FOXC2 mRNA Expression and a 5′ Untranslated Region Polymorphism of the Gene Are Associated With Insulin Resistance

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The human transcription factor FOXC2 has recently been shown to protect against diet-induced insulin resistance in transgenic mice. We investigated the expression of *FOXC2* in fat and muscle and performed a genetic analysis in human subjects. *FOXC2* mRNA levels were increased in visceral compared with subcutaneous fat from obese subjects (12 ± 4-fold; $P = 0.0001$), and there was a correlation between whole-body insulin sensitivity and *FOXC2* mRNA levels in visceral fat (fS-insulin $R = -0.64$, $P = 0.01$, and homeostasis model assessment of insulin resistance [HOMA-IR] $R = -0.68$, $P = 0.007$) and skeletal muscle (fS-insulin $R = -0.57$, $P = 0.03$, and HOMA-IR $R = -0.55$, $P = 0.04$). Mutation screening of the FOXC2 gene identified a common polymorphism in the 5′ untranslated region (C-512T). The T allele was associated with enhanced insulin sensitivity (HOMA-IR $P = 0.007$) and lower plasma triglyceride levels in females ($P = 0.007$). Also, the higher expression of *FOXC2* in visceral than in subcutaneous fat was restricted to subjects homozygous for the T allele ($P = 0.03$ vs. $P = 0.7$). Our data suggest that increased *FOXC2* expression may protect against insulin resistance in human subjects and that genetic variability in the gene may influence features associated with the metabolic syndrome. *Diabetes* 51:3554–3560, 2002

The metabolic or insulin resistance syndrome (1,2) is a common companion of conditions such as impaired glucose tolerance (IGT) and type 2 diabetes (3). Presence of the syndrome is associated with reduced survival, particularly due to cardiovascular mortality (3). Insulin resistance and additional features of the metabolic syndrome, including abdominal obesity, hypertension, and dyslipidemia, are under strong genetic influence and cluster in families (3–6). Obesity, particularly abdominal obesity, usually precedes insulin resistance and type 2 diabetes (3,4). Genetic factors are considered to explain 60% of the variance in abdominal fat (7,8). Obesity, type 2 diabetes, and the metabolic syndrome are considered a paradigm for a multifactorial, polygenic disease in which several genetic variants interact with environmental factors to cause the disease. Despite increased knowledge of the underlying genetic causes for monogenic forms of obesity, there has been limited success in identifying genetic variants contributing to the metabolic syndrome (9–11).

Recently, the human forkhead family transcription factor FOXC2 was shown to protect against diet-induced obesity and insulin resistance in transgenic mice overexpressing FOXC2 in adipose tissue (12). There was a dose-dependent reduction in visceral adipose tissue in the transgenic mice, and this otherwise white adipose tissue had adopted a more brown fat–like histology and gene expression profile. Compared with wild-type animals, the transgenic animals also exhibited significantly reduced levels of serum triglycerides, plasma glucose, and insulin, and they were less insulin resistant as judged by intravenous glucose tolerance tests, both on standard and high-fat diets. Expression of the mouse homolog *Foxc2* was induced in wild-type animals on a high-fat diet. These observations suggested a role for FOXC2 in human obesity and insulin resistance, a hypothesis that was tested in the current study.

RESEARCH DESIGN AND METHODS

Study subjects. All subjects gave informed consent and the studies were approved by the local ethics committees. Diagnosis of type 2 diabetes and IGT was performed according to the World Health Organization criteria from 1998 (1) or by a previous diagnosis of diabetes and treatment with oral agents and/or insulin.

Obese subjects undergoing adipose tissue biopsies at bariatric surgery. Abdominal visceral and subcutaneous adipose tissue biopsies were available from 38 obese nondiabetic subjects undergoing bariatric surgery at Landskrona Hospital, Sweden (5 men and 33 women; age 36 years [30–47]; weight 112 kg [102–124]; BMI 41 ± 5 kg/m^2; and total body fat mass 68 ± 14 kg). Biopsies were immediately frozen in liquid nitrogen and stored at −80°C. Data on glucose homeostasis and energy balance could be obtained from 14 of the obese subjects (1 man and 13 women). They exhibited the following characteristics: age 38 ± 12 years, weight 117 ± 17 kg, BMI 42 ± 6 kg/m^2, waist-to-hip ratio (WHR) 0.86 (0.83–0.91), fasting plasma glucose 4.8 ± 0.3 mmol/l, fasting serum insulin 12.8 mU/l (9.3–15.9), fasting plasma triglycerides 1.4 ± 0.6 mmol/l, and resting metabolic rate (RMR) 2,075 ± 213 kcal/24 h. All subjects were insulin resistant as judged from a HOMA-IR index of 2.73 (1.91–3.31).

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α-FOXC2, anti-FOXC2; HOMA-IR, homeostasis model assessment of insulin resistance; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OSD, observed sum of differences; RMR, resting metabolic rate; SAT, subcutaneous adipose tissue; SSCP, single-strand conformational polymorphism; VAT, visceral adipose tissue; WHR, waist-hip-ratio.

Subjects undergoing muscle biopsy in combination with insulin clamp. Fourteen nondiabetic subjects participated in the clamp studies with biopsy. Seven of them had normal glucose tolerance (NGT) and no family history of diabetes, whereas seven had IGT and a family history of type 2 diabetes. They exhibited the following characteristics: age 55 ± 1.3 years, BMI 32.3 ± 0.9 kg/m^2, fasting plasma glucose 6.1 mmol/l (5.9–6.4), fasting serum insulin 9.5 mU/l (7.1–14.0), and HOMA-IR 2.41 (1.92–4.07). The only significant differences between subjects with NGT and IGT were 2-h plasma glucose during an oral glucose tolerance test (75 g glucose), 5.6 ± 0.8 vs. 9.0 ± 0.9 mmol/l ($P = 0.0012$), and glucose uptake during a hyperinsulinemic-euglycemic clamp, 21.4 ± 2.6 vs. 11.4 ± 1.9 mg · min^{-1} · kg^{-1} fat-free mass/steady-state insulin (nmol/l) ($P = 0.01$). Muscle biopsies were obtained from the vastus lateralis muscle using a modified Bergström's needle under local anesthesia at −40 min before a hyperinsulinemic-euglycemic clamp and at the end of the clamp (+120 min) (13). Biopsies were frozen in liquid nitrogen and stored at −80°C.

Mutation screening. *FOXC2* was screened by single-strand conformational polymorphism (SSCP) analysis followed by conventional DNA sequencing (14) in 32 nondiabetic obese subjects (5 men and 27 women, age 41 ± 11 years, BMI 41 ± 4 kg/m^2) and 38 subjects with type 2 diabetes—13 subjects from 7 families with early-onset diabetes cosegregating with obesity (5 men and 8 women, age 43 ± 12 years, age at onset of diabetes 37 ± 7 years, BMI 32 ± 4 kg/m^2) and 25 subjects (12 men and 13 women, age 50 ± 19 years, age at onset of diabetes 43 ± 17 years, BMI 28 ± 5 kg/m^2) from families showing putative linkage to a region on chromosome 16q harboring the FOXC2 gene in a previous genome wide scan (15)—and in 15 healthy lean control subjects (1 man and 14 women, age 36 ± 5 years, BMI 17 ± 1 kg/m^2). The following primers (covering 999 bp 5′ upstream of ATG and 78 bp 3′ downstream of TGA) and PCR conditions were used, with fragment size, annealing temperature, and number of PCR cycles in parentheses: F1: 5′-ATCCAGGAGAGGC CCTGCCATTC-3′ and R1: 5′-TTGGTGAATGGGCCCACATGCCC-3′ (261 bp, 70°C, 35 cycles); F2: 5′-GCTACTTACAAGGTCTTTGGGAGAG-3′ and R2: 5′-TCCCGGAACTTTGAGCCAATCACC-3′ (310 bp, 63°C, 30 cycles); F3: 5′-GTC TTAGAGCCGACGGATTCCTG-3′ and R3: 5′-TGGGGACCAAGGTGGACCCTC G-3′ (306 bp, 63°C, 30 cycles); F4: 5′-ATTCGCTGGGGGCTTGGAGAGCC-3′ and R4: 5′-AGCGAGAGAGCGCGAGAGAGCC-3′ (339 bp, 62°C, 30 cycles); F5: 5′-CGAGGGTCCACCTTGGTCCCCAG-3′ and R5: 5′-CTGCATGCTGCTTCCGAG ACGGC-3′ (369 bp, 64°C, 30 cycles); F6: 5′-TCTGGCTCTCTCGCGCTCT CTCG-3′ and R6: 5′-AGGGCGGCTTCACCAGGTCCTTAG-3′ (319 bp, 70°C, 35 cycles); F7: 5′-TATTCCGGCCACCCGGAGCAGTAC-3′ and R7: 5′-TAACTGCCC TTGCCGGGCTTCTTG-3′ (314 bp, 70°C, 32 cycles); F8: 5′-TGGCAGAACAGC ATCCGCCACAAC-3′ and R8: 5′-GCTCTTGATCACCACCTTCTTCTCG-3′ (300 bp, 60°C, 30 cycles); F9: 5′-AAGGAGGAGCGGGCCCACCTCAAG-3′ and R9: 5′-ATGATGTTCTCCACGCTGAAGCCAG-3′ (299 bp, 70°C, 35 cycles); F10: 5′-TCATCACCAAGGTGGAGACGCTGAG-3′ and R10: 5′-ATCGCTCGCATGCTG CACTGGTAG-3′ (337 bp, 60°C, 30 cycles); F11: 5′-CTGCCTGGCTTCAGCGTG GAGAAC-3′ and R11: 5′-TTGAGAGCGCTCAGGGGCGACG-3′ (320 bp, 62°C, 30 cycles); F12: 5′-CATGCGAGCGATGAGCCTGTACAC-3′ and R12: 5′-CGC TGTGGTTGAGATACCAGGAG-3′ (293 bp, 60°C, 35 cycles); F13: 5′-TCCTGG TATCTCAACCACAGCGGG-3′ and R13: 5′-AGCCCCTTAATTGTCTGGTGGGG TC-3 (318 bp, 65°C, 35 cycles); F14: 5′-ACGTCGCCCCTGAGCGCTCTCAAC-3′ and R14: 5′-CACGTTGGGGAAAGTTTGCTGCTG-3′ (255 bp, 67°C, 35 cycles). All PCR reactions were performed with initial denaturation (96°C for 3 min.) followed by x number of cycles of denaturation (96°C for 30 s), annealing (30 s), and extension (72°C for 30 s), followed by final extension (72°C for 10 min). The reactions were performed in a total volume of 20 μl with 0.13 mmol/l dNTP; 1.5 mmol/l $MgCl_2$; 0.2 μmol/l of both primers; 0.5 μCi [α-^{32}P]dCTP; 3% DMSO (dimethylsulphoxide), x1 Pharmacia Amersham (Uppsala, Sweden) (F1-R1, F2-R2, F7-R7, F9-R9, F14-R14) or NH4 buffer [16 mmol/l $(NH_4)_2SO_4$; 67 mmol/l Tris (pH 8.8); 0.01% Tween 20], and 0.5 units *Taq* polymerase using 21 ng genomic DNA as template. Reactions were stopped with 95% formamide buffer (1:1) and were then denatured, cooled, and electrophoresed on glycerol-free (35 W for 4 h at 4°C) and 5% glycerol (8 W for 15 h at room temperature) nondenaturing 5% polyacrylamide gels (acrylamide/bisacrylamide 49:1); the migration of bands was visualized by autoradiography.

Allele frequency and genotype-discordant siblings. Allele and genotype frequencies were determined from normal-weight control subjects selected from southern Sweden, none of whom had any first-degree relative with type 2 diabetes; all were older than 40 years and had a BMI <25 kg/m^2. Subjects for a genotype-discordant sibling pair analysis were selected from families with type 2 diabetes from southern Sweden and western Finland and included 798 nondiabetic siblings from 218 families (300 sib-ships), thus comprising 517 sibling pairs matched for age and sex (3). Of these subjects, 213 had IGT.

Extraction of total RNA. Total RNA was extracted from the muscle biopsies by the guadinium thiocyanate method (16) and from adipose tissue biopsies and adipocytes using the RNeasy Mini Kit according to the manufacturer's instructions (Quiagen, Hilden, Germany). cDNA was synthesized using Superscript II RNase H$^-$ Reverse Transcriptase and random hexamer primers (Life Technologies, Frederick, MD). Real-time semiquantitative PCR was performed in the ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA). All samples were run in triplicate and data were calculated using the standard curve method and expressed as a ratio to the *Cyclophilin A* reference (arbitrary units).

Cell culture experiments. Preadipocytes of human origin were treated as described previously (17). Insulin stimulation (at the physiological concentration of 1 nmol/l) was performed after 11–13 days when the cells had adopted an adipocyte-like phenotype. Cells were serum-starved overnight and then incubated with insulin (Novo Nordisk, Bagsvaerd, Denmark) and total RNA extracted after 1, 3, and 6 h. Whole-cell lysates were prepared in a radioimmunoprecipitation (RIPA) homogenization buffer, and proteins (30 μg per lane) were separated on 10% SDS-PAGE gels and blotted to Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Western blotting was performed using affinity-purified rabbit antisera (0.8 μg/ml) raised against a peptide corresponding to amino acids 484–499 of FOXC2 (α-FOXC2; Biovitrum, Uppsala, Sweden). Anti–rabbit-HRP (1:10,000; Bio-Rad Laboratories, Hercules, CA) and an enhanced chemiluminescence kit (Pierce, Rockford, IL) were used to visualize immunocomplexes. To control for specificity, membranes were routinely stripped according to the manufacturer's instructions and reblotted with an irrelevant antisera (anti–SOCS-3 at 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Genotyping. DNA was extracted using conventional methods (14). Genotyping was performed with a single-base extension kit (SNaPshot; Pharmacia, Uppsala, Sweden) using PCR-amplified DNA as a template and laser detection on an ABI377 automated sequencer (PE Biosystems, Foster City, CA).

Laboratory chemistry and anthropometric measures. Assessment of phenotypic characteristics has been described elsewhere for most subjects (3). Briefly, plasma glucose was determined using an automated glucose oxidase method (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA). Serum insulin was determined using immunoassays: 1234 AutoDELFIA (Wallac Oy, Turku, Finland) for the muscle biopsy study subjects and DAKO Insulin (DAKO, Ely, U.K.) for all other subjects. Body weight and height were measured with subjects in light clothing without shoes and BMI calculated as weight (kg) divided by height (m) squared. Fat-free mass was determined by either electrical bioimpedance or dual-energy absorptiometry scans (muscle biopsy subjects) using a Norland XR-36 scanner. Resting metabolic rate (RMR) was determined by indirect calorimetry using a Deltatrac monitor (Datex, Helsinki, Finland) (3,18,19).

Data analyses. Statistical calculations were performed using Number Cruncher Statistical Systems 2000 software (NCSS, Kaysville, UT). Since *FOXC2* mRNA levels and some of the phenotypic variables did not exhibit a normal distribution, Wilcoxon's signed-rank test was used for paired comparisons, Mann-Whitney was used for unpaired comparisons, and Spearman correlations or multiple correlations were used for estimating relationships between variables. Variables that were not normally distributed were log-transformed for graphs of correlation (*FOXC2* mRNA and fasting serum insulin). Descriptive data displaying normal distribution are given as mean ± SE. Data that were not normally distributed are given as median and interquartile range in parentheses. P values <0.05 were considered statistically significant. The primary hypothesis was to test the association of *FOXC2* expression or gene polymorphism with features of insulin resistance (HOMA-IR, insulin, and triglycerides) (12). Therefore, we did not consider it necessary to adjust HOMA-IR or triglycerides for multiple comparisons. Adjusting them for multiple comparisons therefore may seem overly orthodox, particularly as they are strongly interrelated, but these adjustments (Bonferroni) are presented along with the unadjusted P values. HOMA-IR was calculated as fasting plasma glucose × fasting serum insulin ÷ by 22.5 (20). Phenotypic differences between genotype-discordant sibling pairs were compared using a simulation-based permutation test for paired replicates (21). The permutation test does not make any assumptions about the normality, homogeneity of the variance, or the precise form of the underlying distribution. In the permutation test for x included pairs (in this case 169 pairs), there are 2^x equally likely outcomes for each variable under the assumption of no difference between the paired siblings. The two-tailed P values were estimated using a large (10^6) random sample of all the possible permutations. The differences in phenotypic values were calculated as the value in the sibling with more C alleles (wild-type genotype of the *FOXC2* C-5126T polymorphism) minus the value in the sibling with less C alleles. If the observed sum of differences (OSD) entered the 5% region of rejection, the differences between pairs was considered significant.

Accession numbers refer to the accession number at the nucleotide and protein databases at http://www.ncbi.nlm.nih.gov/. Oligonucleotide primers and PCR conditions are available upon request.

RESULTS

***FOXC2* mRNA is increased in visceral compared with subcutaneous adipose tissue.** To investigate whether *FOXC2* is involved in human obesity, we obtained biopsies from abdominal visceral and subcutaneous fat from obese nondiabetic patients. *FOXC2* mRNA was 12 ± 4 times more abundant in visceral than in subcutaneous adipose tissue (0.47 [0.18–1.18] vs. 0.18 [0.03–0.42]; $n = 38$, $P = 0.0001$). The difference was significant in both male (0.81 [0.21–3.04] vs. 0.04 [0.025–0.39]; $n = 5$, $P = 0.04$) and female subjects (0.46 [0.17–1.14] vs. 0.19 [0.035–0.43]; $n = 33$, $P = 0.0009$). As in the transgenic mice (12), we found an inverse relationship between visceral adipose tissue *FOXC2* mRNA and total body fat mass ($R = -0.37$, $P = 0.032$). Visceral and subcutaneous *FOXC2* mRNA levels were also correlated to each other ($R = 0.41$, $P = 0.01$), but there was no correlation between subcutaneous *FOXC2* mRNA and fat mass ($R = -0.03$, $P = 0.9$).

***FOXC2* mRNA levels in visceral adipose tissue and skeletal muscle are correlated to whole-body insulin sensitivity.** Along with enhanced insulin sensitivity the transgenic mice overexpressing FOXC2 exhibited increased adipocyte oxygen consumption and this may have contributed to the protective effect against weight gain and insulin resistance induced by a high-fat diet (12). Data on glucose homeostasis and energy balance could be obtained from 14 of the obese subjects. Although there was no correlation between *FOXC2* mRNA and RMR ($R = -0.3$, $P = 0.3$), there was an inverse relationship between visceral adipose tissue *FOXC2* mRNA levels and fasting serum insulin concentrations ($R = -0.64$, $P = 0.013$; Fig. 1*A*). This correlation remained significant after adjusting for the prevailing plasma glucose (HOMA-IR $R = -0.68$, $P = 0.007$) as well as abdominal obesity (WHR $R = -0.66$, $P = 0.01$). There were no significant correlations between subcutaneous *FOXC2* mRNA and RMR ($R = 0.1$, $P = 0.7$), HOMA-IR ($R = -0.2$, $P = 0.4$), or fasting insulin with ($R = -0.2$, $P = 0.5$) or without ($R = -0.2$, $P = 0.5$) correction for WHR.

Skeletal muscle is a key player in the development of insulin resistance and accounts for a large proportion of insulin-stimulated glucose uptake (22). Since the mouse homolog *Foxc2* is expressed in skeletal muscle (12), we investigated the possible role of *FOXC2* in human skeletal muscle. We obtained muscle biopsies from subjects with either NGT or IGT, before and after a hyperinsulinemic-euglycemic clamp. There was no significant upregulation of *FOXC2* mRNA in human skeletal muscle during the 2-h clamp in the NGT (from 0.62 [0.30–0.74] to 0.64 [0.30–3.93]; $P = 0.3$) or in the IGT group (from 0.41 [0.22–0.63] to 0.55 [0.25–0.92]; $P = 0.2$), and there were no differences in *FOXC2* mRNA between the groups ($P = 0.3$ and $P = 0.4$ for pre- and postclamp biopsies, respectively). Although there was only a borderline correlation between muscle *FOXC2* mRNA levels and insulin-stimulated glucose uptake during the clamp in all subjects ($R = 0.49$, $P = 0.072$), skeletal muscle *FOXC2* mRNA independently explained >50% of the variance in insulin sensitivity ($R^2 = 0.55$, $P = 0.03$) in a multiple regression model including age and BMI. In support of this, there was an inverse correlation between *FOXC2* mRNA and fasting insulin concentrations

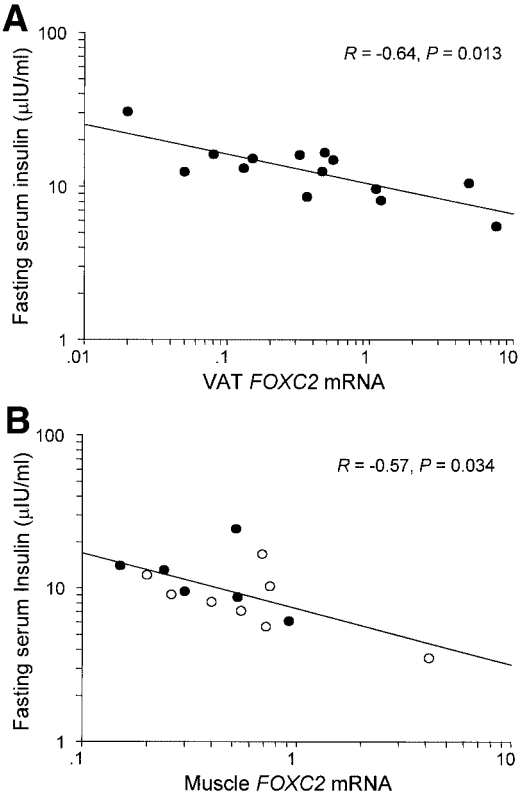

FIG. 1. *FOXC2* and insulin sensitivity. *FOXC2* mRNA was quantified by Real-Time PCR, normalized to *Cyclophilin A* mRNA, and log-transformed for normality. *A*: Correlation between visceral adipose tissue (VAT) *FOXC2* mRNA and fasting serum insulin. *B*: Correlation between skeletal muscle *FOXC2* mRNA and fasting serum insulin concentrations in subjects with NGT (○) and IGT (●).

($R = -0.57$, $P = 0.034$; Fig. 1*B*) as well as HOMA-IR ($R = -0.55$, $P = 0.04$).

***FOXC2* is regulated by insulin in human adipocytes.** From the data presented above, it cannot be deduced whether variation in *FOXC2* mRNA is the cause or consequence of either obesity or hyperinsulinemia. To investigate whether the gene is regulated by insulin, we used a human preadipocyte cell line (17). Insulin upregulated *FOXC2* mRNA within a few hours in mature adipocytes, suggesting that *FOXC2* is actively regulated by insulin in human adipocytes (Fig. 2*A*). In accordance with recent findings in the mouse preadipocyte cell line 3T3-L1 that demonstrated a correlation between *FOXC2* mRNA and protein levels (23), we could also confirm the presence of the FOXC2 protein in human adipocytes by Western blotting (Fig. 2*B*).

A common *FOXC2* 5′ UTR C-512T polymorphism is associated with insulin resistance. *FOXC2* consists of a single exon encoding a transcript of 1,507 bp and a 501–amino acid protein (accession numbers NM 05251 and NP 005242, respectively). The human FOXC2 gene is located on chromosome 16q22–24 (24). We screened the FOXC2 gene for variants in nondiabetic lean and obese subjects and in patients with type 2 diabetes by SSCP. Since homozygous disruption of the gene is lethal in mice (25,26), we did not expect major disturbances in gene structure. Two rare silent single nucleotide polymorphisms [at nucleotide positions C107T (Ser) and C1481T (Tyr)] were identified in one obese subject each. We also

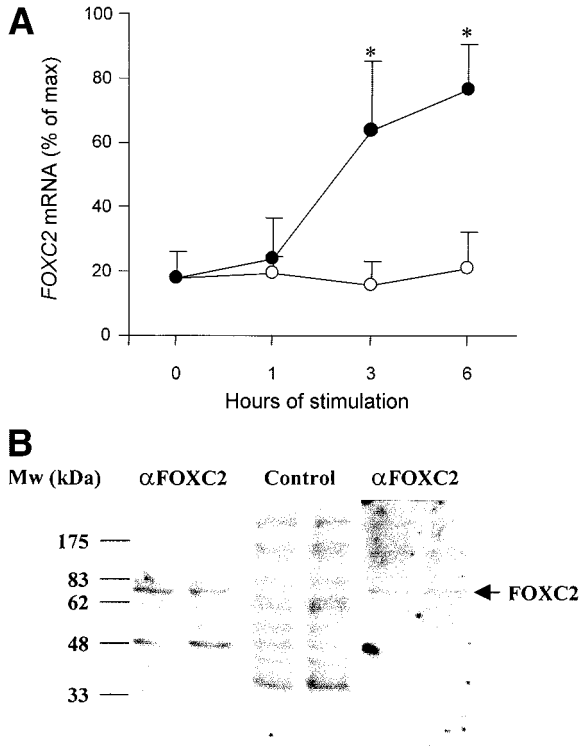

FIG. 2. Insulin stimulation of *FOXC2* expression in adipocytes. *A*: Human adipocyte *FOXC2* mRNA levels in response to insulin stimulation (1 nmol/l; ●) and vehicle (○) for up to 6 h. **P* < 0.05 by one-way ANOVA. *FOXC2* mRNA was quantified by Real-Time PCR and normalized to *Cyclophilin A* mRNA. *B*: Western blotting of whole-cell lysates from human adipocytes. Nonstimulated lysates were loaded in duplicates and (from left to right) blotted with α-FOXC2, and membranes were stripped and reblotted with a control antisera (control) and then stripped and reblotted with α-FOXC2. Molecular weight markers are indicated on the left, and the migration of FOXC2 is indicated by an arrow on the right. Results are representative of three separate cell cultures.

identified a common C/T polymorphism in the 5′ untranslated region (5′UTR) of the gene, 512 bp upstream of ATG, which was used for further investigations (C-512T). In a control population of normal-weight nondiabetic subjects (39 men and 57 women, age 56 ± 11 years, BMI 23 ± 1 kg/m^2), allele frequencies were 0.35 and 0.65 for the C and T alleles, respectively (genotype frequencies CC = 0.18, CT = 0.35, and TT = 0.47).

Since case-control studies are hampered by problems in selecting an appropriate control group, we preferred to investigate whether this polymorphism is associated with features of the metabolic syndrome in siblings discordant for the genotype. Of 517 subjects, 169 genotyped nondiabetic sibling pairs matched for sex and age (250 male and 267 female pairs, age 45 years [37–58], and BMI 25 kg/m^2 [23–28]) were discordant for the number of T alleles at this locus. Since there are sex-specific differences concerning some of the components of the metabolic syndrome, male and female pairs were analyzed separately. Pairwise comparisons (i.e., CC vs. CT, CC vs. TT, and CT vs. TT) showed that female siblings who carried more T alleles were more insulin sensitive (HOMA-IR P = 0.007) and had lower fasting plasma triglyceride levels (P = 0.007) than their sisters who carried less T alleles, thus identifying the T allele as a potential genetic factor protecting against insulin resistance (Table 1). These differences remained significant after adjusting for multiple comparisons (P = 0.021). There were no significant differences concerning these parameters between the male sibling pairs. When both sexes were analyzed together, the difference in HOMA-IR was of only borderline significance (P = 0.052) and no difference was found in fasting plasma triglycerides (P = 0.9). The number of discordant homozygous pairs (i.e., CC vs. TT) was small (10 male and 7 female pairs), but they showed the same, albeit nonsignificant, trends concerning HOMA-IR (2.34 [1.71–3.04] vs. 2.13 [1.32–2.72]; OSD = 2.28, P = 0.7) and fasting plasma triglycerides (1.42 ± 0.14 vs. 1.35 ± 0.14 mmol/l; OSD = 1.13, P = 0.6). When the means of all siblings with the different genotypes were compared, there was a significant difference between the groups with respect to HOMA-IR using BMI (P <

TABLE 1
FOXC2 C-512T genotype-discordant sibling pair analysis identifies the T allele as a potential genetic factor protecting against insulin resistance

	Sibling 1		Sibling 2		
	CC CC CT	vs. vs. vs.	CT TT TT	OSD	*P*
Female pairs (*n*)	82		82		
Age (years)	50.1 ± 1.7		49.5 ± 1.8	46.6	0.1
BMI (kg/m^2)	25.0 ± 0.4		24.9 ± 0.4	10.8	0.7
HOMA-IR	1.99 (1.32–2.82)		1.66 (1.08–2.49)	39	0.007
fP-Triglycerides (mmol/l)	1.18 (0.85–1.70)		1.05 (0.90–1.28)	16.4	0.007
Male pairs (*n*)	87		87		
Age (years)	45.3 ± 1.6		45.1 ± 1.6	25.3	0.4
BMI (kg/m^2)	25.7 (24.0–27.2)		25.2 (23.8–27.2)	3.82	0.9
HOMA-IR	1.80 (1.40–2.72)		2.10 (1.51–2.67)	2	0.9
fP-Triglycerides (mmol/l)	1.16 (0.85–1.57)		1.23 (0.92–1.64)	−20.9	0.2
All pairs (*n*)	169		169		
Age (years)	47.2 (36.0–60.0)		44.6 (35.1–60.2)	71.8	0.07
BMI (kg/m^2)	25.4 (23.5–27.2)		25.1 (23.3–27.3)	14.6	0.8
HOMA-IR	1.94 (1.35–2.76)		1.86 (1.23–2.50)	41	0.052
fP-Triglycerides (mmol/l)	1.16 (0.85–1.62)		1.14 (0.9–1.47)	−4.5	0.9

Data are means ± SE or median (interquartile range).

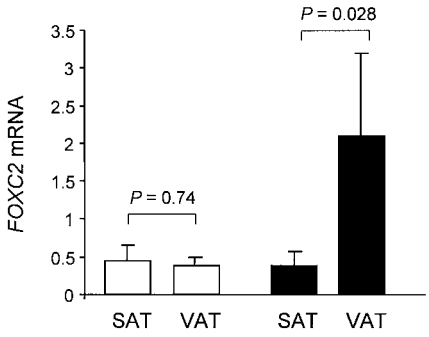

FIG. 3. Effect of the common *FOXC2* 5′ UTR C-512T polymorphism. *FOXC2* mRNA levels in VAT and subcutaneous adipose tissue (SAT) from obese subjects according to C–512T genotype. *FOXC2* mRNA levels were higher in VAT than in SAT in subjects homozygous for −512T (■, $n = 7$) but not in subjects carrying the −512C allele (□, $n = 7$). *FOXC2* mRNA was quantified by Real-Time PCR and normalized to *Cyclophilin A* mRNA.

0.0001), age ($P = 0.2$), and sex ($P = 0.7$) as covariates: 1.96 (1.26–2.74), 1.85 (1.25–2.68), and 1.60 (1.12–2.32) for CC ($n = 100$), CT ($n = 324$), and TT ($n = 294$), respectively, $P = 0.02$. No significant difference in BMI ($P = 0.5$) or fasting plasma triglycerides ($P = 0.4$) was observed in the ANCOVA analysis.

Since the C-512T polymorphism is located in a putative promoter region, we investigated if there was any correlation between genotype and *FOXC2* mRNA expression in 14 obese subjects with data on *FOXC2* mRNA in visceral and subcutaneous adipose tissue and in 11 subjects with available data on skeletal muscle *FOXC2* mRNA levels. The difference between visceral and subcutaneous adipose tissue *FOXC2* mRNA levels was significant only in subjects homozygous for the T allele (0.04 [0.03–0.44] vs. 0.48 [0.13–4.9], $P = 0.028$ for TT carriers [$n = 7$] compared with 0.28 [0.12–0.47] vs. 0.32 [0.08–0.55], $P = 0.74$ for CC/CT carriers [$n = 7$]) (Fig. 3). These subjects also exhibited significantly lower fasting plasma triglyceride levels compared with carriers of the C allele (0.91 [0.83–1.19] vs. 1.51 [1.38–2.42] mmol/l; $P = 0.018$), although when adjusting for multiple comparisons this difference was no longer significant ($P = 0.072$) and there were no significant differences concerning age ($P = 0.9$), BMI ($P = 0.7$), fasting insulin ($P = 0.4$), HOMA-IR ($P = 0.3$), or RMR ($P = 0.3$). There were no differences between the male NGT/IGT muscle biopsy subjects homozygous for the T allele and carriers of the C allele concerning BMI ($P = 0.9$), fasting insulin ($P = 0.1$), HOMA-IR ($P = 0.1$), fasting triglycerides ($P = 0.4$), or glucose uptake during the euglycemic clamp ($P = 0.6$). *FOXC2* mRNA levels increased only barely significantly in response to insulin during the hyperinsulinemic clamp in subjects homozygous for the T allele (from 0.40 [0.20–0.64] to 0.70 [0.24–3.65]; $n = 5$, $P = 0.043$), whereas this effect was clearly not significant in carriers of the C allele (from 0.62 [0.47–0.77] to 0.72 [0.35–3.32]; $n = 6$, $P = 0.29$).

DISCUSSION

This is the first study investigating the putative role of *FOXC2* for glucose metabolism in human subjects. The main findings were that *FOXC2* mRNA is present in both human adipose tissue and muscle and that its expression is sensitive to upregulation by insulin in human adipocytes in vitro, but that *FOXC2* mRNA levels in vivo in both adipose tissue and skeletal muscle correlate negatively with insulin levels and whole-body insulin sensitivity. It must be stressed that the data presented here come mainly from obese or very obese individuals. It may well be that *FOXC2* is upregulated in visceral adipose tissue due to obesity itself, although the negative correlation with fat mass and insulin levels speaks strongly against this reasoning. Additionally, variation in a common 5′ UTR C/T polymorphism of the gene was associated with features of the metabolic syndrome such as insulin resistance and dyslipidemia. This finding originated from a comparison of sibling pairs who were matched for sex and age but discordant for the genotype. Since many of the components of the metabolic syndrome exhibit sex differences, males and females were analyzed separately and it was only in females that siblings carrying more T alleles were more insulin sensitive and had lower plasma triglycerides than their corresponding siblings carrying less T alleles. Sex specificity is not uncommon in the context of obesity, as illustrated by findings concerning leptin and its receptor (27,28), and further studies are needed to explore this possible difference between males and females. In adipose tissue biopsies from mostly female obese subjects, *FOXC2* mRNA was much less abundant in subcutaneous than in visceral fat, the amount of which has been shown to strongly correlate with insulin resistance (5,29). This difference was, however, significant in both males and females. Interestingly, the higher expression of *FOXC2* in visceral adipose tissue was seen only in carriers of the T allele of the 5′ UTR polymorphism. In essence, human carriers of the C-512T-polymorphism T allele to some extent resemble the transgenic mice overexpressing FOXC2 (12). The initial screening for polymorphisms in the gene included subjects from families with type 2 diabetes and some evidence of linkage (15) to the region on chromosome 16q, where *FOXC2* is located but no additional variants were detected. Differences in expression due to allelic variation have recently been evaluated in humans and are relatively common (30). It should be noted that the interindividual differences in the relative abundance of the *FOXC2* mRNA transcript were greater in visceral than subcutaneous adipose tissue, indicating differential regulation. This great variance in gene transcription is uncommon but has been described previously (31). It is premature to conclude that this would represent an effect of a marked regulation by insulin or degree of insulin sensitivity.

The physiological role of FOXC2 in human glucose and energy metabolism is not known and can only be speculated on from data in transgenic mice overexpressing FOXC2. These mice are protected from developing insulin resistance and gaining weight during fat feeding. The effects have been ascribed to upregulation of several "master genes" involved in adipocyte differentiation and glucose and energy metabolism, along with an increased sensitivity in the β-adrenergic/cAMP/protein kinase A pathway and an increase in oxygen consumption (12). We did not observe any significant correlation with RMR and *FOXC2* mRNA expression in visceral adipose tissue, but estimates of metabolic rates by indirect calorimetry represent a relatively crude measure of energy expenditure.

In adipocytes from transgenic mice overexpressing FOXC2, there was also a parallel upregulation of key components of the insulin signaling chain, such as the insulin receptor, the insulin receptor substrates, and GLUT4, that would favor glucose uptake and triglyceride formation (12). In humans these changes might ultimately result in an obese energy-conserving yet insulin-sensitive phenotype.

Much remains to be learned about the role of FOXC2 in adipose tissue, and its role in skeletal muscle is even less certain. There was no significant upregulation of *FOXC2* mRNA levels in skeletal muscle in response to insulin after a 2-h hyperinsulinemic-euglycemic clamp in subjects with either NGT or IGT. It was only after analyzing subjects who were homozygous for the T allele of the 5′ UTR polymorphism that this effect barely reached statistical significance. One possible explanation for this lack of upregulation in response to insulin could be the time-course data that we obtained in the human adipocytes where the effect of insulin was not seen until after several hours. Another explanation may be tissue specificity, but we do not have in vivo data for adipocytes. There was only a borderline correlation between muscle *FOXC2* mRNA and whole-body glucose uptake during a hyperinsulinemic-euglycemic clamp. All the same, there was a strong correlation between *FOXC2* mRNA, fasting serum insulin levels, and HOMA-IR in the obese study subjects. It is unlikely that this correlation is a corollary of increased abdominal obesity or glucose intolerance as the correlation remained significant after adjusting for abdominal adiposity or plasma glucose. The data on FOXC2 in muscle still leaves a number of questions open, and the better correlation with insulin levels than with direct estimates of insulin sensitivity could suggest that a putative interaction occurs between FOXC2 in adipose tissue and the β-cells in a way proposed for leptin (32).

The thrifty gene hypothesis put forward already in 1962 (33) states that genetic selection would favor energy-conserving genotypes in harsh environments with unstable food supply. At the same time these genes would constitute a risk for developing obesity-related conditions, such as insulin resistance and type 2 diabetes, when food is freely available. The present data raise the question whether *FOXC2* serves as an "antithrifty" gene (34–36) and whether *FOXC2* would be a putative target for treatment aiming at protection from developing insulin resistance and gaining weight when exposed to the affluent environment of western societies.

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