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Original Article

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.

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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

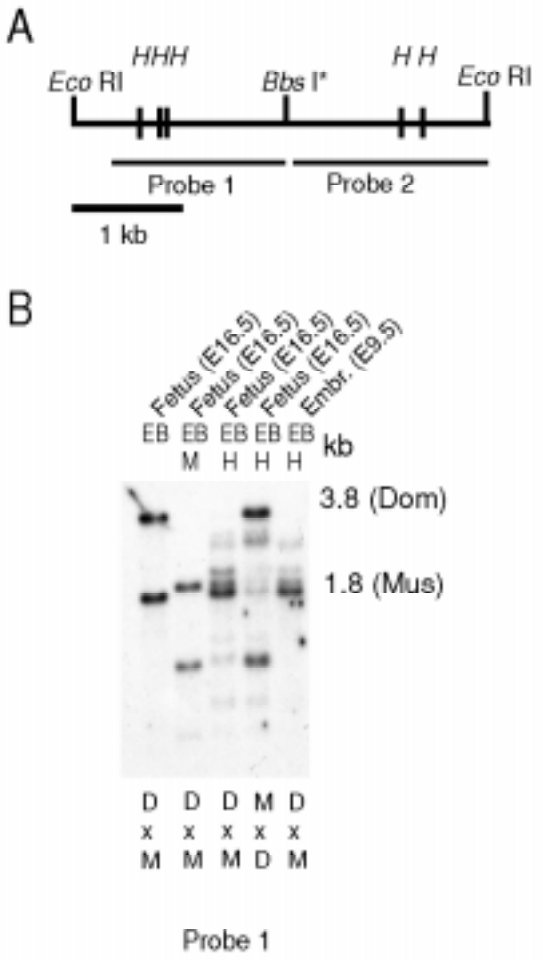

Fig. 1. Methylation analysis of the *H19* 5′-flank in *in vivo* developing mouse embryos. **(A)** *Restriction map of the* H19 *5′-flank. The polymorphic Bbs I site is M. m. musculus-specific.* **(B)** *Southern blot hybridisation analysis of genomic DNA from E16.5 foetuses (D x M and M x D) and E9.5 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.*

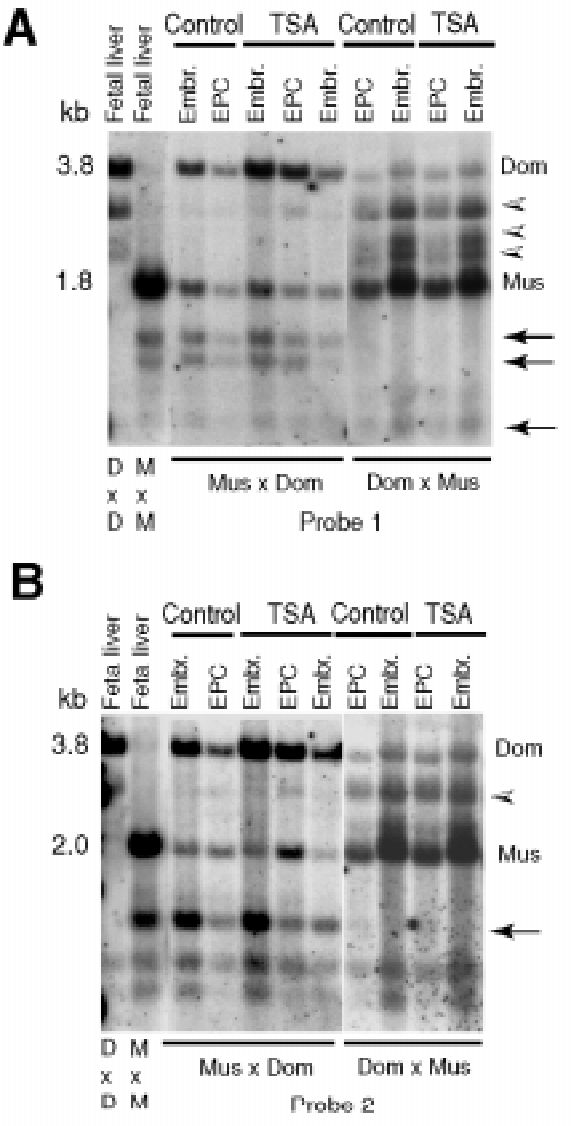

Fig. 2. *In vitro* organogenesis perturbs parental methylation marks in the *H19* 5′-flank. *The figure panels show Southern blot hybridisation analysis of genomic DNA prepared from fetal liver (E16.5) and mouse embryos cultured in vitro, in the presence or absence of trichostatin A. DNA, which was derived 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). Embr., embryo proper; EPC, ectoplacental cone.*

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). We also note, however, that the maternal *H19* DMD allele is methylated at all *Hpa* II sites in a subpopulation of cells in the M x D crosses, but not in the D x M crosses (Fig. 1). This data, which could be reproduced on several different occasions, suggests that the methylation status of the parental alleles of the *H19* DMD is subject to genetic background effects.

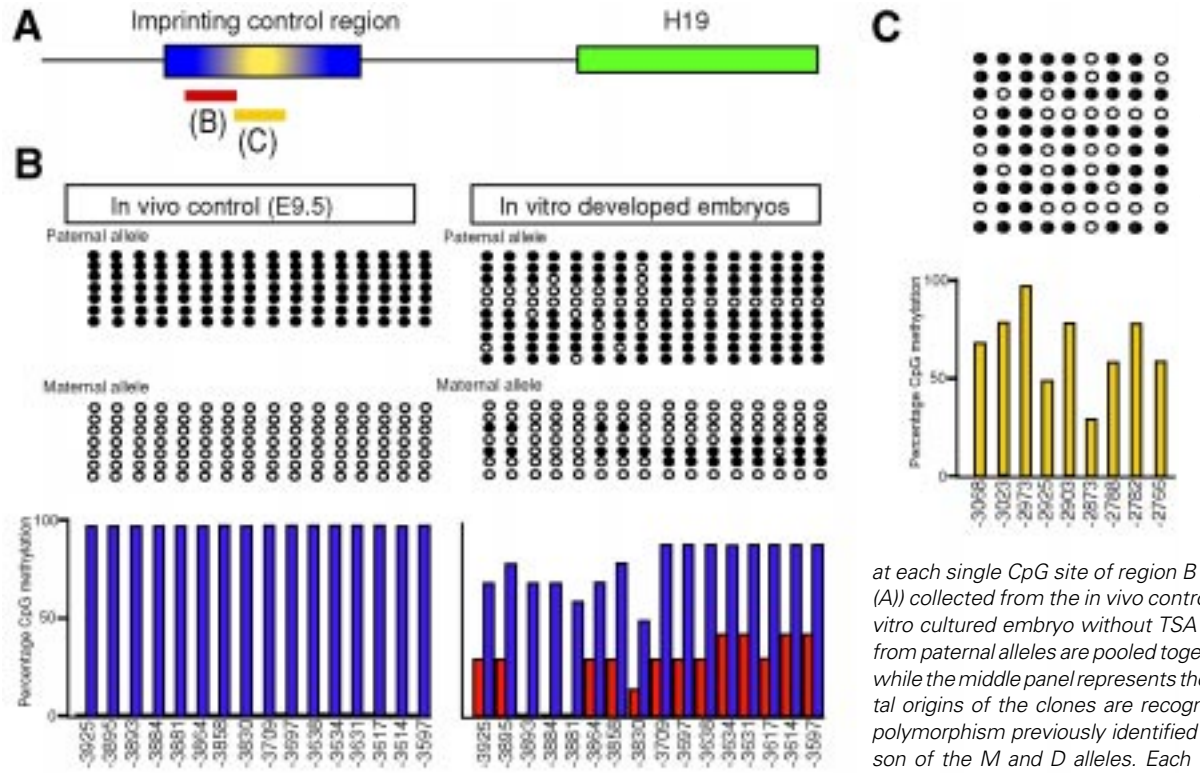

Fig. 3. Bisulfite sequencing data and clonal percentage presentations of methylation levels at single CpG doublets on paternal and maternal *H19* DMD alleles. **(A)** *Schematic illustrations of the* H19 *gene (green bar) position and the upstream imprinting control region (DMD, purple and yellow bar). The thin red and yellow bars depict the sequences that were examined by bisulfite genomic sequencing.* **(B)** *presents the methylation status at each single CpG site of region B (depicted as red bar in (A)) collected from the in vivo control embryo (E9.5) and in vitro cultured embryo without TSA (E9.5). Clones derived from paternal alleles are pooled together in the upper panel, while the middle panel represents the maternal ones. Parental origins of the clones are recognised by the sequence polymorphism previously identified by sequence comparison of the M and D alleles. Each filled circle indicates a methylated CpG site whereas each open circles represents an unmethylated CpG site. The total level of the methylation at each CpG position is summarised in the lower panel of the diagram, with maternal clones (dark red bars) localised to the left of their corresponding paternal clones (dark blue) for each CpG site. The numbers below the diagram show the relative position of each site to the transcription start site of* H19 *gene. The height of each bar shows in percentage the level of the methylated clones.* **(C)** *illustrates the methylation status of the core region of the DMD as depicted in (A). Due to the lack of polymorphic information between D and M alleles, we were unable to determine the parental origins of the clones. The clones were pooled together in spite of their parental identities. The symbols used in this figure are the same as in (B), except that each bar stands for the methylation level at each site comprising both paternal and maternal alleles.*

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 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

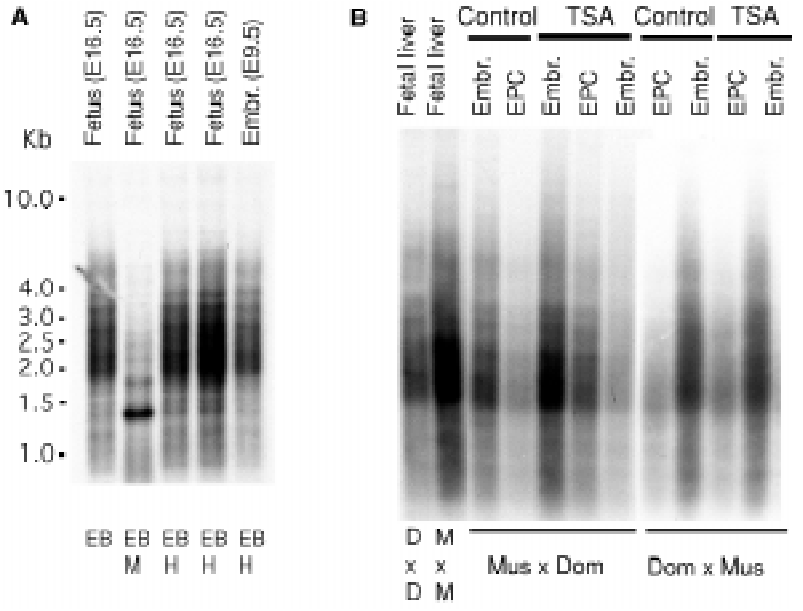

Fig. 4. CpG methylation analysis of Intracisternal A Particle LTRs. (A,B) *Blots used in Figure 2 (A,B) respectively, were rehybridised to an IAP-specific LTR probe. Comparison of the Hpa II restriction pattern among all the samples showed no observable difference in the methylation level of the highly repeated endogenous retrovirus sequences, which are normally known to be heavily methylated in the genome.*

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 vitro* 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 vitro* development is concomitant with genome-wide *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 *H19* DMD allele may, however, reflect a perturbance at another level of control. We have earlier shown that the maternal DMD allele normally adopts a chromatin conformation which displays multiple nuclease 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* devel-

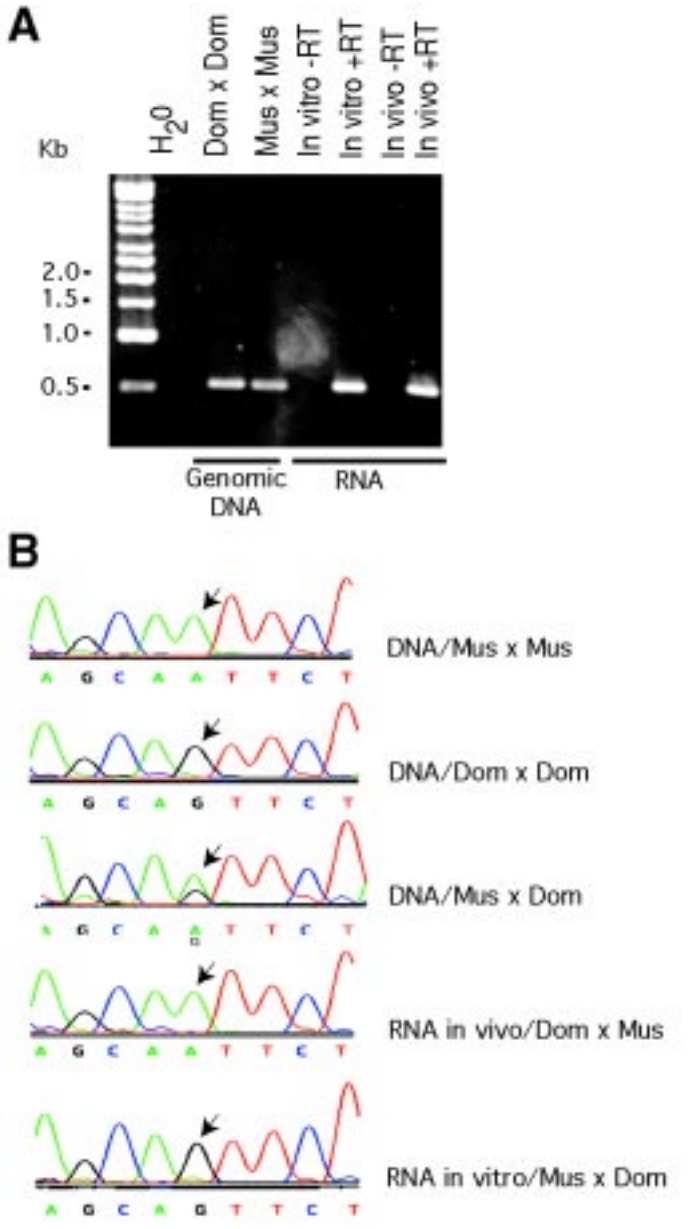

Fig. 5. The imprinted state of *Igf2* is maintained in *in vitro* developing mouse embryos. *The allelic expression pattern of* Igf2 *was examined by sequencing RT-PCR products of total cellular RNA derived from intraspecific hybrid embryos maintained both in vivo and in vitro. The single nucleotide polymorphism in exon six of* Igf2 *was used for distinguishing the maternal and paternal origins.* **(A)** *Agarose analysis of amplified DNA from genomic DNA (M x M; D x D; M x D) and reverse transcribed mRNA derived from M x D and D x M crosses, as indicated in the Figure. The size of the expected PCR fragment is 519 bp. –RT and H_2O depict controls that address contamination of genomic DNA in the RNA preparations and PCR reagents, respectively.* **(B)** *shows the sequencing results of the PCR products. The short stretch of the sequence AGCAGTTCT (Dom) or AGCAATTCT (Mus) depicts the polymorphic site used to determine the parental origin of the* Igf2 *transcripts, as indicated in the Figu*re.

oped mouse embryos defy regular chromatin analyses, it remains to be shown how the maternal *H19* DMD allele gains a methylation mark during *in vitro* organogenesis.

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 *H19* DMD 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-^{32}P-dCTP (Amersham) to a specific activity of more than 1 x 10^8 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 *Bgl* II to generate suitably small fragments containing the target sequence. One μg of digested DNA was denatured with 0.3 M NaOH at 37ºC 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 51ºC 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): BMsp2t1, BhhaIt3, BMSP2t2, BhhaIt4 (the final PCR product covers bases –3952 to –3530 relative to the transcriptional start site), BMsp4t1 and Bhha4t2. The PCR product of the last two primers covers bases –3150 to –2696 relative to the transcriptional start site of the *H19* gene. 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 μg 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 primers were designed to amplify exon six of *Igf2,* where a single nucleotide polymorphism (G-A) facilitated the discrimination of parental allelic usage, as accounted for above. Amplified PCR products were sequenced directly using the BigDye-tm Terminator Cycle Sequencing Kit

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