Dynamic readjustment of parental methylation patterns of the 5′-flank of the mouse H19 gene during in vitro organogenesis

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ABSTRACT Gametic marks are stably propagated in order to manifest parent of origin-specific expression patterns of imprinted genes in the developing conceptus. Although the character of the imprint has not yet been fully elucidated, there is compelling evidence that it involves a methylation mark. This is exemplified by a region upstream of the H19 gene, which is not only methylated in a parent of origin-specific manner, but also regulates the silencing of the maternal Igf2 and paternal H19 alleles, respectively. We show here that the parental-specific methylation patterns within the differentially methylated domain (DMD) are perturbed in the soma during in vitro organogenesis. Under these conditions, the paternal DMD allele becomes partially demethylated, whereas the maternal DMD allele gains methylation. Despite these effects, there were no changes in allelic Igf2 or H19 expression patterns in the embryo. Finally, we show that although TSA derepresses the paternal H19 allele in ectoplacental cone when in vitro developed, there is no discernible effect on the methylation status of the paternally inherited 5′-flank in comparison to control samples. Collectively, this data demonstrates that the parental mark is sensitive to a subset of environmental cues and that a certain degree of plasticity of the gametic mark is tolerated without affecting the manifestation of the imprinted state.

KEY WORDS: Genomic imprinting, methylation, epigenetic mark, in vitro organogenesis, H19.

Introduction

Over the past decade, it has become increasingly clear that the regional methylation status may serve as a gamete-specific imprint to regulate the expression of a subset of autosomal genes in the soma (Bartolomei and Tilghman, 1997; Li et al., 1993; Ohlsson, 1999; Tucker et al., 1996). The parent of origin-specific methylation patterns are established not only by differential methylation of the parental alleles during gametogenesis (Tucker et al., 1996), but are also influenced by two distinct epigenetic reprogramming events in the early zygote (Monk et al., 1987). The differentially methylated domains (DMD) stand out from non-imprinted adjacent sequences due to regional-specific protection from either demethylation during preimplantation development or remethylation during early postimplantation (depending on the sex of the transmitting parent) (Olek and Walter, 1997; Tremblay et al., 1997). It appears clear, therefore, that the stable propagation of both the unmethylated and methylated status of the DMDs constitute parental marks which manifest imprinted gene expression patterns.

By using cultured mouse embryos, which complete their organogenesis in vitro, we have previously reported that TSA, an inhibitor of histone deacetylases, derepresses the paternal H19 allele in the ectoplacental cone, but not in the embryo itself (Svensson et al., 1998). Given that TSA treatment demethylates some DNA sequences in Neurospora (Selker, 1998), our initial objective considered the possibility that TSA-dependent derepression of the paternal H19 allele depended on tissue-specific demethylation events. We show here that although there is a certain degree of demethylation of the paternal H19 DMD allele in both the ectoplacental cone and the embryo during in vitro development, this is independent of TSA. Contrary to our expectations, the maternal allele of the H19 DMD became methylated in both the ectoplacental cone and the embryo during in vitro development. The underlying causes of the modifications in the gametic marks during in vitro developments are discussed.

Abbreviations used in this paper: DMD, differentially methylated domain; IAP, Intracisternal A particles; Igf2, insulin-like growth factor 2; TSA, trichostatin A.
Results

Changes in allelic DNA methylation patterns during in vitro organogenesis

To examine parent of origin-specific methylation patterns of in vitro developed mouse conceptuses, we initially performed Southern blot hybridisation analysis of reciprocal intra-specific hybrid conceptuses (Mus musculus musculus, or “M”, and Mus musculus domesticus, or “D”), using the methylation sensitive restriction enzyme Hpa II. We focused on the H19 5’-flank, since it is methylated in a parent of origin-dependent manner (Olek and Walter, 1997; Tremblay et al., 1997) and regulates the repressed states of the maternal Igf2 and paternal H19 alleles (Thorvaldsen et al., 1998). The region under examination encompassed not only the H19 DMD (3 Hpa II sites) but also the region separating the DMD from the H19 promoter (2 Hpa II sites, Fig. 1A). The parental origin of the alleles was assessed by exploiting a polymorphic Bbs I site situated at the 3-end of the DMD (Fig. 1A) (Kanduri et al., 2000). Figure 1B shows the result of this analysis, obtained with in vivo developed control conceptuses. As could be predicted from previously published observations, the paternal allele of F1 hybrid embryos specifically resisted Hpa II digestion at two different developmental time points (E9.5 and E16.5) and mouse embryos cultured in vitro, in the presence or absence of trichostatin A. DNA, which was derived from reciprocal (M x D; D x M) crosses of intraspecific hybrid conceptuses, was restricted with Eco RI, Bbs I and Hpa II. The arrows depict undermethylated M. m. musculus sequences, whereas the arrowheads indicate undermethylated M. m. domesticus sequences. (A) shows the DMD region (probe 1) and (B) shows the region separating the DMD from the H19 promoter (probe 2). Emb., embryo proper; EPC, ectoplacental cone.

Fig. 1. Methylation analysis of the H19 5’-flank in in vivo developing mouse embryos. (A) Restriction map of the H19 5’-flank. The polymorphic Bbs I site is M. m. musculus-specific. (B) Southern blot hybridisation analysis of genomic DNA from E16.5 foetuses (D x M and M x D) and E9.5 embryo (D x M). DNA was restricted with Eco RI and Bbs I (EB) and by Hpa II (H) or Msp I (M) restriction, as indicated in the Figure.

Fig. 2. In vitro organogenesis perturbs parental methylation marks in the H19 5’-flank. The figure panels show Southern blot hybridisation analysis of genomic DNA prepared from fetal liver (E16.5) and mouse embryos cultured in vitro, in the presence or absence of trichostatin A. DNA, which was derived from reciprocal (M x D; D x M) crosses of intraspecific hybrid conceptuses, was restricted with Eco RI, Bbs I and Hpa II. The arrows depict undermethylated M. m. musculus sequences, whereas the arrowheads indicate undermethylated M. m. domesticus sequences. (A) shows the DMD region (probe 1) and (B) shows the region separating the DMD from the H19 promoter (probe 2). Emb., embryo proper; EPC, ectoplacental cone.
When we compared the methylation patterns of the parental DMD alleles between in vivo and in vitro maintained mouse conceptuses, a notable difference could be observed: Whereas the paternal H19 DMD allele showed some loss of methylation at the diagnostic Hpa II sites, the maternal H19 DMD allele displayed a gain of methylation, as revealed by a reduced sensitivity to Hpa II digestion (Fig. 2A). The attenuation of the methylation difference, as determined by Hpa II, extends from the DMD into the spacer region that separates the DMD from the H19 promoter (Fig. 2B). This data could be obtained on reciprocal crosses although we observed that in vitro maintenance of the mouse conceptuses had a stronger effect on the maternal-specific gain of methylation of the DMD allele when this was derived from Mus musculus.

To gain a more detailed insight into the CpG methylation patterns, we exploited the bisulphite sequencing technique. The analyses were limited to a region of 426 bases, which encompasses bases −3978 to −3505 from the transcriptional start site of the H19 gene, since we were unable to detect polymorphisms in other regions of the H19 DMD in our mouse strains. On the other hand, the selected region is not only part of the region which is differentially methylated in a parent of origin-specific manner (Olek and Walter, 1997; Tremblay et al., 1997), but is also included in the stretch of sequences which regulates the silencing of the maternal Igf2 and paternal H19 alleles, as shown by targeted deletion analysis (Thorvaldsen et al., 1998). Due to the methylation background of the maternal H19 DMD allele in the M x D crosses (see above), we examined alleles derived from only D x M crosses.

Sequence analysis of cloned parental alleles of in vivo developed mouse embryos at E9.5 confirmed previous reports (Olek and Walter, 1997; Tremblay et al., 1997); this region is persistently methylated on the paternal allele and unmethylated on the maternal allele (Fig. 3). A different pattern emerged, however, when in vitro developed mouse embryos (E9.5) were examined. Whereas the paternal allele of the H19 DMD was generally less methylated, the maternal allele gained methylation during in vitro development compared to in vivo developed control specimens (Fig. 3). Although we were unable to trace the parental identity of any other region within the H19 DMD, encompassing bases −3150 to −2696 from the transcriptional start site, the methylation pattern was again mosaic (Fig. 3C). Collectively, the data indicate a dynamic readjustment of the parental methylation marks within the H19 DMD.

**No evidence of genome-wide de novo (de)methylation during in vitro development**

In order to understand the underlying cause of the readjustment of the parent of origin-specific methylation changes during in vitro development, we aimed to discriminate between a regional effect and a more general breakdown of methylation patterns. It has previously been shown that a substantial fraction of the methylated CpGs in the genome can be attributed to endogenous proviral sequences, such as IAP (Walsh et al., 1998). We rehybridised the
membranes of Figs. 1 and 2, therefore, with a probe specific for the LTR region of IAP. Fig. 4 A,B shows that there is no significant modification in the methylation patterns in IAP sequences between mouse embryos developed in utero or in the test tube. Similar data was obtained by rehybridising the membranes with a 0.2 kb probe, derived from Hpa II-digested genomic DNA (data not shown). These results indicate that the modification of the methylation pattern in the H19 DMD is diagnostic for regional, rather than genome-wide events.

**Trichostatin A treatment does not modify the methylation status within the H19 DMD**

As noted above, our initial objective of examining methylation patterns in mouse conceptuses undergoing in vivo development was triggered by the observations that TSA generates demethylated DNA in Neurospora crassa (Selker, 1998) and that TSA reverses the silenced state of the paternal H19 allele in the ectoplacental cone (Svensson et al., 1998). Fig. 2B show that the degree of Hpa II digestion of the paternal H19 DMD allele is very similar between reciprocal crosses of TSA-treated and control specimens, in both the ectoplacental cone and in the embryo. We conclude that the derepression of the paternal H19 allele, which was previously reported by us, does not involve any significant TSA-dependent effect on the paternal-specific methylation status. Given that the methylation patterns of multiple IAP genomes appear to be similarly unaffected by TSA-treatment during in vitro organogenesis (Fig. 4), this conclusion is not restricted to the paternal H19 locus, but apparently applies on a more genome-wide scale.

**The imprinted state of Igf2 is maintained during in vitro development**

Despite the partial demethylation of the paternal DMD allele during in vitro organogenesis, which we describe here, the stringency of the repressed state of the paternally derived H19 allele is maintained in the embryo (Svensson et al., 1998). To examine whether or not the same conclusion applies to the repressed state of the maternally derived Igf2 allele, we exploited a strand-specific sequence difference in exon 6 of Igf2. The allelic Igf2 expression status was examined by sequencing PCR-amplified fragments derived from reverse-transcribed total cellular RNA. Fig. 5 shows that the sequencing strategy of the D and M alleles, which differ by a G→A transition, enabled us to accurately determine the parental origin of the Igf2 allele. When reverse-transcribed cDNA (Fig. 5A) was similarly analysed, it appeared that the paternal Igf2 allele was preferentially expressed both during in vivo and in vitro development (Fig. 5B). We conclude that the imprinted state of Igf2 is maintained, despite the gain of methylation during in vitro organogenesis.

**Discussion**

We document here that the parental methylation mark, which is involved in the regulation of parent of origin-dependent expression of the neighbouring Igf2 and H19 loci, is plastic during in vitro organogenesis. This data suggests that the absence of in vivo factors and/or the presence of in vitro factors dynamically modulates the methylation status of the H19 DMD. Despite this, the imprinted status of both Igf2 and H19 is maintained, showing that a certain degree of plasticity of the parental mark can be tolerated. Whether or not this implies the existence of a threshold of methylation changes; at which the imprinted state can be lost and/or if the repressed status of the paternal H19 allele and maternal Igf2 allele can be differentially relaxed, remains to be established. Although the causes underlying both the loss and gain of methylation marks on the parental DMD alleles are unknown, our methylation analyses indicate that this effect represents a regional, rather than a genome-wide, break-down in the regulation of methylation status. This deduction is supported by the absence of any marked up- or downregulation of expression of the Dnmt3a and b genes, which are the major de novo methyltransferase genes (Reik et al., 1999), during in vitro organogenesis (data not shown).

The dynamic readjustment of the methylation status of the H19 DMD during in vivo development is concomitant with genomewide de novo methylation in vivo (Monk et al., 1987). It is conceivable, therefore, that the limited demethylation of the paternal DMD allele results from active demethylation events. This deduction is in line with the observation of a culturing medium-dependent demethylation of the paternal H19 DMD allele during in vitro preimplantation development (Doherty et al., 2000). The gain of methylation on the maternal H19DMD allele may, however, reflect a perturbation at another level of control. We have earlier shown that the maternal DMD allele normally adopts a chromatin conformation which displays multiple nucleosome hypersensitive sites at linker regions between positioned nucleosomes (Kanduri et al., 2000). This unusual chromatin structure, which can be observed both in the soma and in embryonic stem cells, would presumably protect against de novo methylation during normal postimplantation development, but is perturbed during in vitro organogenesis. Since the limited amounts of tissue available from in vitro develop-
We have earlier shown that TSA-treatment of in vitro developing mouse conceptuses derepresses the paternal H19 allele. This report documents that this effect does not depend on any TSA-dependent demethylation event (Svensson et al., 1998). It remains a distinct possibility, therefore, that TSA inhibits histone deacetylase activity that is associated with the chromatin of the paternally inherited DMD allele in cells of the ectoplacental cone. This notion is supported by our observation that antibodies against MeCP1 and MeCP2, which attract histone deacetylases, specifically pull down the paternal H19DMD allele in chromatin immunopurification analyses (Kanduri et al., unpublished observation).

Here, we have documented an unexpected plasticity in the parental methylation mark in the differentially methylated domain in the 5'-flank of the H19 gene in response to a subset of environmental cues. Although the methylation difference between the parental DMD alleles is attenuated to various degrees during in vitro organogenesis, the imprinted expression patterns of the Igf2 and H19 genes is maintained. This data suggests that an absolute methylation difference between the parental DMD alleles is not necessary for manifesting the imprinted state and raises the question: what minimal methylation difference is required to maintain the imprinted state. It may now be essential to find the agent(s) that induces the methylation modifications. Such information could facilitate our understanding of the character of the parental imprints and the mechanisms underlying loss of imprinting in neoplasia.

Materials and Methods

Mouse strains and in vitro organogenesis

Mus musculus musculus (M or Mus; CZECH II, Jackson laboratory) were mated with Mus musculus domesticus (D or Dom; NMRI) to create intra-specific F1 hybrid conceptuses. These are referred to as D x M or M x D conceptuses in the order of mother-father. At E7.5, the embryo was explanted from the uterus, and maintained in vitro for two days, as described previously (New, 1978; Svensson et al., 1998).

Genotyping

DNA was prepared from in vivo and in vitro embryo specimens using standard procedures (Svensson et al., 1998). To determine the Igf2 genotype, genomic DNA fragments, derived from M or D, were PCR amplified using primers migf2ex6270 (CCATCAATCTGTGACCTCCTC) and migf2ex6789 (CACTGAAGCAATGACATGCC) which cover the midportion of exon 6 (position 24626 to 25152 in GenBank file: Mus musculus insulin-like growth factor II (Igf2 ) gene, complete cds., accession number U71085) followed by DNA sequencing (ABI 377). The mouse strains differ at position 3695 (A in Mus and G in Dom) with respect to the Igf2 transcriptional start site.

Probes and Southern blot hybridisation analyses

Probe 1: A 1.6 kb Bcl I/Bbs I fragment, covering only the H19 DMD; probe 2: A 1.8 kb Bbs I/Eco RI fragment, covering the spacer region between the H19 DMD and H19 transcriptional start site. The IAP LTR probe (a 0.7 kb fragment) was a kind gift of Dr T. Bestor (Walsh et al., 1998). All probe fragments were radiolabeled by using a multiprime labelling kit and α-32P-dCTP (Amersham) to a specific activity of more than 1 x 10^9 cpm/µg. The restriction enzyme-digested DNA samples were electrophoresed in 1.0 % agarose gels, depurinated and blotted to Hybond N+ membranes followed by hybridisation according to routine protocols.

CpG methylation analysis

The methylation status of the H19 DMD region was determined both by Southern blot hybridisation analysis of Hpa II-restricted DNA and by the bisulphite treatment protocol. Southern blot analysis was carried out using
a standard protocol: membranes were prehybridised and subsequently hybridised with \(^{32}P\)-dCTP-labeled DNA probe for 16-24 hours at 42°C. DNA probes were labelled with Random Primers DNA Labelling system (Gibco BRL). Bisulphite treatment of DNA was carried out using established protocols (Olek et al., 1996), with the following adaptations: DNA was restricted with excess amount of BglII to generate suitably small fragments containing the target sequence. One \(\mu\)g of digested DNA was denatured with 0.3 \(\mu\)M NaOH at 37°C for 15 minutes, then mixed with 2 volume of 2% low-melting agarose dissolved in water. The mixture was pipetted into pre-chilled mineral oil to form DNA- agarose beads. The prepared beads were then incubated with 1.2 ml 5.0 M NaHSO\(_3\)/20 mM Hydroquinone solution covered by mineral oil and incubated at 51°C for 6 hours. Treated DNA beads were equilibrated with TE (1 mM EDTA; 10 mM Tris-HCl, pH 8.0), 6 x 15 minutes. Following desulphonation with 0.2 M NaOH, 2 x 15 minutes and equilibration with MilliQ water, 2 x 15 minutes at RT, the treated DNA beads were subjected to PCR amplification reactions, using the following published primers and conditions (Tremblay et al., 1997); BMsp211, Bhhalt3, BMSP2t2, BhhaIt4 (the final PCR product covers bases –3952 to –3530 relative to the transcriptional start site), BMSP2t2, BhhaIt4 (the final PCR product covers bases –3150 to –2696 relative to the transcriptional start site). Amplified fragments were cloned into the PCR2.1 vector (Invitrogen); and subsequently sequenced with the T7 sequenase kit (USB., Amersham) or the BigDye-tm Terminator Cycle Sequencing Kit.

**RNA extraction and Igf2 expression analysis**

*In vitro* (E9.5) and *in vivo* (E9.5 and E16.5) maintained embryos were harvested, dissected and total RNA was extracted using the Tripure reagent (Boehringer Mannheim). About 15 \(\mu\)g of total RNA was subjected to RT treatment (Promega) and the resulting cDNA was used as template for PCR reaction (Expand-tm High fidelity, Boehringer Mannheim). The 5’-flank of the murine H19 gene in an unusual chromatin conformation unidirectionally blocks enhancer-promoter communication. *Curr. Biol* 10: 449-457.

**References**


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