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Gamete-specific methylation correlates with imprinting of the murine *Xist* gene

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We have investigated the potential role of DNA methylation as a regulator of imprinted *Xist* expression in mouse preimplantation embryos. The active paternal allele was found to be unmodified in sperm at CpG loci near the 5' end of the gene transcription unit. In contrast, on the inactive maternal allele, these same sites are initially methylated in the oocyte and then remain modified in the early embryo. In the male germ line, these methyl moieties are removed during spermatogenesis, and this occurs before the programmed reactivation of *Xist* in the testis. This represents a clear-cut example of a potential methylation imprinting signal that is reprogrammable and gamete derived.

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Studies on both endogenous and exogenous imprinted genes in the mouse have shown that these loci are characterized by regions of allele-specific methylation, which suggests that DNA modification may play a role in the regulation of genomic imprinting[1]. It is very likely that some of these methyl moieties are involved in maintaining differential expression from individual parental alleles in embryonic and adult cells of the organism. Although this has not been demonstrated directly for any specific gene, massive undermethylation of the genome, such as that observed in methyltransferase-deficient mice, has been shown to cause the elimination of imprinted expression for insulin-like growth factor II (*Igf2*), insulin-like growth factor II receptor (*Igf2r*) and *H19* (ref. 2).

Another possible function of DNA modification may be to mark imprinted genes in the gametes and in this way to provide a signal for distinguishing the two parental alleles in the embryo. Although this model is attractive, the facts are that most methyl moieties associated with imprinted genes are actually removed from zygotic DNA in the morula and blastula and that the allele-specific pattern seen in later development is in a large part established *de novo* in the post-implantation embryo[3]. These somatically generated differentially modified CpG sites cannot therefore serve as imprinting signals. In contrast, well delineated loci in the *Igf2r* gene domain[4] and the TgA imprinted transgene[5] clearly do have methylation patterns that are directly derived from the gametes and preserved through pre-implantation development. There is as yet no proof that these sites play a role in the imprinting process, but the very fact that they survive the massive genome-wide demethylation that occurs in the morula[6,7] suggests that they are specifically recognized at this critical stage.

The *Xist* gene provides a good system for studying the role of modification in imprinting. Because this gene sequence maps to the Xic region and is expressed exclusively from the inactive X chromosome in female cells, it has been suggested that it may play an active role in the inactivation process itself[8–10]. In keeping with this hypothesis, it has been shown that random X inactivation which occurs in the inner cell mass of the late blastocyst, is preceded by stochastic *Xist* expression[11] as is X inactivation during spermatogenesis[12–14]. Furthermore, in the very early embryo, transcription of *Xist* begins at about the 4-cell stage and is imprinted, with only the paternal allele being active[15]. Assuming that *Xist* is indeed a regulator gene, this differential expression pattern may represent a critical event that leads to paternal-specific X inactivation in extraembryonic tissues of the mouse. In this paper we have investigated the possibility that a DNA methylation signal inherited directly from the gametes may be responsible for directing this imprinting process.

Xist methylation in gametogenesis

Many housekeeping genes on the X chromosome in somatic cells have upstream CpG islands that are differentially methylated on the inactive homologue, and it is this modification which presumably maintains its repressed state throughout the lifetime of the organism[16–17]. A similar pattern of differential methylation has also been observed for the *Xist* gene. Both in the promoter region and within the 5' portion of the transcribed sequences, the active allele is unmethylated, whereas the inactive copy is fully modified in somatic and extraembryonic tissues[18]. In order for these gene loci to be a part of an imprinting signal, however, they should be unmethylated in sperm

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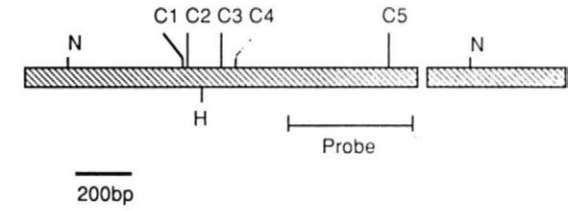

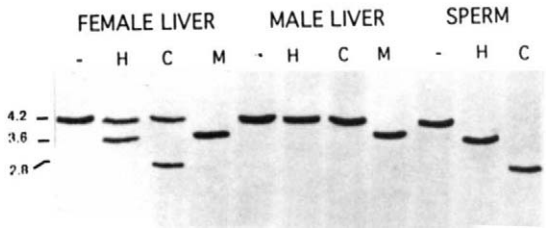

Fig. 1 *Xist* methylation pattern. DNA from either male or female liver and from sperm was digested with *Nco*I(N) and additionally with *Hpa*II(H), *Cfo*I(C) or *Msp*I(M), electrophoresed on 1% agarose gels, blotted and hybridized with an *Xist*-specific probe. The map shows the 5' portion of the *Xist* cDNA sequence. It is shown to scale, except for the gap 3' to C5. The *Nco*I sites are at GenBank positions 624 and 4810 relative to the start of transcription. The locations of the methyl-sensitive restrictions sites are 1108 (C1), 1113 (C2), 1193 (H), 1239 (C3), 1322 (C4) and 1963 (C5). It should be noted that in molecules where C5 is unmethylated, the modification state of C1–C4 cannot be ascertained using this probe. All of the sites shown in the map are differentially methylated on the maternal allele in extraembryonic tissues[18]. CpG residues further 3' from this region are not differentially modified in the adult (data not shown), and were consequently not assayed during gametogenesis and embrygenesis.

DNA, but modified in mature oocytes. The promoter sequences, therefore, are an unlikely candidate , because they appear to be at least partially modified in sperm[18]. DNA at the 3' end of the transcript is probably not involved in imprinting at all, since this region is methylated on both alleles in female somatic cells (data not shown). In contrast, the short ~800 bp domain at the start of the *Xist* coding sequence does have properties consistent with an imprinting signal, since it is 50% methylated in female somatic cells, but fully modified on the single transcriptionally inactive *Xist* allele in the male, and unmethylated in sperm (Fig. 1).

As imprinting signals must be erased from the genome of the previous generation during early gametogenesis and then reestablished in the mature gmaetes, we followed the methylation pattern at this specific 5' locus throughout spermatogenesis and oogenesis. To this end, cell fractions from sequential stages of the germ cell lineage in the embryo and adult gonads were isolated, and purified DNA samples were then subjected to polymerase chain reaction (PCR) analysis for evaluating the degree of methylation at each *Hpa*II or *Cfo*I (*Hha*I) restriction site. We have previously demonstrated that when properly calibrated, this method serves as an accurate and extremely sensitive measure of DNA modification at CpG residues[7,19]. As shown in Fig. 2, these loci are already modified in 12.5–13.5 days post coitum (d.p.c.) germline cells and maintain this methylated state almost throughout embryonic gonadal development until 18.5 d.p.c. in both sexes. By 21.5 d.p.c., however, these sites undergo specific demethylation in the developing spermatogonia, whereas they remain modified during the continuation of oogenesis. This process thus results in a clear difference between the two parental alleles, with the 5' locus being methylated in the mature oocyte and unmethylated in sperm DNA (Fig. 3).

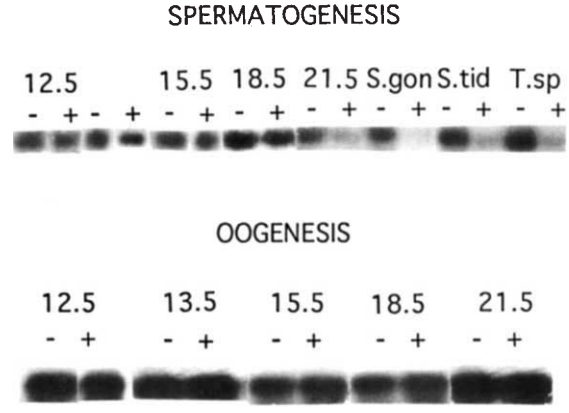

Fig. 2 Analysis of DNA methylation during gametogenesis. Uncut (–) and *Hpa*II digested (+) DNA (1 ng) from embryonic stages (12.5–21.5 d.p.c.) of spermatogenesis and oogenesis and from spermatogonia (S. gon), spermatids (S. tid) and testis sperm (T. sp) were subjected to PCR analysis as described in Methodology using the appropriate primers for site H. Similar results were obtained for sites C3 and C4 using these same primers and for site C5.

Maintenance of methylation

Although these results suggest that the differential methylation pattern seen in extraembryonic tissues may be initiated in the gametes, it must still be demonstrated that allele-specific modification is actually maintained during early development. This is especially critical in light of the genome-wide demethylation that normally occurs in the early pre-implantation embryo at about the 8 to 16-cell stage[7]. As shown in Fig. 4, CpG sites in the critical region actually remain methylated throughout this period in 4-, 8- and 16-cell embryos from a mixture of male and female embryos. Furthermore, in order to ascertain that the maternally derived *Xist* gene specifically remains methylated, we analyzed the oocyte-derived allele exclusively by assaying *Xist* methylation in 32-cell male-specific morulae obtained by screening individual embryos with a Y chromosome specific PCR probe. Here also, the 5' end of the gene remains fully modified at this stage, even though two control genes were clearly unmethylated in the same DNA samples. These results indicate that the maternally derived *Xist* gene maintains its methylated state in the male embryo. Since female-only morulae at this stage are also modified (data not shown), we assume that the same is true during female development as well. If this is the case, it then appears that the differential methylation pattern of the 5' portion of the *Xist* gene coding sequences seen in extraembryonic tissues is initiated in the gametes and maintained in the embryo.

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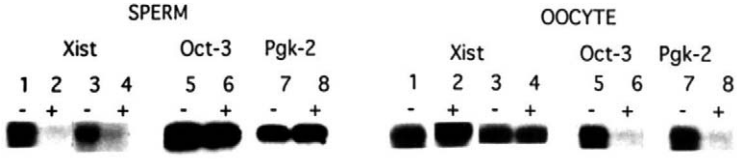

Fig. 3 Methylation state of *Xist* in oocyte and sperm DNA. Uncut (–) and *Cfo*I or *Hpa*II-digested (+) DNA from purified oocytes or vas deferens sperm was subjected to PCR analysis as described in Methodology using the primers for *Xist* sites H (lanes 1 & 2) and C5 (lanes 3 & 4). Similar results were obtained for sites C3 and C4, as well (data not shown). The same DNA was analyzed for methylation at the *Hpa*II site in Oct-3 and the 5' *Cfo*I *(Hha*I) site in Pgk-2 (ref. 7).

Discussion

Differential expression patterns characteristic of genomically imprinted genes are usually initiated sometime during embryogenesis. It is thus clear that there must be some molecular mechanism that marks each parental allele in the gametes and then preserves their identity through development. DNA methylation represents a reasonable candidate for marking and distinguishing individual parental alleles at the molecular level, since CpG residues can undergo either *de novo* methylation or demethylation at specific sites, and the resulting modification patterns can be faithfully preserved through many cell generations by means of post-replication maintenance methylation[1].

Molecular studies on individual genes have indeed shown that differential parent-of-origin-specific modification is a common feature of imprinted sequences. By analysing how these patterns are established in the embryo, however, it has become clear that most differential methylation, such as that seen associated with *Igf2* and the *H19* genes, is not derived from the gametes, but rather is added to these genes after implantation[3]. Thus, although these methyl moieties may play a role in the maintenance of allele-specific expression, it is clear that they do not take part in the actual imprinting signal. In striking contrast to these examples, maternal-specific modification of the *Xist* gene in extraembryonic tissues is obviously established in the gametes of the parents and appears to be maintained during early embryogenesis.

In order for the differential modification pattern of the *Xist* gene to serve as an imprinting signal, it must be established in the mature gametes, but it must also be reversible in the next generation when a maternal X is inherited by a son or a paternal X is inherited by a daughter. In the male, the dynamics of *Xist* methylation appear to be quite simple. These embryos always inherit the X chromosome from the mother and therefore carry a modified *Xist* gene that escapes the generalized early embryonic wave of demethylation. This allele most likely then remains modified in the inner cell mass, which includes the cells destined to generate the germ line. Although we have not examined the initial stages of germ cell development in the primitive ectoderm and yolk sac, the 5' *Xist* sequences, unlike sites in many other genes[7], appear to remain modified in 12.5 d.p.c. primordial germ cells of both sexes as gonads begin to differentiate, and in prospermatogonia throughout most of the fetal stages of spermatogenesis. Erasure of this pattern and the simultaneous generation of the male imprint is then carried out, accoding to our results, in perinatal prospermatogonia between 18.5 and 21.5 d.p.c. where these 5' sites undergo specific terminal demethylation. This finding differs from a previous study[18] based on mutations that disrupt spermatogonia, which suggested that demethylation first appears several days later in meiotic spermatocytes. In contrast to these data our results are in keeping with the timing of demodification of other testis-specific genes[7] and are also consistent with the fact that *Xist* transcription is already active in primitive type A spermatogonia by six days after birth[12–14].

The sequence of methylation events is quite different during female development. It should be noted that imprinting of the *Xist* gene is strictly tissue-specific, being restricted exclusively to extraembryonic tissues. These imprinted molecules are thus part of a dead-end pathway and are never rechanneled into the germ line. In the embryo proper, however, the differential methylation initially present in the early embryo must be erased to clear the slate for the random X inactivation that occurs in the late blastocyst[20]. It is not known how the parent-specific methylation pattern is neutralized in the early inner cell mass. One possibility is that both *Xist* alleles are equally modified at this stage, and this type of erasure

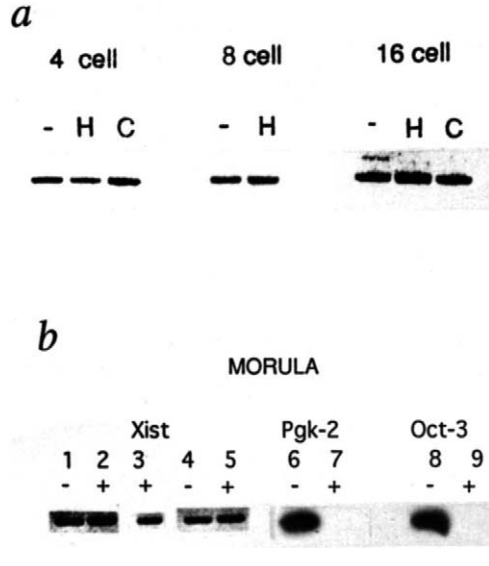

Fig. 4 Methylation state of *Xist* in early embryos. *a*, Uncut (–) *Hpa*II (H)- or *Cfo*I (C)-digested DNA from pools of 4-, 8- and 16-cell embryos was assayed for *Xist* methylation at sites H, C3 and C4 as described in Methodology. These specific samples were previously analyzed using other gene probes[7], and those results thus serve as restriction enzyme controls for the *Xist* gene. *b*, DNA from individual morulae was screened by Y specific Zfy PCR analysis. Male morulae were then pooled and assayed for methylation as in figure 3. Sites H (lane2), C3 and C4 (lane 3) and C5 (lane 5) were studied. Amplification from the uncut samples is shown in lanes 1, 4, 6 and 8. Female embryos from the morula stage also showed a similar degree of methylation at these sites (data not shown).

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would be consistent with the observation that both X chromosomes are active in the morula. Following random inactivation in the blastocyst, *Xist* methylation is associated with either the maternal paternal allele depending on which is inactive in that individual cell[18]. Since X inactivation also occurs in the female germ line, it is reasonable to assume that only one *Xist* gene is modified in each cell during early oogenesis. The PCR assay used in this study is probably not sensitive enough to accurately measure 50% methylation[7], so we have not been able to confirm this hypothesis directly. Our results do demonstrate, however, that the *Xist* gene is indeed modified throughout oogenesis. The one inactive X chromosome present in early oogonia cells is known to undergo reactivation at about 13.5 d.p.c. in the mouse[21,22], and this is associated with the cessation of *Xist* expression in these cells[12]. It is thus likely that at this stage of oogenesis, all copies of *Xist* become methylated at the 5' sites, and this has been partially confirmed by the observation that the maternal *Xist* gene is methylated in all mature oocytes and in early embryos.

Additional examples of methylation imprints include the 1.5 kb intronic region of the *Igf2r* gene[4] and pBR-322 sequences in the imprinted TgA transgene[5]. In both of these cases, a short CpG island-like locus becomes methylated during late stages of oocyte maturation, but remains unmethylated in sperm, and this pattern is then maintained in the early embryo. Although there is, as yet, no direct proof that these methyl moieties actually serve as imprinting signals, the fact that there are parent-specific processes for erasing and resetting these methylation patterns during gametogenesis, and that these modifications are protected from the massive demethylation which occurs in the morula, all support the idea that they may indeed play a special role in the imprinting process. The differential methylation seen on the *Xist* gene also satisfies these criteria. In addition, this modification pattern appears to be restricted to a small region near the start of transcription (see Fig. 1), and thus could play a part in the repression of the maternal allele at the 4 to 8 cell stage. This may thus represent a methylation signal imprinted in the gametes, which has a direct effect on expression in the offspring.

Methodology

Cells from various stages of gametogenesis, mature oocytes and individual morulae were isolated from the outlived CD-1 mouse strain, and DNA was purified as previously described[7,19]. From each morula sample, 40 % was used for PCR analysis using Y-chromosome specific primers for Zfy[23], and the remaining DNA from male (Zfy^+) and female (Zfy^-) embryos pooled. Each sample was divided into three aliquots and digested with *Pvu*II alone, *Pvu*II+*Hpa*II or *Pvu*II+*Cfo*I. *Pvu*II was used since the PCR reaction apparently works more efficiently on low molecular weight DNA. This enzyme does not cut within any of the fragments produced by the primers used in this paper. Approximately 1 ng of genomic DNA was amplified by PCR in a reaction mix (50–100 µl) containing 0.2 µM primer, 0.2 mM nucleotides and 2 U of *Taq* polymerase (Boehringer). After an initial denaturation for 4 min at 95 °C, each cycle consisted of 1 min at 95 °C, 2 min at 55 °C and 3 min at 72 °C. Most samples were amplified for 25 cycles. For oocyte and morula DNA, amplification was carried out on 100 pg DNA for 40 cycles. Following amplification, the samples were electrophoresed in 1.5% agarose gels, blotted onto Hybond membrane filters (Amersham) and hybridized with specific probes. Under these conditions, the degree of DNA methylation could be measured accurately over a 10 to 50 fold range of DNA concentrations[19].

The following *Xist* primers were employed (according to the GenBank cDNA sequence): Sites C3, C4 and H (position 1145, 5'–TTGCGGGATTCGCCTTGATT–3'; position 1351, 5'–TGAGCAGCCTTAAAGCCAC–3'), site C5 (position 1571, 5'–AGATGGCGGAAGTCATGTGA–3'; position 2130, 5'–ATGAACGCAAGCGGATGA GT–3') and the *Hpa*II site at 7631 (position 6981, 5'-GGATATGCTGATCAACAA GG–3'; position 7678, 5'–AGAGAGCAGGTCATTCGTCA–3'). The primers for the *Hpa*II site in Oct-3 and the *Cfo*I (*Hha*I) site in Pgk-2 have already been described[7].

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