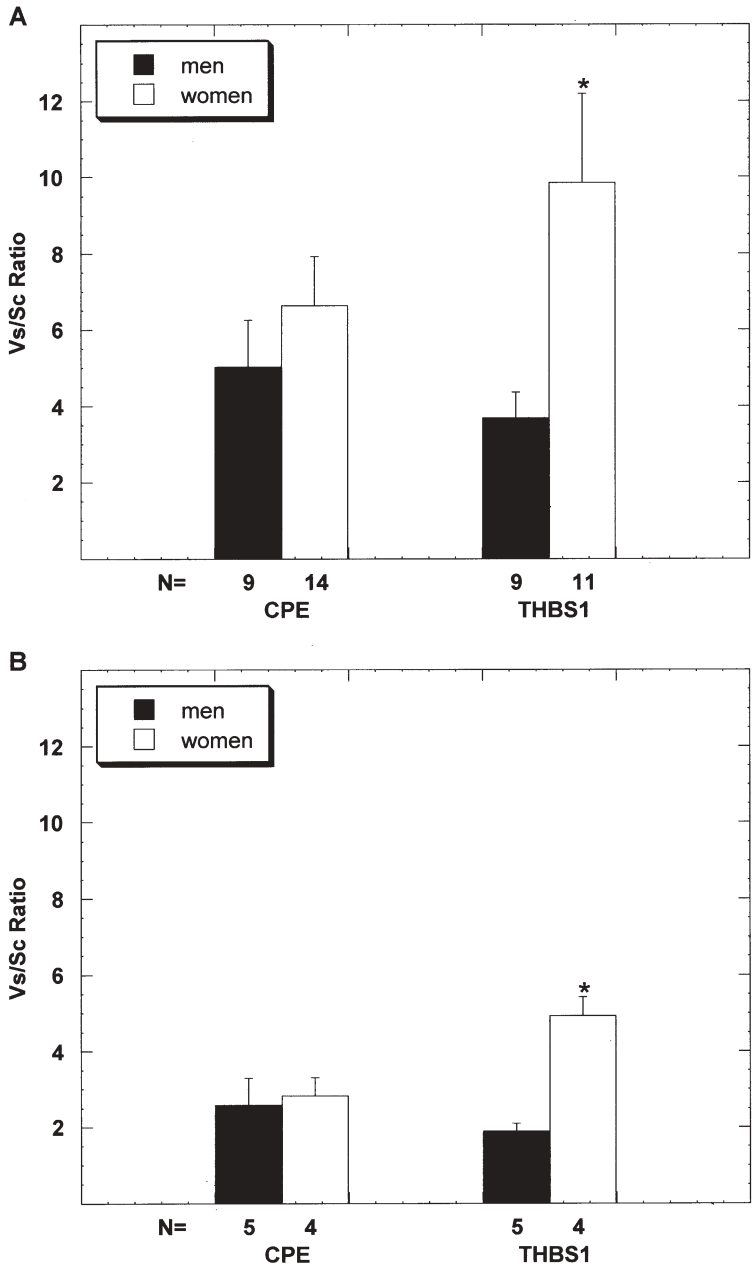

Figure 3. Vs/Sc ratio. Gene expression ratio between visceral and subcutaneous fat depots (Vs/Sc) from adipose tissue (*A*) and isolated adipocytes (*B*) from morbidly obese men and women for CPE and THBS1. Data are the mean ± SE. Results were statistically compared by Student t test (*$p < 0.03$).

2B) followed the same pattern seen in the tissue samples (paired t test $p < 0.03$). Moreover, the significant gender difference for the Vs/Sc ratio found in the tissue for THBS1 was also found in isolated adipocytes (fig. 3B) (Student's t test $p < 0.03$).

Discussion

Subcutaneous and visceral adipose tissues are differently involved in the risk for obesity-related chronic disorders. In our attempt to contribute to elucidating of the molecular basis underlying the metabolic differences between these depots, we used a strategy that combined cDNA microarray screening and real-time RT-PCR. In this study, CPE and THBS1 were found to be more highly expressed in visceral than in subcutaneous adipose tissue from morbidly obese subjects.

From the cDNA microarray screening, six candidate genes were identified with a putativly higher expression in the visceral depot (listed in table 2). Genes that are causally involved in visceral obesity are expected to be

consistently different in all individuals examined. Of the six candidate genes, CPE and THBS1 were selected for further analysis and their differential expression in both depots was confirmed by real-time RT-PCR in a larger number of subjects. Interestingly, differential expression levels were higher in the real-time RT-PCR than in the array experiments. This is consistent with the findings for Rajeevan et al. [29] who showed for some genes that array results pointed in the same direction, but were not identical to real-time results. In our case, the discrepancy may be caused by the small amount of mRNA that was used in the labelling protocol and subsequent hybridisation. However, this does not influence the validity of the data obtained with real-time RT-PCR. In our screening, only some of the spots on the array were informative, which raises the possibility that a number of differentially expressed genes could have been overlooked. This fact is substantiated by the observation that leptin was not identified as a differentially expressed gene in our overall analysis due to the stringent criteria that we set. However, when it was further examined with less stringent criteria, differential expression was found, in agreement with the literature [10, 11, 15–18].

CPE was one of the genes confirmed to be more highly expressed in visceral than in subcutaneous adipose tissue. CPE is a prohormone-processing exopeptidase found in secretory granules of endocrine and neuroendocrine cells [30], and is strongly implicated in obesity. The autosomal recessive fat mutation, which produces maturity-onset obesity, hyperglycaemia and infertility in Cpefat/Cpefat mice, was identified as a single mutation Ser202Pro in the CPE gene that results in a fully inactive enzyme [31]. Development of obesity in the Cpefat/Cpefat mice is attributed to the pleiotropic disturbances in the full maturation of a variety of peptide hormones associated with the control of body fat mass and nutrient partitioning [32]. CPE has been shown to play a role in the conversion of proinsulin to fully mature insulin. The R238W mutation that results in a mutant protein with altered enzymatic properties has been identified in humans [33]. This mutation occurred in four Ashkenazi NIDDM families of which the heterozygous members displayed an earlier age of onset for NIDDM, which is characterised by elevated levels of proinsulin.

To date, only one study has reported the expression of the CPE gene in human adipose tissue [34]. In this study, three CPE clones were present among 1422 randomly sequenced clones of a human adipose tissue cDNA expression library. The differential expression of CPE that we found in visceral as compared to subcutaneous adipocytes might reflect an altered endocrine activity of these cells – supported by the fact that neuroendocrine cells abundantly express CPE [34] – and might explain the elevated release of certain biomolecules from the visceral ompared to the subcutaneous depot.

THBS1 (EST N73406) was the second gene that was confirmed as being more highly expressed in visceral versus the subcutaneous adipose tissue and isolated adipocytes. THBS1 is an extracellular matrix glycoprotein that interacts with structural macromolecules as well as growth factors, cytokines and proteases forming multiprotein complexes [35]. Among the reported functions of THBS1, its ability to inhibit angiogenesis and to activate transforming growth factor-β (TGF-β) are the better-documented ones [36]. TGF-β has been shown to be a strong inducer of PAI-1 mRNA synthesis and PAI-1 release by human adipose tissue explants [12] and human adipocytes [37]. One can speculate that the elevated plasma levels of PAI-1 that are found in obesity and correlate strongly with parameters that define the insulin resistance syndrome may be due to TGF-β activation by THBS1. A factor pointing in this direction is the higher expression of THBS1 in the visceral adipose tissue, in agreement with the higher levels of PAI-1 in this depot [37]. THBS1 is also more directly implicated in abdominal obesity. In the analysis of differential gene expression between visceral adipose tissue of Otsuka Long-Evans Tokushima fatty (OLETF) rats, a model for NIDDM, and its diabetes-resistant counterpart, the Long-Evans Tokushima Otsuka (LETO) rats, Hida et al. [38] found THBS1 as a gene specifically expressed in accumulated visceral adipose tissue of obese OLETF rats. This suggests that it may participate in the pathogenesis of both abdominal obesity and its vascular complications, possibly due to the inhibitory angiogenic activity. Moreover, THBS1 has been recently identified as a possible downstream target of PPARγ in adipocyte differentiation [39]. Our result that THBS1 (EST N73406) was differentially expressed in the visceral and the subcutaneous adipose tissues of morbidly obese subjects may therefore constitute a highly relevant finding. Regional adipose tissue differences in both metabolic activity [40, 41] and gene expression [10] are gender dependent. In line with this, of interest is the lower expression of THBS1 (EST N73406) in the subcutaneous fat of women, which results in a significant gender difference for the Vs/Sc ratio. This agrees with the observation in human endometrium cells that THBS-1 is hormonally regulated [42].

In conclusion, the use of a cDNA microarray as a screening tool combined with the validation by real-time RT-PCR was useful for identifing the differential expression of CPE and THBS1 (EST N73406) in visceral and subcutaneous adipose depots from morbidly obese subjects. Although the expression of these genes remains to be investigated in persons with normal weight and different degrees of obesity, the involvement of both genes in obesity together with the differential expression of THBS1 between men and women allow us to suggest that these genes are contributing factors for depot and gender-related differences in the metabolic complications of obesity.

Acknowledgements. We thank Drs J. Moreiro, J. A. Soro, M. García-Sanz and R. Salinas (Hospital Universitari Son Dureta, Palma de Mallorca, Spain) for providing the human adipose tissue biopsies for this study. We thank Prof. Dr. D. Strossberg (Hybrigenetics, Paris, France) for providing the Paz-6 cell line. We thank C. Gelly and S. Raimbault (CNRS) for help in construction of the adipose cDNA libraries. We thank Prof. Dr. S. Klaus (German Institute for Human Nutrition, Potsdam, Germany) and E. A. van Beek (RIKILT) for constructive discussions. This study was supported by the Spanish Government (Dirección general de Investigación Grant BFI2000-0988-C06-01 and Ministerio de Sanidad y Consumo Grant FIS01/1379), the European Union (COST Action 918) and RIKILT. J. M. Ramis is funded by a doctoral fellowship of the Spanish government.

Appendix 1. List of named genes present on the array

Gene	Symbol	Accession number
Leptin	Leptin	XM_004625
(Liver) fatty acid-binding protein	L-FABP	M10050
uncoupling protein 2	UCP2	XM_006363
Peroxisome proliferator etc gamma	PPARgamma	X90563
Beta adrenergic receptor 2	BAR2	Y00106
Beta adrenergic receptor kinase 1	BARK1	X61157
Ornithine decarboxylase	ODC	XM_068675
Cholecytokinin	CCK	L00354
Estrogen receptor	ESR	XM_045967
X-linked chronic granulomatus disease	CGD-X	X04011
Trefoil factor 1	PS2(TFF1)	X52003
G-protein alpha s	G alfa s	X04408
Adenomatous poly[posis coli	APC	M74088
Sucrose isomaltase	SI	X63597
Amino peptidase N		M22324
Alkaline phosphatase	AP	M31008
Pro galanin	GAL	M77140
Melanocorticoid receptor 4	MC4R	L08603
Melanocorticoid receptor 5	MC5R	Z25470
Dopamine receptor	DRD2R	M30625
Neuropeptide Y	NPY	K01911
Leptin receptor	LEPR	U66497
Glucocorticoid receptor alpha	GRL	U25029
Insulin-like growth factor 2 receptor	IGF2R	Y00285
Hormone-sensitive lipase	LIPE	L11706
Lipoprotein lipase	LPL	M15856
NA-K ATPase	ATP1B1	M25160
BCL2	BCL2	M14745
BCLX1	BCLX1	Z23115
Bcl2-associated X protein	BAX	L22473
Bcl2 killer 1	BAK1	BC004431
Retinoblastoma	RB	L11910
Tumor protein 53	TP53	U94788
Kras	Kras	M54968
Cytochrome P450, subfamily 1, polypeptide 1	CYP1A1	XM007727.2
Microsomal glutathione S trans. 1	MGST1	U77604
Cox 1, prostaglandin-endoper. synth. 1	PTGS1	M59979
mutL (E. coli) homolog 1 (colon cancer, nonpolyposis type 2	MLH1	U07343
mutS (E. coli) homolog 2 (colon cancer, nonpolyposis type 1)	MSH2	NM000251.1
Uracil DNA glycolase	UDG	Y09008
Insulin-like growth factor 1 receptor	IGF1R	X04434
Mahogany, attractin	Mahogany	NM_012070.1
Uncoupling protein 3	UCP3	U84763
Fatty acid-binding protein 3	FABP3	U57623
Fatty acid-binding protein 4	FABP4	NM_001442.1
Fatty acid-synthase	FAS	S80437
Carboxypeptidase-1	CPE1	X51405
Adipsin, complement factor D precursor	DF	M84526
Glucose transporter gene	HepG2	K03195
Glucose transporter 3 (GLUT3)	SLC2A3	M20681
Glucose transporter 4 (GLUT4)	SLC2A4	M91463
Cytochrome P450, subfamily 1, polypeptide 2	CYP1A2	XM_007726.3
Proopiomelanocortin	POMC	J00292
Placenta-specific growth hormone	GH2	M38451
Glucagon receptor	GCGR	L20316
Thyroid hormone receptor alpha	THRA1	X55005
Thyroid stimulating hormone rec.	TSHR	A34990
Parathyroid hormone	PTH	N92572
Insulin-like growth factor-binding prot. 1	IGFBP1	X81579
Peroxisome proliferator etc alpha	PPARa	Y07619
Na-K ATPase 1A2	ATP1A2	J05096
Phosphodiesterase	PDE3	AA135497
Folate-binding protein		AF000380
Transcobalamin 2	TCNII	M60396
Cubilin	CUBN	NM_001081.1
Tumor necrosis factor	TNF	XM_011402.2
Glucagon	GCG	J04040
Alpha1C adrenergic receptor	ADRA1C	U02569
Alpha2C adrenergic receptor	ADRA2C	M38742
Ubiquitin	Ubiquitin	U49869
Phospholipase A2, group V	PLA2G5	U03090
Hypoxanthine phosphoribosyl-transferase	HPRT	J00423
Aryl hydrocarbon receptor	AHR	NM_001621.2
Cytochrome P450	CYP2E1	J02843
Glutathione S-transferase theta 1	GSTTI	X79389
Glutatione S-transferase M1B	GSTI	J03817
Crystallin, zeta	CRYZ	L31526
Retinoic acid receptor, beta	RARB	Y00291
Matrix metalloproteinase I	MMP-1	X54925
DNA damage-inducible transcr. 3	DDIT-3	XM_058528
gadd45 gene (DNA damage-inducible transcript)	gadd45	L24498
Metallothionein isoform 2	MT-2	X97260
MHC class III Hsp7-Hom gene	HLA	M59829
Glutathione peroxide 1	GPX1	M21304
Catalase	CAT	NM_001752.1
Superoxide dismutase	SOD	X02317
Superoxide dismutase 2	SOD2	X65965
HALPHA44 gene for alpha tubilin	HALPHA44	X06956
PeptideYY	PYY	D13902
Angiotensinogen	AGT	M69110
Angiotensinogen-converting enzyme (ACE)	ACE	X16295
Preadipocyte EGF-like protein	dlk1	U15979

Gene	Symbol	Accession number
PPAR coactivator 1	PPARGC1	XM_003396.1
Phosphoribosyl pyrophosphate amidotransferase	PPAT	D13757
CCAAT/enhancer-binding protein alpha	C/EBPa	U34070
CCAAT/enhancer-binding protein beta	C/EBPb	X52560
Prostaglandin-endoperoxide synthase 2	PTGS2	XM_051899
Peroxisome proliferator-activated receptor, delta	PPARD	XM_004285.3
Methionine adenosyl-transferase I, alpha	MAT1A	NM_000429.1
Methionine adenosyl-transferase II, alpha	MAT2A	L43509.1
S-adenosylhomocysteine hydrolase	AHCY	M61831
Cystathionine beta synthase	CBS	L14577
Serine hydroxymethyl-transferase 2	SHMT2	U23143
Methionine synthase receptor	MTRR	AF121214
Cytosine methyltransferase 1	DNMT1	NM_001379.1
Cytosine methyltransferase 2	DNMT2	NM_004412.1
Cytosine methyltransferase 3A	DNMT3A	AF067972
RNA(guanine-7-)methyl-transferase	RNMT	NM_003799.1
Dutp pyrophosphatase	DUT	NM_001948.1
Signal transducer and activator of transcription	STAT5b	U47686
Signal transducer and activator of transcription	STAT3	BC000627
Fork-headed transcription factor	AFX	X93996
Acetyl coenzymeA carboxylase alpha	ACACA	NM_000664.1
Phosphoenolpyruvatecarboxyl-kinase 1	PCK1	NM_002591.1
Phosphoenolpyruvatecarboxyl-kinase 2	PCK2	NM_004563.1
Acyl CoA oxidase 2	ACOX2	X95190
Proprotein convertase subtilisin/kexin type 1	PCSK1	NM_000439.2
Phosphorylase, glycogen: muscle	PYGM	NM_005609.1
Cholesterylestertransferprotein	CETP	M30185
Phospholipid transfer protein	PLTP	L26232
Pyruvate kinase, liver and RBC	PKLR	D13243
Hexokinase 1	HK1	X66957
Hexokinase 3	HK3	U51333
Glucokinase	GCK	M90299
SGLT1, human Na^+/glucose cotransporter 1 mRNA	SLC5A1	M24847
Caudal-related gene 2	cdx2	AF007886
Na^+/H^+ exchanger	SLC9A3	NM_004174.1
Ileal lipid-binding protein (fatty acid-binding protein 6)	FABP6	NM_001445.1
Arachidonate 5-lipoxygenase	ALOX5	NM_000698.1
Solute carrier family 2	GLUT2A2	NM_000340.1
Solute carrier family 16	GLUT16A3	NM_004207.1
Epidermal growth factor receptor	EGFR	NM_005228.1
Epidermal growth factor	EGF	NM_001963.2
Transforming growth factor alpha	TGFA	NM_003236.1
Transforming growth factor beta 2 precursor	TGFB2	NM_003238.1
Betaglycan (TBRIII gene)	TBR3	AJ251961.1
Hepatocyte growth factor	HGF	M73240
IGF-binding protein 4	IGFBP4	M62403
IGF-binding protein 5	IGFBP5	L27560
Dipeptidyl peptidase IV	DPP4	X60708
Apolipoprotein C-III	APOC3	M28614
N-acetyltransferase 1	NAT1	X17059.1
Sulfotransferase (estrogen preferring)	STE	U55764
Sulfotransferase family 1A	SULT1A1	N47673
Occludin	OCLN	U53823
Interleukin BSF-2 (B cell differentiation factor)		X04602
Inducible nitric oxide synthase	NOS	AF068236
Solute carrier family 15	SLC15A1	NM_005073.1
Interferon gamma receptor 1	IFNGR1	AA101236
Interleukin 1 receptor antagonist	IL1RN	U65590.1
Plasminogen activator, urokinase	PLAU	AA192627
Intercellular adhesion 1 molecule	ICAM-1	X59288

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