responsive construct (kindly provided by C. Li, University of Texas Southwestern, Dallas) and a β-galactosidase reporter construct for normalization. Recombinant human leptin (0.5 nM, Sigma) and rObRe (5 and 50 nM) were preincubated for 30 min at room temperature before addition to the cells for 3 h. Luciferase and β-galactosidase activities were measured according to standard protocols (8).

Gel Chromatography. ^{125}I-leptin was used to trace bound and free leptin from serum after fractionation by FPLC (Amersham Pharmacia FPLC system) on a High load Superdex 200–16/60 column (Amersham Pharmacia) calibrated by using Bio-Rad gel filtration standards. A detailed procedure is available in *Supporting Text*.

Histology. Histological analyses were performed on undecalcified specimen as described (1). Static and dynamic histomorphometric analyses were performed according to standard protocols (10) using the Osteomeasure Analysis System (Osteometrics, Atlanta). Four to 10 animals were analyzed for each group. Controls for each group were WT littermates of the same sex, age, and genetic background. Statistical significance was assessed by Student's *t* test. Results are expressed as mean ± SE.

Serum Chemistry. Total leptin serum levels were measured by using an immunoassay kit from Linco Diagnostics, following the manufacturer's instructions.

Osseous Age Measurements. Osseous age was rated by a single radiologist unaware of the patient's chronological ages on radiographs of left hands and wrists, using the method of Greulich and Pyle (11).

Results

Comparison of Antiosteogenic and Anorexigenic Functions of Leptin. The existence of a high bone mass phenotype in the face of an increase in bone resorption in leptin signaling-deficient mice suggests that leptin antiosteogenic activity is a major function of this hormone. To test this contention *in vivo* we used ICV infusion of leptin in WT mice as a bioassay. We infused different amounts of this hormone for 1 month and used body weight and bone volume as indicators of leptin bioactivity (Fig. 1*A*). As reported (12), ICV infusion of leptin at 8 and, to a lesser extent, 4 ng/h significantly decreased body weight, whereas lower leptin doses failed to affect it significantly (Fig. 1*B*). ICV infusion of leptin at 8, 4, and even 2 ng/h significantly reduced bone mass of WT mice (Fig. 1*C*). The fact that similar amounts of leptin were needed to affect body weight and bone mass suggests that leptin anorexigenic and antiosteogenic functions are of equal importance *in vivo*. This is consistent with the fact that the antiosteogenic function of leptin was uncovered in a situation strongly favoring bone loss, not bone gain.

Absence of Leptin Production in the Brain. Different observations raised the formal hypothesis that serum leptin may not regulate bone mass. For instance, it has been difficult to date to correlate bone density, an indirect assessment of bone mass, and serum leptin level in humans (13–15). Moreover, leptin antiosteogenic action was more easily achieved by ICV infusion than by peripheral injection, and lastly, leptin expression has been detected by RT-PCR and immunocytochemistry in the mouse hypothalamus (16), where leptin antiosteogenic neurons reside (2). Together, these observations suggested the possibility that leptin was produced locally in the brain and that this local production was important in understanding leptin antiosteogenic function.

To test this hypothesis we first assessed leptin expression in various parts of the brain and in fat tissue by *in situ* hybridization

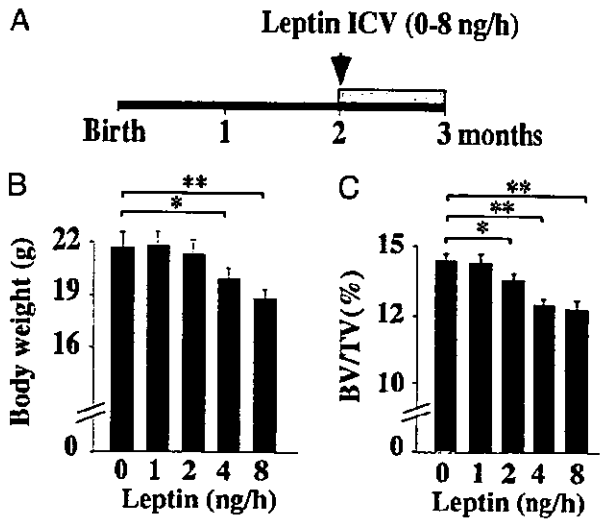

Fig. 1. Comparison of leptin antiosteogenic and anorexigenic functions. (*A*) Two-month-old C57BL6J females were infused ICV for 28 days with the indicated amount of leptin. (*B*) The lowest leptin amount that significantly decreased body weight was 4 ng/h. (*C*) The lowest leptin amount that significantly decreased bone volume over tissue volume (BV/TV, %) was 2 ng/h (n = 8; *, $P < 0.05$; **, $P < 0.005$).

(ISH). Despite several attempts, we failed to detect expression of *Leptin* in any brain area investigated at different developmental stages or postnatally, whereas fat tissues constitutively gave a strong signal (G. Eichele, personal communication). To rule out the possibility that a low level of *Leptin* transcripts, undetectable by ISH, was present in the brain as reported for other genes (9), we knocked-in the *LacZ* gene in the *Leptin* locus through homologous recombination in ES cells (Fig. 2*A*). $Lep^{LacZ/LacZ}$ mice were obese and had a high bone mass phenotype (data not shown). $Lep^{LacZ/LacZ}$ and WT littermates were analyzed for the presence of β-galactosidase activity in brain and adipose tissues. Strong X-Gal staining was observed in adipose tissue of homozygote animals (Fig. 2 *C* and *E*), whereas no staining could be detected in any brain area analyzed macroscopically and histologically (Fig. 2 *D* and *F*). Taken together, these results indicate that leptin is not produced locally in the brain, and thereby imply that circulating leptin must account for its antiosteogenic function.

Raising Serum Leptin Level Decreases Bone Mass. If circulating leptin affects bone mass, then transgenic mouse models with high serum leptin level should have a low bone mass. To determine whether this is the case, we studied two transgenic mouse strains with different serum leptin levels.

The first strain of mice was generated by using the liver-specific human serum amyloid P component (*SAP*) promoter to drive *Leptin* cDNA expression in liver (6). As reported, *SAP-leptin* transgenic mice had a moderate, i.e., 4-fold, increase in their serum leptin level compared to WT littermates (Fig. 3*A*). This resulted in a lower body weight and a complete absence of fat pads (6). Histological examination revealed that *SAP-leptin* transgenic mice also displayed a significant decrease in bone mass when compared to WT littermates, thus suggesting that serum leptin controls bone mass (Fig. 3*A*). The second leptin overexpressing mouse model was generated by using the Apolipoprotein E (*ApoE*) promoter and liver-specific enhancer (Fig. 3*B*). Northern blot analysis verified that the transgene was strongly expressed in liver (Fig. 3*B*). Accordingly, serum leptin level in *ApoE-leptin* transgenic mice was 200- to 300-fold higher than what was seen in WT littermates, at all time points analyzed

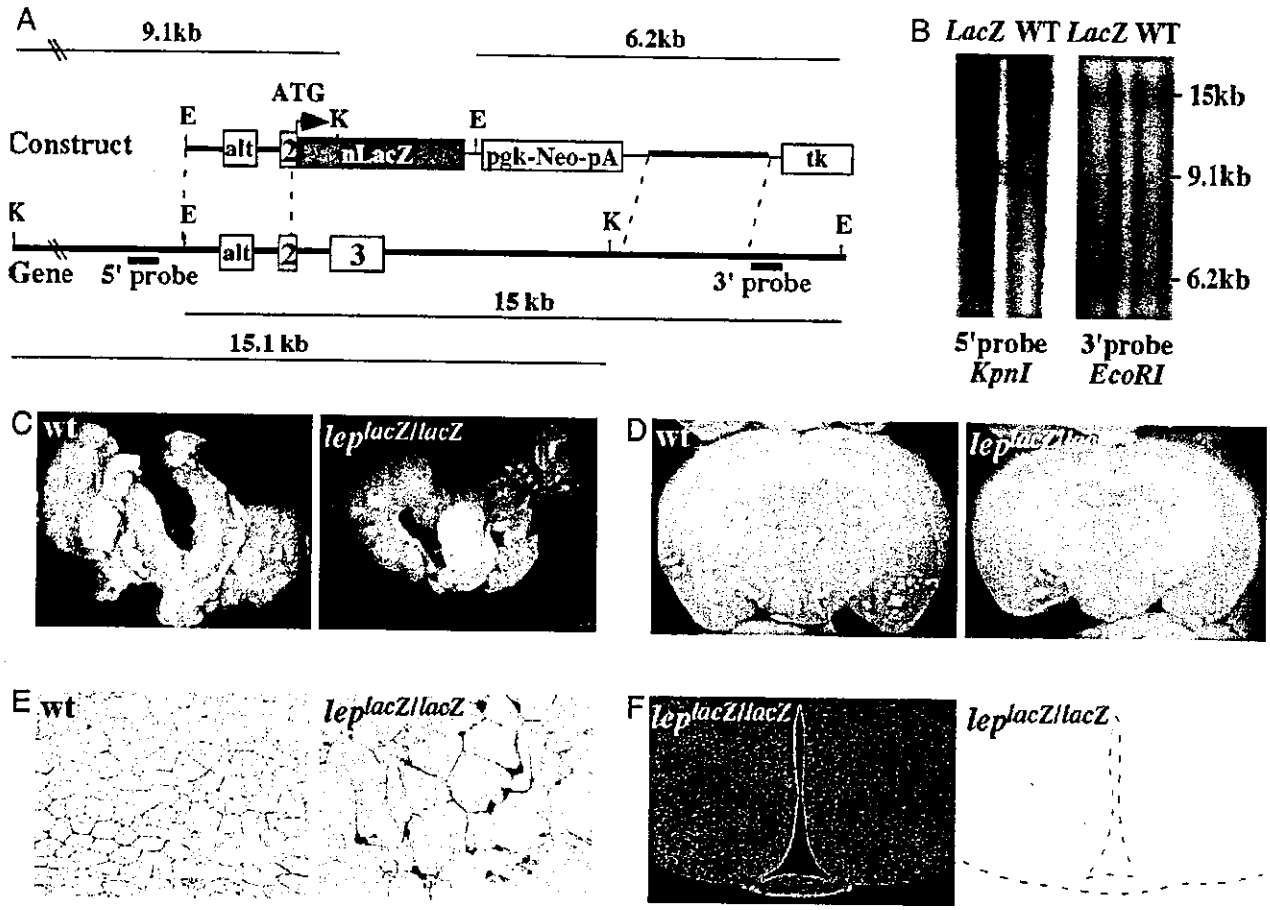

Fig. 2. Adipose tissue as the only detectable source of leptin. (*A*) Genomic organization of the *Leptin* locus and structure of the targeting vector. Probes used for Southern blots and restriction fragments are indicated. (*B*) Southern blots. The WT and *lacZ* knock-in loci gave, respectively, 15.0- and 6.2-kb bands with the 3' probe, and 15.1- and 9.1 kb bands with the 5' probe. E, *Eco*RI; K, *Kpn*I. (*C* and *D*) X-Gal-stained gonadal adipose tissue and brain. Staining was detected exclusively in adipose tissue in $Lep^{LacZ/LacZ}$ mice. No staining was detected in brain. (*E* and *F*) Histological examination of adipose and brain tissues after X-Gal staining. Nuclear staining was readily detectable in adipocyte nuclei, but no staining was observed in any brain section. A representative brain section containing arcuate and ventromedial hypothalamus neurons counterstained with Hoescht (*Left*) and stained with X-Gal (*Right*) are shown.

(Fig. 3 *C* and *D*). This marked elevation of serum leptin level in *ApoE-leptin* transgenic mice resulted in a complete disappearance of fat pads (Fig. 3*B*) and in a decrease in food intake (data not shown), demonstrating that leptin signaling was increased in these animals. Likewise, we observed a significant decrease in bone mass in 3- and 6-month-old *ApoE-leptin* mice, regardless of the sex of the animals (Fig. 3 *C* and *D*). The low bone mass phenotype observed in *ApoE-leptin* mice was accompanied by a reduction in the bone formation rate (Fig. 3*D*). Taken together, these results demonstrate that increasing serum leptin level, even to very high levels, results in a reduction of osteoblastic activity and a subsequent low bone mass.

Reducing Serum-Free Leptin Level Increases Bone Mass. To further address the role of circulating leptin and to determine whether free, i.e., unbound, serum leptin is a determinant of bone mass, we attempted to decrease the level of circulating free leptin by transgenesis. *ObR*, the leptin receptor gene, encodes five different forms of the leptin receptor through alternative splicing. One of these forms, *ObRe*, lacks the transmembrane domain found in all other isoforms, and therefore behaves as a soluble receptor for leptin (17). By analogy with other physiological regulatory loops in bone, we reasoned that *ObRe* could serve as a decoy receptor for leptin. This would be reminiscent of the role that osteoprotegerin plays as a decoy receptor for RANK-L, a critical regulator of bone resorption (18). The potency of ObRe to inhibit leptin signaling was first established *in vitro* by showing that recombinant ObRe inhibits the activity of a STAT3-dependent luciferase reporter construct, induced by leptin treatment of 293 cells expressing the ObRb receptor (Fig. 4*A*). We then used ApoE regulatory elements to overexpress *ObRe* in liver and systemically (Fig. 4*B*). *ApoE-ObRe* transgenic mice had a moderate but nonsignificant increase in body weight, fat pad weight, and bone mass (data not shown). We reasoned that this relative lack of efficacy of the *ObRe* transgene could be explained by a serum leptin level still too high relative to the level of *ObRe* expression in *ApoE-ObRe* transgenic mice. If this was the case, then transferring the *ObRe* transgene to mice with a lower leptin level should uncover any phenotypic abnormalities secondary to the decrease in circulating free leptin. Serum leptin level is lower in *Ob*/+ mice than in WT mice (4.01 ± 0.3 ng/ml in *Ob*/+ mice vs. 5.7 ± 0.1 ng/ml in WT mice, $P < 0.05$, $n = 12$ per group). We therefore crossed them with *ApoE-ObRe* mice to generate *ApoE-ObRe;Ob*/+ mice.

Protein size fractionation through gel filtration indicated that serum-free leptin level was reduced in *ApoE-ObRe;Ob*/+ compared to *Ob*/+ mice. As shown in Fig. 4*C*, free leptin (fraction 90, 16–17 kDa) was efficiently separated from the bound form of leptin (fraction 58, 310 kDa). The size of the bound form of leptin is consistent with leptin binding to dimers of ObRe (19).

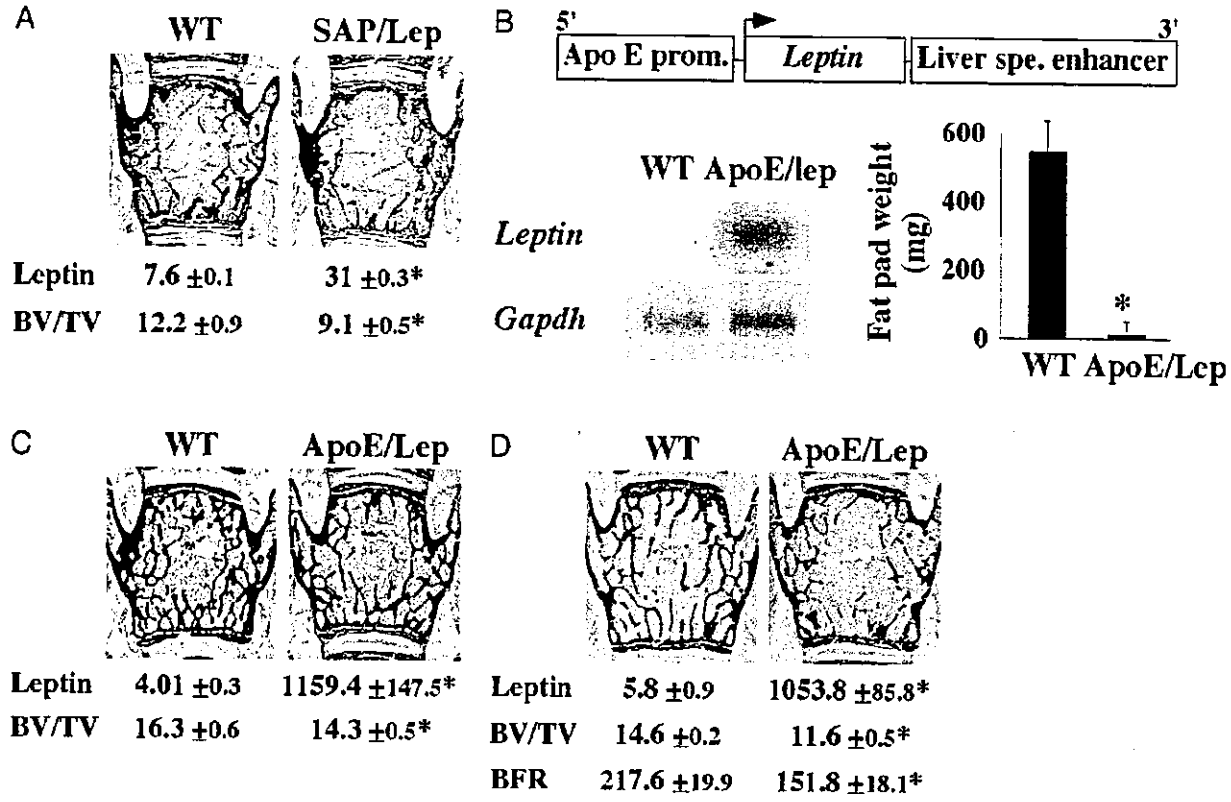

Fig. 3. Increasing serum leptin reduces bone mass. (*A*) Six-month-old *SAP-Leptin* mice are mildly hyperleptinemic (leptin, ng/ml) and display a low bone volume over tissue volume (BV/TV, %) compared to controls ($n = 4$; *, $P < 0.05$). (*B*) Schematic representation of the *ApoE/Lep* transgene. Northern blot analysis confirmed the expression of the transgene in liver. *ApoE/Lep* mice have no fat pads ($n = 8$; *, $P < 0.01$). (*C* and *D*) Compared to controls, *ApoE/lep* mice at 3 (*C*) and 6 (*D*) months of age displayed a reduced bone volume and a decreased BFR/BS ($\mu m^3/\mu m^2$ per yr) despite a 200- to 300-fold increase in serum leptin level (ng/ml) ($n = 8$; *, $P < 0.05$).

Free leptin fraction represented ≈65% of total leptin in the *Ob/+* mouse serum and only 5.4% in *ApoE-ObRe;Ob/+* mouse serum. This decrease in circulating free leptin led to a significant increase in the fat pad weight of these mice (Fig. 4*D*) and, as hypothesized, to a significant increase in bone mass compared to *Ob/+* mice (Fig. 4*E*). These results establish that circulating free leptin is a determinant of bone mass.

Leptin, Lipodystrophy, and Bone Mass. We have previously shown that *A-ZIP/F1* lipodystrophic mice have a high bone mass phenotype, indicating that adipocytes are necessary for the control of bone mass (1). To test whether leptin is the adipocyte-derived gene product responsible of this phenotype, we crossed the *A-ZIP/F1* mice with the *SAP-Leptin* overexpressing mice and compared the bone volume of the double transgenic mice to the one of *A-ZIP/F1* and WT mice. In contrast to *A-ZIP/F1* mice, *A-ZIP/F1;SAP-Leptin* mice had a normal bone mass (Fig. 5), indicating that leptin is an adipocyte gene product that is both necessary and sufficient to control bone formation.

The critical role of leptin in the control of bone mass in mice raised the question of its role in humans. The high bone mass phenotype observed in lipodystrophic mice suggested that, if leptin antiosteogenic function was conserved, lipodystrophic patients should have evidence of an increased bone formation. This is obviously a difficult question to address for several reasons. First, ethical considerations prevent the collection of bone biopsies from these patients. Second, there are no weight- and age-matched controls that can be used in indirect methods to measure bone density. Because of these limitations, we used as an indirect but suggestive indicator of bone formation the osseous age of prepubertal patients affected by congenital generalized lipodystrophy. All lipodystrophic patients displayed low to undetectable circulating levels of leptin (Table 1) and had, regardless of their sex, a marked advance in bone age. The same advance in bone age was also observed in a single leptin-deficient child to whom we had access to (J. Licino, personal communication). These correlative data are in agreement with what was observed in lipodystrophic mice, and suggest that serum leptin in humans also controls bone homeostasis.

Discussion

Our studies show that leptin is equipotent in the control of body weight and bone mass and that circulating leptin, at low or high concentrations, regulates bone mass. Furthermore, available evidence suggests that this function of leptin is, like its other functions, conserved between mouse and human.

The fact that leptin antiosteogenic function was uncovered in animals that have an increased bone resorption was already a suggestive indication that it should be a major function of this hormone. That leptin antiosteogenic function could be achieved with a dose similar to the one needed to affect body weight in WT mice confirms experimentally this contention. This observation, and others previously reported, suggest that leptin is a general neuroendocrine regulator of body weight, reproduction and bone mass (1). A parallel exists between the pleiotropic functions of leptin and for example the multiple functions of insulin. Indeed, besides its crucial role in the regulation of blood glucose level that led to its identification, it is now evident that insulin has other important functions, such as regulating life span in invertebrates and vertebrates (20–22).

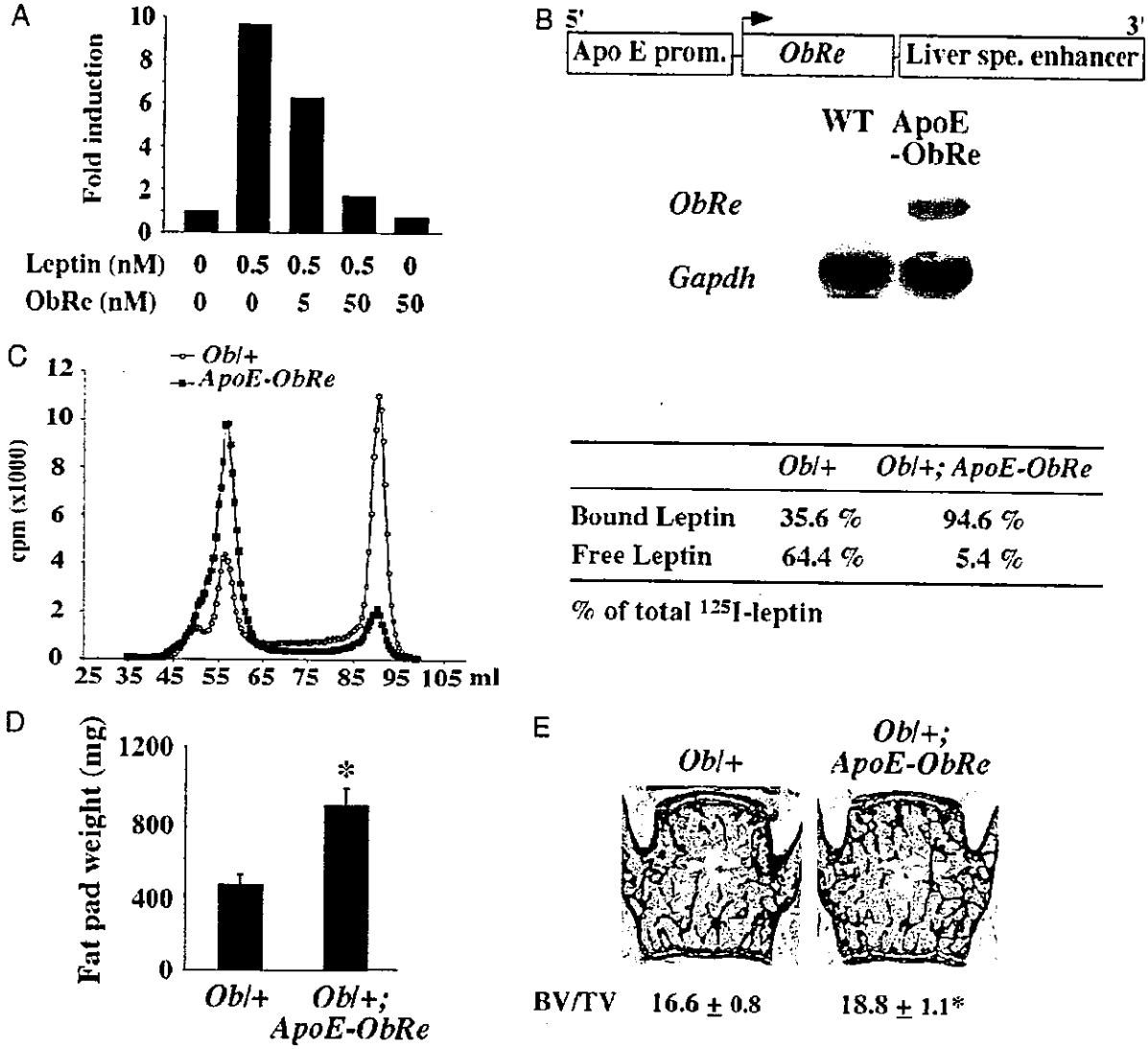

Fig. 4. Decreasing serum free leptin level increases bone mass. (*A*) Leptin-induced STAT3 reporter activity is decreased by ObRe treatment. (*B*) Schematic representation of the *ApoE-ObRe* transgene. Northern blot analysis confirmed the expression of the transgene in liver. (*C*) Size fractionation chromatogram. Marked reduction in serum free leptin level in *Ob/+; ApoE-ObRe* mice compared to *Ob/+* mice (a representative chromatogram is shown, $n = 4$). (*D* and *E*) Three-month-old *Ob/+; ApoE-ObRe* mice had a significant increase in gonadal fat pad weight (*D*) and in bone volume over tissue volume (BV/TV, %) (*E*) compared to controls ($n = 8$; *, $P < 0.05$).

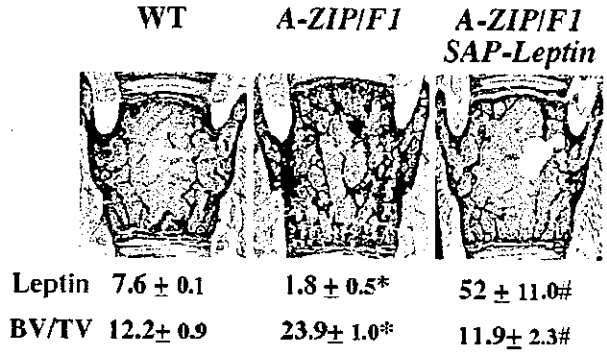

Fig. 5. Leptin and bone mass in lipodystrophic mice. Expression of the *SAP-leptin* transgene in the *A-ZIP/F1* genetic background increased serum leptin levels (ng/ml) and corrected the high bone mass of *A-ZIP/F1* mice ($n = 4$; *, controls versus *A-ZIP/F1*, $P < 0.05$; #, *A-ZIP/F1* versus *A-ZIP/F1;SAP-leptin*, $P < 0.05$).

Several lines of evidence establish that neuronal antiosteogenic pathways are controlled by serum leptin level. First, we failed to detect *Leptin* transcripts in the hypothalamus or elsewhere in the brain. Second, increasing serum leptin level decreased bone mass. This was true even with an extreme elevation of serum leptin level, indicating that hypothalamic neurons remain sensitive even when serum leptin level is increased >200-fold. However, the sensitivity of ventromedial hypothalamus neurons to leptin must be partially abrogated because *ApoE-leptin* mice did not have a lower bone mass than *SAP-leptin* mice. In that respect, it should be noted that A^y/a mice, which are resistant to leptin administration, have a decreased expression of neuropeptide Y (NPY) and Agouti-related peptide mRNA compared to leptin signaling-deficient mice, indicating that, despite the absence of effect on body weight, the leptin signal is still sensed to a certain extent by hypothalamic neurons in these mice (23, 24). Third, decreasing serum-free leptin level by overexpressing the leptin-soluble receptor ObRe led to an

Table 1. Lipodystrophic patients have low to undetectable serum leptin levels and an advanced bone age

	Patient							
	1	2	3	4	5	6	7	8
Sex	M	M	M	F	F	F	F	F
Leptin, ng/ml	0.23	ND	ND	0.68	1.4	0.22	0.37	0.25
Chronological age	6.0 w	5.0 m	1.7 y	1.9 y	3.0 y	4.0 y	8.0 y	13.0 y
Bone age	4.5 m	1.0 y	2.3 y	2.6 y	7.5 y	5.0 y	11.0 y	15.0 y

M, male; F, female; ND, not determined; w, weeks; m, months; y, years.

increase in bone mass. Finally this role played by circulating leptin is important to interpret other data present in the literature. For instance, although NPY-deficient mice have no bone phenotype (25), mice lacking the Y2 or both Y2 and Y4 receptors have a high bone mass phenotype and a marked decrease in serum leptin level (3, 4).

Interestingly, in terms of bone biology, a parallel can be made between ObRe, which modulates the function of leptin, the major hormonal regulator of bone formation, and osteoprotegerin, another decoy receptor regulating the activity of RANK-L, the major regulator of bone resorption (26). These results suggest that bone formation and bone resorption are controlled, at least in part, by the interplay of circulating factors and decoy receptors.

Several observations suggest that leptin antiosteogenic function, like its other functions, is conserved during evolution. First, lipodystrophic mice have a high bone mass phenotype and, as shown here, lipodystrophic patients have an advanced bone age, an indirect evidence of premature bone formation. Second, beta-blockers increase bone formation and bone mass in mice and, in humans, beta-blockers have recently been shown to reduce the incidence of fracture in osteoporotic patients (27). Taken together, these observations suggest that leptin antiosteogenic function has been conserved during evolution.

We thank Drs. Li, Eichele, Ducy, Fauré, Maachi, and Mégarbané for reagents, patients, expertise, and critical reading of the manuscript. This work was supported by National Institutes of Health Grant DK5883 and National Space Biomedical Research Institute Grant NCC9-58 (to G.K.), the Children's Nutrition Research Center (G.K. and F.E.), and the Arthritis Foundation (S.T.).

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and Decherd do not provide robust outcome data on the procedures they advocate. I consider an average fee of more than $5,000 for an operation lasting several hours to be expensive and profitable.[1]

The experience of a mystery shopper who consulted five established plastic surgeons with the same requests illustrates the lack of consensus among cosmetic surgeons about optimal therapy.[2] Both the recommended treatments and their costs ($2,900 to $14,150) varied greatly among the surgeons. Given the lack of good studies quantifying the benefits and risks of many of the cosmetic procedures and products that leading plastic surgeons now advocate and sell, and given the even scantier information on individual surgeons' outcomes, I hope the American Society of Plastic Surgeons will take a leading role in filling this void in the data.

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Long-Term Leptin-Replacement Therapy for Lipoatrophic Diabetes

TO THE EDITOR: Oral et al. reported in 2002 that leptin treatment for a period of four months improved hyperglycemia and hypertriglyceridemia in nine female patients with lipodystrophy.[1] We report the efficacy of leptin-replacement therapy given for 12 months with the use of the same protocol in two Japanese patients. Patient 1 was an 11-year-old girl with acquired generalized lipodystrophy. She was normal at birth and in her growth. At nine years of age, cervical lymphangitis and panniculitis developed. After that, she had systematic fat loss, her body weight decreased from 38.5 kg to 26.0 kg, and diabetes emerged within two months. Treatment with pioglitazone (15 mg per day) for 10 months did not improve glycemic control. Just before starting leptin-replacement therapy, she had marked hyperglycemia (glycosylated hemoglobin, 10.0 percent), hypertriglyceridemia (1941 mg per deciliter), and severe fatty liver. Patient 2 was a 29-year-old man with congenital generalized lipodystrophy. A generalized deficiency of body fat was noticed from birth, with the onset of diabetes when he was 11 years old. Glyburide (glibenclamide) (2.5 mg per day) and voglibose (0.6 mg per day) were ineffective. Just before leptin-replacement therapy was initiated, his glycosylated hemoglobin level was 10.3 percent. He had neither hypertriglyceridemia nor fatty liver.

Patients 1 and 2 had extremely low values for body fat (5.2 percent and 4.7 percent, respectively, as measured by dual-energy x-ray absorptiometry)

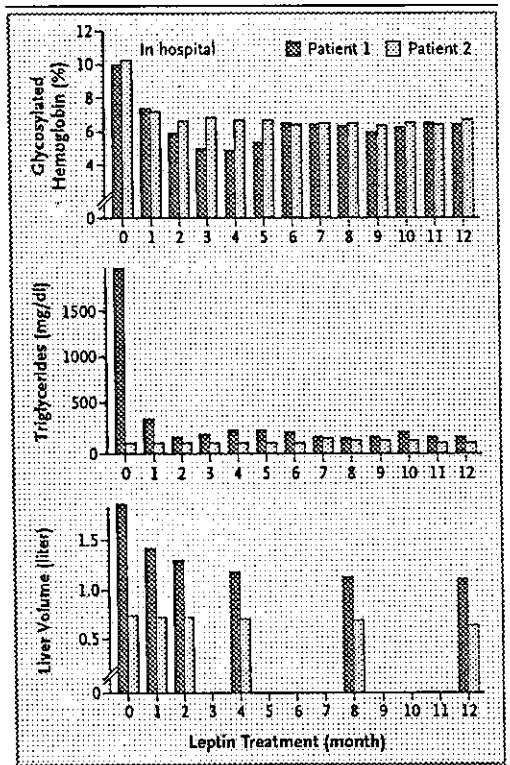

Figure 1. Glycosylated Hemoglobin Levels, Fasting Serum Triglyceride Levels, and Liver Volume in Patients 1 and 2 during 12 Months of Leptin-Replacement Therapy.

Liver volume was calculated with the use of computed tomographic imaging.

and plasma leptin concentrations (0.92 and 0.82 ng per milliliter, respectively). During leptin-replacement therapy, the serum leptin concentrations increased to 7.9 and 26.7 ng per milliliter at 4 months and 5.9 and 35.4 ng per milliliter at 12 months in Patients 1 and 2, respectively.

Both patients received leptin as inpatients for the first four months of therapy. After the initiation of leptin treatment, fasting plasma glucose levels normalized (208 mg per deciliter in Patient 1 and 142 mg per deciliter in Patient 2). Without the use of oral antidiabetic agents, the glucose levels in both patients were well controlled. The glycosylated hemoglobin levels were reduced to 4.8 percent and 6.5 percent, respectively, at four months and remained below 6.5 percent over a full year (Fig. 1). The elevated fasting triglyceride level and severe fatty liver in Patient 1 normalized (Fig. 1). During the year of treatment, we observed no adverse effects of leptin-replacement therapy and no skin reactions at the injection sites.

Our clinical trials in Japan show that leptin-replacement therapy is highly effective for a full year in patients with generalized lipodystrophy. Leptin-replacement therapy appears to be safe and effective for long-term treatment of lipoatrophic diabetes.

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INSTRUCTIONS FOR LETTERS TO THE EDITOR

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Reduction of diet-induced obesity in transgenic mice overexpressing uncoupling protein 3 in skeletal muscle

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Abstract

Aims/hypothesis. It has been suggested that uncoupling protein 3 (UCP3) can increase energy expenditure, thereby regulating body weight. Although studies on UCP3 knock-out mice suggest that lack of UCP3 function does not cause obesity or Type 2 diabetes, it is possible that up-regulation of UCP3 function improves these disorders or their clinical sequelae. A 10- to 20-fold increase of UCP3 gene expression is achievable through physiological or pharmacological stimuli. We examined the phenotype of transgenic mice with approximately 18-fold overexpression of mouse UCP3 mRNA in skeletal muscle.

Methods. We generated transgenic mice with approximately 18-fold overexpression of mouse UCP3 mRNA in skeletal muscle under control of the skeletal muscle-specific muscle creatine kinase gene promoter. The phenotype of these mice was analysed either on a standard diet or on a 4-week high-fat diet.

Results. In mice on standard chow, there was no difference in body weight, oxygen consumption and mitochondrial protonmotive force between transgenic mice and non-transgenic littermates. However, transgenic mice tended to have lower body weight, increased oxygen consumption and decreased mitochondrial protonmotive force than the control mice. Transgenic mice on a 4-week high-fat diet consumed much more oxygen and had noticeably less weight gain and less epididymal fat, as well as better glucose tolerance than non-transgenic littermates.

Conclusions/interpretation. Our study shows that 18-fold overexpression of UCP3 mRNA in the skeletal muscle reduced diet-induced obesity. An 18-fold increase of UCP3 mRNA can be attained by physiological or pharmacological stimuli, suggesting that UCP3 has therapeutic potential in the treatment of obesity. [Diabetologia (2004) 47:47–54]

Keywords Uncoupling protein 3 · skeletal muscle · transgenic mice · high-fat diet · obesity

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Abbreviations: UCP3, uncoupling protein 3; UCP2, uncoupling protein 2; UCP1, uncoupling protein 1; PPAR, peroxisome-proliferator-activated receptor; MCK, muscle creatine kinase; Δp, mitochondrial protonmotive force.

Uncoupling protein 3 (UCP3), which has been identified by several groups [1, 2, 3, 4], is reported to be involved in energy metabolism by uncoupling electron transport from ATP synthesis in mitochondria and is expressed at high levels in the skeletal muscle [3], an important organ in glucose and lipid metabolism [5]. Uncoupling protein 3 mRNA is expressed at much higher levels than uncoupling protein 2 (UCP2) mRNA in the skeletal muscle in vivo although accurate comparison is difficult [1, 2, 3, 4]. As uncoupling protein 1 (UCP1) gene expression is almost undetectable in the skeletal muscle [6], UCP3 is considered to be the most relevant UCP in the skeletal muscle. Several studies have reported that UCP3 gene expression

is up-regulated by triiodothyronine, catecholamines, fatty acids and peroxisome-proliferator-activated receptor (PPAR) agonists [4, 7, 8, 9].

It has been suggested that UCP3 is involved in energy expenditure, possibly leading to regulation of body weight. In two studies on UCP3 knock-out mice [10, 11] there was no difference between UCP3 knock-out and wild-type mice with regard to obesity, body temperature and serum concentrations of insulin, glucose, triglycerides and fatty acids. No obvious phenotype of UCP3 knock-out mice was observed when comparing their responses to fasting, exposure to cold, a high-fat diet and treatment with thyroid hormones with those of the control mice. This suggests that lack of UCP3 is not a major determinant of energy expenditure and obesity.

Although loss of the UCP3 function does not cause obesity or Type 2 diabetes, it is still possible that up-regulation of the UCP3 function improves these disorders. One study reported that glucose transport and GLUT4 translocation to the cell surface were increased in L6 myotubes in which UCP3 was overexpressed in vitro by adenovirus-mediated gene transfer [12]. Another study reported that transgenic mice overexpressing human UCP3 in the skeletal muscle weighed approximately 30% less than their wild-type littermates and that these transgenic mice were insulin-sensitive, with lower fasting plasma glucose and insulin concentrations [13]. These data suggest that drugs increasing UCP3 could have anti-obesity and/or antidiabetic effects. However, the levels of UCP3 overexpression in this study were very high (approximately 66-fold at the mRNA level), and no known stimulus will increase UCP3 gene expression by that amount. Several reports noted that UCP3 gene expression increased by a maximum of 10- to 20-fold in response to physiological or pharmacological stimuli in vivo [14]. We have reported that an agonist for PPARδ increased UCP3 gene expression by approximately 20-fold using L6 myotubes in vitro [15]. In this context we produced transgenic mice that overexpress mouse UCP3 mRNA in the skeletal muscle by approximately 18-fold. Furthermore, to explain the effect of the increased UCP3 on diet-induced obesity, we examined the phenotype of transgenic mice on high-fat diet.

Materials and methods

Generation of transgenic mice overexpressing UCP3. Based on the non-coding sequence of rat UCP3 cDNA [3], sense and antisense primers (sense: 5′-CAA AGG AAC CAG GCC ATC CTC CGG AAC C-3′; antisense: 5′-AA AGT ACC AAG CGG CCT GCT TGC CTT GT-3′) were prepared. Reverse transcription-PCR was done using Superscript (Invitrogen, Carlsbad, Calif., USA) with 10 μg of total RNA from mouse gastrocnemius muscle [16]. The PCR products were subcloned and sequenced (Accession ID: AB008216) by the dideoxy method [3]. A fusion gene comprising the mouse muscle creatine kinase (MCK) promoter and mouse UCP3 cDNA coding sequences was designed to enable UCP3 expression to be targeted mainly to the skeletal muscle (Fig. 1a) [17]. The purified *Hind* III-*Sac* I fragment (10 μg/ml) was microinjected into the pronucleus of fertilised BDF 1 (DBA2 X C57/B6) mouse eggs (Japan SLC, Hamamatsu, Japan). The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (Japan CLEA, Osaka, Japan) using standard techniques [18]. Transgenic founder mice were identified by Southern blot analysis of tail DNAs using the mouse UCP3 cDNA fragment as a probe [18]. Transgenic mice were used as heterozygotes. Transgenic mice were back-crossed with C57/B6 mice. Mice of F4 generation or later were used in this study. Animals were housed in a temperature-, humidity-, and light-controlled room (temperature: 22±0.5°C, humidity: 50%, 12-h light, 12-h darkness) with free access to water and food. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research and carried out in accordance with the Declaration of Helsinki as revised in 2000. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985); were followed, as well as any specific national laws.

RNA extraction and northern blot analysis. Total RNA was extracted from tissues using Trizol reagent (Invitrogen) as described previously [19]. Filters containing 30 μg of total RNA were prepared. Northern blot analyses were done using cDNA probes of mouse UCP3 [9]. The density of 18S rRNA stained with ethidium bromide was used to monitor the amount of total RNA in each sample.

Western blot analysis. The gastrocnemius muscle was removed and homogenised in ice-cold buffer (50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing 1 mmol/l phenylmethlysulfonyl fluoride, 0.01 mmol/l leupeptin and 5 μg/ml aprotinin. Homogenates were centrifuged and supernatants were used for analysis of total protein. Mitochondrial fraction was obtained from gastrocnemius muscle as described previously [20]. Antibody for rat UCP3, its inhibitory peptide and antibody for human UCP3 were purchased from Alpha Diagnostic International (San Antonio, Tex., USA). Total and mitochondrial protein were used for SDS-PAGE and western blot analyses using antibodies for rat UCP3 or human UCP3 [21].

Isolation of mitochondria. Mitochondria were isolated from gastrocnemius skeletal muscle (n=7) as described previously [22, 23]. Mice were decapitated before removal of skeletal muscle. The skeletal muscle was homogenised in buffer containing 250 mmol/l sucrose, 1 mmol/l HEPES and 0.2 mmol/l EDTA (pH 7.2 with KOH). Fractionation of the homogenate was achieved by spinning for 10 min at 1500 g and at 4°C. The supernatant was then poured through a 250 μmol/l Nitex screen (Dynamic Aqua-Supply, British Columbia, Canada) and re-spun for 14 min at 16 000 g and at 4 C to obtain a mitochondrial pellet. The pellet was re-suspended on ice in 175 μl of suspension medium containing 120 mmol/l KCl, 20 mmol/l sucrose, 3 mmol/l HEPES, 2 mmol/l $MgCl_2$, 2 mmol/l EGTA and 0.5% BSA (pH 7.2 with KOH). Stock 9% BSA was defatted by the Chen method [24] and dialysed against 153 mmol/l NaCl and 11 mmol/l KCl. The protein concentration of the mitochondrial suspension was assayed by the modified Lowry method using BSA as the reference standard.

Measurement of mitochondrial oxygen consumption and mitochondrial protonmotive force (Δp). Mitochondrial suspensions

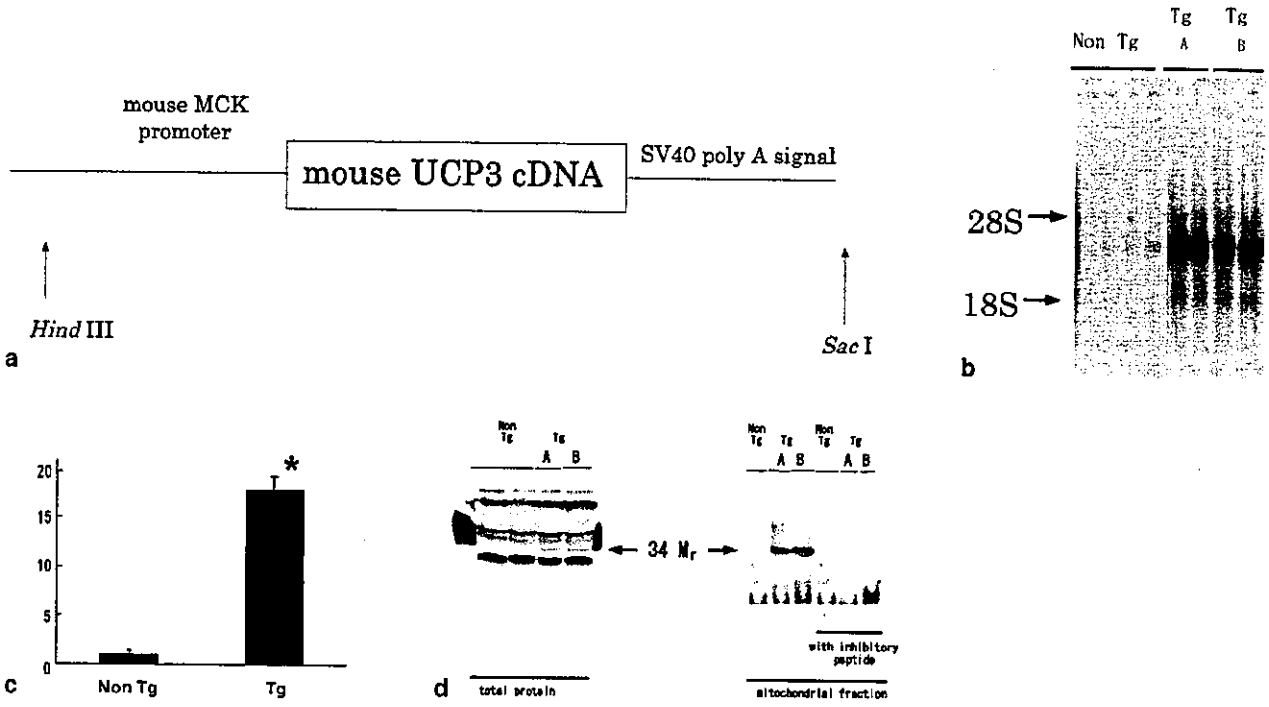

Fig. 1a–e. Generation of transgenic mice overexpressing UCP3. Schematic representation (**a**) of the mouse creatine kinase promoter and mouse UCP3 fusion gene. The coding region of mouse UCP3 cDNA is denoted by the closed box. Northern blot analysis (**b**) of transgene expression in the gastrocnemius muscle from 8-week-old transgenic mice of Line A and Line B, and UCP3 mRNA expression in the gastrocnemius muscle from 8-week-old non-transgenic littermates. Total RNA analysed: 30 µg. Tg, transgenic mice; non Tg, non-transgenic littermates. The bar graph (**c**) shows quantitative analysis of UCP3 mRNA expression. Levels of UCP3 mRNA expression increased approximately 18-fold in transgenic mice Line A in comparison with the non-transgenic littermates. Data are expressed as means ± SE (n=5). Western blot analysis (**d**) using antirat UCP3 antibody. Amounts analysed were: 100 µg of total protein and 600 ng of protein from mitochondrial fraction. No added peptide or inhibitory peptide (to which the antibody was raised) was included during the first antibody incubation. Western blot analysis (**e**) using human UCP3 antibody. Amounts analysed were: 100 µg of total protein and 600 ng of protein from mitochondrial fraction

(0.5 mg protein/ml) were placed in the incubation chamber (incubation temperature: 37°C) and magnetically stirred. Succinate-driven respiration was assessed by using 5 µmol/l rotenone to block complex 1 and 0.4 µg nigericin to assess total protonmotive force through measurements of membrane potential. State 4 respiration (maximum non-phosphorylating or leak-dependent respiration) was measured in the presence of saturating amounts of the ATP synthase inhibitor, oligomycin (10 µg/mg protein). Mitochondrial proton leak kinetics were assessed by adding malonate in increments (0.3 mmol/l to 3.6 mmol/l). The respiration rate of muscle mitochondria was measured using a Clark-type oxygen electrode (Hansatech, Norfolk, UK) [22]. Protonmotive force was measured using a methyltriphenylphosphonium-sensitive electrode as described previously [22].

Measurements of body weight and cumulative food intake. Body weight was measured daily, beginning at 4 weeks of age. Food intake was measured daily over a 1-week period at the age of 26 weeks. It was measured in male mice, which were maintained in individual metabolic cages.

Measurement of oxygen consumption. The gas analyser used to assess the metabolic rate consisted of six acrylic metabolic chambers, CO_2 and O_2 analysers (RL-600, AlcoSystem, Tokyo, Japan), and a switching system (ANI6-A-S, AlcoSystem) to sample gas from each metabolic chamber. Each metabolic chamber had 125.4 cm^2 floor space and was 6.5 cm in height. Oxygen consumption was calculated by measuring the O_2 and CO_2 concentrations of each chamber [25].

Glucose and insulin tolerance tests. For the glucose tolerance test 20-week-old transgenic mice and their non-transgenic littermates were treated with an intraperitoneal injection of 2.0 mg/g glucose after an overnight fast [18]. For the insulin tolerance test, mice were injected intraperitoneally with 0.5 mU/g human regular insulin (Novolin R, Novo Nordisk, Bagsvard, Denmark) [18]. Blood samples were taken from the

mouse tail vein before and 30, 60 and 90 min after the injection. Plasma glucose concentrations were measured by the glucose oxidase method with a reflectance glucometer (One Touch II, Lifescan, Milpitas, Calif., USA).

High-fat diet study. To examine the effect of UCP3 overexpression on diet-induced obesity, UCP3 transgenic mice and non-transgenic littermates were fed a high-fat diet for 4 weeks from 24 weeks of age. The high-fat diet was produced by Oriental Bioservice (Kyoto, Japan) [26]. Its fat content was 23%, whereas that of standard chow was 7%.

Histology. We did histological examinations of skeletal muscle, white and brown adipose tissues, heart, liver, and pancreas. This was done by light microscopy, using 5-μm-thick sections from 10% buffered, formalin-fixed, paraffin-embedded specimens which were stained with hematoxylin and eosin [18].

Measurement of plasma concentrations of glucose, triglyceride, fatty acids, cholesterol and insulin. Blood samples were taken from the retro-orbital sinus of mice at 9:00 hours after overnight fasting or at 14:00 hours. Plasma glucose, triglyceride, and insulin concentrations were measured by the glucose oxidase method with a reflectance glucometer, the enzymatic kit (Wako Pure Chemical, Osaka, Japan) and by RIA with mouse insulin standards (Morinaga BioLab, Yokohama, Japan), respectively [18].

Statistical analysis. Data were expressed as means ± SE. Statistical significance was tested by one-way ANOVA. If F was found to be significant, the Student's t test was used to test individual differences. A p value of less than 0.05 was considered statistically significant.

Results

Generation of transgenic mice overexpressing UCP3. A fusion gene comprising the mouse MCK promoter and mouse UCP3 cDNA coding sequence was designed to enable UCP3 expression to be targeted to the skeletal muscle (Fig. 1a). Several transgenic lines with different copy numbers of the transgene were obtained. With the mouse UCP3 cDNA probe, northern blot analysis showed that an mRNA species of 2.4 kb in size, which was expected from the fusion gene of MCK promoter and mouse UCP3 cDNA, was expressed abundantly in the skeletal muscle from several lines of the transgenic mice (Fig. 1b). The highest UCP3 mRNA levels were obtained in Line A of the transgenic lines and were approximately 18-fold higher than in non-transgenic littermates (Fig. 1b,c). The second highest mRNA levels were approximately 15-fold higher (Line B; Fig. 1b).

Western blot analysis of protein expression in the skeletal muscle of these two lines detected multiple bands, separated by electrophoresis, of total protein extracted from the skeletal muscle (Fig. 1d). When protein of mitochondrial fraction was electrophoresed, antirat UCP3 antibody detected, as expected from the molecular mass of mouse UCP3, a single band near 34 M_r (Fig. 1d). This band at 34 M_r disappeared when

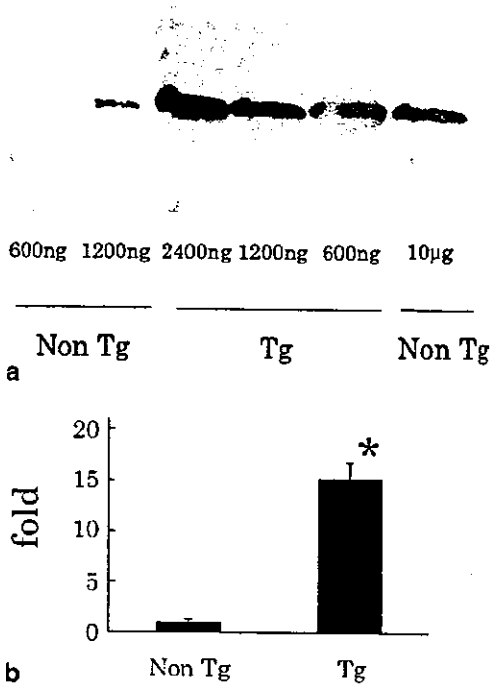

Fig. 2a, b. Quantification of UCP3 protein in mitochondria from skeletal muscle of transgenic mice. Representative result of western blot analysis (a) using antirat UCP3 antibody. Line A transgenic mice were examined. Various amounts of protein from mitochondria were analysed. The density of bands of 600 ng of protein from UCP3 transgenic mice was nearly equal to that of 10 μg of protein from non-transgenic littermates. The bar graph (b) shows quantitative analysis of UCP3 protein, which increased approximately 15-fold in transgenic mice as compared with non-transgenic littermates. Data are expressed as means ± SE (n=5)

inhibitory peptide was simultaneously incubated (Fig. 1d). Similar results were obtained using anti-human UCP3 antibody (Fig. 1e).

To quantify UCP3 concentrations in the skeletal muscle of transgenic mice, we examined transgenic mice of Line A. Western blot analyses were done using various amounts of protein from skeletal muscle mitochondria. Figure 2a is a representative result. It shows that the density of bands of 600 ng of protein from a UCP3 transgenic mouse was nearly equivalent to that of 10 μg of protein from a non-transgenic littermate. UCP3 protein was approximately 15 times higher in transgenic mice than in nontrasgenic littermates (n=5; Fig. 2b). As the MCK promoter also induces gene expression in the cardiac muscle, we examined the UCP3 gene expression there, finding that UCP3 mRNA levels were slightly higher in the cardiac muscle of Line A and Line B than in those of non-transgenic littermates. In Line A and Line B, UCP3 mRNA levels in the cardiac muscle were less than 1% of those in the skeletal muscle. UCP3 protein levels were not detected in the cardiac muscle either of

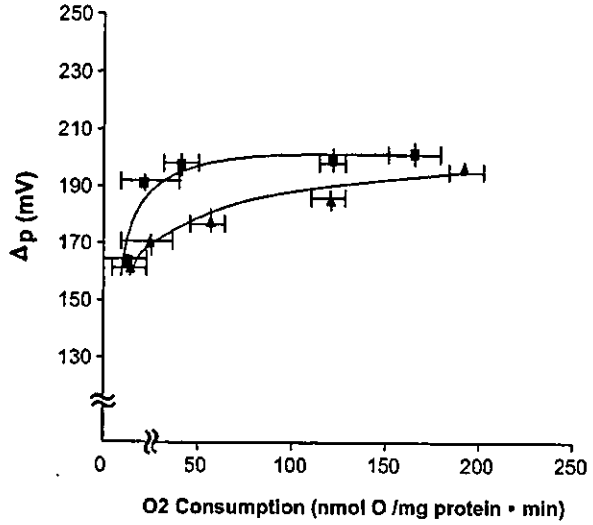

Fig. 3. Kinetic response of the proton leak to protonmotive force in mitochondria isolated from hindlimb muscle of UCP3 transgenic mice (▲) and non-transgenic littermates (■) (n=7, each). State 4 respiration was established using a saturating concentration of oligomycin (10 μg/mg protein). Subsequent additions of malonate (0.3 mmol/l to 3.6 mmol/l) provided the overall kinetics of the proton leak reactions. Data are expressed as means ± SE. Statistical significance was determined by Student's t test. A p value of less than 0.05 was considered statistically significant

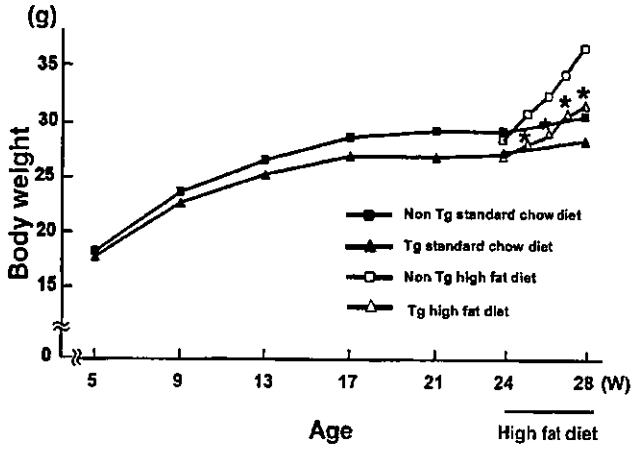

Fig. 4. Changes in body weight of male transgenic mice and non-transgenic littermates on standard chow and on high-fat diets. UCP3 transgenic mice and non-transgenic littermates were fed a standard chow diet for the first 24 weeks (n=22 to 25). For the following 4 weeks, some were fed a high-fat diet (n=7 to 9), while others continued on the standard chow diet (n=16 to 17). Data are expressed as means ± SE. *p<0.05 vs non-transgenic littermates on high-fat diet

transgenic mice or of non-transgenic littermates. UCP3 mRNA levels in the brown adipose tissue of transgenic mice were not significantly different from those of non-transgenic littermates (107±12% in Line A and 112±9% in Line B, p>0.05). Northern blot analysis with 30 μg of total RNA failed to detect any band of UCP3 mRNA in tissues of transgenic mice other than skeletal muscle, cardiac muscle and brown adipose tissue. The transgenic mice of both lines were fertile, and viable throughout adulthood with no appreciable complications. All these results were observed in transgenic mice of each sex.

Mitochondrial proton leak. The kinetic response curve for the proton leak reactions showed trends towards increased mitochondrial oxygen consumption over a range of values for Δp (Fig. 3). The absolute values for oxygen consumption and Δp at State 4 (the point on each curve at the furthermost point on the right) were not statistically different between groups (Fig. 3). However, UCP3 transgenic mice tended to have higher State 4 oxygen consumption than the non-transgenic littermates (202.9±10.1 nmol O_2 mg^{-1} min^{-1} vs 193.1±7.1 nmol O_2 mg^{-1} min^{-1}, p=0.3). UCP3 transgenic mice also tended to have a lower State 4 Δp value (196.5±2.2 mV for transgenic mice vs 201.3± 4.1 mV for non-transgenic littermates, p=0.2).

Phenotype of UCP3 transgenic mice on standard chow diet. In transgenic mice and non-transgenic littermates on a standard chow diet no significant difference in body weight was observed, although the transgenic mice tended to be lighter (27.6±0.6 g vs 29.9±0.6 g at 21 weeks, p=0.07; Fig. 4). No differences in food intake (2.8±0.1 g/day vs 2.7±0.1 g/day, p>0.05), oxygen consumption (46.1±2.6 ml kg^{-1} min^{-1} vs 41.3±1.1 ml kg^{-1} min^{-1}, p>0.05), rectal temperature (36.1±0.6 C vs 35.9±0.5°C, p>0.05) and histological analysis were observed between transgenic and non-transgenic littermates. No significant differences were noted in serum concentrations of glucose, insulin, cholesterol, triglyceride and fatty acids, although these tended to be lower in transgenic mice than in non-transgenic littermates (Table 1). In glucose and insulin tolerance tests on 28-week-old transgenic mice and non-transgenic littermates no significant differences were noted (Fig. 5a, Fig. 6a). As the phenotype described above was noted in transgenic mice of Line A and Line B, we used Line A, the line expressing the highest levels of UCP3, for further examination.

Body weight of transgenic mice on high-fat diet. One week after beginning the high-fat diet, the transgenic mice were approximately 10% less obese than the non-transgenic littermates (28.3±0.6 g vs 31.6±0.8 g, p<0.05; Fig. 4). After 4 weeks of a high-fat diet, the transgenic mice were approximately 14% less obese than the non-transgenic littermates (32.4±1.3 g vs 37.5±1.3 g, p<0.05; Fig. 4). Body weight gain for this 4-week period was nearly 49% less in transgenic mice than in non-transgenic littermates (4.4±1.1 g vs 8.6±0.8 g, p<0.05). The weight of the epididymal fat pad was approximately 20% lower in transgenic mice than in non-transgenic littermates after the 4-week

Table 1. Chemical profiles of UCP3 transgenic mice on standard chow and high-fat diets

	Standard chow diet		High-fat diet	
	UCP3 transgenic mice	Non-transgenic littermates	UCP3 transgenic mice	Non-transgenic littermates
Glucose (mmol/l)				
Fasting	6.4±0.2	6.8±0.4	8.9±0.4	10.3±1.1
Non-fasting	8.2±0.6	8.4±0.6	9.9±1.2	11.3±0.9
Insulin (pmol/l)				
Fasting	44±10	48±17	215±32	224±99
Non-fasting	95±20	91±13	638±77	649±170
Cholesterol (mmol/l)	2.15±0.08	2.25±0.10	3.88±0.18	4.22±0.47
Triglyceride (mmol/l)	0.27±0.05	0.36±0.07	0.41±0.05	0.45±0.06
Fatty acids (mmol/l)	1.27±0.14	1.25±0.23	0.83±0.06	0.90±0.07

Data are means ± SE (n=5 to 7)

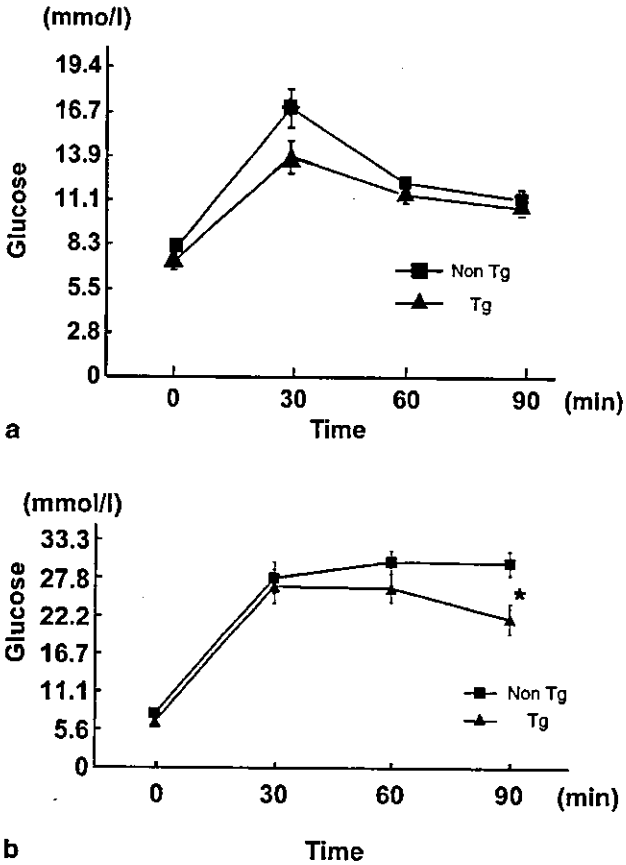

Fig. 5a, b. Glucose tolerance test in (a) male 28-week-old transgenic mice and non-transgenic littermates on a standard chow diet and (b) in male 28-week-old transgenic mice and non-transgenic littermates after 4 weeks on a high-fat diet. Data are expressed as means ± SE (n=7 to 9). *p<0.05 vs non-transgenic littermates

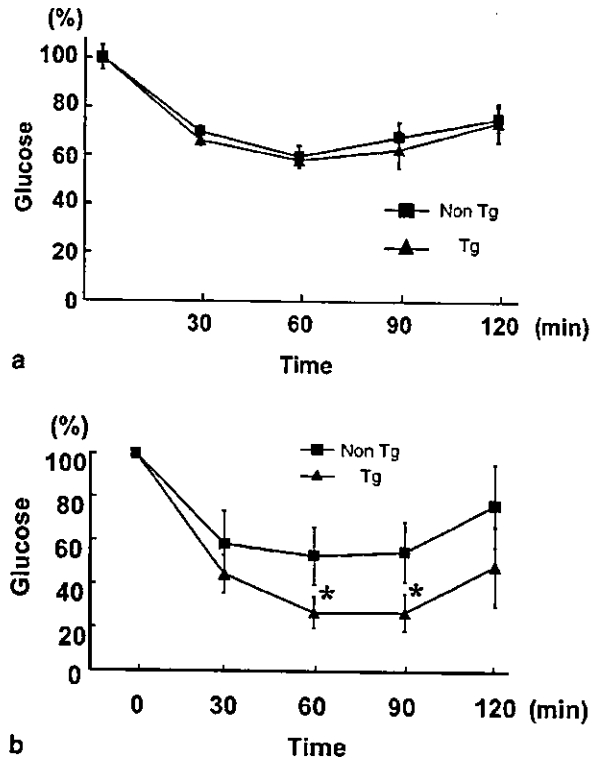

Fig. 6a, b. Insulin tolerance test in (a) male 28-week-old transgenic mice and non-transgenic littermates on a standard chow diet and (b) in male 28-week-old transgenic mice and non-transgenic littermates after 4 weeks on a high-fat diet. Serum glucose concentrations are expressed as percentages of the initial values of transgenic mice and non-transgenic littermates. Data are expressed as means ± SE (n=7 to 9). *p<0.05 vs non-transgenic littermates

high-fat diet (1693±131 mg vs 2110±17 mg, p<0.05). No difference was observed in the weight of livers or other organs. Histological analyses did not show any obvious difference between transgenic mice and non-transgenic littermates after the 4-week high-fat diet.

Food intake and oxygen consumption of transgenic mice on high-fat diet. No difference in food intake and rectal temperature was noted between transgenic mice and non-transgenic littermates (food intake: 2.5±0.15 g/day vs 2.3±0.08 g/day, p>0.05; rectal tem-

perature: 35.8±0.5°C vs 35.6±0.7°C, $p>0.05$). However, total oxygen consumption for 24 h was higher in transgenic mice than in non-transgenic littermates (54.1±4.5 ml kg^{-1} min^{-1} vs 43.1±1.6 ml kg^{-1} min^{-1}, $p<0.05$).

Blood analysis of transgenic mice on high-fat diet. No significant differences in serum concentrations of glucose, insulin, cholesterol, triglyceride and fatty acids were found between transgenic mice and non-transgenic littermates. However, the relevant values tended to be lower in transgenic mice (Table 1).

Glucose homeostasis in transgenic mice on high-fat diet. In the glucose tolerance test, increases in serum glucose were less marked in transgenic mice than in non-transgenic littermates ($n=5$ to 7, $p<0.05$; Fig. 5b). In the insulin tolerance test, hypoglycaemic responses 60 and 90 min after the injection were more pronounced in transgenic mice than in non-transgenic littermates ($n=5$ to 7, $p<0.05$; Fig. 6b).

Discussion

We generated transgenic mice that overexpressed mouse UCP3 in the skeletal muscle by using MCK promoter. In these mice UCP3 overexpression was targeted mainly to the skeletal muscle. Levels of UCP3 mRNA and protein were approximately 18-fold and 15-fold higher in the lines with the highest expression. Northern blot analyses detected a slight increase of UCP3 mRNA in the cardiac muscle. UCP3 mRNA levels in the cardiac muscle of transgenic mice were less than 1% of those in their skeletal muscle. Western blot analyses failed to detect the band of UCP3 in the cardiac muscle in our study. UCP3 mRNA levels in the brown adipose tissue of transgenic mice were not significantly different from those of non-transgenic littermates. No band of UCP3 mRNA was detected in tissues of transgenic mice other than skeletal muscle, cardiac muscle and brown adipose tissue. The phenotype described here therefore results primarily from the expression of the transgene in the skeletal muscle.

Expression of the UCP3 gene has been reported to increase 10- to 20-fold in response to physiological stimuli such as fasting [14]. An approximately 18-fold increase of UCP3 mRNA, as observed in transgenic mice in our study, seems to be feasible by physiological or pharmacological stimuli in vivo.

Our findings for mice on a standard chow diet, with no significant differences across a wide range of factors studied, indicate that an approximately 18-fold overexpression of UCP3 mRNA does not strongly affect energy expenditure on a standard chow diet. However, after a 4-week high-fat diet, transgenic mice were approximately 14% less obese than non-transgenic littermates. This finding is consistent with the tendency of transgenic mice to weigh less on a standard chow diet than their non-transgenic littermates. The increase in body weight of transgenic mice for this 4-week period was nearly 50% less than that of non-transgenic littermates. Moreover, the mean weight of the epididymal fat pad was approximately 20% lower in transgenic mice than in non-transgenic littermates after a 4-week high-fat diet. As no significant difference in food intake was noted between transgenic mice and non-transgenic littermates, the reduction of body weight and epididymal fat pad can be explained by the significantly increased oxygen consumption of transgenic mice on a high-fat diet. The reason why oxygen consumption was significantly increased on a high-fat diet but not on a standard chow diet is not clear. It is also not clear whether the improved glucose tolerance and insulin sensitivity seen in transgenic mice as compared with non-transgenic littermates can be explained by the reduction of obesity or by any other mechanisms.

In one study on transgenic mice which overexpressed human UCP3 mRNA by approximately 66-fold in the skeletal muscle [13] transgenic mice on standard chow were leaner and had decreased adipose tissue mass, improved glucose metabolism and increased metabolic rates (oxygen consumption). The phenotype of transgenic mice on a standard chow diet in this study was consistent with that of our transgenic mice on a high-fat diet. The discrepancy between their and our phenotypes on a standard chow diet could be explained by the difference in mRNA levels of overexpressed UCP3 (66-fold vs 18-fold). Recently the same authors reported that the increase in protein levels of overexpressed UCP3 in their transgenic mice was approximately 23-fold [27], which was also larger than in our transgenic mice (23-fold vs 15-fold). Although this difference in the UCP3 protein levels is not so large, it is possible that a small difference of UCP3 protein concentration in this range is critical for the discrepancy between phenotypes on a standard chow diet. Alternatively, the discrepancy could be due to the difference in the species of overexpressed transgene (human UCP3 vs. mouse UCP3). Taken together with these other findings, our study suggests that overexpression of UCP3 can cause increased energy expenditure, resulting in less adipose-tissue mass and improved glucose metabolism.

In conclusion, our transgenic mice with approximately 18-fold overexpression of UCP3 mRNA did not show any obvious phenotype on a standard chow diet, but were less obese and had improved glucose tolerance on a high-fat diet. As an 18-fold increase of UCP3 mRNA could be attained by physiological or pharmacological stimuli, our results suggest that drugs which increase UCP3 could be effective against obesity and Type 2 diabetes, with few adverse effects.

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Gene and Phenotype Analysis of Congenital Generalized Lipodystrophy in Japanese: A Novel Homozygous Nonsense Mutation in Seipin Gene

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Congenital generalized lipodystrophy (CGL), Berardinelli-Seip syndrome, is a rare metabolic disorder characterized by a near total lack of adipose tissue from birth or early infancy. Recently, *seipin*, encoding a 398-amino acid protein of unknown function, and *AGPAT2*, encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase 2, were identified as causative genes for CGL. *Seipin* mutations were found in patients from families originating from Europe and the Middle East. *AGPAT2* mutations were found predominantly in African ancestry. However, no information is available on these genes in the pathogenesis of CGL in Asian ancestry. We examined the sequences of the entire coding region of *seipin* and *AGPAT2* in four Japanese CGL patients from independent families. Their average body fat content was 4.7 ± 0.5%, and the plasma leptin level was 1.15 ± 0.14 ng/ml. We identified a novel nonsense mutation of *seipin* at codon 275 (R275X). Of four CGL patients, three were homozygous for R275X. No *seipin* mutation was found in any exon in one patient. We did not find any *AGPAT2* mutations in our Japanese patients, suggesting that *AGPAT2* is a minor causative gene, if any, for CGL in Japanese. This is the first report on gene and phenotype analysis of CGL in Japanese. (*J Clin Endocrinol Metab* 89: 2360–2364, 2004)

CONGENITAL GENERALIZED LIPODYSTROPHY (CGL), Berardinelli-Seip syndrome, is characterized by a near total lack of adipose tissue from birth (1–3). Patients with CGL show severe insulin resistance, hypertriglyceridemia, and fatty liver. These metabolic abnormalities develop as a consequence of mass reduction of adipose tissue (4, 5). Leptin is an adipocyte-derived hormone that plays an important role in the regulation of glucose and lipid metabolism (6–8). Plasma leptin concentrations are markedly reduced in patients with lipodystrophy (9, 10). In this context, we and others (11, 12) demonstrated that transgenic overexpression of leptin or exogenous leptin administration reverses the metabolic abnormalities in different mouse models of lipoatrophic diabetes, indicating that the metabolic abnormalities in patients with lipodystrophy are caused mainly by a lack of leptin. However, the genetic defect that causes a failure of adipogenesis or adipocyte differentiation in CGL had long been unknown.

CGL has been suggested to be an autosomal recessive disorder. Recently, two causative genes for CGL were identified. One is a gene encoding a protein named seipin, whose function is unknown (13). The other is the *AGPAT2* gene encoding 1-acyl-*sn*-glycerol-3-phosphate acyltransferase 2 that belongs to the family of acyltransferases and catalyzes the biosynthesis of glycerophospholipids and triglyceride (14). The *seipin* gene is located in chromosome 11q13, and the *AGPAT2* gene is located in chromosome 9q34. Although there is no difference in the prevalence of metabolic disorders such as insulin resistance, hypertriglyceridemia, and fatty liver between patients with the *seipin* or *AGPAT2* mutation, CGL due to *seipin* mutation appears to be a more severe disease than that due to *AGPAT2* mutation, with a higher incidence of premature death and a lower prevalence of partial and/or delayed onset of lipodystrophy (15). Furthermore, patients with the *seipin* mutation have a higher prevalence of intellectual impairment than those with the *AGPAT2* mutation (15).

Seipin mutations were found in patients originating from Europe and the Middle East. *AGPAT2* mutations were found predominantly in African ancestry. However, no information is available about these genes in the pathogenesis of CGL in Asian subjects. The present study is the first report on gene and phenotype analysis of CGL in Japanese.

Subjects and Methods

Study subjects

Sequence analyses of *seipin* and *AGPAT2* were performed in four Japanese CGL patients (two men and two women). The clinical features and laboratory data of these patients are presented in Tables 1 and 2. All of these patients had a near total lack of body fat from birth. The study was approved by the ethical committee of Kyoto University Graduate School of Medicine. All subjects gave written informed consent for participating in the study.

Materials and methods

Genomic DNA was isolated from blood using InstaGene Whole Blood kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Abbreviations: ANP, Atrial natriuretic peptide; BMI, body mass index; BNP, brain natriuretic peptide; CGL, congenital generalized lipodystrophy; IQ, intelligence quotient; MRI, magnetic resonance imaging; SNP, single nucleotide polymorphism.

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The coding regions of *seipin* and *AGPAT2* were amplified by PCR using gene-specific primers (13, 14) in seven and six fragments, respectively. PCR products were separated by electrophoresis in 2% agarose gel, purified, and sequenced directly by the dideoxy chain termination method with both forward and reverse primers on an ABI PRISM 310 Genetic Analyzer (PerkinElmer, PE Applied Biosystems, Foster City, CA). Genotyping of the patients with highly polymorphic microsatellite markers in chromosome 11q13 for the *seipin* locus and chromosome 9q34 for the *AGPAT2* locus was conducted with an ABI PRISM 310 Genetic Analyzer equipped with GeneScan analysis software (version 2.1, PerkinElmer).

The body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Body fat was determined by dual energy x-ray absorptiometry. Body fat distribution was assessed using the whole body magnetic resonance imaging (MRI). MRI was performed using a 1.5-Tesla imaging device (Phillips Medical Systems, Best, The Netherlands). The entire body was surveyed using contiguous axial 10-mm slices and a relatively T1-weighted spin echo sequence. Blood samples were obtained after an overnight fast. Plasma leptin levels were determined by immunoassay using a commercial kit (Linco Research, Inc., St. Charles, MO). Blood glucose and triglyceride levels were determined according to standard methods with the use of automated equipment. Hemoglobin A_{1c} values were measured by ion exchange HPLC. Serum insulin levels were determined by immunoassays using reagents provided by Shibayagi Co. Ltd. (Gunma, Japan). The presence of hypertrophic cardiomyopathy was assessed using echocardiography, electrocardiography, and plasma atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) levels. Plasma ANP and BNP levels were determined by immunoradiometric assay (Shionogi, Osaka, Japan) (16, 17). Fatty liver was diagnosed by both ultrasound and computed tomographic imaging. Intelligence quotient (IQ) was assessed using the Wechsler Adult Intelligence Scale-revised (18).

Statistical analysis

The average BMI in patient 1's family members with or without R275X heterozygous mutation were expressed as the mean ± SE. Comparison among groups was assessed by ANOVA and was completed by Fisher's probable least significant difference test.

Results

We studied four Japanese CGL patients from independent families. Clinical features of the patients are provided in Tables 1 and 2. These four patients showed generalized reduction or a near total lack of adipose tissue from birth. They presented with low body fat content and plasma leptin levels. Whole body MRI scans were available for patients 1, 3, and 4 (Fig. 1). All of these patients showed nearly total absence of sc fat throughout the body, including palm, sole, and head. They also showed near absence of ip fat. Retroorbital and bone marrow fat were preserved only in patient 4, not in patients 1 and 3. Patients 1, 3, and 4 had overt diabetes and markedly elevated hemoglobin A_{1c}values. Patient 1 was receiving insulin therapy. Patient 2 was not diabetic, although she presented with hyperinsulinemia. Patients 1 and 3 had both hypertriglyceridemia and fatty liver. In patient 2, hypertriglyceridemia and fatty liver had been observed in childhood. However, her fatty liver was improved on a strict low fat diet. Neither hypertriglyceridemia nor fatty liver was seen in patient 4. The prevalence of hypertrophic cardiomyopathy in CGL was reported as approximately 20% regardless of genotype (15). We assessed the presence of hypertrophic cardiomyopathy using echocardiography, electrocardiography, and plasma ANP and BNP levels. These results are presented in Table 3. Echocardiography indicated no apparent sign of hypertrophy in interventricular septal wall and left ventricular posterior wall. Electrocardiography indicated no sign of left ventricular hypertrophy. Plasma levels of ANP and, especially, BNP were elevated in patients with hypertrophic cardiomyopathy (19). Although the plasma BNP level in patient 3 was slightly elevated, all patients' levels of both ANP and BNP were almost within normal range. None of the four CGL patients had obvious hypertrophic cardiomyopathy. IQ was assessed in patients 1 and 4. Their IQs were 76 and 80, respectively, which were relatively low, but in the normal range. A formal assessment was not available for patients 2 and 3, but they also showed no distinct intellectual impairment that interfered with daily or school life. All four patients had mild to moderate acanthosis nigricans. Patient 2, who was 12 yr of age, had still not experienced menophania. Patient 3 experienced menophania at the age of 12 yr, but presented with oligomenorrhea and polycystic ovary in our study.

Sequence analysis of the entire coding regions of *seipin* and *AGPAT2* disclosed a novel homozygous C to T mutation at nucleotide 823 in exon 8 of *seipin* in patients 1, 2, and 3 (Fig. 2). This mutation predicts the substitution of arginine at codon 275 by the stop codon (R275X). Patient 4 had no mutation in the coding regions of the *seipin* gene. We did not find any AGPAT2 gene mutation in all four Japanese patients. The pedigrees of the patients are illustrated in Fig. 3. Consanguinity was recognized in the pedigrees of the patients with the *seipin* mutation, but not in the pedigree of patient 4. Although a DNA sample from the father of patient 3 was not available, the remaining five parents of the three patients with the *seipin* gene mutation were all heterozygous for the mutation. Parents showed no CGL phenotypes. Their BMI ranged from 23.5–26.3, and they were not diabetic. We also examined 10 family members of patient 1 for the R275X mutation and found six of them to be heterozygous (Fig. 2A). We found no significant difference in the average BMI be-

TABLE 1. Clinical features of patients with CGL

Patient no.	Sex	Age (yr)	BMI (kg/m^2)	Body fat (%)	Leptin (ng/ml)
1	M	19	20	5.0	1.23
2	F	13	16	3.3	1.05
3	F	23	21	5.7	1.50
4	M	29	14	4.7	0.82

TABLE 2. Metabolic characters of patients with CGL

Patient no.	DM	Age of onset of DM (yr)	Glucose (mg/dl)	Insulin (μU/ml)	HbA_{1c} (%)	Triglyceride (mg/dl)	Fatty liver
1	+	10	238	ND	8.8	547	+
2	−	−	82	49.0	6.0	180	−
3	+	5	268	17.2	11.8	318	+
4	+	12	142	5.2	10.3	69	−

+, Present; −, absent; DM, diabetes mellitus; HbA_{1c}, hemoglobin A_{1c}; ND, not determined.

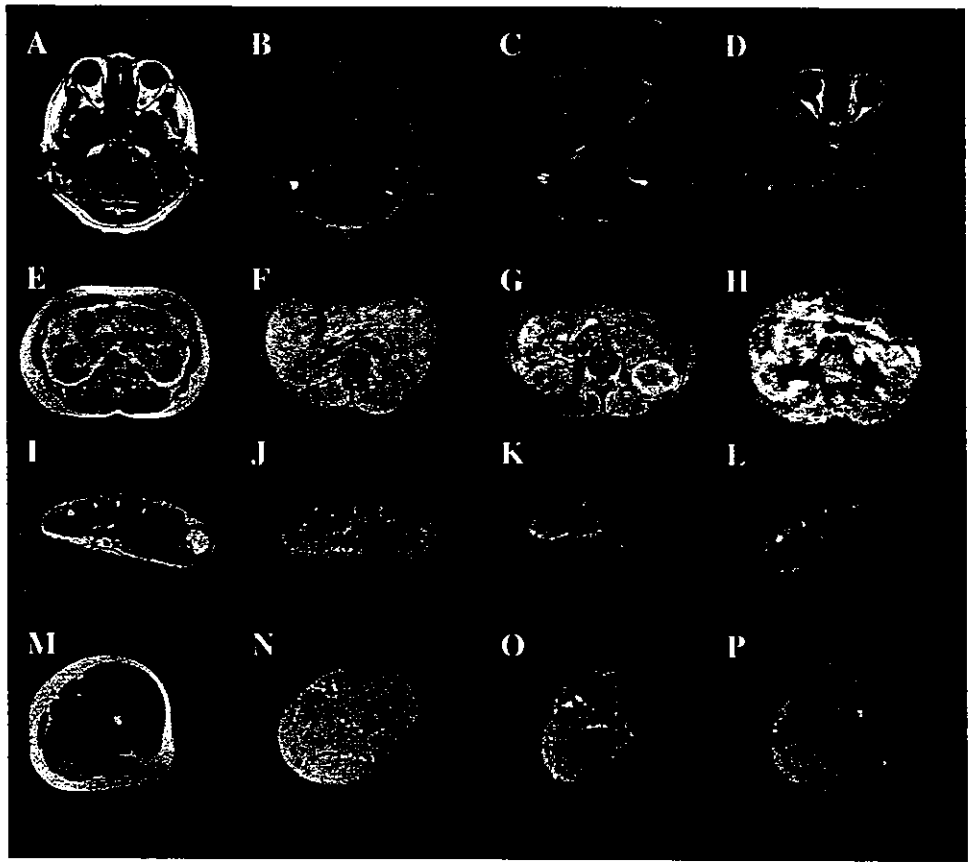

FIG. 1. T1-weighted magnetic resonance images at the levels of orbits (A–D), umbilicus (E–H), palm (I–L), and thigh (M–P) in the control (A, E, I, and M) and in patients 1 (B, F, J, and N), 3 (C, G, K, and O), and 4 (D, H, L, and P).

TABLE 3. Clinical parameters related to hypertrophic cardiomyopathy in patients with CGL

Patient no.	Echocardiography		ECG	ANP	BNP
	IVST (mm)	PWT (mm)	LVH	(pg/ml)	(pg/ml)
1	10	8	–	7.1	5>
2	9	8	–	9.4	9.2
3	9	9	–	14.5	16.3
4	7	7.5	–	15.0	5>

IVST, Interventricular septal wall thickness; PWT, left ventricular posterior wall thickness; ECG, electrocardiogram; LVH, left ventricular hypertrophy diagnosed based on the presence of tall left precardial R waves and deep right precardial S waves (SV1 + RV5 or RV6 >35 mm); –, absence.

tween the family members with or without the R275X heterozygous mutation (25.5 ± 3.6 and 27.8 ± 3.3 kg/m^2, respectively; $P = 0.33$). We also found no difference in the prevalence of diabetes mellitus between these two groups. Of 10 family members, one for each genotype had diabetes mellitus. These observations indicate that CGL caused by the *seipin* R275X mutation is inherited as an autosomal recessive fashion.

We investigated the genotype using microsatellite markers on *seipin* and *AGPAT2* loci and single nucleotide polymorphisms (SNPs) within the *seipin* gene (13, 20) (Table 4). Patients 1–3 were homozygous for the microsatellite markers flanking the *seipin* gene and the SNPs within the gene. In addition, they were disclosed to have the same genotype of microsatellite markers in this region and the SNPs. On the other hand, patient 4 was heterozygous for both *seipin* and *AGPAT2* loci.

Discussion

Seipin is homologous to cDNA of the mouse *Gng3lg* with unknown function, which is localized in the region of mouse chromosome 19 that is orthologous to human 11q (13, 21–23). Comparison between mouse and human sequences showed that the human cDNA contained an open reading frame of 1196 nucleotides, starting at position 345. Seipin is a predicted protein with 398 amino acids and more than two hydrophobic amino acid stretches, suggesting that it could be a *trans*-membrane protein. However, seipin has no similarity with other known proteins or consensus motif that could predict its function. Fourteen different *seipin* mutations, including R275X, have been identified to date (13, 15). Of these mutations, R275X is the most C-terminally located mutation. R275X results in a deletion of 124 amino acids of its C-terminal region. The patients with the homozygous R275X mutation show typical CGL phenotypes, suggesting that the C-terminal region of 124 amino acids could be important for the protein function, especially in adipogenesis or adipocyte differentiation. The metabolic phenotypes including insulin resistance and dyslipidemia in our subjects were similar to those observed in CGL subjects with the *seipin* mutation from other ethnic groups (13, 15). This suggests that the *seipin* gene mutation alone is sufficient for the onset of these metabolic abnormalities.

Of four patients we investigated, three had a homozygous mutation in the *seipin* gene, and their mutation was the same

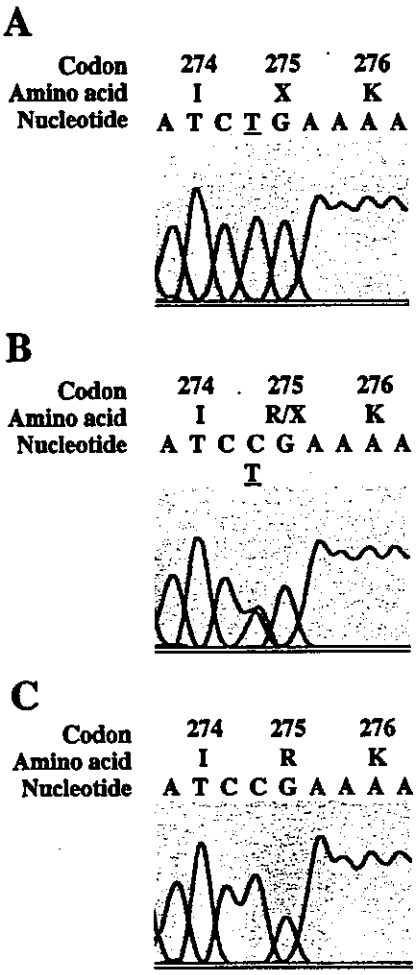

FIG. 2. Sequence analyses of the *seipin* gene. A, B, and C, Homozygous and heterozygous C to T mutations and normal sequence at nucleotide 823 in patient 1, his father, and his aunt.

R275X mutation in all of them. Although they were from independent families living in different remote regions in Japan, they had the same genotype of the microsatellite markers flanking the *seipin* gene and the SNPs within the gene. This demonstrates that they have a common ancestor in whom the R275X mutation was originated.

We did not find any mutations in the coding regions of *seipin* and *AGPAT2* in patient 4. Genotype analysis using microsatellite markers and SNPs revealed that he is heterozygous for *seipin* and *AGPAT2* loci. His phenotype, without hypertriglyceridemia and fatty liver, is atypical for CGL with *seipin* or *AGPAT2* mutation. Further, retroorbital and bone marrow fats are preserved only in patient 4 in the present study. Taken together, it is unlikely that *seipin* and *AGPAT2* genes link to his disease, although we cannot completely exclude the possibility that he has compound heterozygous mutation in noncoding regions of the genes. In addition, the generalized deficiency of body fat and the typically lipoatrophic face were noticed at birth, and autoimmune or causative disease has not been demonstrated in patient 4. These findings indicate the possible existence of another locus for CGL.

We did not find any *AGPAT2* mutations in these four

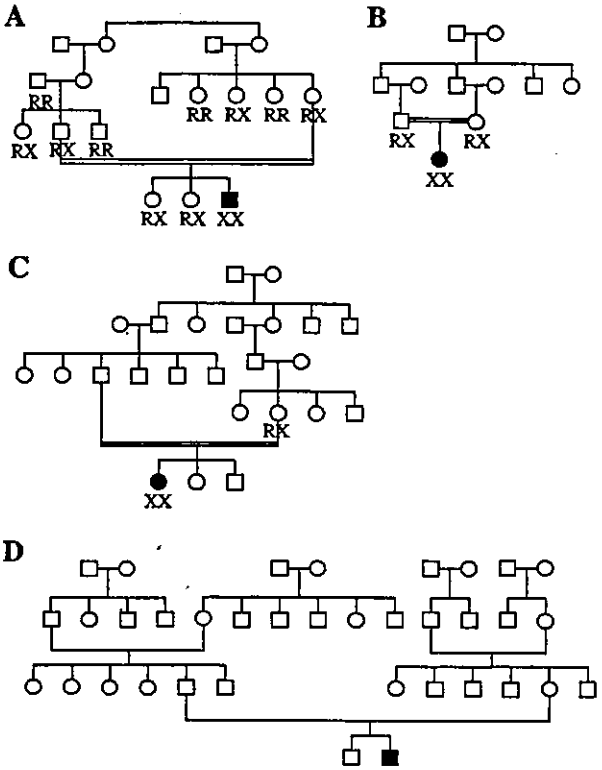

FIG. 3. Pedigrees of patients 1 (A), 2 (B), 3 (C), and 4 (D). *Squares* and *circles* indicate males and females, respectively. The probands are shown as *filled symbols*, and other family members are shown as *unfilled symbols*. The R275X homozygous mutation is shown as XX, the heterozygous mutation is shown as RX, and the wild type is shown as RR.

TABLE 4. Status of microsatellites and SNPs at the *seipin* and *AGPAT2* loci in patients

Microsatellites	Patient no.			
	1	2	3	4
Seipin				
D11S986	1,2	4,4	2,2	1,4
D11S4191	2,5	6,6	5,5	2,7
D11S1765	1,1	1,1	1,1	1,1
D11S4076	2,2	3,3	2,2	2,3
CA10	6,6	6,6	6,6	1,5
CA9	1,1	1,1	1,1	1,2
D11S480	1,1	1,1	1,1	1,1
D11S1883	2,2	2,2	1,1	1,2
D11S4205	1,1	3,3	3,3	3,3
PYGM		2,2	3,4	4,5
SNPs				
Int5/+69	G,G	G,G	G,G	A,G
Int5/−49	C,C	C,C	C,C	T,C
Ex9/65	A,A	A,A	A,A	A,G
AGPAT2				
D9S164	4,5	1,4	3,4	3,5
D9S1818	2,3	2,3	1,1	2,3
D9S1826	2,7	1,4	4,5	2,7
D9S1838	2,2	2,6	1,3	2,4
D9S158	1,1	1,1	3,3	2,5

patients. Although the number of subjects we examined was small, these observations indicate that *AGPAT2* is a minor causative gene for CGL in the Japanese people. We also elucidated that one of four patients is without *seipin* or

AGPAT2 mutations. He did not present a lipid metabolic disturbance until now, although dyslipidemia is one of the major phenotypes of CGL caused by *seipin* or *AGPAT2* mutations. This observation is consistent with the presence of another CGL-associated gene (20). This is the first report of gene and phenotype analysis of CGL in the Japanese population.

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