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ORIGINAL PAPER

Gene expression of mesoderm-specific transcript is upregulated as preadipocytes differentiate to adipocytes in vitro

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Abstract Mesoderm-specific transcript (*Mest*) is a distinct gene associated with adipocyte differentiation and proliferation. The mechanisms regulating expression of the *Mest* gene are not established. Therefore, we investigated *Mest* gene expression during adipogenic differentiation in murine 3T3-L1 preadipocytes and adipose-derived stromal cells (ADCs) from C57BL/6J mouse adipose tissue. Expression of *Mest* mRNA increased significantly in 3T3-L1 cells during differentiation. Additionally, *Mest* mRNA expression levels were additively enhanced by the inhibition of DNA methylation. Expression levels of the *Mest* gene were also markedly elevated in differentiating ADCs in vitro. Additionally, we showed that *Mest* mRNA can be upregulated by increasing intracellular cAMP, and that *Mest* expression is suppressed by inhibition of protein kinase A (PKA). *Mest* expression was regulated through cAMP-dependent PKA pathways during differentiation of preadipocytes into adipocytes in vitro, supporting the critical role of *Mest* in proliferation and differentiation of adipocytes.

Keywords Mesoderm-specific transcript · Adipocytes · Adipogenic differentiation · Obesity · Protein kinase A

Introduction

Obesity increases the risk of metabolic disorders, including type 2 diabetes, atherosclerosis, hypertension, and hyperlipidemia [1]. The fat mass expansion that is associated with the progression to obesity results from adipocyte hypertrophy and hyperplasia [2]. Although microarray analyses conducted in white adipose tissue (WAT) have characterized some gene expression profiles linked to obesity [3–5], the roles of individual genes in the process of fat mass expansion have not yet been elucidated. Clarification of the mechanisms of regulation of relevant genes in vitro is key to understanding the mechanisms underlying the development of obesity.

Mesoderm-specific transcript/paternally expressed gene 1 (*Mest*, also called *Peg1*) is an imprinted gene transcribed only from the paternal allele [6]. The expression of *Mest* mRNA is markedly upregulated ($\sim$50-fold) in obese WAT mice, and *Mest* gene expression positively correlates with adipocyte size [4, 7, 8]. Additionally, the overexpression of *Mest* increases the size of adipocytes in vivo and promotes adipocyte differentiation in vitro [8].

Although *Mest* gene expression also regulates preadipocyte proliferation and adipocyte differentiation in 3T3-L1 cells [9, 10], the mechanisms regulating expression of the *Mest* gene are not established in vitro. In this research, we analyzed *Mest* expression patterns during preadipocyte-to-adipocyte differentiation in both 3T3-L1 and adipose-derived stromal cells (ADCs) from C57BL/6J mice.

Materials and methods

Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): 3-isobutyl-1-methylxanthine (IBMX), 5-aza-2′-deoxycytidine (5-aza-dC), H89 dihydrochloride hydrate and Oil Red O. RNAiso Plus, a

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FastPure DNA Kit, a MethylEasy *Xceed* Rapid DNA Bisulphite Modification Kit and TaKaRa EpiTaq HS (for bisulfite-treated DNA) were obtained from Takara Bio (Otsu, Japan).

Animals

C57/BL6J mice were purchased from Japan SLC (Hamamatsu, Japan). All experimental procedures were approved by the Animal Care and Use Committee of Tokushima Bunri University, and conformed to the guidelines of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Cultivation of ADCs from mouse adipose tissue

There are many possible binding sites for the sex-determining region of the Y chromosome (SRY) protein to the *Mest* gene 5′-upstream [11]. Therefore, female mouse ADCs were used to eliminate the male-specific influence of the SRY protein. Preparation and culture of ADCs were performed as previously described [12]. Briefly, ADCs were isolated from the murine fat pads of 5-day-old female C57BL/6J mice. ADCs that had been cultured in DMEM containing 10 % calf serum (CS; MP Biomedicals, Solon, OH, USA) for more than ten passages were used. To determine the sex of the ADCs, PCR analysis of the *Sry* was conducted on genomic DNA isolated from tail samples of the mice.

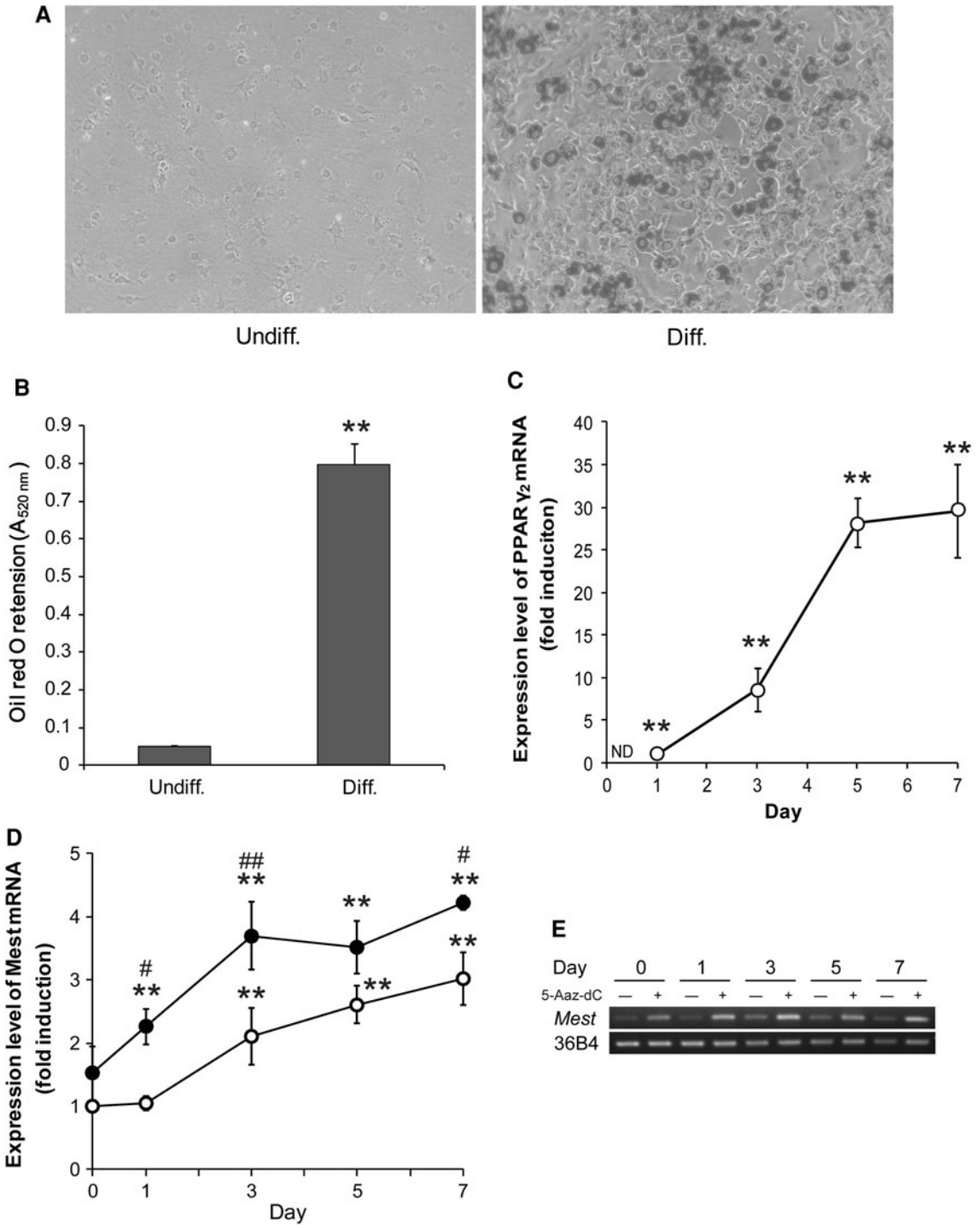
Induction of preadipocyte-to-adipocyte differentiation

ADCs and 3T3-L1cells were seeded at a density of 3×10^5 cells/well onto a 6-well plate (in 2 ml of DMEM containing 10 % CS), and precultured under these conditions for 2 days. After this preculture, adipogenic differentiation in the ADCs was induced on day 0 by replacing the original culture medium with DMEM containing 10 % fetal bovine serum (FBS) supplemented with a DMI cocktail (1 μg/ml insulin (INS), 1 μM dexamethasone (DEX), and 0.5 mM IBMX). Two days after stimulation of differentiation (day 2), the culture medium was changed to DMEM containing 10 % FBS supplemented with 1 μg/ml INS, and cells were cultured for two more days. At day 4, the medium was replaced with DMEM containing 10 % FBS. During all these stages of culture, the medium was changed every 2 days. To study the effects of the inhibition of DNA methylation, a subgroup of 3T3-L1 cells was pretreated with 5 μM 5-aza-dC during from day −1 to day 0. For cAMP-dependent protein kinase A (PKA) pathways, ADCs were treated with adenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP; Merck, Nottingham, UK) for 24 h. H89 was added 2 h prior to ADCs that had been given a single treatment of 0.5 mM IBMX for 24 h. An MTT assay was employed to confirm that no cellular cytotoxicity was caused by any of the reagents used.

Fig. 1 Expression of *Mest* in 3T3-L1 cells during adipogenic differentiation. **a** Representative oil red O staining of lipid droplets on day 7 in undifferentiated (*undiff.*) and differentiated (*diff.*) 3T3L1 cells. **b** Quantitative determination of Oil Red O (a measure of lipid accumulation) in undifferentiated (*undiff.*) and differentiated (*diff.*) 3T3-L1 cells. Data are expressed as mean ± SD ($n = 6$). *Double asterisks* represents significant oil red O retention in differentiated cells at the $P < 0.01$ level. **c** The levels of *PPARγ2* expression in 3T3-L1 cells during adipogenic differentiation were measured by RT-PCR and expressed as ratios of the values measured in 3T3-L1 cells on day 1 ($n = 3$). *ND* not detected. *Double asterisks* represents significant differences in untreated cells on day 0 at the $P < 0.01$ level. **d** The gene expression values in control 3T3-L1 cells and in cells that had been pretreated with 5 μM 5-aza-dC (denoted by *open circles* and *closed circles*, respectively) are expressed as ratios of values measured in untreated 3T3-L1 cells at day 0, normalized against *36B4* mRNA levels. Data are expressed as mean ± SD ($n = 3$). *Double asterisks* represents significance compared to untreated cells at day 0 ($P < 0.01$); *single* and *double hash symbols* represent significant differences in gene expression levels in cells pretreated with 5-aza-dC compared with untreated cells ($P < 0.05$ and $P < 0.01$ respectively). **e** Representative RT-PCR results of Mest expression in 3T3-L1 cells. **f** The genomic region around the transcriptional start site of the Mest gene (−175 to +92 bp) shows as bisulfite-modified DNA (cytosines are converted to thymines). Methylated and unmethylated CpGs are shown as *boxed* YGs (*Y* pyrimidine). The *Fnu*4HI cutting site (GC/NGC) at the *gray highlighted area* is maintained, since the methylated cytosines in CpG islands are not converted to uracils by bisulfite treatment. The PCR products (319 bp) are cleaved into 222- and 97-bp fragments by *Fnu*4HI. However, *Fnu*4HI will not digest if the site is converted to any one of the sequences GTGGT, GTGGC or GCGGT by bisulfite-PCR. *Underlined* are primer sequences for PCR amplification. Exon1 begins at the double underline. **g** Genomic DNA was prepared from control 3T3-L1 cells or 3T3-L1 cells pretreated with 5 μM 5-aza-dC on days 0 and 7 after the induction of differentiation. The DNA was treated with bisulfite and amplified by PCR using a specific primer set. The PCR products were incubated in the presence or absence of *Fnu*4HI. The fragments were subjected to electrophoresis on 2 % agarose gel stained with ethidium bromide. Representative results from the COBRA assay are shown. **h** DNA methylation state of CpG islands around the *Mest* gene transcription starting site (TSS) (−175 to +92 bp) in control 3T3-L1 cells and 3T3-L1 cells that had been pretreated with 5 μM 5-aza-dC. Data points are taken from days 0 and 7 after the induction of differentiation. The percentage of methylated DNA was calculated from the band intensity ratio between the *Fnu*4HI-cleaved PCR fragments and the total amount of PCR product. Data are expressed as mean ± SD ($n = 3$). *Double hash symbols* indicates significant difference compared to control ($P < 0.01$)

Oil red O staining

Lipid accumulation was evaluated by Oil Red O retention. The cells were fixed with 4 % paraformaldehyde and stained with 3 mg/ml Oil Red O in 60 % isopropyl alcohol. To quantify the retention of Oil Red O, stained adipocytes were extracted with 4 % Nonidet P-40 (Nacalai Tesque, Kyoto, Japan) in isopropyl alcohol for 15 min, and the

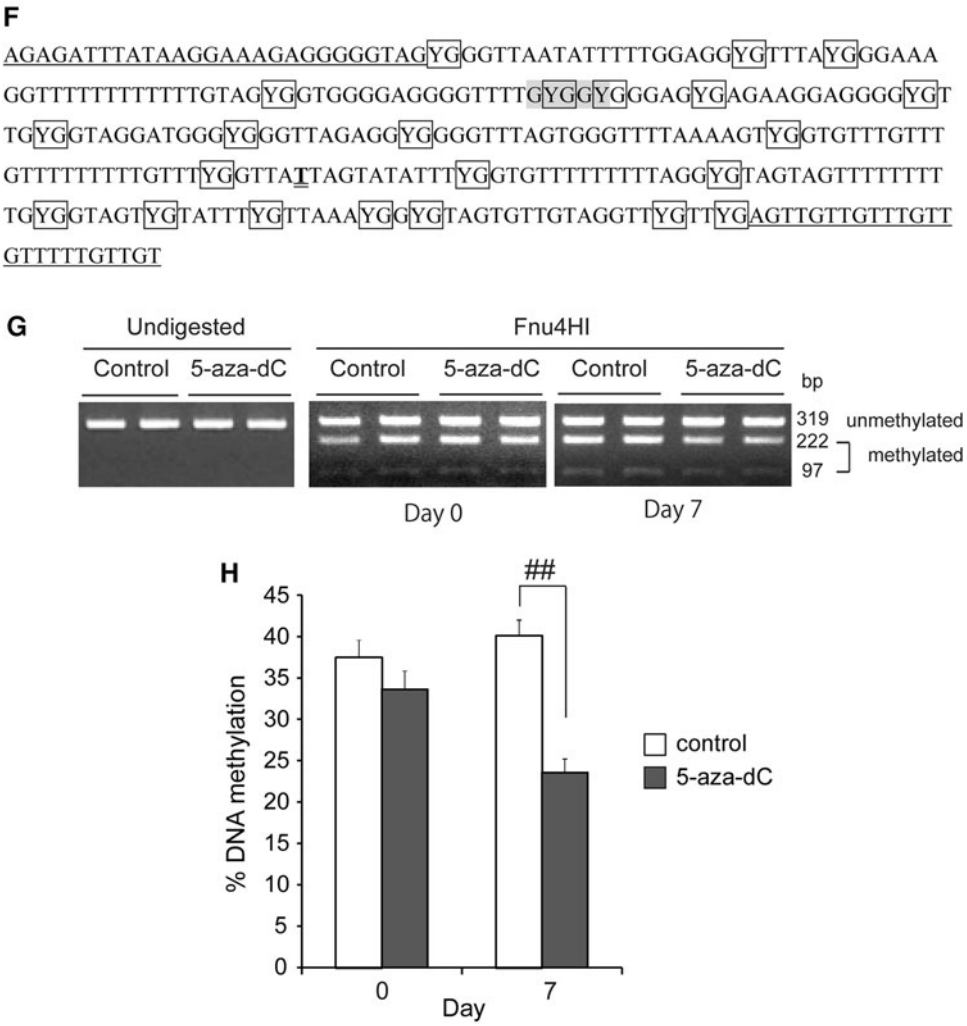


absorbance of the eluted Oil Red O was measured at 520 nm with a microplate spectrophotometer.

RNA isolation and RT-PCR analysis

Total RNA was isolated from the cells using RNAiso Plus. For reverse transcription (RT), 2 μg of RNA from each sample was reverse-transcribed with a High-Capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA). PCR was performed within a linear range of amplification using the following selected primer sets: 5′-AA CCGCAGAATCAACCTGCT-3′ and 5′-CGAAGAAA TTCATGAGCCTGG-3′ for mouse *Mest* (GenBank ID: NM_008590); 5′-GGTGAAACTCTGGGAGATTC-3′ and 5′-TAATAAGGTGGAGATGCAGG-3′ for mouse peroxisome proliferator-activated receptor (*PPAR*) γ2 (GenBank

Fig. 1 continued



ID: NM_011146); 5′-ATCCGGATCAAACGTGGCT-3′ and TGCTCGAAACGGAAAAGGTT-3′ for mouse CCAAT/enhancer binding protein (*C/EBP*) *β* (GenBank ID: NM_009883); 5′-GAGATTCGGGATATGCTGTTGG -3′ and 5′-GTTGTCAAACACCTGCTGGATG-3′ for *36B4* (GenBank ID: NM_007475) as an internal control housekeeping gene. PCR products were analyzed by 2 % agarose gel electrophoresis and visualized with ethidium bromide with an LAS 4000 mini image analyzer (Fujifilm, Tokyo, Japan).

Combined bisulfite restriction analysis (COBRA)

Genomic DNA was isolated from 3T3-L1 cells using a FastPure DNA Kit. Then, 1 μg of genomic DNA was bisulfite-converted using a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit. Bisulfite-modified DNA was amplified by TaKaRa EpiTaq HS (for bisulfite-treated DNA) using the following selected primer sets, which were prepared according to the method of Koza et al. [13]: 5′-GAGATTTATAAGGAAAGAGGGGGTAG-3′ and 5′-ACAACAAAAACAACAAACAACAACT-3′. Amplified products were digested for 12 h at 37 °C with the restriction enzyme *Fnu*4HI (New England BioLabs, Ipswich, MA, USA). The DNA fragments that had been treated with the restriction enzyme were then analyzed by 2 % agarose gel electrophoresis and visualized with ethidium bromide using a CS-Analyzer ver. 3.0 (ATTO, Tokyo, Japan). The percentage of methylated DNA was calculated from the band intensity ratio between the *Fnu*4HI-cleaved PCR fragments and the total amount of PCR product.

Statistical analysis

Ekuseru-Toukei 2006 for Windows (Social Survey Research Information, Tokyo, Japan) was used for statistical analysis. Datasets were compared for significant differences by one-way analysis of variance using Dunnett's test or a paired Student's *t* test.

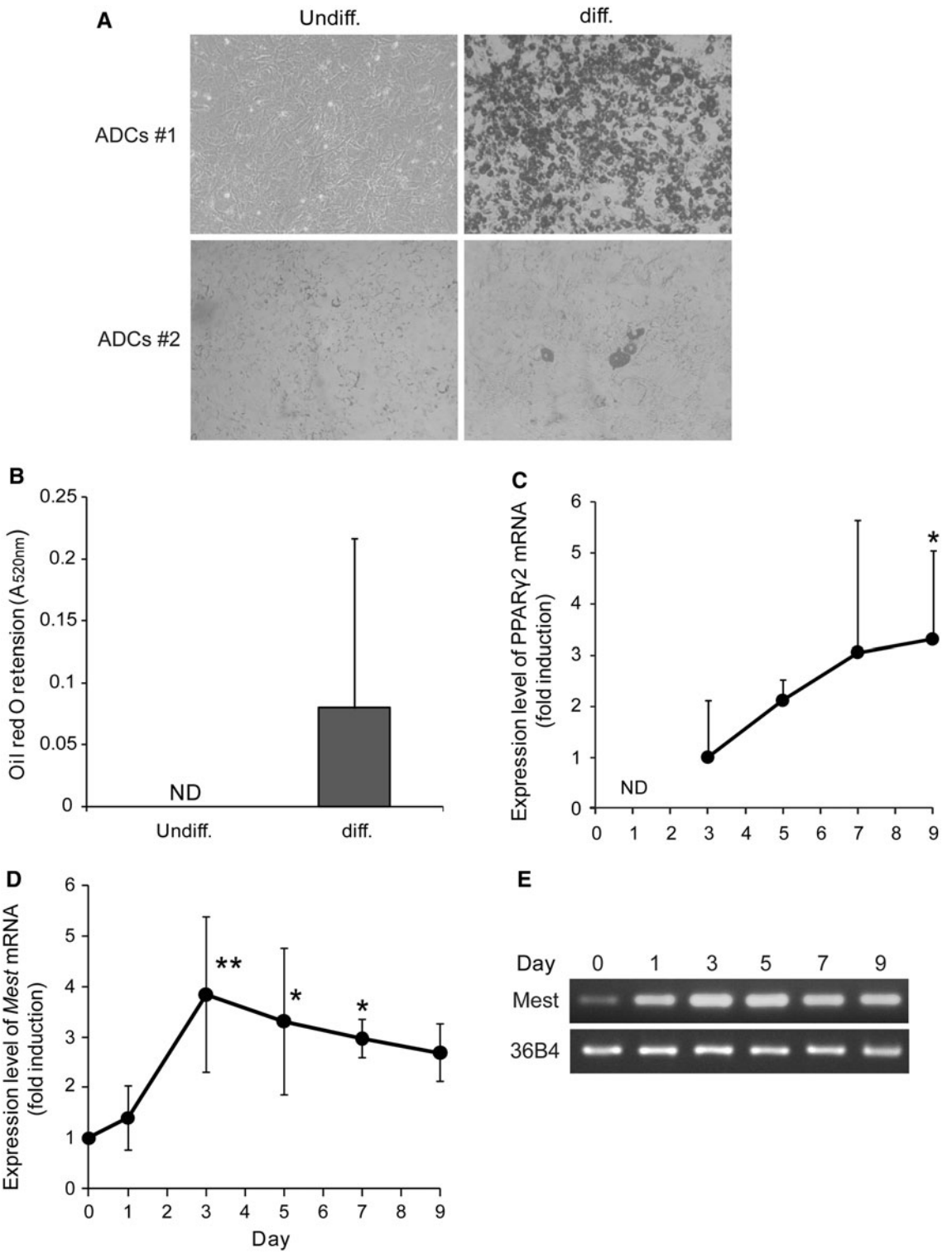


Fig. 2 Expression of *Mest* in ADC cells during adipogenic differentiation. **a** Representative Oil red O staining on day 9 in undifferentiated (*undiff.*) and differentiated (*diff.*) ADCs derived from 2 mice (ADCs #1 and ADCs #2). Data were taken from two C57BL/6J mice. **b** Retention of Oil Red O (a measure of lipid accumulation) in undifferentiated (*undiff.*) and differentiated (*diff.*) ADCs on day 9. **c** *PPARγ2* and **d** *Mest* gene expression in ADCs during adipocyte differentiation. The levels of gene expression measured in ADCs are the combined results from 4 female pups during adipogenic differentiation; these results were normalized against *36B4* mRNA expression. The ratios of test values to control values were then calculated from measurements taken on day 0 for *Mest* and day 3 for *PPARγ2*, and expressed as mean ± SD ($n = 4$). *ND* = not detected. *Single* and *double asterisks* represent significance in the test values compared to control values measured on day 0 ($P < 0.05$ and $P < 0.01$ respectively). **e** Representative RT-PCR results for *Mest* and *36B4* expression in ADCs

Results

Induction of *Mest* gene in 3T3-L1 cells and ADCs during adipogenic differentiation

3T3-L1 adipocytes exhibited significant lipid accumulation when differentiation was stimulated with a DMI cocktail (Fig. 1a, b). The cells also showed increased expression of the adipogenic marker *PPARγ2* (Fig. 1c). Under these conditions, RT-PCR analyses revealed that the expression level of *Mest* mRNA increased more than twofold between days 0 and 3 after stimulation of 3T3-L1 differentiation (Fig. 1d, e), and that these levels remained steady until day 7. *Mest* is an imprinted gene that is expressed on the paternal allele. The expression of *Mest* from the maternal allele in mice is completely silenced by DNA hypermethylation of the 5′-region. In order to further investigate the effect of DNA methylation on *Mest* gene expression, we looked at the level of DNA methylation of CpG islands around the *Mest* gene transcription starting site (TSS) using COBRA (Fig. 1f–h). The percentage of DNA methylation around the *Mest* gene TSS was not significantly different from day 0 to day 7 in untreated 3T3-L1 cells or between control 3T3-L1 cells and 3T3-L1 cells that had been pretreated with 5 μM 5-aza-dC, a DNA methylation inhibitor, on day 0 (Fig. 1g–h). 3T3-L1 cells pretreated with 5 μM 5-aza-dC showed a 1.4- to 2-fold increase in *Mest* gene expression compared to the untreated control group 3 days after the induction of differentiation in the two treatment groups (Fig. 1d, e). This additional increase in *Mest* gene expression stimulated by 5-aza-dC was negatively correlated with the methylation state around the TSS of the *Mest* gene at day 7 (Fig. 1g, h); the methylation rate of the control cells was 1.7-fold greater than that of the 5-aza-dC-pretreated cells. Although the DMI cocktail also stimulated lipid accumulation (Fig. 2a, b) and *PPARγ2* expression (Fig. 2c) in ADCs derived from C57/BL6J mice, each individual ADC had a different response to the adipogenic inducer. However, *Mest* mRNA expression was significantly upregulated by induction of adipocyte differentiation, and mRNA expression peaked on day 3 (Fig. 2d, e).

IBMX stimulates expression of *Mest* gene in 3T3-L1 cells

In order to determine which component of the DMI cocktail was required for *Mest* gene upregulation, 3T3-L1 preadipocytes pretreated with 5 μM 5-aza-dC and ADCs were incubated in the presence or absence of 1 μg/ml INS, 1 μM DEX, or 0.5 mM IBMX for 24 h. As shown in Fig. 3a, b, the *Mest* mRNA level was significantly elevated by IBMX.

Mest gene expression was upregulated via cAMP-dependent PKA pathways

IBMX is one of the most potent inhibitors of cyclic nucleotide phosphodiesterases (PDEs), which are of key importance in cAMP-dependent signaling pathways. Therefore, we next examined the effect of 8-Br-cAMP, a membrane-permeable cAMP analog, and H89, a specific inhibitor of PKA, on *Mest* gene expression. One ADC cell

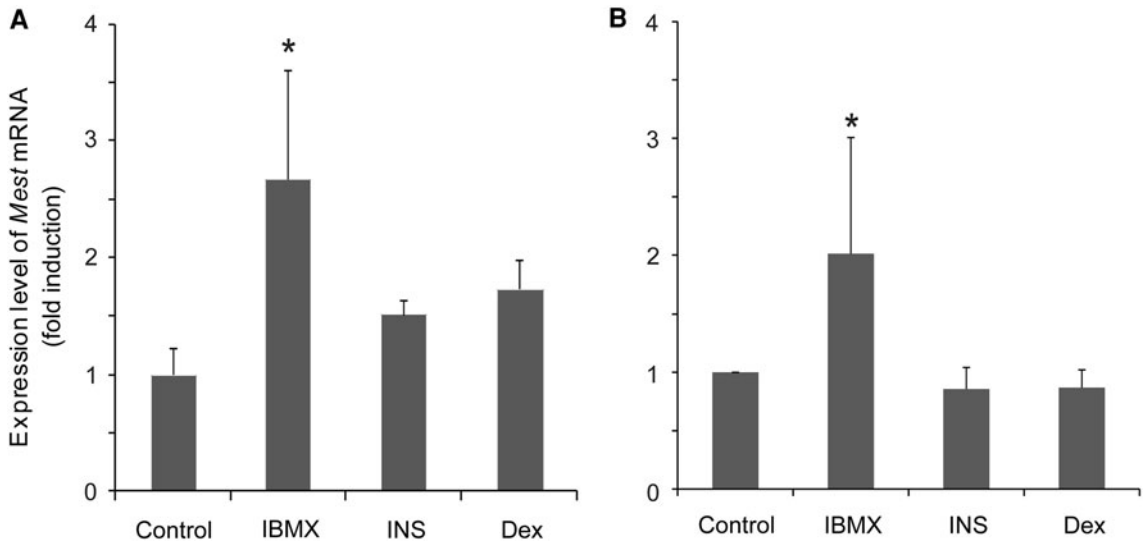


Fig. 3 Effect of the components of the adipogenic inducer on expression of *Mest* in 3T3-L1 cells and ADCs. **a** 3T3-L1 preadipocytes and **b** ADCs were incubated in normal culture medium (*control*) in the presence or absence of *IBMX* (0.5 mM), *DEX* (1 μM), and *INS* (1 μg/ml) for 24 h. The gene expression values are expressed as ratios of values measured in controls, and normalized against *36B4* mRNA levels. Data are expressed as mean ± SD ($n = 3$). *Asterisk* indicates significant difference compared to control at the $P < 0.05$ level

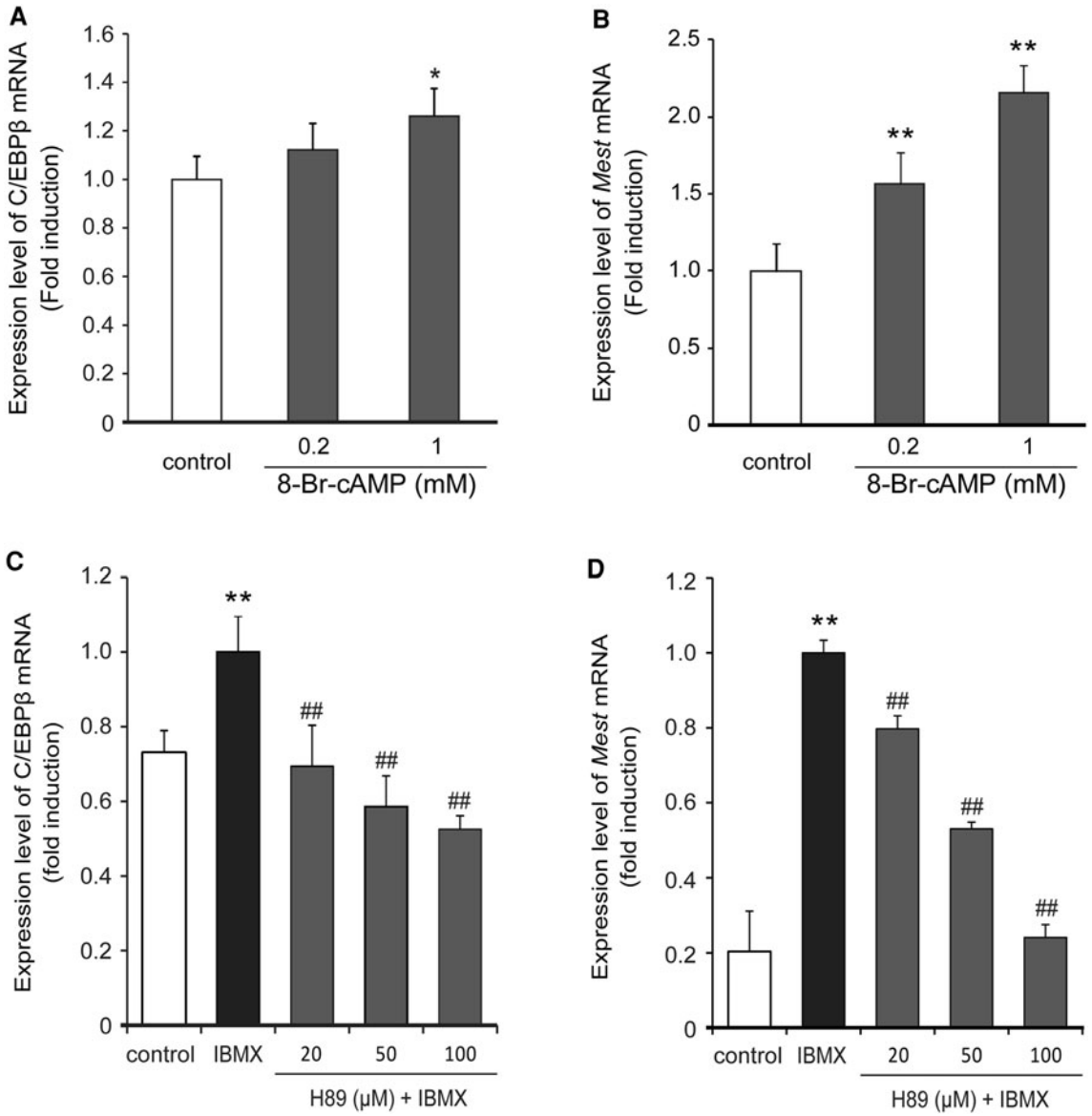


Fig. 4 Effects of the cAMP analog and PKA inhibitor on expression of *Mest* mRNA in ADCs. **a**, **b** ADCs were treated with 8-Br-cAMP at the indicated concentration for 24 h. **c**, **d** ADCs were pre-treated with H89 at the indicated concentration for 2 h. Following this, ADCs were incubated in culture medium containing 0.5 mM IBMX for 24 h. The expression of *C/EBPβ* (**a**, **c**) and *Mest* (**b**, **d**) in ADCs was calculated as the ratio of treatment values to control values, normalized against *36B4* mRNA levels and expressed as the mean ± SD ($n = 3$). *Single* and *double asterisks* indicate significant differences compared to controls at the $P < 0.05$ and $P < 0.01$ levels, respectively. *Double hash symbols* represents significance differences in gene expression levels in cells treated with 0.5 mM IBMX compared with untreated cells ($P < 0.01$)

line was used because it have been shown to be the most sensitive to IBMX when *Mest* expression was measured. The expression level of *C/EBPβ* mRNA is known to be regulated by the cAMP/PKA pathways. Both *C/EBPβ* and *Mest* mRNA increased in a dose-dependent manner in ADCs when treated with 8-Br-cAMP (Fig. 4a, b). In addition, IBMX-induced *C/EBPβ* and *Mest* expression in ADCs was significantly suppressed in a dose-dependent manner by pretreatment with H89 (Fig. 4c, d).

Discussion

Microarray analysis has revealed that the expression of *Mest* increased 24-fold in obese mice, the largest increase seen in 792 upregulated WAT-related genes [4]. Additionally, Nikonova et al. [7] have shown that *Mest* expression is strongly correlated with fat mass, and is induced within 2 days in mice by feeding them high-fat diets, occurring even before the mice become obese. In in

vitro 3T3-L1 cultures, *Mest* gene overexpression facilitates preadipocyte proliferation and differentiation into adipocytes, and *Mest* siRNA suppresses adipogenic differentiation [8–10]. Because obesity results from fat mass expansion, which occurs through the proliferation, differentiation and hypertrophy of adipocytes, it is possible that *Mest* is important in the development of adipose tissue and fat mass. Thus, it may be a useful diagnostic marker in evaluating the risk of obesity or for the establishment of preventive weight targets in the management of adult-onset metabolic diseases such as metabolic syndrome.

In the present study, *Mest* mRNA levels were increased in mouse preadipocytes during adipogenic differentiation (Figs. 1, 2). In addition, we also observed increased expression levels of the *Mest* gene in differentiating primary preadipocytes derived from another mouse strain, 129/SV [12]. These data raise the possibility that *Mest* mRNA levels vary in differentiating preadipocytes in mouse adipose tissue. Although *Mest* overexpression augmented the expression levels of *PPARγ2*, a master regulator of adipogenesis and lipid accumulation [8], and *Mest* knockdown by RNAi suppressed adipocyte differentiation in 3T3-L1 cells [10], *Mest* expression levels were poorly correlated with both $PPAR\gamma_2$ mRNA levels and lipid accumulation in ADCs (Fig. 2). This suggests that the *Mest* gene may only contribute indirectly to adipogenic differentiation. In fact, the *Mest* gene is not essential for adipogenesis in vivo, as indicated by the fact that significant adipose tissue formation still occurs in *Mest*-deficient mice [7, 14].

In recent years, the possible role of epigenetic mechanisms in metabolic disorders has been recognized [15]. *Mest* is an imprinted gene that is exclusively transcribed from the paternal allele, while the maternal allele is frequently hypermethylated. Koza and Nikonova and colleagues [4, 7] have shown that the variation in body weight in diet-induced obesity in male B6 mice strongly correlated with *Mest* expression levels in the adipose tissue of the mice. Although loss of imprinting of the *Mest* gene has been observed in some human tumor cells [16, 17], it has been shown that significant DNA methylation does not occur with the upregulation of *Mest* gene expression in the obese mouse model WAT [13, 18]. *Mest* gene expression may contribute to individual differences in obesity mediated by other epigenetic events. In this study, *Mest* gene expression in 3T3-L1 cells was significantly increased by adipogenic stimulation, but the degree of elevation of expression was lower than that in ADCs. We evaluated the level of DNA methylation around the *Mest* gene TSS in 3T3-L1 cells (Fig. 1g, h). The DNA methylation state of 3T3-L1 cells that were not treated with 5-aza-dC did not change before or after adipogenic differentiation. This suggests that DNA demethylation does not contribute to the increase in *Mest* mRNA levels seen in 3T3-L1 cells undergoing differentiation to adipocytes. 5-aza-dC induced DNA demethylation of a silent allele of the *Mest* gene, and the methylation levels around the *Mest* gene TSS negatively correlated with the level of *Mest* gene expression. Therefore, the *Mest* expression level, which is increased by pretreatment with 5-aza-dC during adipogenic differentiation, would be a combination of the expressed *Mest* mRNA derived from both the paternal and demethylated maternal alleles. These results differ from the data presented by Kamei et al. [19] and Okada et al. [18], in which *Mest* gene expression did not increase during 3T3-L1 differentiation, although upregulation of *Mest* mRNA levels was seen in response to pretreatment with 5-aza-dC. One observation of relevance to this is that epigenetic mutations may occur frequently in cultured 3T3-L1 cells. Therefore, we suggest that the 3T3-L1 in vitro model of adipogenesis might warrant further investigation via an epigenetic study, with particular reference to the role of DNA methylation.

In many experimental designs, the induction of adipogenesis in vitro is initiated by treating 3T3-L1 cells with medium containing a DMI cocktail. This mixture of reagents induces two transcription factors, *C/EBPβ* and *C/EBPδ*. These transcription factors then induce the expression of the key adipogenic transcription factors, *C/EBPα* and *PPARγ2* [20, 21]. IBMX increases intracellular cAMP levels by the inhibition of PDEs, and activates the PKA/cAMP-responsive element binding protein (CREB) signaling cascade, which, in turn, induces expression of *C/EBPβ* and *C/EBPδ* [22, 23]. Conversely, cAMP-dependent PKA pathways are also involved in the suppression of adipocyte differentiation, and cAMP-induced Epac activation may instead be required for differentiation [24, 25]. In this study, *Mest* gene expression was upregulated by treatment with IBMX, a PDE inhibitor, and 8-Br-cAMP, a cAMP analog. The IBMX-induced increase of *Mest* mRNA was suppressed by treatment with the PKA inhibitor, H89. *Mest* knockdown in differentiating 3T3-L1 cells has been shown to decrease expression levels of *C/EBPα* and *PPARγ2* through activation of the Wnt/β-catenin signaling pathway, leading to the suppression of differentiation [10]. PKA activates the Wnt signaling pathway by inhibiting the degradation of β-catenin [26]. Therefore *Mest* gene upregulation by the cAMP/PKA cascade may contribute to the inhibition of some pathways antagonistic to adipogenic differentiation, such as PKA-induced Wnt/β-catenin. Further analysis of the expression pattern of the *Mest* gene in adipocytes may provide clues to the mechanism of adipocyte hypertrophy and hyperplasia in obesity.

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