Published in final edited form as:
Epigenetics. 2009 October 1; 4(7): 512–518.

Inter-individual variation of dietary fat-induced mesoderm specific transcript in adipose tissue within inbred mice is not caused by altered promoter methylation

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Abstract

Mesoderm specific transcript (*Mest*), an imprinted gene associated with fat mass expansion under conditions of positive energy balance, shows highly variable expression (~80-fold) in white adipose tissue (WAT) of C57BL/6J (B6) mice fed an obesogenic diet. Since B6 mice are essentially genetically invariant and *Mest* is known to be regulated by CpG methylation within its immediate proximal promoter, the large variability in its expression in adipose tissue has the hallmarks of being controlled via an epigenetic mechanism. In this study, bisulfite sequencing and allelic discrimination analyses were performed to determine whether variations in CpG methylation within the *Mest* promoter were associated with its expression. Results showed no relationship between CpG methylation in the *Mest* promoter and high versus low expression in either WAT or isolated adipocytes; and, experiments using a single nucleotide polymorphism in the *Mest* promoter region between B6 and *Castaneus* mice showed the expected pattern for an imprinted gene with all maternal alleles being methylated. These data suggest that mechanisms independent of the CpG methylation status of the *Mest* promoter must underlie the control of its expression during adipose tissue expansion.

Keywords

imprinting; methylation; epigenetics; genetics; obesity; adiposity

Introduction

Studies as early as the 1930's with *Axin*^{Fu}, and more recently with agouti (*A*^{vy}), have shown that variable expressivity of genes are not always linked to genetics [1-3]. The *A*^{vy} and *Axin*^{Fu} genes are regulated by CpG methylation within an intercisternal A particle that is associated with promoter function [4-6]. Non-genetic variations of complex metabolic phenotypes such as obesity have also been described [7, 8]. We have identified a number of genes expressed in adipose tissue that are associated with variations in the development of adiposity among individual mice within a genetically invariant mouse population; however, the gene with the most variable expression (~80-fold) was mesoderm specific transcript (*Mest*)[8].

During development the paternal allele of *Mest* is unmethylated and the maternal allele is methylated within a CpG island encompassing the promoter region and part of exon 1 [9, 10]. Repression of *Mest* transcription by methylation of the immediate proximal promoter on the paternal allele has been shown to occur during embryonic metanephric development [11] and in studies with reporter constructs [12, 13]. Although the biochemical function of *Mest* has not

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been elucidated, it has sequence and structural homology to α/β hydrolases, a family of proteins including lipases and acyltransferases that have a wide variety of catalytic activities [14, 15]. MEST has been localized to the endoplasmic reticulum/Golgi apparatus where it may function to promote lipid accumulation under conditions of excess caloric intake [16].

Because of the high variability of adipose tissue *Mest* expression, its association with susceptibility for the development of diet-induced obesity in the absence of genetic variation and molecular evidence that the methylated state of regulatory elements in the promoter region control transcription, *Mest* is an ideal target gene to investigate epigenetic regulation of gene expression via methylation. In this study we focused our investigation on determining whether variations of CpG methylation within the immediate proximal *Mest* promoter are associated with its expression in adipose tissue of mice fed a high fat diet. Bisulfite sequencing analyses of DNA from adipose tissue or adipocytes from adipose tissue with low and high *Mest* expression showed no evidence of an association between the degree (characteristics) of promoter methylation and *Mest* gene expression. Additionally, *Mest* shows the normal allelic methylation pattern for an imprinted gene in both the adipocyte and SV fractions of adipose tissue. Thus, the 80-fold range of *Mest* expression among individual mice must be determined by a transcriptional mechanism that over-rides the gene dosage effects that would result from a simple loss-of-imprinting.

Methods

Animals

C57BL/6J (B6) and *Castaneus* (CAST) mice were bred at the Pennington Biomedical Research Center from stocks that originated from The Jackson Laboratory (Bar Harbor, ME, USA). Animal rooms were maintained at 22-24°C with a 12 hour light/dark cycle. Breeders housed in plastic pens with corncob bedding were fed a breeder diet (LabDiet 5015; PMI, St Louis, MO, USA) *ad libitum*. After weaning, mice were fed a standard low fat chow diet (LabDiet 5053; PMI) *ad libitum* until 8 weeks of age. At 7 weeks of age mice were singly housed and at 8 weeks of age mice were fed *ad libitum* a high saturated fat diet (D12331; Research Diets, New Brunswick, NJ, USA) for periods of time ranging from 1 to 4 weeks. Hybrid (CAST X B6)F1 and (B6 X CAST)F1 mice were fed a high saturated fat diet (D12331) for 2 weeks prior to sacrifice and isolation of adipocyte and SV cell fractions for analyses. All protocols have been approved by the Pennington Biomedical Research Center's Institutional Animal Care and Use Committee.

Tissue fractionation

Inguinal or epididymal adipose tissue was minced and digested with collagenase class I (2 mg/ml; Worthington Biochemical Corp., Freehold, NJ, USA) in prewarmed 37°C HBSS in a shaking water bath for 1 hour at 37°C. The tissue homogenate was filtered through a 100 μm cell strainer (Becton Dickinson Labware, NJ, USA) and the cells isolated by centrifugation at 360 x g for 10 minutes. The pellet containing the stromal-vascular (SV) cells and the upper part (floating) mature adipocytes (AD) were separated and washed 1X with HBSS (5ml) followed by centrifugation at 360x g for 10 min. Supernatants were removed and DNA was isolated from the purified SV and AD cells.

Phenotyping and sample preparation

RNA was isolated from adipose tissue using TRI-Reagent (Molecular Research Center Inc. Cincinnati, OH, USA) with modifications to remove DNA as described by Koza et al [8]. Quantitative RT-PCR using TaqMan probes and primers (Applied Biosystems, Foster City, CA, USA) for murine *Mest* and cyclophilin B (*Ppib*) was performed as previously

described [8]. Briefly, purified RNA diluted to 5 ng/ul in RNase-free H_20 and pooled adipose tissue RNA that was serially diluted for use as a standard curve and quality control were loaded as 3 ul aliquots onto a 384-well optical assay plate. Following the addition of 7 ul of an assay mix containing ABI One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) and gene-specific primers and probe quantitative gene expression was analyzed using an ABI 7900HT Sequence Detection System. Analyses of gene expression were performed as single assays and gene expression was normalized to cyclophilin b (*Ppib*). Probes and primers were used at a concentration of 0.775 uM and 0.2 uM respectively for all reactions. Probe and primer sequences were as follows: *Mest*: probe, 5′-*FAM*-CCG CGG TCC ACA GTG TCG ATT CT-*BHQ*-3′; forward primer, 5′-GAT CCT ATA AAT CCG TAT CCA GAG TTT T-3′; reverse primer, 5′-GGG TAG TGG CTA ATG TGG TCA TC-3′; and for cyclophilin b: probe, 5′-*FAM*-ATC CTT CAG TGG CTT GTC CCG GCT-*BHQ*-3′; forward primer, 5′-GGT GGA GAG CAC CAA GAC AGA-3′; reverse primer, 5′-GCC GGA GTC GAC AAT GAT G-3′. DNA for bisulfite sequencing analyses was isolated from adipose tissue, or the AD and SV fractions of adipose tissue by digesting tissue or cells overnight at 55°C in digestion buffer (50 mM Tris pH 8, 100 mM EDTA, 100 mM NaCl and 1% SDS) containing 40 ug/ml proteinase K. DNA was extracted by mixing an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; Amresco, Solon, CA USA) to the digested tissue/cells and centrifuging in a microfuge at 4°C for 20 min at 20,000 x g to separate the aqueous from the organic phases. The aqueous phase was extracted with an equal volume of chloroform/isoamyl alcohol (24:1; Amresco) and re-isolated from the organic phase after centrifugation as above. DNA was precipitated from the aqueous phase with addition of 2V 100% EtOH, pelleted by centrifugation, washed 1X with 600 ul 70% EtOH, and air dried for 15 min. The DNA pellet was re-solubilized in 300 ul 0.1X Tris-EDTA (TE) buffer (pH 8.0) and re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) as described above. Prior to precipitation of DNA, 50 ul of 3M sodium acetate (pH 5.2) was added to the re-extracted aqueous fraction and the volume was adjusted to 0.5 ml. DNA was precipitated and washed as previously described and the dried DNA pellet was dissolved in 10-20 ul 0.1X TE. DNA concentration and purity was determined using a NanoDrop Analyzer (NanoDrop Technologies, Wilmington, DE, USA).

Bisulfite Sequencing

Bisulfite sequencing was performed essentially as described by Olek et al [17] with some modifications. Purified DNA was denatured at 50°C in the presence of 0.3M NaOH followed by addition of hot low melting temperature agarose. DNA-agarose beads were produced by adding 5 ul aliquots of this mixture into cold mineral oil. The beads were incubated at 50°C for 4 hours in presence of 2.5M sodium metabisulfite/125 mM hydroquinone, washed 4 times (15 min each) with 1 ml 0.5X TE, desulphonated by washing 2X (15 min each) with 0.5 ml 0.2M NaOH, and washed 3X (15 min each) with 1 ml 0.5X TE followed by 2X (15 min each) with dH_2O. Primers designed in regions containing no CpGs were be used for the PCR amplification reaction. The primers for each of the 3 genomic regions are as follows: *Region I*; forward, 5′-GGTAT TTTTA GTGTT AGTTG GGTGG T-3′; reverse, 5′-CCTTA AAAAT CATCT TTCAC ACCTT-3′: *Region II*; forward, 5′-GAGAT TTATA AGGAA AGAGG GGGTA G-3′; reverse, 5′-ACAAC AAAAA CAACA AACAA CAACT-3′: *Region III*; forward, 5′-GAGTT GTTGT TTGTT TTTGT TG-3′; reverse, 5′-CAACC TTTAA ATAAA AATTT TTACC TCC-3′. To encompass a SNP polymorphism located +366 bp downstream from the TSS of Mest, an alternative reverse primer with the sequence 5′-CAC CAA ACA TTA CCA AAA TTC TC-3′ was used in the amplification reaction with the forward primer for genomic *Region III*. PCR amplicons were TA cloned into pCR4-TOPO as described by the manufacturer

(Invitrogen Life Technologies, Carlsbad, CA) and individual clones sequenced to detect C/T conversions in non-methylated CpG dyads.

Results

Non-Genetic Variability of Adipose Tissue *Mest* expression in Mice

To determine whether differences in CpG methylation are associated with variations of *Mest* expression in adipose tissue, *Mest* was measured in RNA from inguinal fat of 112 male B6 mice after feeding a high fat diet for 4 weeks. From this cohort, 14 mice with the lowest (1.61 ± 0.21 AU/cyclo) and highest expression (37.2 ± 2.6 AU/cyclo) were selected for further analyses (Fig 1).

To aid in the identification of genomic regulatory sequences to be analyzed by bisulfite sequencing, conserved genomic regions were identified within ~16 kb of the promoter region of *MEST* from both mouse and human. This analysis detected at least 18 genomic regions having significant sequence homology with 5 of these regions, including those known to be in the differentially methylated region (DMR) of *Mest*, having conserved CpG motifs (Fig 2). Three genomic regions (I, −1100 to −818 bp; II, −175 to +92 bp; and III, +116 to +334 bp) within the DMR of the *Mest* promoter that encompass and are immediately upstream from the transcriptional start site (TSS) and the open reading frame were selected for bisulfite sequence analyses based on high conservation between the human and mouse sequences and density of CpG islands (Fig 2).

DNA from intact adipose tissue shows no variation in *Mest* promoter methylation

Oligonucleotide primers (see Methods) were designed to amplify *Mest* promoter regions I, II and III in bisulfite-treated inguinal fat DNA as shown in Figure 2. Bisulfite sequencing results in Figure 3 show the CpG methylation patterns across all 3 *Mest* promoter regions for each allele as well as the average among the DNA's selected for the low (LM) and high (HM) *Mest* expression groups. The data for *Mest* promoter region II, which encompasses the TSS, contains the average of 14 mice that were selected for the LM and HM groups (Fig. 1) whereas promoter regions I and III used 6 animals per group. A minimum number of 11 clones were sequenced for each DNA. Results showed no association ($P \geq 0.45$) between methylation for any of the CpG dyads within the 3 genomic regions of the *Mest* promoter in DNA isolated from adipose tissue with *Mest* gene expression (Fig 3). Furthermore, approximately 50% of the alleles were methylated in all of the genomic regions resulting in a pattern consistent for an imprinted gene.

Mest promoter methylation patterns differ between adipocytes and SV cells

Since adipocytes have been estimated to account for less than 20% of the total number of cells within an adipose tissue depot in rodents [18-20]; and, *Mest* is predominantly expressed in mature adipocytes [8, 21], CpG methylation profiles of the *Mest* promoter in adipose tissue fractions enriched with adipocytes or stromal-vascular (SV) cells were determined. Initial experiments comparing methylation profiles from pooled adipocyte and SV DNA from fractionated inguinal fat of 7-C57BL/6J male mice (Fig. 4) showed decreased methylation in the adipocyte fraction compared to the SV fraction for *Mest* promoter regions I (~17% vs ~51%) and III (~9% vs ~37%), but not for region II (~34% vs ~29%). An independent experiment using DNA isolated from adipocyte-enriched and SV fractions of inguinal fat from male A/J mice showed similar patterns of methylation (data not shown). Although the reasons for the enrichment in the number of unmethylated 'paternal-like' alleles for *Mest* promoter regions I and III in adipocytes compared with SV cells is unclear, one explanation is that loss of DNA methylation occurs in selected genomic regions of the *Mest* promoter within the maternal allele in adipocytes. The apparent under-representation of methylated

alleles in *Mest* promoter regions I and III of adipocytes could indicate a mechanism associated with high variability in *Mest* expression. An experiment to test this by using purified adipocytes from adipose tissue with low and high *Mest* expression is described below.

Mest expression in adipocytes is not associated with variations in promoter methylation

To investigate whether mature adipocytes derived from adipose tissue depots with low or high *Mest* expression show differences in CpG methylation in *Mest* promoter regions I and III, experiments were performed using mice fed dietary fat for 1 week since it has been previously shown that a broad range of *Mest* expression can be induced in adipose tissue of B6 mice in as little as 2 to 7 days [16]. Bisulfite sequencing of DNA from adipocytes of epididymal fat from mice with low (n=4; 2.07 to 3.53 AU/cyclo) or high (n=4; 14.26 to 21.95 AU/cyclo) *Mest* expression selected from a cohort of 46 animals showed no differences in methylation in promoter regions I and III (Fig. 5). Interestingly, consistent with the data shown in Figure 4, adipocytes show a greater than expected number of unmethylated (65.8% and 82.4%) vs methylated alleles (34.2% and 17.6%) for genomic regions I and III of the *Mest* promoter respectively. It's possible that the changes in allelic DNA methylation observed in adipocytes, especially in *Mest* promoter region III, could be associated with an 'active' state of the *Mest* promoter that is primed for transcriptional activation when given the proper stimulus. An alternative explanation could be that the under-representation of methylated alleles is a PCR-generated artifact caused by impurities in the DNA isolated from lipid-containing adipocytes. To test this, an experiment based on allelic discrimination was performed to determine whether the maternal alleles of *Mest* show evidence of being hypomethylated.

Allelic discrimination analyses indicates that the maternal allele of *Mest* is not hypomethylated in adipocytes

A single nucleotide polymorphism (SNP) with a C>A (effectively a T>A variation in bisulfite converted DNA) variation between B6 and CAST/EiJ (CAST) mice within genomic region III of the *Mest* promoter (located in position +366 relative to the TSS and within intron 1 of *Mest*) makes it possible to determine whether loss-of imprinting occurs within maternal alleles of adipocytes. To discriminate maternal from paternal alleles, bisulfite sequencing was performed on DNA from adipocyte and SV cell fractions of epididymal fat from dietary fat-fed male progeny from reciprocal crosses between B6 and CAST. Results comparing allelic lineage and methylation patterns of *Mest* promoter region III in DNA from adipocytes and SV cells from (B6 X CAST)F1 and (CAST X B6)F1 mice indicate that the maternally-derived allele of *Mest* was predominantly methylated and the paternally-derived allele was not (Fig. 6). These results suggest that the *Mest* promoter shows the expected representation of methylated and unmethylated alleles in adipocytes and variations in allelic methylation do not contribute to differences in *Mest* expression in adipose tissue. The apparent increase in the number of hypomethylated alleles in adipocytes observed in our studies appears to be an artifact caused by the enrichment of non-methylated alleles in the PCR amplification reaction of *Mest* promoter regions I and III. Although we did not measure *Mest* expression in adipose tissue from the hybrid progeny derived from B6 and CAST, all mouse strains we have examined including B6 and CAST show induced adipose tissue *Mest* to varying degrees (>2 to more than 10-fold) when fed an obesogenic diet (unpublished observations). Thus we would expect that the hybrid progeny from a cross between B6 and CAST to show a comparable response.

Discussion

The identification of gene targets associated with fat mass expansion in an obesogenic environment could lead to new pharmacological approaches for the treatment of obesity. Because genetically inbred male B6 mice show large variations in dietary fat-induced obesity when raised in seemingly identical conditions, it was anticipated that this would be an ideal model to identify genes and epigenetic determinants that predispose mice to becoming obese. We previously showed that phenotypic variability of fat mass expansion among genetically inbred B6 mice fed a high fat diet is positively associated with the expression of *Mest* in adipose tissue. A number of studies have shown that *Mest*, a maternally imprinted gene, can be regulated by methylation within its promoter region and; in some cases bi-allelic expression can occur due to loss of imprinting [22]. Since *Mest* expression varies up to 80-fold among individuals within a genetically homogenous mouse population, even after a relatively short exposure to dietary fat, it seemed highly plausible that variations in DNA methylation could be involved in its regulation. However, in this study, extensive bisulfite sequencing analyses of DNA from adipose tissue and adipocytes of mice with low and high *Mest* expression showed no differences in methylation that could account for variability in gene expression. Furthermore, what appeared to be hypomethylation of the maternal allele in adipocyte DNA, as evidenced by enrichment of non-methylated DNA fragments, was likely caused by a PCR-derived artifact. These data are consistent with recent observations of Kamei et al[23] who used a single nucleotide polymorphism between B6 and Japanese Fancy mice within the coding region of *Mest* to show that adult adipose tissue *Mest* mRNA was only derived from the paternal allele. Additionally, a recent manuscript by Okada et al used MALDI-TOF mass spectrometry to show that no changes in methylation occurs within the *Mest* promoter after induction of *Mest* gene expression in adipose tissue of mice by feeding them a high fat diet [24]. In studies to determine whether isoforms of *Mest* are derived from alternative transcriptional start sites (promoter switching) in adipose tissue from mice fed a high fat diet showed that *Mest* transcripts that were predominantly transcribed contained the ‘authentic’ exon 1 which is used in development [23].

Although our experiments, and those of Kamei et al [23] and Okada et al [24], clearly show that the maternal allele of *Mest* in adipose tissue of adult mice is not transcriptionally functional; the mechanism by which variability in *Mest* expression occurs within a genetically identical population of mice remains intriguing. As an alternative to a DNA methylation based mechanism, epigenetic control of the regulation of transcription factor(s)/ enhancer(s) could indirectly regulate *Mest* via interaction with its regulatory elements; or, variations in the acetylation and /or methylation of histones could repress or promote *Mest* transcription. Recent studies suggest that activation of transcription from the non-imprinted paternal allele of *Mest* may occur via disruption of the interaction between TIF1β (transcriptional intermediary factor 1β) and HP1 (heterochromatin protein 1) and a switch from DNA hypermethylation and histone H3K9 trimethylation to DNA hypomethylation and histone H3K27 trimethylation [25]. Because our bisulfite sequencing analyses of DNA does not show evidence of hyper-methylation within genomic regions that overlap those analyzed by Riclet et al [25], it is likely that the *Mest* promoter of the paternal allele in adipocytes of adult mice is maintained in a transcriptionally permissible state.

A number of studies have failed to demonstrate epigenetic changes within CpG islands of genes associated with complex metabolic phenotypes. Recently, transgenerational amplification of body weight was shown to be prevented in agouti viable yellow (A^{vy}) mice fed diets supplemented with methyl donors; however, no association with changes in the methylation state of the CpG island in the A^{vy} promoter was observed [26]. These studies and others underscore the complexities of gene regulation as well as the difficulty in being able

to establish the effects of epigenetic variation within CpG islands encompassing the immediate proximal promoter regions of genes. Because the identification of molecular and epigenetic determinants for the regulation of *Mest* provides an opportunity to evaluate it as a target gene for nutritional programming, in depth promoter analyses of *Mest* is now being pursued in our laboratory. The data from these and future studies may be used to develop pharmaceutical interventions that can modulate fat mass expansion and obesity in humans by specifically targeting *Mest* in adipocytes.

Acknowledgments

We thank Tamra Mendoza for technical support, Drs. Kenneth Eilertsen and Jong Seop-Rim for reviewing the manuscript, Susan Newman and Jana Smith of the Pennington Biomedical Research Center's Genomic Core Facility for support in DNA sequencing and quantitative RT-PCR, and Dr. Brenda Smith-Richards for providing reciprocal mouse hybrids generated from B6 and CAST/EiJ. These studies were supported by The Health Excellent Fund of the State of Louisiana (LPK), NIH P30 DK072476 CNRU P&F (RAK), NIH P20-RR021945 COBRE (RAK) and NIH R21 DK074951 (RAK).

Abbreviations

Mest	mesoderm specific transcript
DMR	differentially methylated region
TSS	transcriptional start site
B6	C57BL/6J

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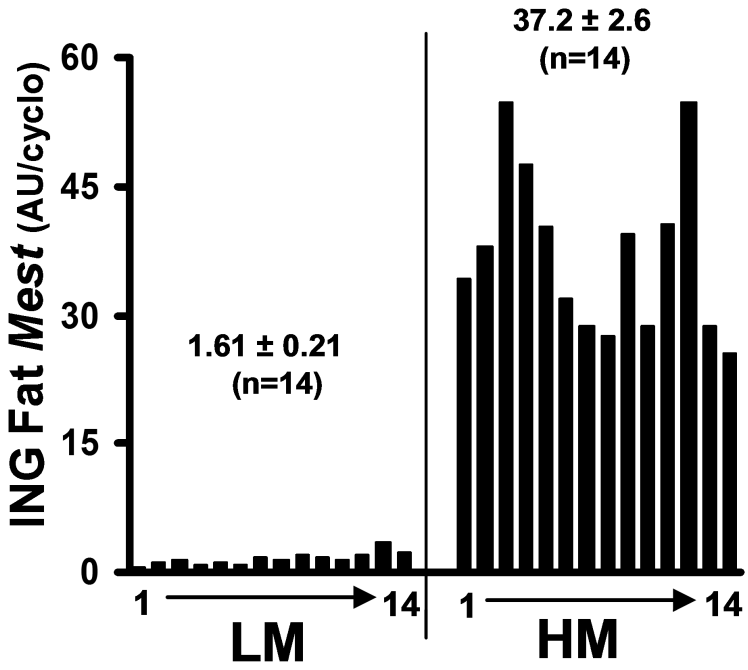

Figure 1.
This figure represents male C57BL/6J mice with the lowest (n=14) and highest (n=14) levels of inguinal (ING) fat *Mest* expression from a cohort of 112 mice fed a high fat diet for 4 weeks. *Mest* mRNA was normalized to cyclophilin B. DNA isolated from the ING fat of these mice was used for the bisulfite sequencing analyses experiments in Figure 3.

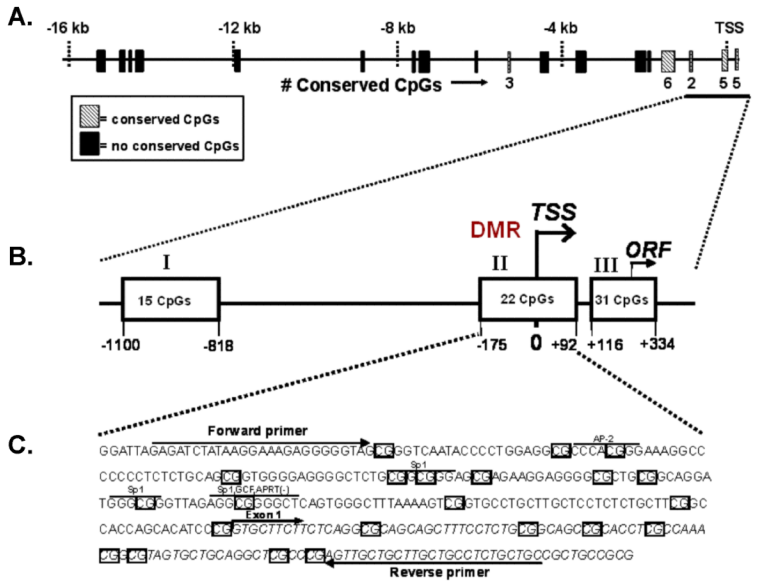

Figure 2.

A schematic illustrating: (A) Conserved DNA segments between human and mouse located in a 16 kb genomic region upstream from the transcriptional start site (TSS) of *MEST*. Hatched boxes indicate genomic regions with conserved CpGs between human and mouse whereas black boxes indicate genomic regions with no conserved CpGs. (B) Genomic regions (I, II and III) were selected for bisulfite sequencing analyses based upon sequence homology and conservation of CpGs between human and mouse. (C) Example of genomic sequence and primers used for bisulfite sequencing analyses of a genomic region encompassing the TSS. The 22 CpGs within this sequence, start of TSS and location of potential promoter binding sites are indicated.

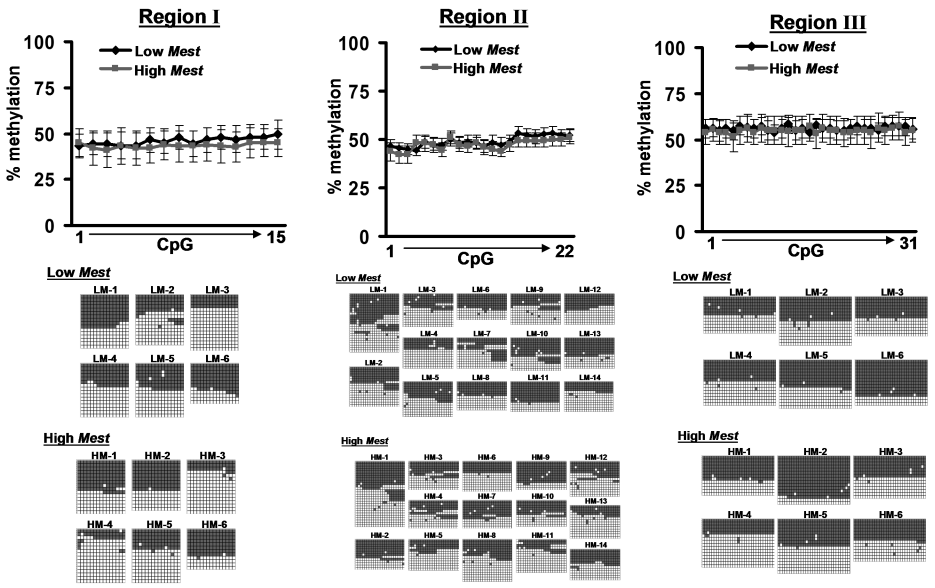

Figure 3.

Bisulfite sequencing analyses of DNA from inguinal fat with low and high *Mest* expression. DNA from inguinal fat of mice with low (LM) or high (HM) *Mest* expression corresponding to the data shown in Figure 1 were subjected to bisulfite conversion and sequencing analyses for genomic regions I, II and III (as shown in Figure 2B). DNA was analyzed from at least 6 animals with low and high *Mest* expression per group with at least 11 clones sequenced per animal. Line graphs show the temporal frequency of methylated CpGs (% methylation) for each genomic sequence. The figures below each line graph indicate the patterns of unmethylated (white squares) and methylated (gray squares) CpGs for each sequenced allele.

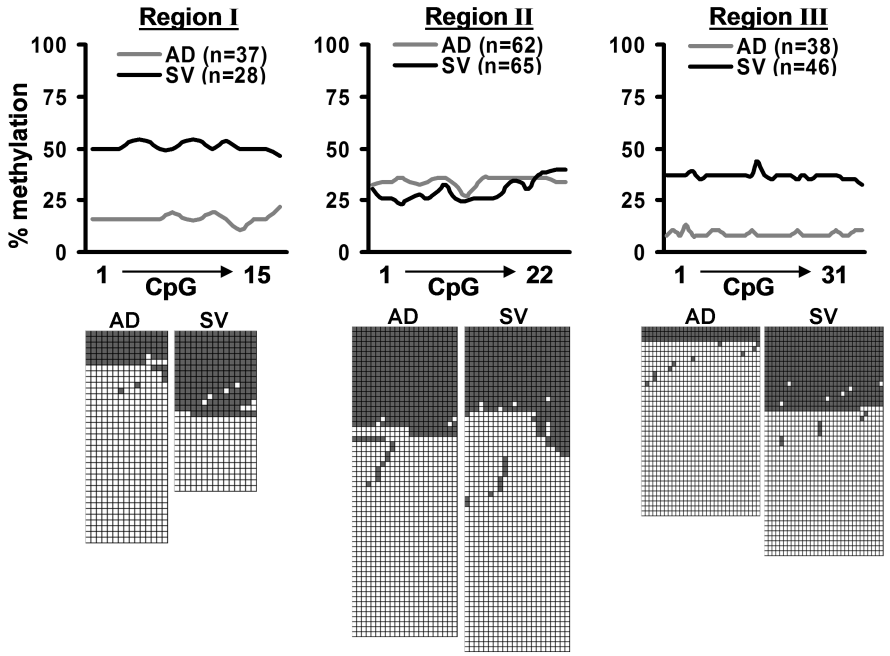

Figure 4.

Comparison of CpG methylation patterns within the *Mest* promoter in DNA isolated from the adipocyte (AD) and stromal-vascular (SV) fractions of adipose tissue show enhancement of hypomethylated alleles in genomic regions I and III of adipocytes. Pooled inguinal fat from B6 mice fed a chow diet were sub-fractionated into fractions that were enriched for mature adipocytes and SV cells. The graphs show the temporal frequency of CpG methylation (% methylation) of genomic regions I, II and III for each cellular fraction. A minimum of 28 clones were sequenced and analyzed for each of the cellular fractions and the individual alleles showing unmethylated (white boxes) and methylated (gray boxes) CpGs within each genomic region are shown under the line graphs.

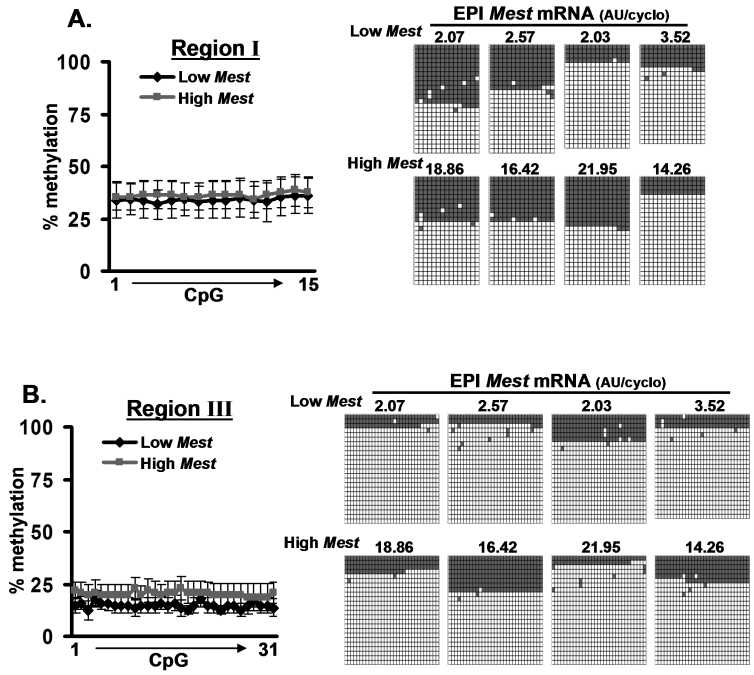

Figure 5.

Enhancement of hypomethylated alleles in adipocytes are not associated with *Mest* gene expression. Methylation patterns within genomic regions I and III of the *Mest* promoter were determined in DNA from epididymal (EPI) fat-derived adipocytes from mice with low (n=4) or high (n=4) EPI fat *Mest* gene expression selected from a cohort of 46 animals fed a high fat diet for 1 week. Line graphs show the temporal patterns of CpG methylation (% methylation) across the genomic regions. Figures on the right-hand side of the of the graphs show the level of *Mest* mRNA expression of the adipose tissue depot from where adipocytes were derived and the individual alleles sequenced for each DNA with gray squares indicating methylated CpGs and white squares indicating non-methylated CpGs. A minimum of 22 clones were sequenced for each DNA analyzed.

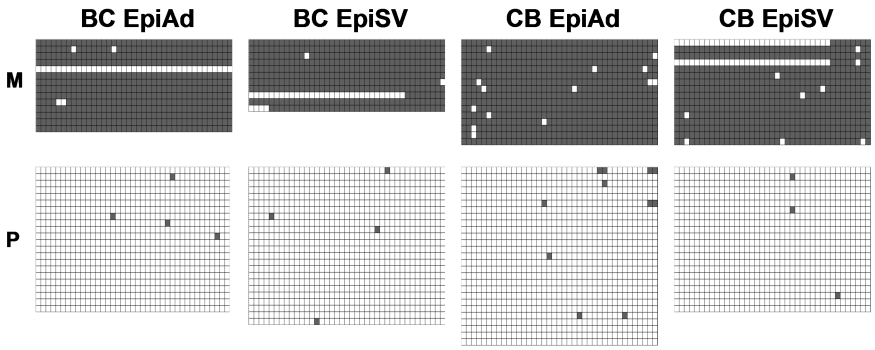

Figure 6.
Allelic discrimination analysis shows normal maternal and paternal allelic methylation patterns in adipocytes. Data shows methylation patterns of DNA from epididymal fat-derived adipocytes (EpiAd) and stromal-vascular cells (EpSV) from F1 progeny derived from crosses between B6 X *Castaneus* (BC) and *Castaneus* X B6 (CB) mice. A single nucleotide polymorphism in genomic region III of the *Mest* promoter was used to identify alleles as being maternally (M) or paternally (P) derived.