Involvement of Mesoderm-specific Transcript in Cell Growth of 3T3-L1 Preadipocytes

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Mesoderm-specific transcript (*Mest*) is an imprinted gene that is predominantly expressed in the embryonic and extraembryonic mesoderm, and its gene expression is the marker of the size of adipocytes. To characterize the expression effect of *Mest* gene products, we transfected mouse *Mest* into the 3T3-L1 cell line, which is a mouse preadipocyte cell line. The proliferation of the transfected 3T3-L1 cells was assessed via 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetry, and cell growth was assessed by counting the nuclei stained by Hoechst 33258. The 3T3-L1 cells transfected with recombinant *Mest* cDNA acquired the increased cell growth ability. Therefore, the gene products of *Mest* could be related to not only adipocyte expansion and adiposity but also mesoderm formation by enhancing the cell proliferation.

Key words —— mesoderm-specific transcript, preadipocyte, 3T3-L1, cell growth

INTRODUTION

White adipose tissue is the organ that not only stores energy in the form of fat but also secretes endocrine hormones, adipokines, to maintain the homeostasis of an organism.[1,2] Obesity is a growing and serious public health problem in developed countries. Obesity harms the homeostatic balance of adipose tissues; eventually, obesity can harm the whole body through the development of type 2 diabetes mellitus, atherosclerosis, hypertension, and hyperlipidemia.[3,4] Adipocytes that ingest large quantities of lipids secrete adipokines, such as TNF-α and resistin, which cause insulin resistance.[5] Various factors such as sterol regulatory element-binding protein-1 (SREBP-1) and peroxisome proliferative activated receptor γ (PPARγ) enhance the proliferation, differentiation, and enlargement of adipocytes in obesity.[4,6] However it has been remained elucidated what kind of molecular affect on adipocyte hypertrophy.

Mesoderm-specific transcript/Paternally expressed gene 1 (*Mest*, also called *Peg1*) is an imprinted gene that is only transcribed from the paternal allele and is expressed in the embryonic and extraembryonic mesoderm.[7–11] Although *Mest* is little expressed in the tissues of adult mice, the loss of *MEST* imprinting occurs in several human cancers.[12–15]

In both dietary and genetically obese mice, the expression level of *Mest* mRNA is markedly upregulated in white adipose tissue and is correlated with the size of adipocytes.[16–18] The overexpression of *Mest* increases adipocyte size *in vivo* and promotes adipocyte differentiation *in vitro*.[16] However, the mechanism by which *Mest* gene products cause the enlargement and differentiation of adipocytes has not been identified. Also, it is unknown if the expression of the *Mest* gene influences other cell types, such as preadipocytes.

In the present study, we examined the effect of recombinant *Mest* overexpression on a mouse fibroblastic preadipocyte cell line, 3T3-L1. We found that the transfected 3T3-L1 cells with *Mest* cDNA acquired an increased cell growth ability.

MATERIALS AND METHODS

Materials —— Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma (St. Louis, MO, U.S.A.). Geneticin (G418) sulfate solution and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Nacalai Tesque (Kyoto, Japan). The pcDNA 3.1/*myc*-His A vector, mouse anti-*myc* antibody, and Hoechst 33258 were obtained from Invitrogen (Carlsbad, CA, U.S.A.) *Trans*IT-LT1 and RNAiso were purchased from TaKaRa Bio (Tokyo, Japan).

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Construction of Expression Vector Plasmids for *Mest* —— We constructed expression vectors for mouse *Mest* (Accession number: NM_008590) and *Mest-myc*-His, which contains a *myc*-polyhistidine tag sequence at the carboxyl terminal. Total RNA from 3T3-L1 adipocytes was used as a template for PCR to amplify the coding region of *Mest* and to create a *HindIII-XbaI* restriction site at the 5′ and 3′ ends with the following primers, respectively: forward primer, 5′-AGTAGTAGAAGCTTGCCATGGTGCGCCGAG-ATCG-3′; reverse primer for the expression vector of wild-type mouse *Mest*, 5′-AGTAGTAGTCTA-GATCAGAAGGAGTTGATGAAGC-3′; and reverse primer for *Mest-myc*-His to eliminate the stop codon, 5′-AGTAGTAGTCTAGATCCGAAGGAG-TTGATGAAGC-3′. The amplified cDNA fragments were subcloned into the *HindIII-XbaI* site of pcDNA 3.1/*myc*-His A.

Cell Culture and Establishment of 3T3-L1 Cell Lines that Stably Express *Mest* —— 3T3-L1 preadipocytes, mock-transfected cells, and transformed 3T3-L1 cells that express *Mest* or *Mest-myc*-His cDNA, designated as L1-*Mest* and L1-MMH, respectively, were maintained in DMEM supplemented with 10% (v/v) calf serum (MP Biomedicals, Irvine, CA, U.S.A.) at 37°C in the presence of 5% CO_2. 3T3-L1 cells (5×10^5) were seeded onto 60-mm dishes one day prior to the addition of plasmid DNA. The lipofection reagent *Trans*IT-LT1 and 5 μg of plasmid were used to transfect the cells. Forty-eight hours after transfection, the media was replaced with fresh DMEM containing 10% calf serum. After 2 days, the cells were trypsinized and seeded into 100-mm dishes; then, G418-resistant cell lines were selected with DMEM containing 10% calf serum and G418 at a final concentration of 800 μg/ml.

RNA Isolation and reverse transcription (RT)-PCR Analysis —— Total RNA was isolated from the cells with RNAiso. For RT, 2 μg of RNA from each sample was transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's protocol. PCR was performed within a linear range of amplification and with the following primers: 5′-AACCGCAGAATCAACCTGCT-3′ and 5′-CGAAGAAATTCATGAGCCTGG-3′ for *Mest*; 5′-GACCCTGAAGTACCCCATTGAA-3′ and 5′-GC-TTCTCTTTGATGTCACGCAC-3′ for *β*-actin, a housekeeping gene used as a control. The PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Immunoblotting for Expressed Mest Protein —— The cells were harvested, sonicated in mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, U.S.A.) containing 2.5 μg/ml leupeptin and protein inhibitor cocktail tablets (Complete mini; Roche Diagnostics. K.K., Basel, Switzerland), and then centrifuged at $15{,}000 \times g$ for 15 min at 4°C. Aliquots of the resultant supernatants were treated with 25 mM mercaptoethanol, boiled 100°C for 2 min, and then 30 μg of proteins from each sample were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% acrylamide gel, and then electroblotting onto polyvinylidene difluoride (PVDF) membranes. Proteins were visualized by immunostaining using a 1 : 1000 dilution of rabbit anti-MEST antibody (ProteinTech Group, Chicago, IL, U.S.A.), a 1 : 1000 dilution of mouse anti-*myc* antibody and a 1 : 5000 dilution of rabbit anti-*β* actin IgG (Abcam Ltd., Cambridge, U.K.) for primary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Millipore, Billerica, MA, U.S.A.) and HRP-conjugated anti-mouse IgG antibody (Dako, Copenhagen, Denmark) for secondary antibodies, and a chemiluminescent substrate (Millipore, Billerica, MA, U.S.A.). The antibodies were diluted in SignalBoost Immunoreaction Enhancer Kit (Calbiochem, San Diego, CA, U.S.A.). Prestained SDS-PAGE standard (Bio-Rad, Hercules, CA, U.S.A.) and a biotinylated protein ladder detection pack (Cell Signaling Technology, Beverly, MA, U.S.A.) were used as molecular mass standard proteins to calculate the molecular weight of Mest protein.

Hoechst Staining for Evaluating Proliferative Activity —— 3T3-L1 parent cells and the transformed 3T3-L1 cell lines were seeded onto 48-well plates (5×10^3 cells/well). After 1, 3, 5, and 7 days, the cells were fixed with 2% paraformaldehyde/phosphate buffered saline (PBS) for 1 hr at room temperature and then stained with Hoechst 33258 at a final concentration of 10 μg/ml for 1 hr at room temperature. After washing with PBS, the cells were observed under a fluorescence microscope BX51 with a digital single-lens reflex camera E-330 (Olympus, Tokyo, Japan) under magnification (× 100). The number of stained nuclei per field was counted by Image J (NIH, Bethesda, MD, U.S.A.), and then the mean number of stained nuclei from 5 or 6 fields was calculated to evaluate cell

proliferation.

MTT Reduction Assay —— 3T3-L1 parent cells and the transformed 3T3-L1 cell lines were seeded onto four 96-well plates at a density of 2×10^3 cells/well in DMEM with 10% calf serum and cultured for 1, 3, 5, and 7 days. Then, the cells were mixed with MTT at a final concentration of 0.75 mg/ml and incubated at 37°C for 4 hr. A 20% SDS solution in 0.01 M hydrogen chloride was added to each well, and then the plate was incubated overnight in a humidified atmosphere. The absorbance ($A_{570\,nm}-A_{670\,nm}$) of the mixture was measured with a microplate spectrophotometer. The percentage of the absorbance of cells as compared with that of the cells cultured for one day was calculated ($n = 5$).

Statistical Analysis —— Statistical analyses were performed by one-way analysis of variance (ANOVA) for multiple groups comparison. When significant effects were detected, Dunnett's test was used to compare the means. Statistical analysis was performed with commercially available software, Ekuseru-Toukei 2006 for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan). The results are presented as the means ±S.D.

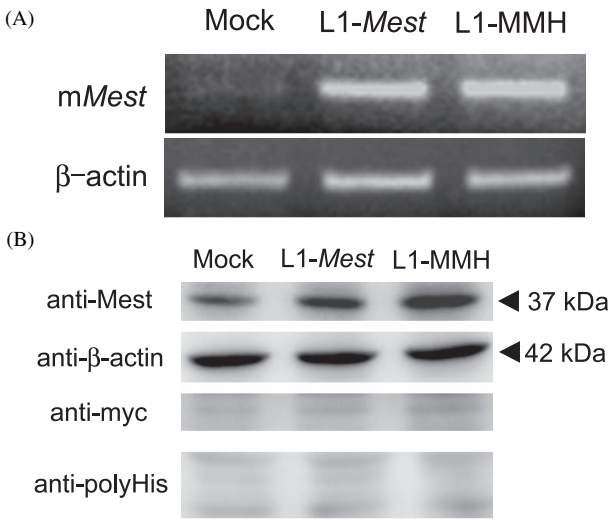

Fig. 1. Comparison of Expression Level of Exogenous *Mest* mRNA and Mest Protein in Transformed-3T3-L1

(A) Mock-transfected cells (Mock), L1-*Mest*, and L1-MMH were harvested and analyzed by RT-PCR for both *Mest* and β-actin mRNA levels. (B) Immunoblot pattern of the recombinant Mest protein expressed in stably transformed 3T3-L1 cell lines with anti-MEST, anti-*myc*, and anti–polyhistdine (polyHis) antibodies. The expressed Mest protein was visualized by immunostaining described under "MATERIALS AND METHODS" section. Immunoblotting for β-actin was detected for loading control. Arrow heads indicate the Mest protein (approximately 37 kDa) and the β-actin (42 kDa).

RESULTS

Expression of Recombinant Mouse *Mest* and *Mest* Protein in Transformed 3T3-L1 Cells

RT-PCR analysis with specific primers revealed remarkable increases in *Mest* cDNAs in transformed 3T3-L1 cells expressing recombinant *Mest* and MMH, designated as L1-*Mest* and L1-MMH, respectively. A slight amount of the cDNA, which is derived from the endogenous *Mest*, was detected in the mock-transfected cells (Fig. 1A). As shown in Fig. 1B, immunoblotting analysis with an anti-MEST antibody revealed significant increase in approximately 37-kDa Mest protein in the L1-*Mest* and L1-MMH compared with the mock-transfected cells. The specific band of C-terminal *myc*-polyhistidine-tagged Mest protein was not detected with anti-*myc* and anti-polyhistidine antibodies in the cell extract of L1-MMH. *Mest* gene contains an open reading frame encoding for 335 amino acids, and the synthesized Mest protein has a theoretical molecular mass of 38.9 kDa. Mest protein observed in this experiment may be subject to post-translational modifications including proteolytic C-terminal cleavage.

Overexpression of *Mest* Promotes Proliferation in the 3T3-L1 Cell Line

We performed nuclear staining with Hoechst33258. 3T3-L1 and Mock cells exhibited a significant increase in the number of nuclei at 3 and 5 days, indicating that the cells were proliferating (Fig. 2A). L1-*Mest* and L1-MMH cells exhibited a greater increase in cell number as compared with the controls (3T3-L1 and Mock cells) (Fig. 2A and 2B). We were unable to accurately count the cells on day 7, because the cells were excessively overlapped. We examined the effect of *Mest* overexpression on proliferative features in 3T3-L1 preadipocytes by using the MTT reduction assay (Fig. 3). The absorbance values of 3T3-L1 and the mock-transfected cells rapidly increased until day 3; then, after day 3, the values increased moderately. These results suggest that the cell growth was becoming arrested. In contrast, L1-*Mest* and L1-MMH rapidly proliferated at least until day 7. The absorbance values of L1-*Mest* and L1-MMH were 1.6 times higher than the absorbance values of 3T3-L1 and mock cells on day 7.

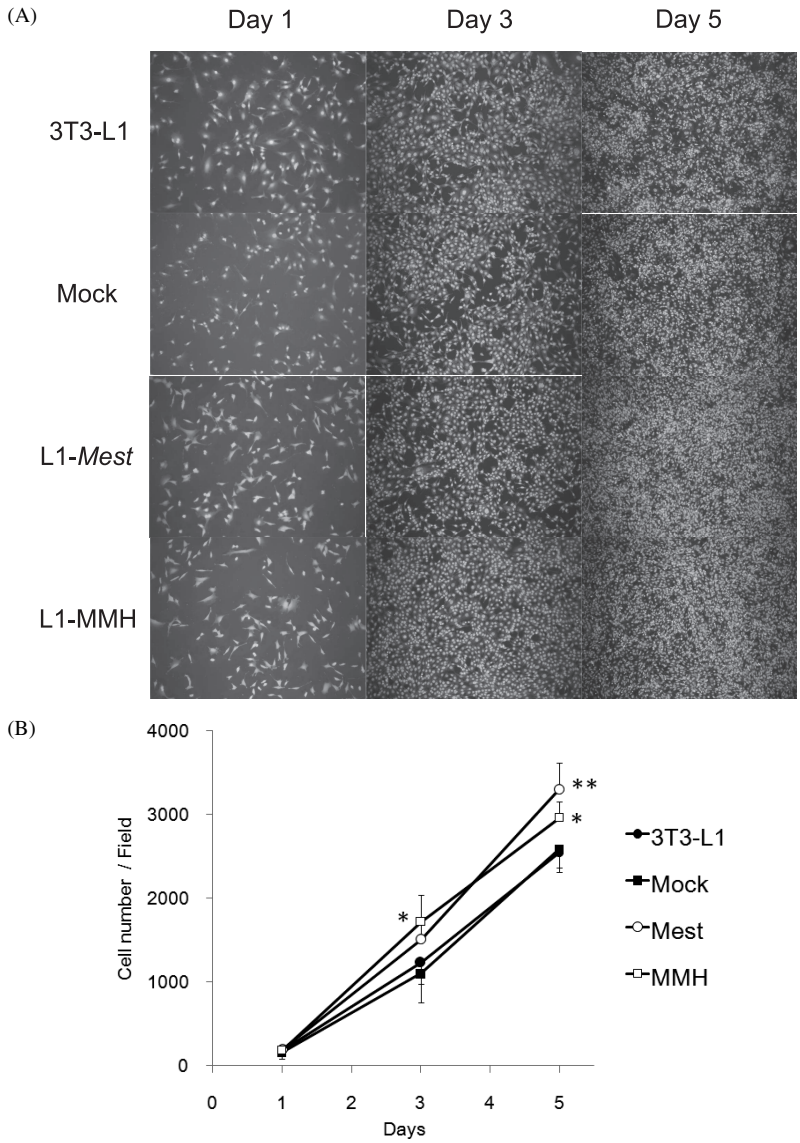

Fig. 2. Nuclear Staining with Hoechst Dye

(A) Nuclear staining patterns in 3T3-L1 parent cells and the transformed cells. The cells were fixed at 1, 3, and 5 days after seeding and then stained with Hoechst 33258 and examined under a fluorescence microscope. Magnification, ×100. (B) Time-dependent changes in the nuclear number per field under magnification (×100). The means ±S.D. of the nuclear numbers determined in 5 or 6 fields are plotted. $^{*}p < 0.05$, $^{**}p < 0.01$ (as compared with 3T3-L1 by Dunnett's test).

DISCUSSION

Mest is an imprinted gene that is expressed from the paternal allele. Although the expression of *Mest* from the maternal allele in mice is completely silenced by DNA methylation of the 5′-region, the transcription rate of *Mest* on the paternal allele was increased in obese mouse tissues.[19] On the other hand, biallelic expression of *MEST* isoform 2,[20] which is non-imprinting, occurs in human organs. But, several human tumor tissues lose the imprinting of the *MEST* isoform 1,[12–15] which can be involved in tumor malignancy. Although multiple variants of mouse *Mest* mRNA with distinct first exons are registered in the Genbank DNA database,[19] the expression regulation-like loss of imprinting has been not reported in mouse *Mest*. *Mest*-deficient mice are characterized by growth retardation, increased perinatal and postnatal lethality, abnormal maternal behavior, and reduced adiposity.[10, 18] In the present study, we showed that 3T3-L1 preadipocytes that express recombinant *Mest* had increased cell proliferation. Therefore, gene products of *Mest* could be related to not only adipocyte expansion and hypertrophy but also to tumor malignancy and the cell proliferation during

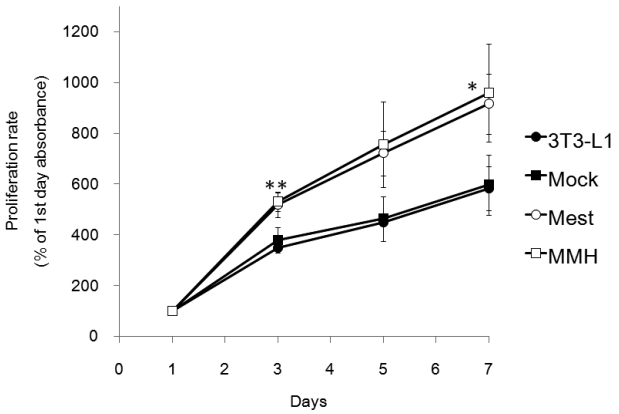

Fig. 3. The Effect of *Mest* Overexpression on Cell Proliferation

Control cells, mock-transfected cells, L1-*Mest*, and L1-MMH were cultured for 1, 3, 5, and 7 days. The proliferative features were determined by MTT assay. Data are the means ±S.D. of measurement for 5 different cell lines. $^{*}p < 0.05$, $^{**}p < 0.01$ (as compared with 3T3-L1 by Dunnett's test).

mesoderm formation. This hypothesis is supported by evidence suggesting that *MEST*/*Mest* is a potential angiogenesis factor in humans and mice.[21, 22]

Although increased levels of *Mest* mRNA have only been found in adipocytes of obese mice,[16–18] the expression level of *Mest* in non-adipocyte cells in the stromal vascular fraction is higher than that of adipocytes from non-obese mice. It has been speculated that a genetic system exists to increase the number of adipocytes after the expansion of adipose tissue in order to control the adipose tissue mass and that then mature adipocytes symmetrically or asymmetrically divide into adipocytes or fibroblastic non-adipocytes.[23] Thus, *Mest* may be the gene regulating the number of the preadipocytes that will later differentiate into adipocytes so that the number of adipocytes does not decrease by cell death from excess lipid accumulation.

Based on the primary sequence of the Mest protein, it appears that the Mest protein is a member of the α/β fold hydrolase family, which includes lipases, epoxidases, acyltransferases, and esterases, and that the enzymatic activity of the Mest protein catalyzes lipid metabolism.[24] Moreover, Mest is exclusively located in the endoplasmic reticulum and Golgi apparatus.[18] In the present study, the proliferation difference between control cells and *Mest*-overexpressed cells as determined by the MTT assay (Fig. 3) was more remarkable than the difference determined by nuclear staining (Fig. 2). This distinction may imply that the cell growth was indirectly promoted by the energy generated in the mitochondria by β-oxidation of fatty acids that are synthesized in lipid metabolism due to the overexpression of recombinant *Mest*, because the MTT assay estimates the number of living cells by measuring the enzyme activity of the mitochondrial respiratory chain. Further studies are needed to elucidate the catalytic function of Mest and the lipid metabolism in *Mest*-expressing cells such as adipocytes, preadipocytes, and tumor cells.

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