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Intronic Polymorphisms within *TFAP2B* Regulate Transcriptional Activity and Affect Adipocytokine Gene Expression in Differentiated Adipocytes

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We have identified a gene encoding transcription factor activating enhancer binding protein-2β (*TFAP2B*) as a candidate for conferring susceptibility to type 2 diabetes. Although we have also found that *TFAP2B* was preferentially expressed in adipose cells in a differentiation-dependent manner, the mechanisms by which the gene and gene polymorphisms contribute to conferring susceptibility to the disease have not yet been elucidated. The aim of this study was to evaluate the impact of the polymorphisms within the *TFAP2B* gene on conferring susceptibility to type 2 diabetes. We identified that a 300-bp DNA fragment in intron 1 of *TFAP2B* had significant enhancer activity, and the variations of this region affected this enhancer activity in differentiated adipocytes. In an experiment using adenovirus vectors encoding *TFAP2B*, the expression of TNF-α gene was shown to be elevated in the *TFAP2B* overexpressing cells compared with those in control cells. Furthermore, we demonstrated that the expression of *TFAP2B* was increased in the adipose tissues of subjects with the disease-susceptibility allele, and the plasma levels of TNF-α and high sensitivity C-reactive peptide were significantly elevated in the patients with the disease-susceptibility allele. These results suggest that *TFAP2B* may contribute to the pathogenesis of type 2 diabetes through regulation of adipocytokine gene expression, and that *TFAP2B* may be a promising target for treatment or prevention of this disease. (*Molecular Endocrinology* 20: 1104–1111, 2006)

TYPE 2 DIABETES AFFECTS more than 100,000,000 individuals worldwide (1). Although the precise mechanism is still not well known, the pathogenesis of type 2 diabetes is thought to be the consequence of insulin resistance in peripheral tissues combined with dysfunction of β-cells in pancreatic islets (2, 3).

Recent studies in the field of obesity research have suggested that adipose tissue functions as an endocrine organ and secretes a number of cytokines known as adipocytokines, and these include TNF-α, IL-6, IL-8, adiponectin, resistin, leptin, and others. These adipocytokines have been reported to be involved in systemic insulin responsiveness and are thus considered to play pivotal roles in the pathogenesis of type 2 diabetes (4–7).

We have identified the activating enhancer binding protein (AP)-2β transcription factor gene (*TFAP2B*) located on chromosome 6p12 as a susceptibility gene to type 2 diabetes by a genome-wide association study using 58,266 gene-based SNPs (single-nucleotide polymorphisms) as genetic markers (8). Several variations within *TFAP2B* were shown to be significantly associated with type 2 diabetes in the Japanese population as well as in the United Kingdom population. This association was especially strong with a variable number of tandem repeat (VNTR) locus in the first intron ($\chi^2 = 11.0$; $P = 0.0009$; odds ratio = 1.53; 95% confidence interval, 1.19–1.98), and a nearby SNP ($\chi^2 = 11.2$; $P = 0.0008$; odds ratio = 1.54; 95% CI, 1.20–1.99). We also reported that mouse transcription factor AP-2β gene (*Tcfap2b*) was preferentially expressed in adipose cells in a differentiation-dependent manner (8). These results suggest that *TFAP2B* plays some roles in the pathogenesis of type 2 diabetes through the regulation of adipocyte functions. However, the precise mechanisms by which the polymorphism within *TFAP2B* confers the susceptibility to the disease have not yet been elucidated.

The AP-2 transcription factor family consists of four members, AP-2α, AP-2β, AP-2γ, and AP-2δ, each encoded by a separate gene (9–12). AP-2 proteins homo- and heterodimerize through a unique C-terminal helix-span-helix motif and bind palindromic DNA recognition sequences (consensus 5′-GCCN$_3$GGC-3′) through the basic domain that lies immediately N-terminal of the dimerization motif (13). The dimerization/

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Abbreviations: AP-2, Activating enhancer binding protein 2; hs-CRP, high-sensitivity C-reactive peptide; SNP, single-nucleotide polymorphism; VNTR, variable number of tandem repeat.

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DNA-binding region is highly conserved among the AP-2 isoforms. AP-2 transcription factors appear to execute crucial, overlapping (14, 15), yet distinct functions during embryonic development (16–18) and malignant transformation (19–21). Although a member of this transcription factor family, AP-2α, has been reported to inhibit adipogenesis (22, 23). Functional roles of AP-2β in the adipocytes have not yet been identified.

In the study reported here, we provide evidence that polymorphisms in the first intron of *TFAP2B* affect the transcriptional activity of the gene in differentiated adipocytes and confer susceptibility to type 2 diabetes through the regulation of adipocytokine gene expression.

RESULTS

Transfection Experiment in 3T3-L1 Cells

Polymorphisms within *TFAP2B* are shown in Fig. 1A. Because the polymorphisms in the first intron were shown to be most strongly associated with type 2 diabetes (8), and no exonic polymorphisms were identified, we examined the function of 300-bp fragments containing both the SNP at intron 1+774 and nearby VNTR for transcriptional activity in 3T3-L1 cells at different stages of differentiation. As shown in Fig. 2A, the 300-bp fragment showed significant enhancer activity both in the undifferentiated and in the differentiated adipocytes, although neither single nor multiple copies of a short DNA fragment (+766 to +785 in the first intron) had enhancer function. We next examined whether the enhancer activity of this fragment differed between the fragment corresponding to the disease susceptibility allele and that of the major allele. Because the two variations (intron 1+774T/G and VNTR) were in almost complete linkage disequilibrium (8), we compared the disease-susceptibility allele that contained both substitutions (S allele: T nucleotide for SNP, nine repeats for VNTR) with the major allele (N allele: G nucleotide for SNP, 10 repeats for VNTR). The results indicated that the fragment corresponding to

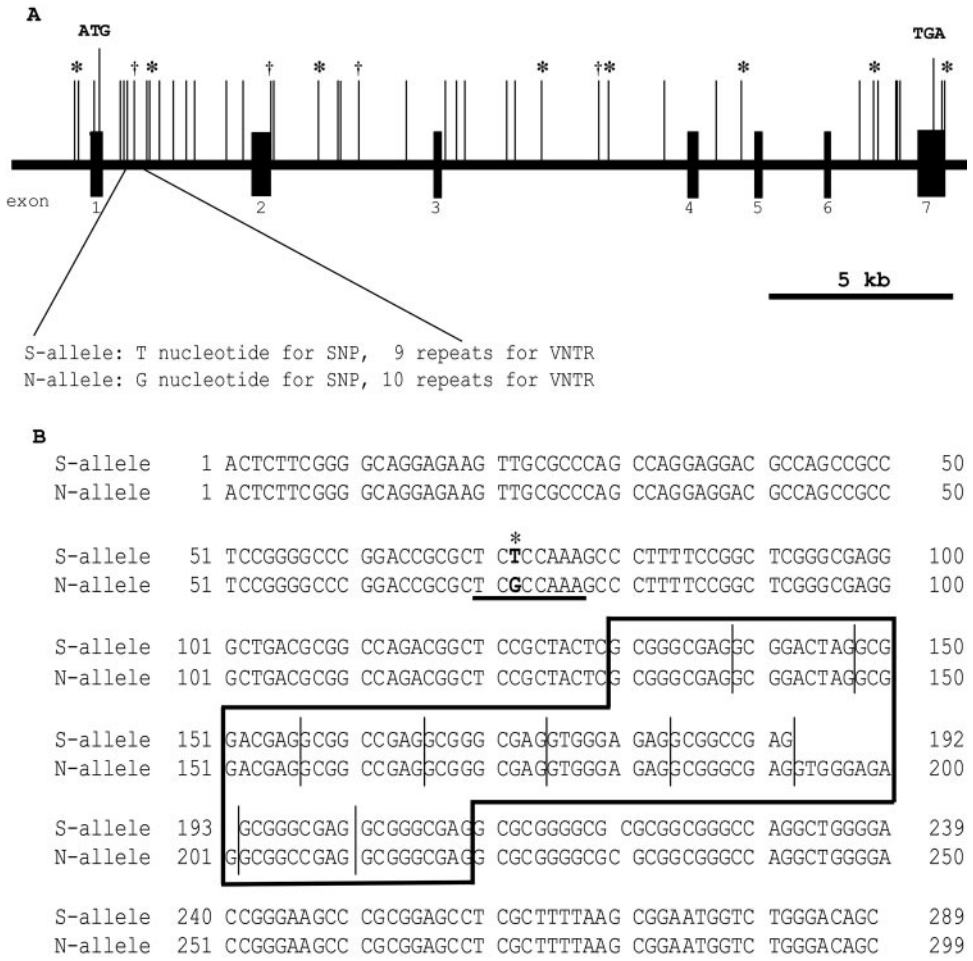

Fig. 1. Genetic Variations within the *TFAP2B*

A, Polymorphisms within the *TFAP2B* gene. *, Insertion/deletion polymorphisms; †, tandem-repeat polymorphisms; no symbol, SNPs. B, Nucleotide sequences of the 300-bp fragment containing the SNP and the VNTR in the first intron. The SNP (+774T/G) is shown by an *asterisk*, and VNTR regions are *boxed*. *Underline* denotes a sequence similar to E2F binding motif.

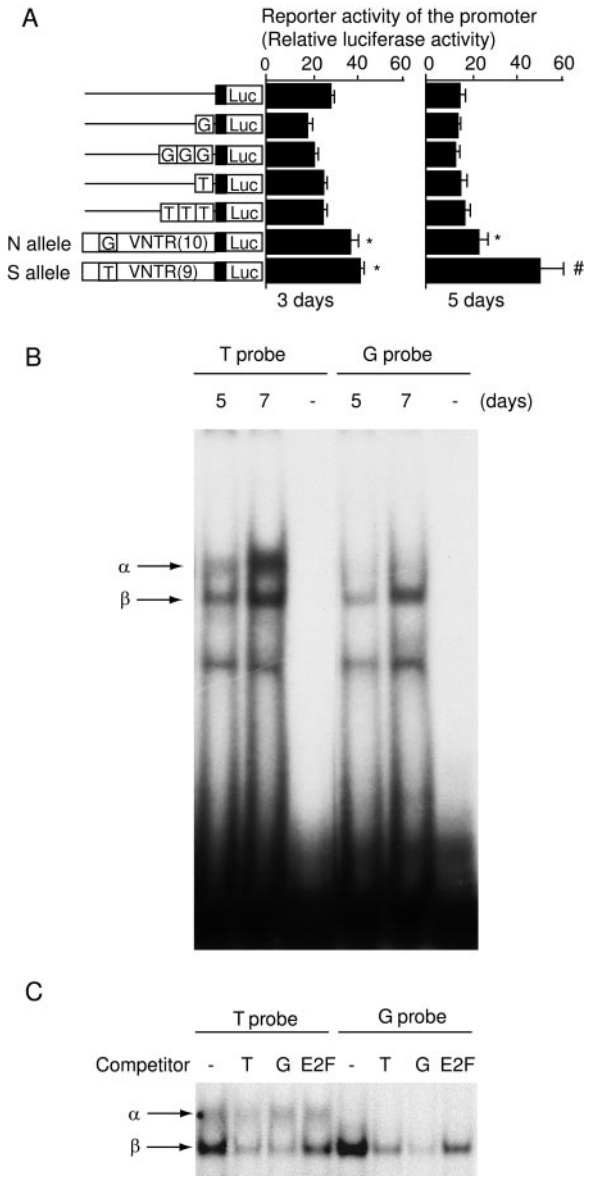

Fig. 2. Functional Analyses of Genetic Variations in the *TFAP2B* Gene

A**,** Enhancer activity of transfected human *TFAP2B* fragments in 3T3-L1 cells 3 and 5 d after induction of differentiation. One or three copies of the sequence corresponding to +766 to +785 in the first intron, or the 289- or 299-bp sequence containing the SNP at +774 and also the VNTR in the first intron, were subcloned upstream of the heterologous promoter of pGL3 vector and transfected to 3T3-L1 cells. Values are expressed as means ± SEM from four independent experiments. *, $P < 0.01$ *vs.* promoter alone; #, $P < 0.0001$ *vs.* other constructs. B, EMSA using nuclear extracts from 3T3-L1 cells, performed on the indicated days after inducing differentiation. The sequence from +766 to +785 in the first intron of human *TFAP2B* was used as a probe. C**,** Competition experiments using nuclear extracts from mature 3T3-L1 cells 7 d after inducing differentiation. The nuclear extracts were preincubated with a 100-fold excess of unlabeled oligonucleotide. T probe, T at position +774; G probe, G at position +774; E2F, E2F consensus oligonucleotide; Luc, luciferase.

the S allele had greater enhancer activity than the N allele in the cells 5 d after inducing differentiation, although there was no significant difference in the cells after 3 d of differentiation.

EMSA

Because the DNA segment corresponding to the particular SNP at +774 of the first intron was similar to the E2F-binding motif (Fig. 1B), we performed an EMSA using a 20-bp probe (+766 to +785 in the first intron) and identified two bands that were likely to reflect specific binding of protein to the DNA (α, β in Fig. 2B). Interestingly, the α-band was seen only when the 20-bp probe containing the S allele (T probe) was incubated with a nuclear extract from differentiated 3T3-L1 cells (Fig. 2, B and C).

Expression of TFAP2B in Human Adipose Tissues

We tested adipose samples and genomic DNA for eight independent individuals obtained from Genomics Collaborative (Cambridge, MA). Among them, only two subjects possessed the T/G genotype for intron 1+774. As shown in Fig. 3, the expression of *TFAP2B* was higher in subjects with the T/G genotype compared with those with the G/G genotype. The expression of *TFAP2B* in adipose tissue from two subjects with the G/G genotype could not be detected.

Effects of TFAP2B on Adipokine Gene Expression

To discover a possible candidate gene(s) that is regulated by *TFAP2B*, we examined the expression of adipocytokine genes in human adipocytes overexpressing the *TFAP2B* gene and identified significant increases in the expression of TNF-α in the *TFAP2B* overexpressing cells compared with those in control cells (Fig. 4, 1.3 ± 0.3 *vs.* 6.2 ± 0.7; LacZ *vs. TFAP2B*; mean ± SD; $P = 0.0004$). Although the expression of IL-6 seemed to be increased in *TFAP2B* overexpress-

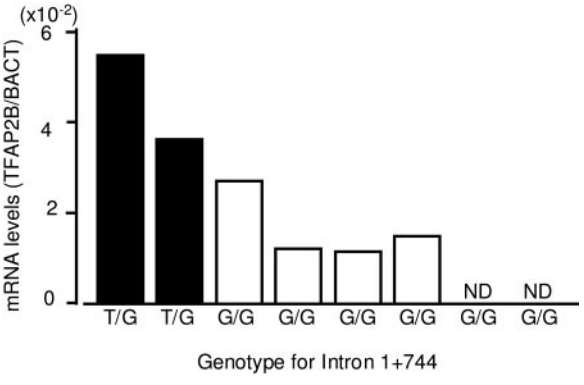

Fig. 3. Expression of *TFAP2B* in Human Adipose Tissue

Genotype for the SNP (+774 T/G in intron 1) is indicated *under each bar*. ND, Not detected; BACT, β-actin.

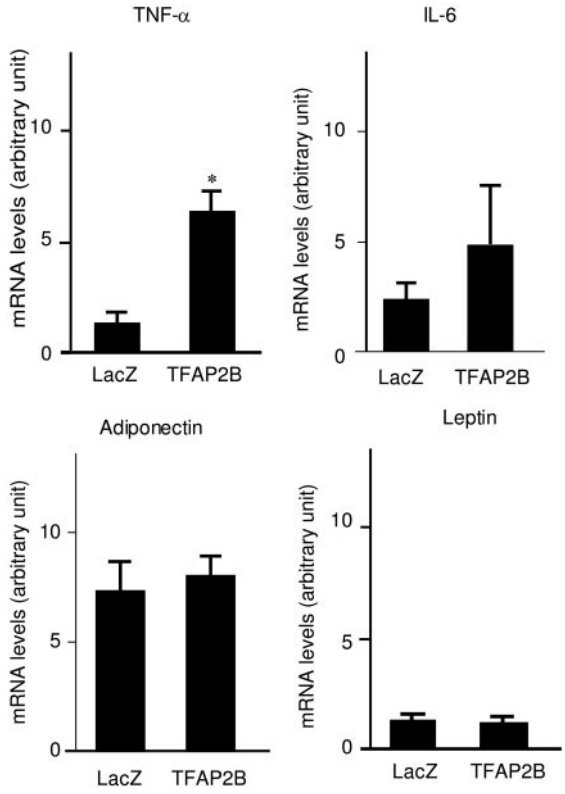

Fig. 4. Results of Quantitative RT-PCR for Adipocytokine Genes in Cultured Human Adipocytes Transfected with adeno-*AP2B* or adeno-LacZ

Values are expressed as means ± SD from three independent experiments. *, $P < 0.001$ *vs.* control.

ing cells, the difference between *TFAP2B* and LacZ overexpressing cells was not statistically significant (2.4 ± 1.5 *vs.* 4.8 ± 3.0; LacZ *vs. TFAP2B*; mean ± SD; $P = 0.29$). The expression of adiponectin and leptin was similar in the overexpressing and control cells (Fig. 4).

Plasma Adipocytokines and High-Sensitivity C-Reactive Peptide

We subsequently measured plasma levels of TNF-α, adiponectin, and hs-CRP in 228 patients and examined the association between their concentrations and *TFAP2B* genotype. Plasma TNF-α, and hs-CRP were significantly elevated in subjects possessing the disease-susceptibility allele (Table 1, TNF-α [pg/ml] 7.1 ± 5.2, 5.4 ± 4.5, 5.0 ± 3.7, T/T, T/G, G/G, respectively, mean ± SD, $P = 0.038$ T/T *vs.* G/G, hs-CRP [μg/ml]: 0.83 ± 0.55, 0.62 ± 0.41, 0.58 ± 0.42, T/T, T/G, G/G, respectively, mean ± SD, $P = 0.038$ T/T *vs.* G/G). The plasma adiponectin, body mass index, HbA1c, and gender distribution were not statistically different among these groups.

DISCUSSION

In the present study, we demonstrated that the polymorphisms in the first intron of *TFAP2B* affected the transcriptional activity of the gene. Our results also indicated that the subjects with the disease-susceptibility allele had higher expression of *TFAP2B* in adipose and had increased expression of adipocytokines, such as TNF-α.

We have identified the *TFAP2B* as a susceptibility gene to type 2 diabetes by genome-wide case-control association studies using gene-based SNPs (8). This approach proved to be successful in the discovery of candidate genes for other diseases, such as myocardial infarction (24) and diabetic nephropathy (25, 26).

AP-2β is a well-known transcription factor and has been reported to play an important role in embryonic development. In mice, expression of AP-2β decreases significantly after birth (10). Mice lacking AP-2β die within 1 or 2 d after birth from renal failure due to polycystic kidney disease (18). In humans, mutation of *TFAP2B* causes Char syndrome, a condition characterized by patent ductus arteriosus and variable degrees of facial dysmorphism and hand abnormalities (27); those features suggest that AP-2β plays an important role in the embryonic development of various tissues. However, to date, no evidence other than our previous report (8) has emerged to suggest that AP-2β has a role in the pathogenesis of type 2 diabetes.

To investigate possible roles of *TFAP2B* in the pathogenesis of type 2 diabetes, we first examined the expression of this gene by quantitative real-time PCR

Table 1. Clinical and Biochemical Parameters in the Subjects Divided According to the Genotype of the Polymorphism at the First Intron of *TFAP2B* (Intron1+774 T/G)

	TT	TG	GG
n	23	99	106
Sex (male:female)	6:17	47:52	44:62
Age (yr)	64.3 ± 9.7	61.6 ± 9.9	61.8 ± 10.6
BMI (kg/m^2)	22.7 ± 6.0	23.7 ± 3.2	23.2 ± 3.2
HbA1c (%)	7.3 ± 1.1	7.2 ± 1.2	7.1 ± 1.2
Plasma TNF-α (pg/ml)	7.1 ± 5.2[a]	5.4 ± 4.5	5.0 ± 3.7
Plasma hsCRP (μg/ml)	0.83 ± 0.55[a]	0.62 ± 0.41	0.58 ± 0.42
Plasma adiponectin (μg/ml)	10.0 ± 5.3	10.3 ± 5.9	9.7 ± 5.8

Values are presented as mean ± SD. BMI, Body mass index.
[a] $P < 0.05$ *vs.* GG.

using RNA from human cultured adipocytes. The expression of *TFAP2B* was significantly higher in differentiated adipocytes compared with undifferentiated preadipocytes; the average increase was 4.94-fold relative to undifferentiated preadipocytes (data from our unpublished observations). Because this result was consistent with our previous observations in mouse 3T3-L1 cells (8), it was suggested that *TFAP2B* had some functions in differentiated adipocytes.

Cumulative evidence has indicated that differentiated adipocytes have an endocrine-related function to secrete several cytokines, called "adipocytokines," which include TNF-α, IL-6, leptin, adiponectin, and others (28–30). These genes are found to contain binding sites for AP-2 in their promoter (31, 32). Given such observations, we suggest that *TFAP2B* plays a key role in the pathogenesis of type 2 diabetes by affecting insulin responsiveness through the transcriptional regulation of these adipocytokine genes in differentiated adipocytes.

To elucidate the possible mechanism by which *TFAP2B* and *TFAP2B* polymorphisms contribute to the susceptibility to type 2 diabetes, we examined the function of the 300-bp DNA fragment containing the SNP site at intron 1+774, and the nearby VNTR, which were both shown to be strongly associated with the disease (8). The result indicated that the 300-bp fragment had significant enhancer activity both in differentiated and immature adipocytes, whereas neither single nor multiple copies of a short DNA fragment (+766 to +785) showed enhancer activity (Fig. 2A). Because the VNTR sequence did not have enhancer activity by itself (data not shown), it is likely that these two fragments cooperate with each other to regulate transcriptional activity. In the present study, we also demonstrated that a DNA fragment containing both substitutions (S allele, T nucleotide for SNP+774, nine repeats for VNTR) had greater enhancer activity than a fragment corresponding to the major allele (N allele, G nucleotide for SNP+774, 10 repeats for VNTR) in differentiated adipocytes (Fig. 2A). We found a specific protein binding to the particular DNA (+766 to +785 in the first intron) that could be observed only when nuclear extracts from differentiated adipocytes were incubated with the S allele (T probe) (Fig. 2, B and C). Therefore, this short core sequence may be critical for the regulation of the transcriptional activity of *TFAP2B*, although the cooperation of this 20-bp fragment with VNTR sequence was necessary for the enhancer activity. Because the sequence around this SNP site was similar to the E2F consensus sequence, we performed super-shift analyses using the antibodies for E2F proteins. However, antibodies for several E2F proteins could not affect the mobility of the DNA-protein complex (our unpublished observations), which suggested the binding protein was a novel protein other than E2F.

These observations suggest that the subjects with the S allele had higher expression of *TFAP2B* in adipose tissues than the subjects with the N allele. Our subsequent human study using RNA from adipose tissues (Fig. 3) could further support this hypothesis.

We then examined the effect of overexpression of *TFAP2B* on the expression of several adipocytokine genes in human differentiated adipocytes. The results indicated that there were clear differences in the expression of TNF-α between *TFAP2B* overexpressing cells and control cells (Fig. 4). Because TNF-α produced in adipose cells has been reported to be implicated in systemic insulin resistance (33), the increase in the expression of the *TFAP2B* in differentiated adipocytes results in the elevation of expression of the TNF-α gene and may contribute to the pathogenesis of type 2 diabetes in individuals having the disease-susceptibility allele.

Finally, to further verify our hypothesis, we examined plasma levels of TNF-α and hs-CRP; the latter was reported to correlate with plasma levels of several adipocytokines (34). Interestingly, the plasma TNF-α and hs-CRP levels were significantly elevated in the patients with the disease-susceptibility allele (Table 1).

In summary, our present study indicated that the polymorphisms in the first intron of *TFAP2B* affect the transcriptional activity of the gene in differentiated adipocytes and confer susceptibility to type 2 diabetes through the regulation of adipocytokine gene expression, such as TNF-α.

MATERIALS AND METHODS

Materials

Poly-(dAdT) was purchased from Sigma-Aldrich Co. (St. Louis, MO), deoxynucleotide triphosphates and EX Taq HS DNA polymerase were purchased from Takara Bio, Inc. (Otsu, Shiga, Japan), and SYBR Green I was purchased from Cambrian Chemicals, Inc. (Oakville, Ontario, Canada).

A mouse 3T3-L1 cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were grown to confluence and induced to differentiate into adipocytes according to methods described previously (35). Human cultured adipocytes were obtained from Zen-Bio, Inc. (Research Triangle Park, NC). Total RNA was extracted from these cells by Trizol Reagent (Invitrogen, Carlsbad, CA).

EMSA for DNA-Binding Protein

Nuclear extracts from 3T3-L1 cells were prepared by a procedure described previously (36). Oligonucleotides comprising the sequences for T probe (5′-CTA GCC GCG CTC TCC AAA GCC CTT-3′) and for G probe (5′-CTA GCC GCG CTC GCC AAA GCC CTT-3′) were end labeled with [γ-^{32}P]ATP (Amersham Biosciences, Piscataway, NJ). Nuclear extract (6 μg) was placed in binding buffer [final concentration: 20 mM HEPES (pH 7.9), 30 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mg/ml BSA, and 1 μg poly(dAdT)] at room temperature for 1 h and then incubated with the radiolabeled probe at room temperature for 20 min. The protein-DNA complexes were analyzed on a 4% polyacrylamide gel. The

Table 2. Human Gene-Specific Primers and Probes Used in this Study

Gene	Sequence (5′–3′)
TFAP2B	
Sense primer	TTGAACCGGCAGCACACA
Antisense primer	CTTGGTGGCCAACAGCATATT
TaqMan probe	(FAM)-CCGAGTGACCTGCACTCCCGAAA-(TAMRA)
β-Actin	
Sense primer	AAAGACCTGTACGCCAACAC
Antisense primer	GTCATACTCCTGCTTGCTGAT
TaqMan probe	(FAM)-AGATCATTGCTCCTCCTGAGCGCAAGTA-(TAMRA)
TNF-α	
Applied Biosystems TaqMan Pre-Developed Assay Reagents	No. 4327055F
IL-6	
Applied Biosystems TaqMan Gene Expression assays	No. 185324329
Adiponectin	
Applied Biosystems TaqMan Gene Expression assays	No. 185323401
Leptin	
Sense primer	TCCAGAAAGTCCAGGATGACACCAA
Antisense primer	TCAGCATTCAGGGCTAACATCCAAC

gel was dried and exposed to BioMax XAR film (Eastman Kodak, Rochester, NY).

Plasmid Construction and Transfection Experiments

Various DNA fragments of the first intron of *TFAP2B* were subcloned into the pGL3-promoter vector (Promega Corp., Madison, WI) at its multiple-cloning site upstream of the SV-40 promoter. On appropriate days after inducing differentiation of the 3T3-L1 cells, we introduced each construct to the cells along with a sea-pansy luciferase control vector, pRL-TK (Promega), using the liposome transfection procedure (FuGene6, Roche, Mannheim, Germany). Forty-eight hours after transfection, luciferase activities were determined by the Dual Luciferase Reporter Assay System (Promega), and the luminescence of firefly luciferase was corrected by that of sea-pansy luciferase, which reflected transfection efficiency.

Preparation of Adenovirus Vectors and Infection

Adenovirus vector encoding human *TFAP2B* was prepared by Takara Bio, Inc. (Otsu, Shiga, Japan). LacZ encoding vector was used for the control. Human differentiated adipocytes were transduced with a multiplicity of infection of 50 plaque-forming units/cell for 16 h. At the indicated time after infection, the total RNA was extracted for RT-PCR.

RT-PCRs

First-strand cDNA was prepared by reverse transcription of total RNA extracted from the human cultured adipocytes, or human adipose tissues obtained from Genomics Collaborative (Cambridge, MA), by oligo-dT priming, using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).

Quantitative RT-PCR was performed by a TaqMan assay or by a method using SYBR Green detection. The amplifications were carried out in a 25-μl reaction volume containing 1× EX Taq Buffer, 200 nM deoxynucleotide triphosphate mixture, 800 nM each primer, 200 nM TaqMan probe (for TaqMan assay) or 1/20,000 SYBR Green (for SYBR Green detection), 0.125 U EX Taq HS DNA polymerase (Takara Bio, Inc.) and 5 ng template. The thermal profile was 50 C for 2 min, 95 C for 10 min followed by 40 cycles of 95 C for 30 sec, 63 C for 30 sec, and 72 C for 30 sec. The amplification and quantification were performed using the Mx3000P Multiplex Quantitative PCR system (Stratagene, CA). Primers and TaqMan probes for amplifications are described in Table 2.

Subjects and DNA Preparations

DNA samples were obtained from patients who regularly visit the outpatient clinics of Shiga University of Medical Science. Written informed consent was obtained from each patient, and DNA extraction was performed using a standard phenol-chloroform procedure. The genotype of *TFAP2B* SNP was determined using the invader assay as previously described (8). The protocol was approved by the ethics committees of the Institute of Physical and Chemical Research and Shiga University of Medical Science.

Measurement of TNF-α, Adiponectin, and hs-CRP in Plasma

Plasma concentrations of TNF-α and adiponectin were measured by ELISAs using an ELISA kit (Human TNF-α Quantikine HS; R&D Systems, Minneapolis, MN; Human adiponectin ELISA kit; Otsuka Pharmaceutical, Inc., Tokyo, Japan). hs-CRP was determined by a latex-enhanced immunonephelometric assay on a BN-II analyzer (Dade Behring, Marburg, Germany).

Statistical Analysis

For transfection experiments and evaluation of TNF-α, adiponectin, and hs-CRP in plasma, comparisons among three or more groups were analyzed by one-way ANOVA, followed by Scheffe's tests to evaluate statistical differences between the two groups.

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