

Diet-Induced Up-Regulation of Gene Expression in Adipocytes Without Changes in DNA Methylation

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The expansion of white adipose tissue (WAT) mass during the development of obesity is mediated in part through an increase in adipocyte size. Although gene expression profiles associated with adipogenesis *in vitro* and the development of obesity *in vivo* have been characterized by DNA microarray analysis, the role of chromatin and chromatin-modifying proteins in the regulation of gene expression related to adipocyte hypertrophy has remained unclear. We have now shown that maintenance of C57BL/6J mice on a high-fat diet for 16 weeks resulted in marked up-regulation of the expression of leptin, Mest (mesoderm specific transcript; also known as paternally expressed gene 1, or Peg1), and sFRP5 (secreted frizzled-related protein 5) genes in WAT. Furthermore, the demethylating agent 5-aza-2'-deoxycytidine increased the amount of Mest/Peg1 mRNA, but not that of leptin or sFRP5 mRNAs, in mouse 3T3-L1 adipocytes. However, analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry revealed that maintenance of mice on a high-fat diet for various times did not affect the level of methylation at specific CpG sites in the promoter regions of leptin, Mest/Peg1, and sFRP5 genes in WAT. Our results indicate that the diet-induced up-regulation of leptin, Mest/Peg1, and sFRP5 gene expression in WAT during the development of obesity in mice is not mediated directly by changes in DNA methylation.

The worldwide epidemic of obesity is a serious threat to public health, in part because the increase in the mass of white adipose tissue (WAT) in obese individuals increases the risk for development of type 2 diabetes mellitus and cardiovascular disease. The expansion of WAT during the development of obesity occurs as a result of increases in cell number (adipocyte hyperplasia) or in cell size (adipocyte hypertrophy) [1, 2]. Analysis of the changes in gene expression in WAT associated with adipocyte hypertrophy are likely to provide insight into the contribution of this process to obesity and metabolic disorders. Although gene expression profiles of adipocytes during their differentiation *in vitro* [3] as well as of WAT during the development of obesity *in vivo* [4] have been characterized with the use of DNA microarrays over the past decade or so, the role of chromatin and chromatin-modifying proteins in the regulation of gene expression during adipogenesis has only become apparent more recently [5].

Among epigenetic modifications of DNA and histones that contribute to the regulation of gene expression [6], DNA methylation plays an important role both during development and in tumorigenesis [7, 8]. DNA methylation consists of the addition of a methyl group to the 5' position of cytosine in a CpG dinucleotide. Whereas most human genomic DNA is deficient in CpG sites, clusters of CpG dinucleotides (CpG islands) are located in the promoter regions of >70% of known human genes [9]. On the basis of the assumption that adipocyte hypertrophy is regulated epigenetically, in particular by DNA methylation, we have investigated whether diet-induced changes in gene expression in WAT during the development of obesity are associated with alterations in DNA methylation.

MATERIALS AND METHODS

Reagents and cells

5-Aza-2'-deoxycytidine (5-aza-C) was obtained from Sigma (St. Louis, MO, USA). 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained as preadipocytes as described previously [10]. Their differentiation into adipocytes was induced by treatment of confluent cells first for 2 days with insulin (5 µg/ml), 0.25 µM dexamethasone, and 0.5 mM isobutylmethylxanthine in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and then for 2 days with insulin (5 µg/ml) alone in the same medium. The cells were then returned to the basal medium, which was replenished every other day.

Animals

Male C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan), and only male animals were studied. For examination of the effects of a high-fat diet, mice were fed from 4 weeks of age with chow containing 30% fat by weight as described previously [11]. Experiments with mice were performed according to the guidelines of the animal ethics committee of Kobe University Graduate School of Medicine.

Determination of adipocyte size

Tissue samples (~100 mg) obtained from epididymal fat pads were fixed with osmium tetroxide (Sigma) as described [11], suspended in isotonic saline, and passed through a nylon filter with a pore size of 250 µm to remove fibrous elements. The filtrate was then passed through a nylon filter with a pore size of 25 µm to trap fixed adipocytes (with a diameter of >25 µm), and the isolated cells were washed extensively with isotonic saline. A total of 10,000 cells was analyzed as described previously [11] with the use of a Coulter counter equipped with a 560-µm aperture tube, a stirred sample chamber, and a multichannel particle analyzer (Multisizer III; Coulter Electronics, Fullerton, CA, USA). Analysis of the distribution of adipocyte size was performed for cells ranging in diameter from 25 to 250 µm.

Oligonucleotide microarray analysis

Total RNA was isolated from epididymal WAT of C57BL/6J mice at 20 weeks of age with the use of an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Portions (20 µg) of the RNA were used to synthesize biotin-labeled cRNA, which in turn was used to probe Murine Genome U74v2 microarrays (Affymetrix, Santa Clara, CA, USA). The arrays were scanned and analyzed as described previously [10].

Quantitative RT-PCR analysis

Reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis was also performed as described [11] with the use of a Sequence Detector (model 7900; Applied Biosystems, Foster City, CA, USA) and with 29B4 mRNA as the invariant control. The primers (upstream and downstream, respectively) were

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5'-CACCAGGCTCCCAAGAATCATGTA-3' and 5'-GGGATGGCTCTTATCTCTACTTGCT-3' for leptin mRNA, 5'-GTGGTTCGGAAGCCCTGAGATAG-3' and 5'-GGGCGATCACTCGATGGAA-3' for Mest/Peg1 mRNA, 5'-TCCTACTCCAAGCCACCGCAGTG-3' and 5'-CCAGGCAGACGGGAGCGAAGAGC-3' for sFRP5 mRNA, and 5'-GGCCCTGCACTCTCGCTTTC-3' and 5'-TGCCAGGACGCGCTTGT-3' for 29B4 mRNA.

Quantitative methylation analysis

Quantitative methylation analysis of the leptin, Mest/Peg1, and sFRP5 gene promoters was performed with the MassARRAY Compact system (Hitachi High-Technologies, Tokyo, Japan). Genomic DNA was extracted from mouse epididymal WAT with the use of a Nucleic Acid Purification Kit (Toyobo, Osaka, Japan). The DNA (1 mg) was treated with sodium bisulfate with the use of an EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), and target regions of the modified nucleic acid were amplified by PCR with the primer pairs shown in Table I. Each forward primer contained a 10-nucleotide tag (5'-AGGAAGAGAG-3') to balance the PCR, and each reverse primer contained a T7 promoter tag and sequence insert (5'-CAGTAATACGACTCACTATAGGGAGAAGGCT-3') for in vitro transcription. The amplification protocol comprised an initial incubation at 94°C for 15 min; 45 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and a final incubation at 72°C for 3 min. Unincorporated deoxynucleoside triphosphates were dephosphorylated by the addition of 2 µl of Premix for in vitro transcription including 0.3 U of shrimp alkaline phosphatase (Sequenom, San Diego, CA, USA). The reaction mixture was incubated at 37°C for 40 min, after which the phosphatase was inactivated by incubation for 5 min at 85°C. A portion of the PCR products (2 µl) was then subjected to in vitro transcription, with RNase A cleavage being used for the T-reverse reaction (Sequenom). Samples were spotted onto a 384-pad Spectro-CHIP (Sequenom) with the use of a MassARRAY nanodispenser (Sequenom), and spectra were acquired with a MassARRAY compact matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Sequenom). The resulting methylation calls were analyzed with EpiTyper software (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

Table I. PCR primers for analysis of the methylation status of sFRP5, Mest/Peg1, and leptin gene promoters. Each forward (L) primer contains a 10-nucleotide tag to balance the melting temperature (5'-aggaagagag-3'), and each reverse (R) primer contains a T7 promoter tag for in vitro transcription (5'-cagtaatcagactcactatagg-3') and an 8-nucleotide insert (5'-agaagct-3').

Gene	Primer names	Analyzed regions relative to transcription start site	No. of analyzed CpG units	Sequences
sFRP5	sFRP5-z1-L	-209 to +94	21	5'-aggaagagagTAGGAAAAGGTATAAGGAGAGGTTTG-3'
	sFRP5-z1-R			5'-cagtaatcagactcactatagggagaaggctAAACCCACCACTAACTAAATATTAC-3'
	sFRP5-z2-L	+138 to +361	16	5'-aggaagagagATTTAGTTATGTGGGTGGTTGGAG-3'
	sFRP5-z2-R			5'-cagtaatcagactcactatagggagaaggctCCAACACTTACTACTACTTCACCTC-3'
Mest/Peg1	Peg1/Mest-L	-175 to +88	15	5'-aggaagagagTATTAGAATTTGGGGTTTAGGATTAG-3'
	Peg1/Mest-R			5'-cagtaatcagactcactatagggagaaggctCAACAAAACAA CAAACAACAACCTC-3'
Leptin	Leptin-L	-374 to -2	15	5'-aggaagagagTGAGTAGTTAGGTTAGGTATGTAAGAGTT-3'
	Leptin-R			5'-cagtaatcagactcactatagggagaaggctCAACACTACTAAACAAAATCCCTC-3'

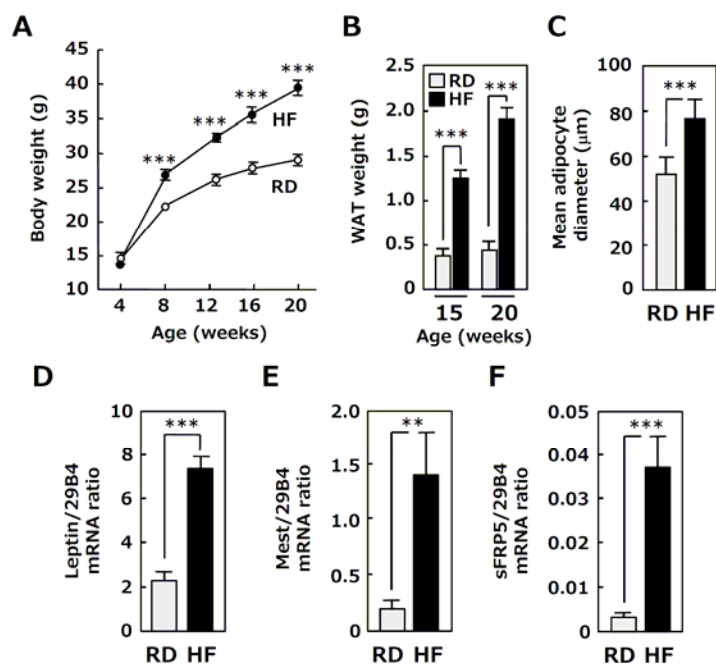
Statistical analysis

Data are presented as means \pm SEM. Differences between two groups were evaluated by Student's unpaired two-tailed *t* test as performed with StatView software (SAS Institute, Cary, NC, USA). A *P* value of <0.05 was considered statistically significant.

RESULTS

Effects of a high-fat diet on adipocyte size and gene expression in WAT

We first examined the size of adipocytes in WAT of C57BL/6J mice fed a high-fat diet or regular chow from 4 weeks of age. Maintenance of mice on the high-fat diet increased body weight and the weight of epididymal WAT by 7.1 and 0.91 g, respectively, after 11 weeks and by 11.5 and 1.4 g, respectively, after 16 weeks, compared with mice fed the normal diet (Fig. 1A, B). The high-fat diet also induced a marked increase in the size of adipocytes (Fig. 1C) in epididymal WAT after 16 weeks. To investigate the factors responsible for this difference in adipocyte size between mice fed the two types of diet, we first analyzed the corresponding gene expression profiles in WAT. Total RNA isolated from WAT of C57BL/6J mice fed a high-fat diet or regular chow for 16 weeks was analyzed with mouse oligonucleotide microarrays, revealing that, among the $\sim 36,000$ genes examined, the expression of 617 genes was up-regulated (\log_2 ratio > 1.0) and that of 476 genes was down-regulated (\log_2 ratio < 0.5) in WAT of mice with diet-induced obesity (data not shown). The genes for leptin, *Mest* (mesoderm specific transcript; also known as paternally expressed gene 1, or *Peg1*), and sFRP5 (secreted frizzled-related protein 5) were among those most differentially expressed, consistent with previous observations [12]. We next confirmed the differential expression of these three genes in obese and nonobese mice by quantitative RT-PCR analysis. Maintenance of C57BL/6J mice on the high-fat diet for 16 weeks indeed resulted in a marked increase in the abundance of mRNAs for leptin, *Mest*/*Peg1*, and sFRP5 in WAT (Fig. 1D–F).



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Figure1 Effects of a high-fat diet on adipocyte size and gene expression in WAT. *A, B*, Body weight (*A*) and epididymal WAT weight (*B*) in C57BL/6J mice of the indicated ages fed a regular (RD) or high-fat (HF) diet from 4 weeks of age. *C*, Mean diameter of adipocytes in epididymal WAT of 20-week-old C57BL/6J mice fed a regular or high-fat diet as in *A*. Data in *A* through *C* are means \pm SEM of values from 18 to 20 mice. *** $P < 0.005$. *D–F*, Quantitative RT-PCR analysis of leptin, Mest/Peg1, and sFRP5 mRNAs, respectively, in epididymal WAT of C57BL/6J mice at 20 weeks of age fed a regular or high-fat diet as in *A*. Data are expressed relative to the abundance of 29B4 mRNA and are means \pm SEM of values from five to seven mice. ** $P < 0.01$, *** $P < 0.005$.

Effects of 5-aza-C on leptin, Mest/Peg1, and sFRP5 gene expression in 3T3-L1 cells

The potential role of DNA methylation in regulation of leptin, Mest/Peg1, and sFRP5 gene expression in adipocytes was evaluated by treatment of cultured 3T3-L1 mouse adipocytes with the demethylating agent 5-aza-C [13] at various concentrations for 2, 4, or 6 days. Quantitative RT-PCR analysis revealed that treatment of the cells with 5-aza-C at 0.5 or 5 μ M induced a time-dependent increase in the amount of Mest/Peg1 mRNA, with this effect being maximal at 4 days (Fig. 2A). In contrast, 5-aza-C did not affect the amount of leptin mRNA (Fig. 2B), and sFRP5 mRNA was not detected in 3T3-L1 adipocytes (data not shown). These results thus suggested that expression of the Mest/Peg1 gene, but not that of the leptin gene, may be regulated by DNA methylation.

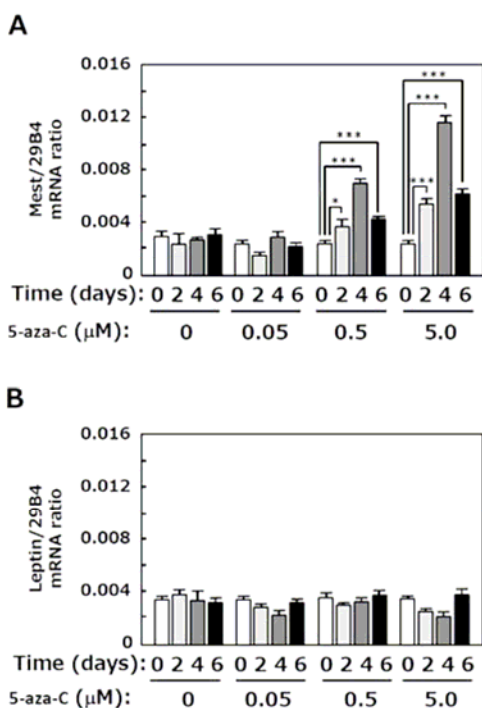


Figure2 Effects of 5-aza-C on the expression of Mest/Peg1 and leptin genes in 3T3-L1 adipocytes. Cells were incubated with 5-aza-C at the indicated concentrations for the indicated times, after which the amounts of Mest/Peg1 (*A*) and leptin (*B*) mRNAs were determined relative to that of 29B4 mRNA by quantitative RT-PCR analysis. Data are means \pm SEM of values from three independent experiments. * $P < 0.05$, *** $P < 0.005$.

Effects of a high-fat diet on methylation of CpG islands in the promoter regions of Mest/Peg1, leptin, and sFRP5 genes in mouse WAT

The effects of a high-fat diet on the methylation of CpG islands in the promoter regions of Mest/Peg1, leptin, and sFRP5 genes in WAT of C57BL/6J mice were examined by quantitative analysis of DNA methylation based on MALDI-TOF mass spectrometry. Such

analysis at 15 CpG sites in the promoter region of the *Mest/Peg1* gene spanning nucleotides -175 to +88 [14] revealed that the mean methylation level for these sites in mice at 10 weeks of age fed a normal diet ranged from 33 to 61% (Table II). Unexpectedly, this pattern of DNA methylation was not affected by maintenance of mice on the high-fat diet for 6 to 21 weeks (Table II).

Age(weeks), diet	CpG site (position)														
	(-175)	(-155)	(-149)	(-124)	(-106)	(-84)	(-67)	(-56)	(+12)	(+29)	(+47)	(+54)	(+61)	(+68)	(+88)
10, RD	33	38	44	33	46	45	40	40	36	52	43	47	56	51	61
10, HF	40	38	54	35	48	50	43	43	39	54	48	50	56	53	69
15, RD	38	39	54	37	50	51	44	44	40	56	49	56	59	55	70
15, HF	37	43	45	35	51	50	44	44	40	56	48	51	60	57	75
20, RD	37	44	48	34	49	48	43	43	38	54	48	46	58	55	67
20, HF	35	57	42	37	48	52	44	44	38	52	48	42	59	56	72
25, RD	33	32	31	34	49	53	43	43	39	51	45	46	66	54	72
25, HF	32	32	43	34	47	50	43	43	38	48	47	44	65	48	70

Table II. Quantitative methylation analysis of the promoter region of the *Mest/Peg1* gene spanning nucleotides -175 to +88 relative to the transcription start site. Genomic DNA isolated from epididymal WAT of C57BL/6J mice of the indicated ages fed a regular (RD) or high-fat (HF) diet from 4 weeks of age was analyzed for methylation status of 15 CpG sites at the indicated positions of the *Mest/Peg1* gene promoter. Data for each CpG site represent the mean percentage methylation level (0% = nonmethylated, 100% = methylated) determined from four or five mice.

The mean methylation level of 15 CpG sites in the promoter region of the leptin gene spanning nucleotides -374 to -2 [15] ranged from 34 to 92% in WAT of C57BL/6J mice fed the normal diet at 10 weeks of age (Table III). Similarly, the mean methylation level of 21 or 16 CpG sites in the promoter region of the *sFRP5* gene spanning nucleotides -209 to +94 or +138 to +361, respectively [16], ranged from 2 to 36% in WAT of 10-week-old C57BL/6J mice fed the normal diet (Tables IV, V). The methylation levels of the leptin and *sFRP5* gene promoters in WAT were also not affected by maintenance of mice on a high-fat diet for between 6 and 21 weeks (Tables III-V). These results thus suggest that the diet-induced changes in leptin, *Mest/Peg1*, and *sFRP5* gene expression in adipocytes are regulated by mechanisms independent of DNA methylation.

Age(weeks), diet	CpG site (position)														
	(-374)	(-265)	(-252)	(-231)	(-224)	(-214)	(-200)	(-182)	(-176)	(-157)	(-152)	(-85)	(-62)	(-52)	(-2)
10, RD	51	52	51	69	92	76	61	34	78	47	73	53	65	73	73
10, HF	55	47	60	61	79	61	52	19	73	33	69	56	65	69	69
15, RD	50	41	45	53	86	49	51	22	65	39	65	48	62	65	65
15, HF	57	46	50	64	88	45	55	26	75	39	69	55	69	69	69
20, RD	55	43	48	60	87	52	51	30	66	32	62	53	61	62	62
20, HF	65	51	58	73	90	68	60	22	83	41	78	62	72	78	78
25, RD	49	45	54	59	83	45	52	18	71	37	65	57	67	65	65
25, HF	67	56	65	78	88	75	65	31	88	42	7	67	73	79	79

Table III. Quantitative methylation analysis of the promoter region of the leptin gene spanning nucleotides -374 to -2 relative to the transcription start site. Genomic DNA isolated from epididymal WAT of C57BL/6J mice of the indicated ages fed a regular (RD) or high-fat (HF) diet from 4 weeks of age was analyzed for methylation status of 15 CpG sites at the indicated positions of the leptin gene promoter. Data for each CpG site represent the mean percentage methylation level (0% = nonmethylated, 100% = methylated) determined from four or five mice.

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Age(weeks), diet	CpG site (position)																				
	(-209)	(-198)	(-191)	(-186)	(-179)	(-170)	(-165)	(-132)	(-124)	(-93)	(-71)	(-48)	(-36)	(-22)	(-16)	(-11)	(+33)	(+55)	(+64)	(+74)	(+94)
10, RD	2	7	7	1	2	2	13	4	8	3	36	2	3	3	7	7	2	10	3	20	8
10, HF	1	3	12	0	0	0	7	2	7	3	33	4	2	5	12	12	2	6	3	22	6
15, RD	2	2	7	1	0	0	8	3	8	3	39	3	2	5	7	7	3	6	3	20	8
15, HF	4	6	9	2	1	1	8	6	9	6	42	2	3	7	9	9	3	9	6	21	9
20, RD	1	5	10	1	1	1	10	4	8	4	37	2	2	5	10	10	2	7	4	21	10
20, HF	6	9	13	7	0	0	12	6	9	7	47	3	5	8	13	13	3	9	7	21	10
25, RD	3	8	6	2	0	0	8	5	7	6	34	3	4	4	6	6	3	9	6	23	9
25, HF	8	7	11	4	0	0	12	6	7	7	38	3	5	7	11	11	4	11	7	23	9

Table IV. Quantitative methylation analysis of the promoter region of the sFRP5 gene spanning nucleotides -209 to +94 relative to the transcription start site. Genomic DNA isolated from epididymal WAT of C57BL/6J mice of the indicated ages fed a regular (RD) or high-fat (HF) diet from 4 weeks of age was analyzed for methylation status of 21 CpG sites at the indicated positions of the sFRP5 gene promoter. Data for each CpG site represent the mean percentage methylation level (0% = nonmethylated, 100% = methylated) determined from four or five mice.

Age(weeks), diet	CpG site (position)															
	(+138)	(+146)	(+185)	(+193)	(+216)	(+228)	(+233)	(+240)	(+263)	(+273)	(+282)	(+293)	(+305)	(+319)	(+325)	(+361)
10, RD	4	3	3	3	3	5	4	4	2	4	11	7	3	4	3	4
10, HF	0	4	10	4	6	3	4	3	2	4	10	8	10	0	4	4
15, RD	7	3	3	3	3	5	6	5	3	6	9	7	3	7	4	3
15, HF	5	4	3	4	3	5	6	4	2	6	10	8	3	5	6	3
20, RD	9	4	3	4	4	5	4	2	5	10	6	3	9	4	3	
20, HF	6	4	3	4	4	5	6	5	3	6	10	9	3	6	7	3
25, RD	5	4	2	4	3	6	6	5	2	6	11	8	2	5	5	3
25, HF	6	4	4	4	3	5	6	6	2	6	11	9	4	6	7	2

Table V. Quantitative methylation analysis of the promoter region of the sFRP5 gene spanning nucleotides +138 to +361 relative to the transcription start site. Genomic DNA isolated from epididymal WAT of C57BL/6J mice of the indicated ages fed a regular (RD) or high-fat (HF) diet from 4 weeks of age was analyzed for methylation status of 16 CpG sites at the indicated positions of the sFRP5 gene promoter. Data for each CpG site represent the mean percentage methylation level (0% = nonmethylated, 100% = methylated) determined from four or five mice.

DISCUSSION

Our results indicate that DNA methylation does not contribute directly to the regulation of leptin, Mest/Peg1, and sFRP5 gene expression in WAT, with the expression of each of these genes having previously been associated with adipocyte hypertrophy [12, 17, 18]. The extent of DNA methylation is highly correlated with heritable gene silencing [19–22], examples of which include methylated CpG islands on the inactive X chromosome of female mammals as well as in genomic regions that carry a parent-specific imprint. In both these instances, DNA methylation of CpG islands has evolved as a developmentally regulated mechanism for reducing the dosage of a gene product by allelic silencing [22–24]. Although the demethylating agent 5-aza-C induced expression of the imprinted gene for Mest/Peg1 in mouse 3T3-L1 adipocytes in the present study, the extent of DNA methylation at specific sites in the Mest/Peg1 gene promoter was not altered in WAT of mice during the development of obesity.

The pattern of DNA methylation in the promoters of the leptin and sFRP5 genes was found to differ from the bimodal methylation patterns observed for genes carrying a parental imprint, such as that for the Mest/Peg1 gene, and for genes on the X chromosome. The leptin gene has previously been shown to be methylated in preadipocytes but to become hypomethylated during adipogenesis in human preadipocytes [25] and mouse 3T3-L1 cells [26]. We found that the methylation levels for CpG sites in the leptin gene promoter were lower in WAT of mice than in 3T3-L1 adipocytes (data not shown). However, the extent of

methylation at such sites in the promoters of the leptin and sFRP5 genes in WAT was not altered in association with the development of diet-induced obesity. Thus, in addition to that of the imprinted gene for Mest/Peg1, expression of the leptin and sFRP5 genes during the development of obesity does not appear to be directly regulated by DNA methylation. It remains possible that epigenetic modification of histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, or isomerization [27–29] contributes to regulation of the expression of genes associated with adipocyte hypertrophy.

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