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Islet-1: A Potentially Important Role for an Islet Cell Gene in Visceral Fat

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Objective: To examine differences in gene expression between visceral (VF) and subcutaneous fat (SF) to identity genes of potential importance in regulation of VF.

Methods and Procedures: We compared gene expression (by DNA array and quantitative PCR (qPCR)) in paired VF and SF adipose biopsies from 36 subjects (age 54 ± 15 years, 15 men/21 women) with varying degrees of adiposity and insulin resistance, in chow and fat fed mice (± rosiglitazone treatment) and in c-Cbl$^{-/-}$ mice. Gene expression was also examined in 3T3-L1 preadipocytes during differentiation.

Results: A twofold difference or more was found between VF and SF in 1,343 probe sets, especially for genes related to development, cell differentiation, signal transduction, and receptor activity. Islet-1 (*ISL1*), a LIM-homeobox gene with important developmental and regulatory function in islet, neural, and cardiac tissue, not previously recognized in adipose tissue was virtually absent in SF but substantially expressed in VF. *ISL1* expression correlated negatively with BMI ($r = -0.37$, $P = 0.03$), abdominal fat (by dual energy X-ray absorptiometry, $r = -0.44$, $P = 0.02$), and positively with circulating adiponectin ($r = 0.33$, $P = 0.04$). In diet-induced obese mice, expression was reduced in the presence or absence of rosiglitazone. Correspondingly, expression was increased in the c-Cbl$^{-/-}$ mouse, which is lean and insulin sensitive (IS). *ISL1* expression was increased sevenfold in 3T3-L1 preadipocytes during early (day 1) differentiation and was reduced by day 2 differentiation.

Discussion: An important developmental and regulatory gene *ISL1* is uniquely expressed in VF, probably in the preadipocyte. Our data suggest that *ISL1* may be regulated by adiposity and its role in metabolic regulation merits further study.

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Type 2 diabetes is a steadily increasing and costly disease closely associated with obesity—especially abdominal obesity (1). Studies by our own (2) and other groups (3,4) have shown strong correlations among abdominal obesity, insulin resistance, and cardiovascular risk factors, and in animals surgical removal of intra-abdominal fat improves insulin sensitivity and delays onset of diabetes (5,6). In humans, when there is discordance between amount of peripheral and visceral fat (VF), as in the HIV lipodystrophy syndrome, insulin resistance is related to VF (7).

A number of important differences between the visceral and peripheral adipocyte have been documented. For instance the visceral adipocyte is more responsive to lipolytic stimuli and less to insulin's anti-lipolytic action; lacks the differentiation and lipogenic response to PPARγ agonists; and has a different pattern of cytokine production compared with the subcutaneous adipocyte (8). Recently it has also been shown that a number of developmental genes are expressed quite differently in VF and subcutaneous fat (SF) and that this difference is maintained during culture *in vitro* (9,10)—suggesting that VF and SF are developmentally different tissues.

To gain further information on differences between human VF and SF, we have examined gene expression in biopsies of both, and have identified the unique expression of a transcription factor, Islet-1 (*ISL1*), in VF. *ISL1* plays an important role in the development of pancreatic islets, motor neurons, and cardiac tissue (11–13). We have shown a high level of expression of *ISL1* in preadipocytes and correlations with adiposity and adiponectin levels in humans and animals.

METHODS AND PROCEDURES

Subjects

A total of 36 subjects (15 men/21 women) undergoing elective abdominal surgery participated in the study. At surgery, biopsies of abdominal

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SF and VF were obtained and immediately frozen in liquid nitrogen. Where possible a number of phenotypic and metabolic measures were made (logistic problems prevented performance of all measurements in all subjects, so the number completing each analysis is given): dual energy X-ray absorptiometry scan for total and regional fat and lean mass (including a "window" of central abdominal fat previously shown to correlate strongly with insulin resistance (2)) was performed on 31 subjects. Computed tomography scan for determination of VF vs. SF (four slices between L2 and L4 (14)) was performed on 27 subjects. Euglycemic hyperinsulinamic clamp, with insulin infusion at 50 mU/kg/h into an antecubital vein with a variable rate infusion of IV glucose to maintain a blood glucose level of 5 mmol/l, sampling of "arterialized" venous blood from a cannula placed retrogradely in the warmed contralateral hand (2), was performed in 13 non-diabetic subjects. In these subjects, *M*-value was inversely correlated ($R = -0.86$; $P = 0.0002$) with $HOMA_{IR}$, an index of insulin resistance, determined as "(fasting plasma glucose × fasting insulin level)/22.5" (15); thus $HOMA_{IR}$ was used as a measure of insulin sensitivity in non-diabetic subjects ($n = 29$). Subjects with $HOMA_{IR}$ < the group mean (4.0) were considered normally insulin sensitive (IS) (Table 1). The study protocol was approved by the Vincent's Hospital Research Ethics Committee and all subjects gave prior written consent.

Animals

Male mice (C57BL/6, 12 weeks) were fed ad libitum a standard chow diet containing 69% carbohydrate, 21% protein, 5% fat, fiber, vitamins, and minerals. Food intake and body weight were monitored. After 2 weeks of adaptation, the animals were fed either the same chow diet (Chow) or a high-fat (HF) diet for 20 weeks. The HF diet was 59% fat, 21% protein, 20% carbohydrate with quantities of fiber, vitamins, and minerals equal to those in chow. One group of HF mice was given rosiglitazone (30 mg/kg/day as a food additive) (HFRosi) for the last 4 weeks. Glucose (2 g/kg glucose IP) tolerance tests were performed in overnight-fasted mice. Blood samples were obtained from tail tip. Glucose levels were measured using a glucometer (ACCU-CHEK II; Roche Diagnostics, Basel, Switzerland). After 3–4 days of recovery, the animals were killed by cervical dislocation. Paired VF and SF adipose samples were removed, immediately frozen in liquid nitrogen, and stored at −80 °C for measurement of gene expression. The percentage of perigonadal fat mass (male epididymal or female ovarian fat mass) was calculated relative to total body weight and expressed as VF%.

VF was obtained from 20- to 24-week-old male c-Cbl (Casitas b-lineage lymphoma) knock out mice (c-$Cbl^{-/-}$) that displayed decreased adiposity and increased energy expenditure (16) and from leptin deficient ($ob^{-/-}$ on C57BL/6J background) mice aged 10 weeks.

All the experiments on mice were carried out with the approval of the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee.

Table 1 Clinic characteristics in subjects with normal insulin sensitivity (IS), insulin resistance (IR), or type 2 diabetes mellitus (TD2)

	IS (8 M/12 F)	IR (4 M/5 F)	TD2 (3 M/4 F)
Age (years)	52.8 ± 15.5	51.0 ± 15.7	61.6 ± 7.4
BMI (kg/m^2)	25.2 ± 4.2	29.3 ± 5.2*	34.8 ± 7.1
WHR	0.88 ± 0.08	0.95 ± 0.05*	1.01 ± 0.13*
F-plasma glucose (mmol/l)	5.1 ± 0.5	5.8 ± 0.5	8.6 ± 1.9*
F-serum insulin (mU/l)	12.1 ± 3.5	26.8 ± 9.8*	18.6 ± 8.0*
$HOMA_{IR}$	2.7 ± 0.7	6.9 ± 2.9*	7.4 ± 3.8*
$HOMA_{IR}$ [a]	1.8 ± 0.4 (1.4–2.2)	7.2 ± 2.8 (4.0–10.8)	
Central fat (%)	34.3 ± 10.6	42.5 ± 4.3*	45.9 ± 4.3*
Triglyceride (mmol/l)	1.2 ± 0.7	1.7 ± 0.8	1.9 ± 1.2
Cholesterol (mmol/l)	4.9 ± 0.9	5.2 ± 0.7	4.8 ± 0.9[b]
LDL (mmol/l)	2.9 ± 0.9	3.2 ± 0.8	2.8 ± 1.2[b]
HDL (mmol/l)	1.5 ± 0.4	1.3 ± 0.3	1.1 ± 0.3
Diastolic blood pressure (mm Hg)	75.0 ± 9.7	78.9 ± 3.9	73.6 ± 8.9
Systolic blood pressure (mm Hg)	126.5 ± 16.9	120.7 ± 6.7	137.8 ± 15.4
Plasma adiponectin (µg/ml)	9.6 ± 3.6	5.7 ± 3.1*	7.7 ± 3.8

Data are mean ± s.d.
F, females; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M, males; WHR, waist-hip ratio.
[a]Subjects included in microarray analysis (IS, 2 M/2 F; IR, 2 M/3 F) (see Methods and Procedures).
[b]Of seven diabetic subjects, five were treated for dyslipidemia.
*$P < 0.05$ compared with IS subjects.

Affymetrix HG_U133_Plus2 chip (DNA microarray)

As an exploratory investigation RNA was prepared from paired tissues samples (VF and SF) of 36 subjects, including 20 subjects who were relatively IS, 9 subjects who were relatively insulin resistant (IR), and 7 subjects with type 2 diabetes mellitus (Table 1). RNA from the two fat depots (VF and SF) in two non-diabetic subject groups (IS and IR, see below) was pooled to give four samples for microarray analysis—IS-VF, IS-SF, IR-VF, IR-SF. This led to identification of genes with differences in expression in SF vs. VF and/or in IR vs. IS subjects (a number of which had been previously documented). A number of genes of interest were followed up with examination by qPCR in each adipose depot of all 36 subjects.

Total RNA extraction. Total RNA was extracted from paired VF and SF samples in subjects with IS (two men/two women; $HOMA_{IR}$, 1.8 ± 0.4, range 1.4–2.2) or IR (two men/three women; $HOMA_{IR}$, 7.2 ± 2.8, range 4.0–10.8) (Table 1) using TRI-reagent (Sigma, St. Louis, MO) followed by phenol–chloroform extraction. RNA was purified with an RNeasy Mini Kit (Qiagen, GmbH, Germany) and digested with RNase-free DNase I (Ambion, Austin, TX) during RNA purification. Equal amount of purified RNA from the two depots in the two subject groups was pooled to generate four samples for subsequent synthesis of complementary DNA (cDNA), complementary RNA (cRNA), and microarray analysis. The integrity of RNA was assessed by RNA 6000 Nano Assay using Agilent 2100 bioanalyzer (Agilent Technologies, Headquarters; Palo Alto, CA) before and after the RNA samples were pooled.

Double stranded cDNA synthesis. Double stranded cDNA was synthesized with 1 µg of purified RNA using a Superscript cDNA Synthesis kit (Invitrogen Life technologies, Carlsbad, CA) in combination with a T7-$(dT)_{24}$ primer (Proligo, St. Louis, MO). Double stranded cDNA was isolated with phenol–chloroform–isoamyl (25:24:1), and purified using Spin Phase-Lock gel tubes (Eppendorf Scientific, Westbury, NY) and Micro Bio-Spin Chromatograph columns (Bio-gel P-6; Bio-Rad).

cRNA synthesis and amplification. Biotinylated cRNA was transcribed and amplified from Double stranded cDNA using GeneChip IVT Labeling kit (Affymetrix, Surry Hills, VIC, Australia). The cRNA was then purified using an RNeasy Mini Kit (Qiagen, GmbH, Germany). Twenty micrograms of purified cRNA was fragmented by incubation with 1× fragmentation buffer at 95 °C for 30 min. The integrity of cRNA was assessed by RNA 6000 Nano Assay using Agilent 2100 bioanalyzer (Agilent Technologies, Headquarters; Palo Alto, CA) before and after fragmentation.

Affymetrix HG_U133_Plus2 chip (Microarray). A 15 µg fragmented biotin-labeled cRNA was added to hybridization cocktail and hybridized to the human Affymetrix HG_U133_Plus2 chip (Microarray) that contains 54,675 gene transcripts (http://www.affymetrix.com/index.affx). The array was incubated for 16 h at 45 °C at 60 r.p.m. rotation in Affymetrix GeneChip Hybridization Oven (Model 640). After hybridization, washing and staining of the hybridized array were performed using an automated fluidics station (Affymetrix GeneChip Fluidics Station FS400) according to manufacturer's protocols. The stained arrays were scanned using Affymetrix GeneChip Scanner (Model GCS3000), and the resultant image was captured as a data image file.

Microarray data analysis. Array data was analyzed using the Affymetrix GeneChip Operating Software (GCOS; Version 1.2) from which Present (P), Absent (A), and Marginal (M) flags were obtained. Four chips had comparable metrics information (with similar background values, RawQ values, percentage of probes present, and average signal present or absent). The .CEL files were RMA (Robust Multichip Average) (17,18) preprocessed using GeneSpring 7.3.1 and the data were normalized to the median. We calculated fold changes between chips of interest and mapped the GCOS calls, for each chip, to the fold changes. Gene lists were then filtered on both fold changes and GCOS flags and their biological processes and molecular functions assigned using gene ontologies. Expression of genes (*LRP10*, *CLN3*, *COBRA1*), previously identified as reference genes (house-keeping genes) in human adipose tissue (19,20), was similar between VF and SF, and between IS and IR subjects (data not shown). The analysis was performed at the Peter Wills Bioinformatics Centre at the Garvan Institute. The experimental data including the Affymetrix .CEL files are available with a guest login at http://gspring.garvan.unsw.edu.au.

Quantification of mRNA expression by qPCR in human subjects and mice

Using TRI-reagent as stated above, total RNA was extracted from paired VF and SF in all subjects, and in C57BL/6 mice fed with Chow, HF, and HFRosi ($n = 5$ in each group). RNA was available from VF in c-Cbl mice (wild type, $n = 5$; c-Cbl$^{-/-}$, $n = 4$) and ob mice (wild type, seven males/three females; ob$^{-/-}$, three males/three females). RNA was quantified using SYBR II by a spectrophotometer (Hitachi Fluorescence spectrophotometer model F-4010). RNA integrity was verified by agarose gel electrophoresis and by OD_{260}/OD_{280} nm absorption ratio >1.90. cDNA was synthesized using Omniscript RT kit (QIAGEN, GmbH, Germany) according to manufacturer's instruction. qPCR was performed using ABI Sequence Detection System (ABI 7900HT; ABI Prism, Foster City, CA) or Rotor-Gene (RG-3000; Corbett Research). Data were expressed as the ratio between expression of the target gene and the house-keeping gene cyclophilin, and given as arbitrary units. In c-Cbl and ob mice, ribosomal protein 36B4 was used as house-keeping gene; its expression level was not affected by adiposity (21). Primer sequences are given in Table 2.

Isolating adipocytes and stromovascular fraction from rat adipose tissue

Adipocytes and stromovascular fraction (SVF) of adipose tissue from Wistar rats were isolated as previously described (22). Briefly, 20-week-old rats ($n = 4$) were killed. Epididymal adipose tissue was removed under sterile conditions and immediately put in prewarmed (37 °C) Krebs-Ringer-Phosphate buffer supplied with 3% BAS and 5 mmol/l D-glucose. Obvious blood vessels and connective tissue, if any, were removed. Adipose tissue was minced and digested with collagenase (Collagenase Type II; C6885; Sigma) for 45 min at 37 °C in a shaking water bath. The undigested tissue was removed by filtration through a nylon mesh with a pore size of 280-µm. The SVF was separated from mature adipocytes by centrifugation. The resulting cell pellet of SVF or floating layer of adipocytes was resuspended and incubated in erythrocyte lysis buffer for 10 min at room temperature. Tri-Reagent was added to both SVF and adipocytes, immediately followed by phenol–chloroform extraction of RNA.

Culture and differentiation of 3T3-L1 fibroblast cells

3T3-L1 fibroblast cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS), 100 units/liter penicillin, 100 µg/liter streptomycin, and 2 mmol/l L-glutamine (DMEM, newborn calf serum and antibiotics were from Invitrogen, Myoclone). The cells were grown to confluence at 37 °C in 10% CO_2. Two days after reaching 100% confluence (day 0), 3T3-L1 fibroblast cells were induced to differentiate into adipocytes using differentiation medium containing 10% fetal calf serum (Trace Scientific, Melbourne, VIC, Australia), 350 nmol/l insulin, 0.5 mmol/l isobutylmethylxanthine, 250 nmol/l dexamethasone, 400 nmol/l biotin for 3 days (day 1–3), with or without the PPARγ agonist ciglitazone (10 µmol/l). On day 4, the medium was changed to DMEM–fetal calf serum supplemented with 350 nmol/l insulin (post-diff medium). On day 7, the medium was changed to DMEM–fetal calf serum. Cells were then maintained in the DMEM–fetal calf serum medium and changed every 3 days thereafter. Before (on day 0), during, and after differentiation (from day 1 to 10), 3T3-L1 cells were washed with ice-cold 1 × PBS in the 10 cm dishes, and collected and extracted for total RNA using 1 ml Tri-Reagent. cDNA was synthesized by reverse transcription of 1 µg of total RNA as described above.

Metabolic measurements and biochemical assays. Plasma glucose was measured by the oxidase method (NOVA 14, Nova Biomedical, Waltham, MA) and serum free insulin by RIA (Linco Research, St. Charles, MO). Total cholesterol, high-density lipoprotein choles-

Table 2 Primer sequences for qPCR

	Accession no.	Forward primer (5′–3′)	Reward primer (5′–3′)
Human *cyclophilin*	BC000689	CTCCTTTGAGCTGTTTGCAG	CACCACATGCTTGCCATCC
Human *ISL1*	NM_002202.1	Hs00158126_ml (Pre-made)	Hs00158126_ml (Pre-made)
Mouse *ISL1*	NM_021459	CGTCTGATTTCCCTGTGTGTTGG	AAGTCGTTCTTGCTGAAGCCTATG
Mouse *aP2*	M13261&2	CATGAAAGAAGTGGGAGTGGGC	GACCGGATGGTGACCAAATC
Mouse *Pref-1*	L12721	GAACCATGGCAGTGCATCTG	CAAGTTCCATTGTTGGCGC
Mouse *Leptin*	BC125245	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
Mouse *LPL*	BC003305	GCAGGAAGTCTGACCAATAAG	GTGCCTCCATTGGGATAAATG
Mouse *cyclophilin*	XM_913899	TGTGCCAGG GTGGTGACTTTAC	TGGGAACTGTTTGTGTTTGG
Mouse *36B4*	NM_007475	CGACCTGGAAGTCCAACTAC	ATCTGCTGCATCTGCTTG
Rat *ISL1*	NM_017339	ACTGAGTGACTTCGCCTTGC	ATCTGGGAGCTGAGAGGACA

terol, and triglyceride concentrations were determined spectrophotometrically at 490 nm using enzymatic colorimetric kits (Roche, Basel, Switzerland). Low-density lipoprotein cholesterol was estimated using the Friedewald formula (23).

In mice, plasma glucose was determined using a YSI 2300 glucose analyzer (Yellow Springs, OH). Plasma insulin and adiponectin concentrations were determined using commercially available radioimmunoassay kits (Linco Research). Plasma leptin was measured by enzyme-linked immunosorbent assay using the Quantikine mouse Leptin Immunoassay kit (Quantikine; RnDSystems, Minneapolis, MN).

Statistical analysis

Data were analyzed using StatView 5 (Abacus Concepts, Berkeley, CA). Data were given as the mean ± s.d. or mean ± s.e. Relationships between gene expression and clinical variables were analyzed with the Spearman rank correlation test. Pearson's correlation coefficients were used to describe the linear association between continuous variables. Logarithmic transformation of data was performed to variables with skewed distribution. Group means were analyzed using the Mann–Whitney or the Kruskall–Wallis tests. Data involving more than two groups were assessed by ANOVA. $P < 0.05$ was considered significant.

RESULTS

Human visceral adipose tissue expression of *ISL1* and its inverse relationship with central abdominal fat

Consistent with previous studies, we observed differential expression (twofold changes or more) of genes related to lipolysis (***ADRA2A***, ***PDE3B***, ***PLAT***), inflammation (***C3***, ***VCAM1***, *SAA1/2*), adipokines (Leptin, Leptin Receptor, *RBP3*, ***RBP4***), and genes involved in embryonic development (*SFRP2*, *NR2F1*, *SHOX2*, *TBX15*) between VF and SF, or between IR and IS subjects (confirmation of these differences by qPCR in the full set of subjects for six (in bold) of these genes). Focusing on genes increased (twofold or more) in VF compared with SF, we found that there was a high proportion of genes related to development, cell differentiation, signal transducer activity, and receptor activity (Table 3). Interestingly, we found that *ISL1*, a LIM-homeobox transcription factor, was uniquely expressed in VF, but not in SF in both IS and IR subjects (Figure 1). qPCR confirmed there was a substantial but variable expression of *ISL1* in VF in 36 subjects, and the expression levels tended to be higher in IS than in IR or type 2 diabetes mellitus subjects (Figure 1). *ISL1* was undetectable in SF in nearly all subjects (only one subject had detectable expression). This 39-year-old thin female subject (BMI, 19.0 kg/m^2; waist-hip-ratio, 0.74; central fat %, 18.3; HOMA 3.5) had the highest VF-*ISL1* expression of all subjects. Visceral *ISL1* mRNA levels were negatively correlated with BMI ($r = -0.37$; $P = 0.03$), body weight ($r = -0.40$; $P = 0.010$), and central abdominal fat (dual energy X-ray absorptiometry scan: $r = -0.44$; $P = 0.02$) (Figure 2), but positively correlated with circulating adiponectin concentration in all subjects ($r = 0.33$; $P = 0.04$), especially in women ($r = 0.54$, $P = 0.01$). Visceral *ISL1* mRNA levels were not correlated with glucose and insulin levels, or insulin sensitivity (assessed by $HOMA_{IR}$ and/or hyperinsulinemic clamp).

Table 3 Function of genes that are increased in visceral fat

GO category of genes	% of total genes examined[a]	% of genes over expressed in VF[b]	*P* value[c]
According to biological process			
Development	15.8	21.7	0.000001
Cell differentiation	4.3	5.8	0.017000
According to Molecular Function			
Signal transducer activity	17.2	24.6	0.000000
Receptor activity	10.6	15.9	0.000000
Receptor binding	3.9	6.9	0.000007
Peptidase activity	3.9	5.8	0.001970

[a]Including only gene ontology (GO) categories with genes that account for >2% of the total genes examined on the chip.
[b]Genes that are increased (twofold or more) in visceral fat (VF) than in subcutaneous fat (SF). Genes were analyzed using the Ontology Browser of GeneSpring (Version 7.3.1) and matched to the generic gene ontology slim ontologies (see Methods and Procedures).
[c]A hypergeometric *P* value was used to estimate the statistical significance of over-representation of genes on each GO term. The table was sorted by increasing *P* values. Only GO categories with $P < 0.02$ were shown.

ISL1 mRNA in rodent visceral adipose tissue was inversely related to adiposity

In agreement with our findings in humans, *ISL1* mRNA was expressed in epididymal fat, but was absent or barely detectable in SF in both Wistar rats and C57BL/6 mice (by qPCR). In C57BL/6 mice fed a HF diet for 20 weeks, which reliably produces obesity and insulin resistance (24), *ISL1* expression in VF was considerably reduced (ninefold; $P = 0.009$) in comparison to animals on a chow diet who are normally IS (Figure 3a).

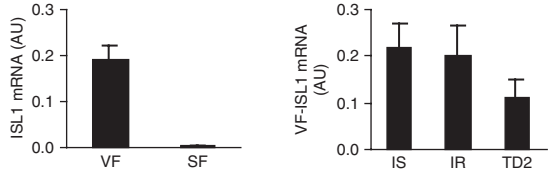

Figure 1 The mRNA expression of Islet-1 (*ISL1*) in only visceral fat (VF) was confirmed in 36 subjects using quantitative PCR (qPCR) by TaqMan Green Expression Assay. Data were expressed relative to cyclophilin, given as arbitrary units (AU; mean ± s.e.) (see Methods and Procedures). The expression levels tended to be higher in insulin sensitive (IS) (0.22 ± 0.05 AU) than in insulin resistant (IR) (0.20 ± 0.07 AU) or type 2 diabetes mellitus (TD2) (0.11 ± 0.04 AU) subjects, but the differences were not statistically significant.

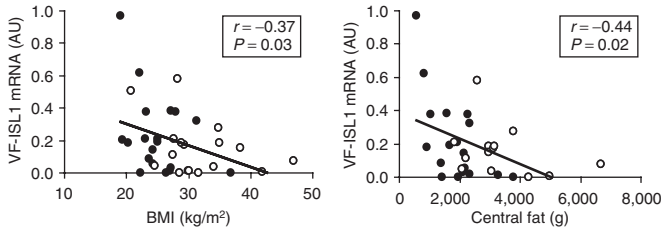

Figure 2 Inverse correlation of visceral fat Islet-1 (VF-*ISL1*) mRNA expression (relative to cyclophilin, expressed as arbitrary units (AU)) with BMI (15 men/21 women) and central fat (13 men/18 women) in human subjects. Filed circles indicate subjects who are insulin sensitive (IS). Open circles indicate subjects with insulin resistance (IR) or type 2 diabetes mellitus (TD2).

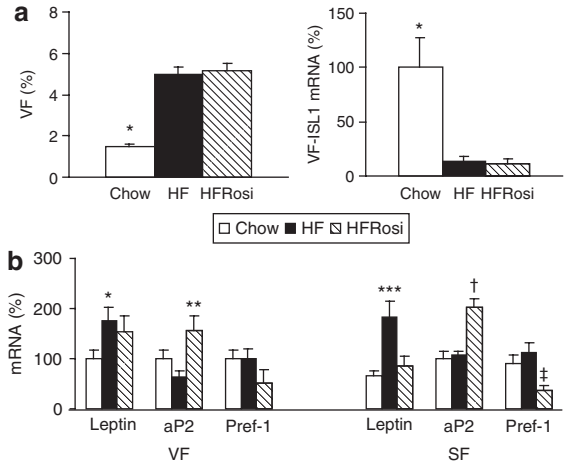

Figure 3 Expression of *ISL1*, *Leptin*, *aP2* and *Pref-1* in VF and SF according to diet and rosiglitazone administration. (**a**) Epididymal fat mass (VF%) and epididymal fat *ISL1* mRNA expression (VF-*ISL1*) in male mice fed with chow (Chow; $n = 5$), high fat (HF; $n = 5$), and HF with rosiglitazone (HFRosi; $n = 5$). *ISL1* mRNA levels are normalized by cyclophilin. VF-*ISL1* levels in chow fed mice are expressed as 100%. Corresponding expression levels in HF and HFRosi mice are the percent of the levels in Chow mice. $^{*}P < 0.05$ compared with HF or HFRosi. (**b**) mRNA expression of *Leptin*, *aP2*, and *Pref-1* in epididymal fat (VF) or subcutaneous fat (SF) in male mice fed with Chow, HF, HFRosi. mRNA levels are normalized by cyclophilin. VF mRNA levels in Chow mice are expressed as 100%. Corresponding expression levels in VF of HF and HFRosi mice, and in SF are the percent of the VF levels in Chow mice. Compared with Chow, $^{*}P = 0.05$; compared with Chow or HFRosi, $^{***}P < 0.05$; compared with HF, $^{**}P < 0.05$; compared with Chow or HF, $^{\dagger}P < 0.001$; compared with Chow or HF, $^{\ddagger}P < 0.001$.

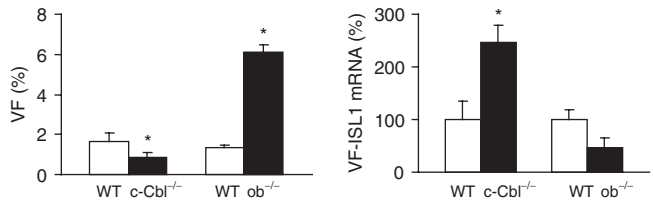

Figure 4 Perigonadal fat mass (VF%) and perigonadal fat *ISL1* mRNA expression (VF-*ISL1*) in c-Cbl$^{-/-}$ (aged 20–24 weeks) or ob$^{-/-}$ (aged 10 weeks) mice. mRNA levels of *ISL1* are normalized by 36B4 (see Research Methods and Procedures). Expression levels in wild type littermates (WT) are expressed as 100%. Corresponding expression levels in mutant mice (c-Cbl$^{-/-}$ or ob$^{-/-}$) are the percent of the VF levels in WT mice. c-Cbl$^{-/-}$ mice (4 males) had an increased (150%) VF-*ISL1* mRNA expression than WT mice (5 males). Four weeks high fat feeding reduced VF-*ISL1* expression (28%) in c-Cbl$^{-/-}$. ob$^{-/-}$ mice (3 males/3 females) had lower (53%) *ISL1* expression than WT mice (7 males/3 females), especially in female group (61%). $^{*}P < 0.05$ compared with WT mice.

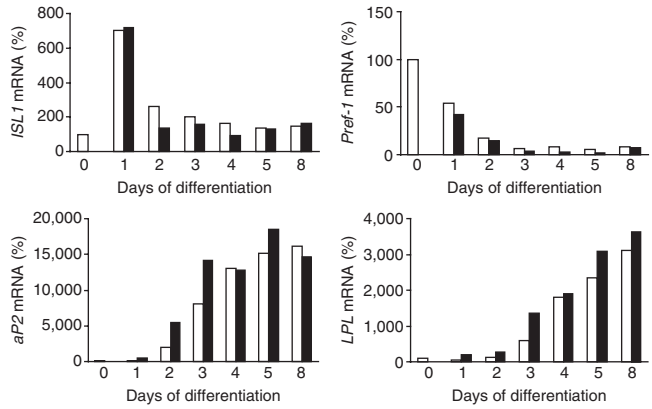

Figure 5 mRNA expression of genes (*ISL1*, *Pref-1*, *aP2*, and *LPL*) in 3T3-L1 cells following differentiation, with (filled bars) or without (open bars) Ciglitazone. mRNA levels of each gene are expressed relative to cyclophilin. Expression levels at day 0 (before differentiation) are expressed as 100%. Corresponding expression levels of each gene at day 1, 2, 3, 4, 5, or 8 of differentiation are the percent of expression levels at day 0.

Rosiglitazone therapy, which increased plasma adiponectin concentration (HF vs. HFRosi: 4.5 ± 0.48 vs. 27.0 ± 3.8 μg/ml; $P < 0.0001$) and improved glucose tolerance (area under the curve during ipGTT; Chow vs. HF vs. HFRosi, mean ± s.e., 554 ± 21 vs. 763 ± 77 vs. 535 ± 54; $P = 0.017$ HF vs. Chow; $P = 0.033$ HFRosi vs. HF), did not increase *ISL1* expression in the fat fed mice who maintained a similar amount of epididymal fat (VF%, 5.1%) as HF mice without rosiglitazone treatment (5.0%, more than threefold higher than the chow fed mice (Figure 3a)). As expected, leptin mRNA expression was increased in HF mice in both VF and SF compared with chow fed mice (Figure 3b) with a significant increase in plasma leptin concentration (Chow vs. HF: 1.7 ± 0.57 vs. 17.2 ± 8.7 ng/ml; $P = 0.001$), which was not decreased with rosiglitazone treatment (14.9 ± 5.9 ng/ml). Rosiglitazone reduced *leptin* mRNA expression in SF, but not in VF (Figure 3b). Similarly, in response to rosiglitazone, the increase in mRNA levels of *aP2* (adipocyte fatty acid binding protein, a mature adipocyte marker) or decrease in *Pref-1* (a preadipocyte marker) tended to be more pronounced in SF than in VF (Figure 3b).

In c-Cbl$^{-/-}$ mice that had lower adiposity (VF%: 0.87% vs. 1.65%) and lower plasma leptin (1.28 vs. 3.82 ng/ml) (16), VF-*ISL1* expression was increased (150%; $P < 0.05$) compared with wild type (WT) mice (Figure 4). In contrast, in leptin deficient ob$^{-/-}$ mice, VF-*ISL1* expression tended to be decreased (53% reduction; $P = 0.13$ compared with WT littermates, Figure 4), particularly in females (61% reduction; $P = 0.05$). These young ob$^{-/-}$ mice (VF%, 6.1%) had comparable obesity to the C57BL/6 mice fed with HF diet for 20 weeks (5.1%).

The cell of origin of *ISL1* in visceral adipose tissue

We separated adipocytes from the SVF of rat epididymal fat (VF) (see Research Methods and Procedures); *ISL1* mRNA was predominantly expressed in the SVF (354% increase vs. VF) with only low expression in mature adipocytes.

ISL1 was expressed in 3T3-L1 cells and upregulated in early differentiation

In cultured 3T3-LI cells, *ISL1* mRNA was expressed in fibroblasts (preadipocytes) before differentiation (day 0), and was increased during early phase differentiation (day 1), but later declined with progression to the mature adipocyte. Treatment with Ciglitazone had no effect on *ISL1* expression in 3T3-L1 cells (Figure 5). The mRNA level of genes involved in adipocyte

differentiation, such as *LPL* and *aP2*, was increased, while the level of *Pref-1*, a preadipocyte marker, was decreased between day 0 and 5 (data not shown), which is in line with the differentiation process of 3T3-L1 cells (25,26).

DISCUSSION

To gain information on the differences between human VF and SF and possible mechanisms by which VF contributes to metabolic dysregulation, we examined gene expression in biopsies of both VF and SF. Multiple differences in gene expression were seen, but in particular we identified the unique expression of a transcription factor, *ISL1* (27) in VF, where it is inversely correlated with BMI and central fat, but positively correlated with plasma adiponectin concentration. *ISL1* plays an important role in the development of pancreatic islets, motor neurons, and cardiac tissue (11–13), but its role in adipose tissue is not known.

Analysis of RNA isolated from rats and mice confirmed our novel observation in humans that *ISL1* is expressed only in VF, not in SF. The expression of *ISL1* in VF was significantly downregulated in mice fed with a HF diet for 20 weeks that induced obesity and insulin resistance. Rosiglitazone treatment increased plasma adiponectin concentration and improved glucose tolerance/insulin sensitivity in HF mice, but *ISL1* mRNA levels were not changed. Neither the amount of epididymal fat in HF mice nor the plasma concentration of leptin, an indicator of adipocyte mass, was reduced following rosiglitazone treatment (28–30), suggesting that *ISL1* expression is related to degree of central adiposity rather than to insulin sensitivity. Although expression of *leptin* in SF was decreased in response to rosiglitazone, VF leptin mRNA was unaltered, consistent with the effect of rosiglitazone in inducing adipose tissue differentiation being relatively specific to SF (31–33). In line with this, the increase in mRNA of *aP2*, a mature adipocyte marker, and decrease in *Pref-1*, a preadipocyte marker, were more pronounced in SF than in VF (25,26).

To further investigate the relationship between *ISL1* expression and adiposity, we studied *ISL1* expression in ob$^{-/-}$ mice, a genetic mouse model of obesity (34), and c-Cbl$^{-/-}$ mice that have a lean phenotype and reduced adiposity (16,35). The expression of *ISL1* in VF was decreased in ob$^{-/-}$ mice compared with lean littermates, but was increased in c-Cbl$^{-/-}$ mice, and 4 weeks HF feeding (35) reduced *ISL1* expression in c-Cbl$^{-/-}$ mice. These data are consistent with a direct or indirect negative effect of adiposity on *ISL1* expression.

When mature adipocytes were separated from the SVF, we found that there was little expression of *ISL1* in mature adipocytes, but strong expression in the SVF, a primary source of preadipocytes (26,36). As the expression of *ISL1* was decreased with central obesity, it is possible that decreased expression of *ISL1* could be related to low preadipocyte numbers in obese humans (37). On the other hand, despite expansion of adipose tissue in obesity, there is a low differentiation potential of preadipocytes in obese subjects (38,39), suggesting a functional defect in preadipocytes, which could be related to an adverse effect on body fat metabolism, and/or abnormal production of adipokines. In humans, a negative correlation between BMI and differentiation capacity in mammary preadipocytes has been reported (38). A relationship between low omental preadipocyte number and high leptin has been suggested, and high leptin secretion was related to low adiponectin secretion in preadipocyte culture (40). Thus, a positive correlation between *ISL1* expression and plasma adiponectin concentration observed in 36 human subjects may relate to increased percentage of body fat (28) and adipocyte size (41,42).

Similarly, in obese rodents, there is reduced differentiation capacity of preadipocytes (43,44), and an increased number of large adipocytes (45). Thus, HF fed obese mice with large adipocytes express less *ISL1*, whereas c-Cbl$^{-/-}$ mice with a large number of small adipocytes (35) overexpress *ISL1*.

Similar to our *in vivo* data, *ISL1* is expressed in 3T3-L1 preadipocytes, and is upregulated during transition from preadipocyte to early adipocyte, but declines with further differentiation, suggesting a role of *ISL1* in early differentiation of adipocytes. Previous study has shown that functional *ISL1* is required for development in both pancreatic mesenchyme and in islet cells (46). In motor neurons, *ISL1* is marker of differentiation (12), including effects on interneurons (47). More recently, *ISL1* expression has been recognized in a group of cardiogenic precursor cells; expression being downregulated as precursors differentiate (13,48), so it seems possible that *ISL1* has an important role in early visceral adipocyte differentiation.

Consistent with previous studies, expression of *Pref-1*, a preadipocyte marker, was decreased in differentiated 3T3-L1 cells (25,26), and in adipose tissues of HF fed mice treated with rosiglitazone (31,49), perhaps related to the adipocyte differentiating effect of rosiglitazone. However, its expression level was not reduced with HF feeding alone.

Neither the upstream control nor the down stream target of *ISL1* is adequately understood at this time. Leptin cannot be the downregulator of *ISL1* in obesity as leptin deficient ob$^{-/-}$ mice have low *ISL1* expression. As far as the down stream target is concerned, *ISL1* has recently been shown to transiently activate the JAK-STAT pathway, increasing STAT3 activity in mammalian cells (50), but the consequences in adipose tissue have not been defined.

In summary, we have identified the expression of *ISL1* in visceral adipose tissue in both humans and rodents. Decreased visceral expression of *ISL1* is associated with increased visceral adiposity and lower adiponectin levels. *ISL1* clearly does not have a direct impact on insulin sensitivity, but a developmental role in the visceral adipocyte could have subsequent impact on metabolic function; this possibility will require further study.

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DISCLOSURE

The authors declared no conflict of interest.

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