Mest/Peg1 imprinted gene enlarges adipocytes and is a marker of adipocyte size

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Obesity, defined as an increase in adipose tissue mass, is the most prevalent nutritional disorder in industrialized countries and is now a growing problem in developing countries. Adipose tissue mass increases with the production of new fat cells through the process of adipogenesis and/or the deposition of an increased amount of cytoplasmic triglyceride per cell. The mechanisms controlling adipose tissue development have been the focus of intense research (8). Adipocyte differentiation is a complex process regulated by various factors (28). On induction of differentiation, a cascade of gene transcription events occurs, leading to the expression of adipocyte-specific genes. Peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer binding protein (C/EBPα) are essential transcription factors involved in the cascade of adipogenesis. PPARγ and C/EBPβ upregulate the expression of adipocyte-specific genes to promote adipose differentiation (19, 32, 33, 36). PPARγ is a member of the nuclear receptor family (20) and is activated by anti-diabetes drugs such as thiazolidinediones (TZDs), which can stimulate the differentiation of preadipocytes (18). Although much has been learned about the differentiation of adipocytes in vitro (28), less is known about the molecular basis for the mechanisms regulating adipogenesis in vivo.

Many reports have been published concerning mice with genetic modifications affecting obesity. Diacylglycerol acyltransferase (DGAT)-1 catalyzes the final step in mammalian triglyceride synthesis. Homozygous Dgat knockout mice are resistant to diet-induced obesity and have smaller adipocytes than wild-type control mice on both chow and high-fat diets (4). Transgenic mice overexpressing PPARγ co-activator (PGC)-1β, a nuclear receptor coactivator, are resistant to obesity because of increased energy expenditure and have smaller adipocytes (12). Heterozygous PPARγ-deficient mice have smaller adipocytes and less fat (15). In the cases described above, a reduction in fat mass is associated with a decrease in the size of adipocytes. Larger adipocytes secrete molecules that cause insulin resistance, such as TNF-α and resistin (7), and smaller adipocytes secrete molecules that increase insulin sensitivity, such as adiponectin (37). Thus the size of adipocytes can be essential to the management of obesity-related diseases.

To further characterize the regulation of gene expression during adipose tissue formation in vivo and in vitro, microarray assays have been conducted (10, 29, 30). In a microarray analysis, we observed that the expression level of an mRNA named mesoderm-specific transcript (Mest)/paternally expressed gene 1 (Peg1), hereafter just Mest, was markedly increased in the white adipose tissue (WAT) of obese mice. The Mest gene has been well studied as an imprinted gene expressed only from the paternal allele during development (13, 16, 17, 22, 24). However, few studies have been done on the function of Mest protein in adult tissue. In this study, we focused on characterizing the function of Mest expression in the formation of adipose tissue, using in vitro and in vivo approaches.

MATERIALS AND METHODS

Animals. C57BL/6J mice were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). The compositions of diets were as described (31). Laboratory chow diet (CE2; Clea, Tokyo, Japan) contained (in %, wt/wt) 6.0 oil, 24.5 casein, 10.0 sucrose, 46.5 α-starch, 1.0 vitamin mix (AIN 76; Oriental Yeast, Tokyo, Japan), 7.0 mineral mix (Oriental Yeast, Tokyo, Japan), and 5.0 cellulose powder. Carbohydrate diet contained (in %) 4.0 oil, 24.0 casein, 10.0 sucrose, 50.0 α-starch, 1.0 vitamin mix, 7.0 mineral mix, and 4.0 cellulose powder. High-fat diet contained (in %) 32.0 oil, 33.6 casein, 17.6 sucrose, 0.0 α-starch, 1.4 vitamin mix, 9.8 mineral mix, and 5.6 cellulose powder. db/db and db/dh mice were obtained from Clea Japan. Mice were exposed to a 12:12-h light-dark cycle and maintained at a constant temperature of 21 °C.

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22°C. All animal procedures were in accordance with institutional guidelines.

RNA preparation and Northern blot analysis. Total RNA was prepared from tissue with TRIzol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA fragments for probes were obtained by RT-PCR and confirmed by sequencing. cDNA of rat C/EBPα and mouse glucose transporter-4 (GLUT4) was provided by Dr. M. D. Lane (The Johns Hopkins University). cDNA of mouse PPARδ was provided by Dr. C. K. Glass (University of California-San Diego). Northern blotting was performed as described (27).

Isolation of adipocytes and nonadipocytes from adipose tissues. Adipocytes and nonadipocytes were prepared by the collagenase method from parametric adipose tissue of 12–18 mice, as described in a previous study by our laboratory (34). The tissue homogenates were fractionated by brief centrifugation (350 g for 20 s) in Krebs-HEPES buffer, pH 7.4, supplemented with 20 mg/ml of bovine serum albumin (fraction V) and 2 mmol/l of glucose. Floating cells were adipocytes, whereas the pelleted cells were nonadipocytes.

Histological analysis and morphometry. Parametrical adipose tissue from lean (db/db−) and obese (db/db+) mice was fixed in 4% paraformaldehyde in phosphate-buffered saline and then processed for paraffin embedding, sectioning, and staining with hematoxylin and eosin. For quantitation of the number and size of adipocytes, the sectional areas of WAT in the hematoxylin- and eosin-stained preparations were analyzed.

Retrovirus-mediated expression. The Mest viral expression vector was constructed by ligating the full-length cDNA into pMX-puro (21) (pMX-Mest). Phoenix-293 packaging cells (11) were transfected with pMX-Mest. Viral supernatants were harvested 48 h after transfection. Viral supernatants were applied to 3T3-L1 cells in DMEM containing 10% FCS and 5 μg/ml of polybrene. The medium was replaced with DMEM containing 10% FCS and 10 μg/ml of puromycin to eliminate uninfected cells. To measure the degree of differentiation to adipocytes, 3T3-L1 cells were stained with Oil Red O (32, 33).

Cell culture and induction of differentiation. Virally infected cells were cultured to confluence in differentiation medium, DMEM con-

Fig. 1. Increased Mest mRNA expression in white adipose tissue (WAT) in mice with high-fat diet-induced obesity. A: parametrical adipose tissue weight of mice fed a carbohydrate diet (Carb.) for 4 and 10 wk and a high-fat diet for 4 and 10 wk. 4W, 4 wk; 10W, 10 wk. B: Northern blot analysis of Mest mRNA in parametrical WAT of mice shown in A. Each lane represents a sample from an individual mouse. C: expression of Mest mRNA in adipocyte and nonadipocyte fractions of parametrical adipose tissue. Lanes 2 and 4 are samples from mice with high-fat diet (10 wk)-induced obesity. Lanes 1 and 3 are samples from age- and sex-matched control lean mice fed a laboratory chow diet. The membrane was rehybridized with the uncoupling protein (UCP)2 probe as a nonadipocyte marker and the GLUT4 probe as an adipocyte marker.

Fig. 2. Increased Mest mRNA expression in WAT in db/db obese mice. A: expression of Mest mRNA in parametrical WAT of db/db mice. Samples from female db/db mice and lean female littermates (db/db−) at 20 wk of age were used. A typical Northern blot is shown. Each lane represents a sample from an individual mouse. B: tissue distribution of Mest mRNA expression in db/db mice. RNA was extracted from the indicated tissue of male (body wt 41 g, 11 wk of age) and female (body wt 41 g, 11 wk of age) db/db mice. BAT, brown adipose tissue. C: expression of Mest mRNA in parametrical WAT of mice with streptozotocin (STZ)-induced diabetes. Samples from STZ-treated mice and age- and sex-matched control mice were used. Each lane represents a sample from an individual mouse.
Fig. 3. Correlation of adipocyte size and Mest expression in pioglitazone-treated db/db mice. A: histology of retroperitoneal WAT. WAT sections were stained with hematoxylin and eosin after fixation in formalin. The adipocytes from db/db obese mice were larger than those from control lean mice. Pioglitazone caused a decrease in the size of adipocytes in both db/db obese mice and control lean mice. B: distribution of the size of adipocytes in retroperitoneal adipose tissue. Diameters of adipocytes in 5 WAT sections of independent mice were measured. In each group, a total of 175 cells were measured. Cell sizes are calculated from the diameter (1/2 of the diameter squared times 3.14), and the relative nos. of cells of different sizes are shown. ○, Lean without pioglitazone; ●, lean with pioglitazone; □, db/db without pioglitazone; ■, db/db with pioglitazone. C: expression of Mest mRNA in retroperitoneal WAT used in A and B. A typical Northern blot is shown. Each lane represents a sample from an individual mouse. The graph is a densitometric analysis of Northern data (n = 4–5 in each group), the value for lean mice without pioglitazone being 100%. *P < 0.05.
taining 10% FCS. At confluence, cells were treated for 42 h with 1 μM dexamethasone and 10 μg/ml of insulin for 42 h. Cells were refed every 2 days. Total RNA (20 μg/lane) was isolated and analyzed by Northern blotting with [32P]-labeled probes. C/EBPα, CCAAT/enhancer binding protein-α; PPARγ, peroxisome proliferator-activated receptor-γ; aP2, adipocyte fatty acid binding protein 2. B: microscopic views of representative 3T3-L1 cells expressing Mest and control cells, stained with Oil Red O. Seven days after the induction of differentiation, cells were fixed with formalin and stained with Oil Red O. Bar, 100 μm.

**RESULTS**

**High-fat diet increases Mest expression in adipocytes.** Microarray analysis showed that levels of Mest mRNA were markedly increased in WAT of obese mice (not shown). To confirm this increase, we performed a Northern blot analysis using samples from WAT of obese mice. Mice were fed a carbohydrate diet or a high-fat diet. The compositions of the diets are described in MATERIALS AND METHODS. The body weights of these mice were as follows: 4-wk carbohydrate diet, 18.6 ± 0.7 g; 4-wk high-fat diet, 19.0 ± 0.4 g; 10-wk carbohydrate diet, 21.5 ± 0.6 g; and 10-wk high-fat diet, 24.0 ± 0.5 g. After 10 wk on the high-fat diet, WAT of mice weighed much more than that of mice on the 4-wk high-fat diet (average 0.6 g at 10 wk and 0.2 g at 4 wk) (Fig. 1A). In parallel with fat weight increases, Mest mRNA levels after 10 wk on the high-fat diet were much higher than those after 4 and 10 wk on the carbohydrate diet and 4 wk on the high-fat diet (Fig. 1B).

Because adipose tissue is composed of adipocytes and nonadipocytes, we then examined whether the increase in expression of Mest mRNA occurred in adipocytes or nonadipocytes. Adipose tissues of mice fed a 10-wk high-fat diet and of age-matched mice fed laboratory chow (a dietary composition similar to carbohydrate diet, see MATERIALS AND METHODS) were digested by collagenase and then separated into adipocytes and nonadipocytes by brief centrifugation, and RNA was extracted. As shown in Fig. 1C, nonadipocytes had fivefold higher levels of uncoupling protein (UCP)2 than adipocytes. GLUT4 was substantially expressed in adipocytes but not in nonadipocytes, suggesting that there was no significant cross-contamination between the nonadipocyte cell fraction and the adipocyte...
fraction. An increased Mest mRNA level was only observed in the adipocyte fraction of obese mice.

A marked Mest expression is observed in WAT but not in other tissues in genetically obese db/db mice. Next, to examine whether Mest expression in WAT was also increased in other types of obesity, Mest mRNA levels in db/db obese mice, which have a leptin receptor mutation (3), and in control lean mice (db/−) were measured. Also in db/db mice, a marked increase in the expression of Mest mRNA in parametrial WAT was observed (Fig. 2A). At that time, the average body weight and blood glucose level were 64.4 ± 0.5 g and ~600 mg/dl (db/db, n = 3) and 25.4 ± 0.5 g and 190 ± 10 mg/dl (lean, n = 3), respectively. To examine the possibility that Mest was also expressed in other tissues, Northern blotting in other tissues of db/db mice was performed. Mest mRNA expression in brain, heart, liver, kidney, stomach, muscle, and brown adipose tissue (BAT) of both male and female db/db mice was very weak, whereas Mest mRNA was highly expressed in WAT of both male and female db/db mice (Fig. 2B). Thus Mest was preferentially expressed in obese white adipose tissues, irrespective of gender.

Mest mRNA expression in WAT is downregulated in streptozotocin-induced diabetic mice. Mice with high-fat diet-induced obesity and db/db mice were diabetic as well as having phenotypes of obesity. To examine whether an increased level of Mest mRNA was associated with obesity or diabetes, Mest mRNA expression in another model of diabetes, streptozotocin (STZ)-induced diabetes, was measured (35). In STZ-treated mice, blood glucose levels were high (524.7 ± 7.3 mg/dl for STZ-treated mice, and 182.7 ± 6.4 mg/dl for control mice) with decreased body weight (16.6 ± 0.2 g for STZ-treated mice, and 19.6 ± 0.4 g for control mice). In parametrical WAT of these diabetic mice, Mest mRNA expression was not increased but rather decreased (Fig. 2C). Thus the increased Mest mRNA expression observed in obese WAT may not be related to an increased blood glucose level.

Pioglitazone administration downregulates Mest expression with a decrease in the size of adipocytes. Troglitazone (TZD), a drug for type 2 diabetes (2, 5), reduces the size of adipocytes in falfa rats (23). To examine the relationship between adipocyte size and Mest expression, we treated db/db mice with pioglitazone, a commercially available TZD (2, 5). Pioglitazone (200 µg/g diet) given as a food admixture for 5 wk caused an increase in the body weight of db/db mice. Final body weights were as follows: lean without pioglitazone, 25.1 ± 0.2 g (n = 5); lean with pioglitazone, 26.7 ± 0.5 g (n = 5); db/db without pioglitazone, 42.0 ± 0.6 g (n = 5); and db/db with pioglitazone, 53.2 ± 1.7 g (n = 4) (P < 0.001 between db/db with and without pioglitazone). Pioglitazone lowered blood glucose levels of db/db mice to normal values: lean without pioglitazone, 126.8 ± 2.8 mg/dl; lean with pioglitazone, 109.8 ± 15.2 mg/dl; db/db without pioglitazone, >600 mg/dl (over the maximum level of the glucose analyzer); and db/db with pioglitazone, 197.3 ± 51.6 mg/dl. At the same time, histological analysis of WAT showed that pioglitazone decreased the size of adipocytes (Fig. 3, A and B), which is consistent with the result that troglitazone reduces the size of adipocytes in falfa rats (23). In WAT of pioglitazone-treated obese db/db mice, Mest mRNA levels were markedly decreased compared with those in untreated db/db obese mice (Fig. 3C). Because pioglitazone increases the body weight of db/db mice, possibly by increasing cell number, Mest mRNA does not need to be expressed during obesity per se but may reflect adipocyte cell size.

Exogenous expression of Mest in 3T3-L1 adipose cells promotes adipocyte differentiation. To gain insight into the role of increased expression of Mest in obese WAT, we stably expressed Mest in 3T3-L1 adipose cells. We cloned Mest cDNA into a retroviral vector and used the virus to infect cells. In 3T3-L1 cells, endogenous Mest mRNA was not expressed during differentiation (not shown). 3T3-L1 cells infected with Mest or vector alone at confluence were cultured in differentiation medium, including 1 µM dexamethasone and 10 µg/ml of insulin. The medium was changed 42 h later to DMEM containing 10% FCS. At the indicated time, the mRNA levels of adipocyte markers were examined. Compared with control vector alone-transfected 3T3-L1 cells, increased mRNA levels
of adipocyte markers were observed in Mest-transfected 3T3-L1 cells (Fig. 4A). An increase in lipid droplets, stained with Oil Red O, in 3T3-L1 cells expressing Mest was also observed (Fig. 4B). These results suggest that Mest augments adipogenic activity. However, a significant difference in cell size between Mest cells and control cells was not detected. This is possibly because of the difference between cultures and whole animals. 3T3-L1 cells do not differentiate to the extent observed in vivo in a week.

Creation of transgenic mice overexpressing Mest in adipose tissues. To investigate the role of increased expression of Mest in vivo, we next established transgenic mice overexpressing the Mest gene in adipose tissue. The aP2 promoter, which is commonly used for driving the adipose-specific expression of a transgene (14, 25), was used to drive the expression of the Mest cDNA (Fig. 5A). Three independent lines of mice were obtained. The transgene copy number of each animal, estimated by Southern blot analysis of tail DNA of the mice, was 3 (line C), 10 (line D), and 12 (line E), respectively. Expression of the Mest transgene was evaluated by Northern blot analysis. Figure 5B illustrates the intensity of the hybridization of a Mest probe to total RNA isolated from tissues of female transgenic mice (lines C–E) and female nontransgenic controls. The use of the aP2 promoter resulted in predominantly high expression levels of the Mest transgene in adipose tissue (Fig. 5B). Lines C and E showed expression levels of the Mest transgene in adipose tissue that were higher than those in line D. A similar preferential expression of the Mest transgene was observed in male transgenic mice (not shown). The increases occurred at the physiological level, since mRNA levels of the Mest transgene in WAT of lines C and E were comparable to those observed in WAT of db/db mice (not shown).

Mice overexpressing Mest showed increases of adipogenic gene expression and adipocyte size. To examine whether the increased adipogenic gene expression observed in 3T3-L1 cells expressing Mest is also observed in WAT of Mest-transgenic mice, a Northern blot analysis was performed. As shown in Fig. 6A, the expression of adipose genes, such as the genes for
aP2, GLUT4, leptin, CD36, and resistin, was significantly enhanced in gonadal WAT of Mest mice (line C, male, and line E, female) compared with their sex-matched wild-type littermates. Moreover, histological analysis showed that the adipocytes of these Mest mice are markedly larger than those of control mice (Fig. 6, B and C). Thus, also in vivo, Mest is functional and increased the size of adipocytes.

**DISCUSSION**

In this study, we found that the Mest mRNA level was markedly increased in WAT of mice with high-fat diet-induced and genetically caused obesity. Experiments using mice with STZ-induced diabetes and/db/db mice treated with pioglitazone have suggested that adipose cell size, but not diabetes or increased body weight, reflects Mest expression. The ectopic expression of Mest increased adipose gene expression both in vitro and in vivo. Moreover, adipose cells were markedly enlarged in the transgenic mice. Although little attention has been paid to the function of Mest protein, the observations described in this study indicate that Mest plays a role in the formation of adipose tissue, including the determination of adipose cell size.

Mest was originally cloned from a mouse embryonal carcinoma cell line (MC12) and expressed in the embryonic and extraembryonic mesoderm but not in adult tissues (26). During a systematic screening of imprinted genes, using subtraction extraembryonic mesoderm but not in adult tissues (26). During a systematic screening of imprinted genes, using subtraction hybridization between cDNAs from normal and parthenogenetic embryos (maternal genome only), Mest was identified as one of the paternally expressed genes (pegs) (13). In a subsequent study, the maternal allele of the Mest gene was found to be fully methylated in its 5′-region and not to be expressed in the embryo, whereas the paternal allele was unmethylated in its 5′-region and expressed (17). In adult tissues, although Mest expression was very weak, imprinting of Mest expression persisted (24). Recently, a luciferase reporter assay of the Mest promoter revealed that a lower level of Mest expression in adult tissues was not related to the methylation status of the Mest promoter (22). The data suggested that the marked upregulation of Mest in obese adipose tissues is due to the demethylation of the Mest promoter of the maternal allele and/or the enhancement or derepression of the Mest promoter via a methylation-independent mechanism. To gain further insight into the regulatory expression of imprinted genes as well as the mechanism of adipocyte enlargement, it is important to elucidate the mechanism(s) behind the marked expression of Mest in obese adipocytes.

How does Mest affect the adipose tissue, including the stimulation of gene expression and enlargement of adipose cells? The function of the Mest protein has not been investigated at all. Considering the amino acid sequence of the protein, Mest is not a transcription factor. A protein homology search (1) has shown that Mest shares a high level of amino acid similarity with the α-B-hydroxylase of a fish, Danio rerio (GenBank accession no. AB042295, 74% identity in 344 amino acids), and the epoxide hydrase of a fungus, Pseudomonas aeruginosa (GenBank accession no. AE004764, 41% in 292 amino acids) (not shown), suggesting that the Mest protein has enzymatic activity. Mest may catabolize reactions that have enzymatic activity. Mest may catabolize reactions that produce an activator molecule for adipogenic transcription factors, such as PPARγ and C/EBPα. In support of this idea, heterozygous PPARγ-deficient mice had smaller adipocytes (15). This possibility remains to be tested.

The human MEST gene is mapped to 7q32.2 (http://www.ensembl.org/Homo_sapiens/). Interestingly, it has been shown that a chromosomal locus influencing body mass index maps to 7q32.3 (6). This locus is near the location of human LEPTIN (7q32.1) (9) (http://www.ensembl.org/Homo_sapiens/). In addition to the LEPTIN gene, MEST could be a new candidate gene in this region. On the other hand, a computer-predicted sequence analysis (http://sosui.proteome.bio.tuat.ac.jp/) has shown that Mest has a signal peptide sequence in its NH2-terminus (not shown), suggesting that the protein might be secreted out of adipocytes of obese subjects. Namely, a test of the blood Mest protein level might provide a novel biological marker of obesity and/or adipose cell size.

Obviously, the next important question is the phenotype of Mest mice in terms of obesity and related diseases such as diabetes. GLUT4 mRNA was increased in WAT of Mest-overexpressed mice (Fig. 6A). Because the amount of GLUT4 protein on plasma membrane is a rate-limiting step of glucose oxidation, enhanced insulin-regulated glucose uptake might occur in adipose tissues from Mest-overexpressed mice. This possibility remains to be examined. Preliminary experiments showed that the body weight of Mest mice was not increased compared with that of wild-type control mice, but their lean body mass was decreased, suggesting increased adiposity and decreased muscle mass in Mest mice. Further studies on Mest will provide fundamental information that may be useful for developing potential drugs targeting Mest, including drugs for obesity and related metabolic disorders.

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