Research Paper

In Vivo Methylation Patterns of the Leptin Promoter in Human and Mouse

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ABSTRACT

Leptin is a fat hormone regulating energy homeostasis. Here, it is reported that the promoter and CpG island of the autosomal gene leptin (LEP) is a tissue-specific differentially methylated region (T-DMR) and subject to dynamic methylation in human and mouse in vivo. Highly variable densities of cytosine methylation were detected by hairpin-bisulfite PCR among cells in human adipose tissue and peripheral blood leukocytes. Intermediate and low levels of methylation characterize the majority of human LEP epialleles. Low-density epialleles are often methylated at a specific CG site within the binding element of the C/EBP-α transcription factor. In the human LEP promoter, the methylation frequency at that site is 1.8-fold as great as the average frequency for all other CG sites analyzed. The LEP promoter has a significantly higher methylation density in mouse somatic tissues than in the human LEP promoter. Though the LEP CpG island is generally unmethylated in both human and mouse sperm, depletion of CG sites within the mouse promoter indicates occasional presence of methylated Lep epialleles in the germline. These findings suggest that LEP promoter methylation is normally imposed during postzygotic development, and that this epigenetic mark may play a role in modulating expression of an important metabolic gene.

INTRODUCTION

Methylation is a nucleotide modification which, in mammalian DNA, is primarily confined to cytosines of 5'-CG-3' dinucleotides. These potential sites of cytosine methylation are not uniformly distributed within the genome. Domains with a high density of 5'-CG-3' dinucleotides, also termed CpG islands, punctuate the mammalian genome that is otherwise characterized by a relative depletion of CGs based on the GC content.1-3 CpG islands frequently harbor promoters and first exons of genes.4,5 Genome analysis suggests that transcription of about 75% of genes is controlled and initiated within CG-rich regions.4-6 It is generally assumed that most CpG islands associated with gene promoters remain completely unmethylated throughout development, and in all tissues of an animal. However, based on data from recent genomic studies on mouse tissues, it has been estimated that as many as 5%-34% of CpG islands constitute tissue-specific differentially methylated regions (T-DMRs).7,8 Although the genetic information of T-DMRs is identical between tissues and cells of an individual, differences in cytosine methylation patterns generate epigenetic variants, which I refer to as “epialleles”.

Dense promoter methylation is highly correlated with heritable gene-silencing,10-12 (reviewed in ref. 13). The best-documented examples are methylated CpG islands on the inactive X-chromosome in females, and genomic regions that carry a parental-specific imprint. In both cases, CpG island-methylation has evolved as a developmentally regulated mechanism participating in the process of reducing the dosage of a gene product by allelic silencing (reviewed in refs. 13-15).

Leptin, the product of the obes gene Leptin (LEP) is a key hormone regulating energy intake and expenditure.16 (reviewed in ref. 17). The LEP promoter is embedded within a CpG island (Fig. 1A). 217 base pairs containing a TATA-box element and a functional
Benign adipose tissues were provided. The hairpin bisulfite PCR method and a 23
is: Chromosome 7: 127474702-127475326; The position of the transcription factor C/EBP-
F1, 61 years of age, abdominoplasty. For each individual sample approximately 5 x 5 mm
methylation patterns are described.

MATERIALS AND METHODS

Criteria used to define the LEP/Lep CpG islands. The properties of these
methylation data obtained from cell lines may not always reflect the in vivo methylation status of a particular locus, since CpG islands of nonessential genes frequently become methylated under tissue culture conditions.22
The aim of this study was to investigate whether the regulatory region of LEP, an autosomal gene not known to carry a parental-specific imprint, is methylated in human and mouse tissues. Here, I establish that the LEP promoter is indeed methylated in both species. The properties of these methylation patterns are described.

Figure 1. The human and mouse Leptin promoters. (A) Plots show the distribution of CG dinucleotides - potential sites of cytosine methylation - within a 1.5 kb genomic region centered around the TATA-box promoter element. DNA segments meeting criteria of CpG islands are depicted in gray; defining criteria are described in the Methods section. (B) Alignment of the core Leptin promoter sequence from human and mouse. CG dinucleotides are highlighted in gray, while TG or CA nucleotide substitutions are highlighted in brown. The methylation status of 21 CG sites was analyzed on the human promoter.

Sequence alignment. Figure 1B shows the result of two sequence alignments: Alignment of mouse and human Leptron promoters sequences covering human CG sites 1 to 4 was obtained from the comparative genome feature of the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). The position of the mouse Leptron CpG island is: Chromosome 7: 127474702-127475326; The position of the mouse Leptin CpG island is: Chromosome 6: 28992101-28992316.

CpG island plots/CG distribution. Plots generated by the online “MethPrimer” program (www.urogene.org/methprimer/index1.html) were used as templates to draw the CG distribution with “Adobe Illustrator” software.

Tissues, cells and DNA. Benign adipose tissues were provided by the Cooperative Human Tissue Network (CHTC), which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. Abdominal adipose tissues were obtained from individuals undergoing reduction mammoplasty. Female F2, 28 years of age, reduction mammoplasty; female F5, 18 years of age, reduction mammoplasty. For each individual sample approximately 5 x 5 mm of tissue with homogenous white adipose appearance was taken for formalin fixation of tissue with homogenous white adipose appearance was taken for

Hairpin bisulfite PCR. The hairpin bisulfite PCR method and protocol is described in detail.23 In brief, ~5 μg of genomic DNA were cleaved with restriction enzymes close to the genomic region of interest: NheI for the human Leptin promoter; StyI (CG sites A–N), or FokI (CG sites M–U) for the mouse lepton promoter. After heat inactivation of restriction enzymes and buffer exchange, synthesized hairpin linkers were ligated onto the sticky ends of the cleaved genomic DNA. For each human DNA sample a hairpin linker with a different “batch-stamp” and “barcode” was used, thereby allowing the accreture of unambiguous sequence and methylation data and exclusion of redundant or contaminant sequences; the concept and use of encoded hairpin oligonucleotides has been described.24 For
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The spleen and brain DNA samples from mouse, the same hairpin linker was used (Fig. 5). Cytosine deamination by sodium bisulfite was performed as described. Ten heat-denaturing steps were included during the sodium bisulfite incubation step to repeatedly denature the DNA and thereby achieve a very high degree (>99%) of C → U conversion of non-CG cytosines. Sequences with more than one unconverted non-CG cytosine were excluded from the data sets. Sequences of the human Lepin hairpin linkers are as follows: 5'-CTAGAGCGATGCDDDDDGGCATCGCT-3' for individual M1; 5'-CTAGACGATGCDDDDDGGCATCGCT-3' for individual F1; 5'-CTAGAGCGATGCDDDDDGGCATCGCT-3' for individual F2; 5'-CTAGACGATGCDDDDDGGCATCGCT-3' for individual F3; 5'-CTAGACGTGCADDDDDGGCATCGCT-3' for individual F4; 5'-CTAGACGTGCADDDDDGGCATCGCT-3' for individual F5; 5'-CTAGACGTGCADDDDDGGCATCGCT-3' for mouse adipose DNA; 5'-CTAGACGTGCADDDDDGGCATCGCT-3' for mouse brain DNA.

Primers to amplify the 464 nt human Lepin hairpin fragment from bisulfite converted genomic DNA: hlept-F: 5'-TTGTTYGGTTAGTTTTAGTTTTTGYGA-3', hlept-R: 5'-CRACCAACCCCAATCCTAC-3'; annealing temperature: 53˚C. Primers to amplify a 435 nt mouse leptin hairpin PCR fragment from bisulfite converted DNA to analyze CG sites A to D: hp-ob3F: 5'-TGGGGYGTTTGGTATGAGTYG-3', hp-ob3R: 5'-ACAATAACCATATAACACTCTCTTCTAC-3'; annealing temperature: 58˚C.

Stringent conditions were used to convert mouse adipose and liver DNA samples, resulting in >99% conversion of non-CG cytosines. Primers to amplify the 429 nt mouse leptin PCR fragment from bisulfite converted DNA to analyze CG sites A to U:

BS-OB1F: 5'-CTATTAAAAAAAACCTAAAAAAACATAC-3',

BS-OB2R: 5'-CTATTAAAAAAAACCTAAAAAAACATAC-3',

annealing temperature: 55˚C.

Typically, PCRs were carried out in a reaction volume of 50 µl containing 25 µl HotStarTaq Master Mix (Qiagen), 1 µl each of forward and reverse primers (50 µM stock), 1–3 µl DMSO, 10–20 µl of bisulfite converted DNA and H₂O to adjust the reaction volume to 50 µl; 41 thermal cycles. Some of the PCR primers were designed using the online MethPrimer program (www.urogene.org/methprimer/index1.html).

Chi-square test. The Chi-square test was used to assess whether significant differences exist in Lepin promoter methylation densities between human and mouse. For this analysis data from epialleles that had 0, or 1 CG site methylated were excluded. The “0” class of epialleles was excluded because only methylated epialleles were examined; the “1” class of epialleles was excluded because a single methylation event lies within the range of “background noise” created by the occasional failure to convert an unmethylated cytosine during the sodium-bisulfite procedure. The remaining methylation data were pooled, independent of origin of tissue. For the human data pool a total of 4074 CG sites were sampled; 1246 methylation events were observed. For the mouse data pool a total of 1009 CG sites were sampled; 782 methylation events were observed.

RESULTS AND DISCUSSION

The autosomal LEP gene is primarily expressed in differentiated adipocytes of white-fat tissue. Therefore, in vivo methylation patterns of the human LEP CpG island were examined in benign abdominal and mammary fat tissue of females using hairpin bisulfite PCR. With this method, the methylation status can be determined on both strands of individual DNA molecules for all cytosines, including complementary, palindromic 5’-CG-3’ structures. Each of the sampled genomic epialleles was tagged with a molecular batchstap and barcode prior to the bisulfite sequencing procedure. This tagging ensures a high level of confidence in PCR-derived sequence data, since PCR contaminations and redundant epialleles can be identified and removed from the data set. As expected, some epialleles are entirely unmethylated and likely derived from differentiated adipocytes expressing LEP (Fig. 2). However, most epialleles are methylated and show surprisingly large variation in methylation densities. The spectrum of methylation densities ranges from 2% (1/42) methylated CG dinucleotides, to 76% (32/42) (Fig. 2).
The wide range of methylation densities at the human LEP promoter is unusual and differs from the bimodal methylation densities observed on genes carrying parental imprints, and from genes on the inactive X chromosome. Epialleles of both imprinted genes and inactive X genes have CpG islands that are either unmethylated and transcriptionally competent, or they are densely methylated and silenced\textsuperscript{23,25,26} (see also Fig. 4). This type of methylation-bimodality is not unlike a control switch with two settings: “on” or “off”. Once established, these epigenetic settings are stably maintained and inherited through many mitotic cell divisions. In contrast, low-density methylation, where only a subset of CG sites within a given locus is modified, appears to be inherently unstable.\textsuperscript{27} Lorincz and colleagues, for example, used proviral reporter constructs in murine erythroleukemia cells and showed that reporter sequences with low-density methylation stochastically gave rise to bimodal populations composed of some unmethylated epialleles, and some densely methylated epialleles.\textsuperscript{27} Consequently, it will be important to establish the origin and fate of moderately and sparsely methylated LEP epialleles. They may be derived from cells that stably maintain these low-density methylation levels, or, alternatively, from preadipocytes undergoing differentiation into mature adipocytes. In the latter case, these epialleles may be intermediates sampled during an epigenetic transition leading to a completely unmethylated LEP promoter. In vitro differentiation of preadipocytes to adipocytes indeed correlates with reduction of methylation at the promoter, and induction of LEP expression.\textsuperscript{20,21} The question arises whether reduction of methylation density at the LEP promoter is a developmentally regulated process that occurs in vivo during adipocyte maturation. Methylation density is overall lower in epialleles recovered from fat tissue when compared with epialleles derived from peripheral blood leukocytes (see Figs. 2 and 3; and text below), supporting the hypothesis that LEP promoter demethylation may occur in adipose tissue. Various somatic cell types, including adipose tissue, will need to be sampled from one and the same individual in order to assess in greater detail the occurrence of tissue-specific demethylation.

To investigate the methylation status in different types of human somatic cells the same LEP promoter region was analyzed in peripheral blood leukocytes (PBLs). Pronounced diversity in methylation densities was observed, ranging from epialleles with 7% (3/42) CG methylation, to epialleles with 88% (37/42) methylated CG dinucleotides (Fig. 3). Only a single unmethylated epiallele was recovered from PBL DNA; intermediate levels of methylation characterize the majority of LEP epialleles (Fig. 4).

**Figure 2.** In vivo methylation patterns of the LEP promoter in human fat tissues. 21 complementary CG dinucleotides were analyzed by hairpin-bisulfite PCR on both strands of individual DNA molecules. The two rows of boxes within each rectangle represent CG sites on either the top or the bottom strand of an individual, double-stranded DNA molecule. Unmethylated CGs are shown in white; methylated CGs are shown in black. The sequences obtained from each tissue are sorted by methylation density. Numbers correspond with the 21 CGs of the human sequence depicted in Figure 1B.
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Methylation data collected in the past from the *FMR1* promoter on inactive X-alleles in female cells enabled comparison with methylation data of the *LEP* promoter, since DNA from PBLs was used for the analysis of both genes. The broad distribution of methylation densities within the population of *LEP* epialleles is significantly different and contrasts sharply with the bimodal distribution of *FMR1* epialleles, whose methylation densities correlate to the stable on-off expression states of *FMR1* (Fig. 4). CpG island methylation typically indicates permanent gene silencing, and is thought to prevent efficient gene reactivation. Overall, the moderate and low levels of promoter methylation detected in human adipose tissues and PBLs suggests that the epigenetic state of *LEP* is generally less stable, perhaps facilitating transitions between active and inactive expression states during the life time of a cell or tissue. Rather than an “on-off” switch, the broad spectrum of methylation densities evokes comparison with a dimmer switch, where methylation density of an epiallele may reflect, or even modulate, the expression level of *LEP*.

Melzer and colleagues have demonstrated that methylation indeed can modulate or silence the activity of the *LEP* promoter when driving the expression of a luciferase reporter gene in a human adipose cell line. Their results showed that luciferase activity from the partially-methylated *LEP* promoter was reduced by one-third relative to activity from the unmethylated promoter. Three specific CG sites within the minimal *LEP* promoter region were found to play an important role in regulating reporter gene expression; Methylation of a CG located within the C/EBP-binding motif caused a decrease in promoter activity, and methylation of two CG sites proximal to the TATA-box strongly diminished expression of the luciferase reporter. The in vivo methylation status of these three CG sites is reported here, as they correspond to CG sites 11, 12 and 13 within the analyzed region of the human *LEP* promoter (see Figs. 1B, 2, 3 and 6). It is apparent that CG sites 11 to 21 are by and large methylated at higher frequency, than are CG sites 1 to 10 (see Figs. 2, 3). CG site 11 is of interest since methylation could affect binding and activity of C/EBP-α, a transcription factor that is known to modulate expression of *LEP*. CG site 11 is methylated at a high frequency (77%), when compared to the regional average (43%) in methylated PBL-epialleles. Similarly, 43% of methylated epialleles from fat tissue are modified at CG site 11, whereas the regional average of methylation density is only 23%. The incidence of methylation at the C/EBP-α binding site is particularly striking in sparsely methylated epialleles from fat tissue, since only one or few additional cytosines appear to remain methylated, along with CG site 11 (Fig. 2). Further research is warranted to determine how methylation density of a given *LEP* epiallele in vivo correlates with: (1) the modification of histones surrounding the promoter and (2) transcription levels of *LEP* RNA.

The mouse *Lep* promoter was analyzed in four somatic tissues. In fat tissue, a mosaic pool of unmethylated and densely methylated *Lep* epialleles was detected (Fig. 5). Cells from brain, liver and spleen carry *Lep* epialleles that are hypermethylated in most cases (Fig. 5). The current data provide evidence that
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Methylation densities are significantly different between human LEP and mouse Lep; murine epialleles are overall more densely methylated than their human orthologues \( (p < 0.001; \chi^2 = 446, \text{df} = 3) \). This finding suggests that the mouse Lep gene may have a higher probability of acquiring dense methylation patterns, which generally correlate with gene silencing.

DNA methylation established at particular genomic loci can be transmitted to offspring.\(^30\)\(^-\)\(^33\) Thus, I was curious to assess LEP methylation status in human sperm DNA. With a single exception, the sampled sperm epialleles were all unmethylated (12), or had a single methylated CG site (2) (Fig. 6). The lone densely methylated epiallele likely derived from a somatic cell that was isolated along with spermatozoa, but the possibility that this sequence came from a gametic cell cannot be excluded. Likewise, predominantly hypomethylated Lep epialleles were recovered from mouse sperm, clearly distinguishing this cohort of molecules from somatic tissue sequences (Fig. 6). A few methylated Lep sequences (5) were also detected in the mouse sperm DNA sample, but ascertain-ment of their somatic or gametic origin was not possible (Fig. 6).

The data from human and mouse sperm suggest that the LEP promoter becomes reprogrammed by demethylation during male gametogenesis. Since there is no indication that LEP/Lep carries a parental- specific imprint, and this locus is generally unmethylated in spermatozoa, de novo methylation of the promoter most often is expected to occur in somatic cells, after fertilization.

While most germ cells have unmethylated LEP epialleles, it is conceivable that some of the sperm cells could carry methylated versions of the LEP promoter. Evidence that the methylation state of a gene can vary in the male germ line is provided by results of recent studies, where intra- and interindividual methylation differences were identified in humans.\(^33\)\(^,\)\(^34\) Future experiments are required to determine whether transgenerational inheritance of methylated LEP epialleles is possible.

Methylation in the germline would be expected to deplete the Leptin promoter of CG dinucleotides over time. Methylated cytosines tend to deaminate at higher frequencies than do unmethylated cytosines, and thereby promote evolutionary C \( \rightarrow \) T and G \( \rightarrow \) A base transitions.\(^35\)\(^,\)\(^36\) I compared 1500 bp of orthologous, genomic sequence surrounding the mouse and human Lep/LEP promoters, and observed a 58% lower density of CG dinucleotides in mouse (34 CGs vs. 81 CGs). Furthermore, the 216 bp mouse CpG island is almost three times smaller than the 625 bp human CpG island sequence (Figs. 1A and 5A). The core of the Lep/LEP promoters is conserved, but numerous CGs in the human sequence are occupied in mouse by TG or CA dinucleotides at the equivalent location; these are likely deamination products of methylated cytosines (Fig. 1B). The “sinking” CpG island in mouse suggests that methylated...
Lep epialleles are present in the germline at a higher frequency than their human orthologs. Depletion of CG dinucleotides is not restricted to the Lep CpG island, and appears to affect many CpG islands in the mouse genome. The question whether increased methylation, as observed for the murine Lep promoter in somatic tissues, is a genome-wide phenomenon in mouse, and a contributing factor to the accelerated loss of CpG islands in rodent cells, calls for comparative epigenomic studies.

In summary, the analysis of somatic and gametic cells demonstrated that the Lep CpG island constitutes a T-DMR, and that the promoter is subject to dynamic, epigenetic programming in human and in mouse. Expression of this metabolic gene therefore has the potential to be modulated by DNA methylation, raising an abundant number of intriguing biologically and clinically relevant questions. Identification of in vivo methylation at the LEP promoter provides a molecular entry point to study the timing, factors and conditions that lead to tissue-specific methylation patterns of gene promoters.

Note added in proof: In a recent study Collas and colleagues also report mosaic cytosine methylation of the LEP promoter in human adipose tissue (Noer A, Sørensen AL, Boquest AC, Collas P; Stable CpG hypomethylation of adipogenic promoters in freshly isolated, cultured, and differentiated mesenchymal stem cells from adipose tissue. Mol Biol Cell 2006; 17:3543-56.)

References