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X Inactivation in the Mouse Embryo Deficient for *Dnmt1:* Distinct Effect of Hypomethylation on Imprinted and Random X Inactivation

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It has been suggested that DNA methylation plays a crucial role in genomic imprinting and X inactivation. Using DNA methyltransferase 1 (*Dnmt1*)-deficient mouse embryos carrying X-linked *lacZ* transgenes, we studied the effects of genomic demethylation on X inactivation. Based on the expression pattern of *lacZ*, the imprinted X inactivation in the visceral endoderm, a derivative of the extraembryonic lineage, was unaffected in *Dnmt1* mutant embryos at the time other imprinted genes showed aberrant expression. Random X inactivation in the embryonic lineage of *Dnmt1* mutant embryos, however, was unstable as a result of hypomethylation, causing reactivation of, at least, one *lacZ* transgene that had initially been repressed. Our results suggest that maintenance of imprinted X inactivation in the extraembryonic lineage can tolerate extensive demethylation while normal levels of methylation are required for stable maintenance of X inactivation in the embryonic lineage.

Key Words: **X inactivation; imprinting; DNA methylation; *Dnmt1;* mouse embryo.**

INTRODUCTION

Female mammals have evolved a mechanism to inactivate one of two X chromosomes during early embryogenesis in order to make X-linked gene dosages equivalent to the ones in males that have only a single X chromosome (Lyon, 1961). X inactivation in mouse is characterized by random inactivation of either X chromosome in the epiblast (primitive ectoderm) lineage, which gives rise to every tissue of the future fetus and the extraembryonic mesoderm, and imprinted preferential inactivation of the paternal X chromosome only in the extraembryonic lineages (Takagi and Sasaki, 1975). In embryos carrying an extra copy of the maternal X chromosome as a result of meiotic nondisjunction in female, both of those maternal X chromosomes remain active in the extraembryonic tissues because of imprinting laid on the X chromosome (Shao and Takagi, 1990; Tada *et al.*, 1993; Goto and Takagi, 1998). All those embryos die soon after implantation primarily due to severe defect of the extraembryonic tissues, suggesting that the presence of multiple copies of the active X chromosome is extremely deleterious to development of the extraembryonic lineages.

It has been suggested that DNA methylation is involved in X inactivation and genomic imprinting. CpG islands of X-linked genes are heavily methylated on the inactive X chromosome but barely methylated on the active counterpart. Differential methylation at the *Hprt* locus does not become evident until several days after the appearance of a cytologically identifiable inactive X chromosome, implying that methylation plays a role in maintenance of X inactivation (Lock *et al.*, 1987). *Xist* (X-inactive specific transcript), which was originally identified as a gene exclusively expressed from the inactive X chromosome in female somatic cells (Brown *et al.*, 1991; Borsani *et al.*, 1991; Brockdorff *et al.*, 1992), was shown to be essential for X inactivation to occur *in cis* (Penny *et al.*, 1996; Marahrens *et al.*, 1997). Targeted disruption of

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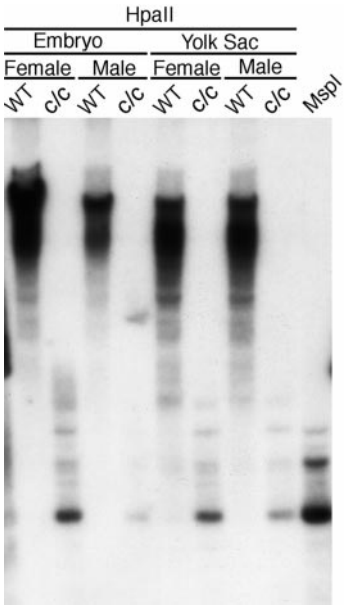


FIG. 1. Methylation levels of genomic DNA in the embryo proper and yolk sac of wild-type and $Dnmt^{c/c}$ embryos at E9.5. A Southern blot was probed with a MoMuLV cDNA probe. An extensive loss of genomic methylation levels was evident in both the embryo proper and the yolk sac of $Dnmt^{c/c}$ embryos in both sexes. WT, wild type; c/c, $Dnmt1^{c/c}$.

DNA methyltransferase 1 (*Dnmt1*) causes ectopic expression of *Xist* in male embryos and differentiating ES cells, which is apparently triggered by demethylation at the 5′ region of *Xist* (Beard *et al.*, 1995). By means of RNA FISH, Panning and Jaenisch (1996) further suggested that such ectopic *Xist* expression might lead to inappropriate X inactivation in *Dnmt1* mutant embryos and ES cells. In contrast to the primary role of *Xist* in initiation of X inactivation, it may not be essential for maintenance of X inactivation in somatic cells, as a human inactive X chromosome lacking *XIST* still maintains the inactive state (Brown and Willard, 1993). This is further supported by a recent conditional knockout of *Xist* in mouse primary embryonic fibroblasts (Csankovszki *et al.*, 1999).

The differentially methylated regions are often found in the vicinity of imprinted genes, which presumably regulate parental-origin-specific expression of imprinted genes. Embryos lacking functional *Dnmt1* have lost such differential methylation by E9.5 with concomitant aberrant expression of those genes, suggesting that DNA methylation is essential for, at least, maintenance of proper imprinting (Li *et al.*, 1993). It is of interest whether preferential X inactivation in the extraembryonic lineages is affected by hypomethylation like other imprinted genes. The embryonic cells, in contrast to the extraembryonic tissues, display random inactivation of the X chromosomes irrespective of their parental origin. It is not known if the loss of Dnmt1 activity may affect the onset or the randomness of the inactivation process and the maintenance of the inactive status of the X-linked gene.

Our results described in this study suggest that, unlike most imprinted genes, the lack of methylation does not perturb maintenance of parent-specific X inactivation in the extraembryonic lineage, whereas it apparently causes reactivation of the inactive X chromosome that has once undergone X inactivation in the embryonic lineage. DNA methylation therefore has different impact in the maintenance of X inactivation in the embryonic and extraembryonic tissues.

MATERIALS AND METHODS

Mice and Embryos

The *Dnmt1* mutant mice carrying the $Dnmt^{c}$ allele were maintained in the (129/SvJae × C57/BL6) mixed background. The H253 mice carry multiple copies of the *lacZ* gene on the X chromosome under the control of regulatory element of the mouse 3-hydroxy-3′-methylglutaryl-coenzyme A reductase with the SV40 nuclear localization signal (Tam and Tan, 1992). The derivation of ES cells with mutation at the *Msg1* locus and an analysis of the knockout mouse will be described elsewhere. In brief, the genomic sequence encoding the most part of *Msg1* was deleted and replaced with a *lacZ-Pgk*neo cassette leaving the promoter, exon 1, and 5′ part of exon 2 intact. The *Msg1* translation initiation codon in exon 2 was also deleted. The $Dnmt^{c}$ heterozygous mice were crossed with either H253 or *Msg1* mutant mice to create females double heterozygous for $Dnmt^{c}$ and either X^{H253} or $Msg1^{lacZ}$ and males heterozygous for $Dnmt1^{c}$ and hemizygous for X^{H253} or $Msg1^{lacZ}$. Genotype was determined by Southern hybridization (Lei *et al.*, 1996).

Embryos were collected at E8.5 or E9.5 from the double-heterozygous females crossed with $Dnmt^{+/c}$ males or from $Dnmt^{+/c}$ females crossed with males heterozygous for $Dnmt^{c}$ and hemizygous for X^{H253} or $Msg1^{lacZ}$. Genotyping of those embryos was carried out by Southern hybridization using DNA isolated from the trophoblast.

X-gal Staining and Histology

Embryos were dissected out from the decidua in PBS and fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40 for 30 min. They were rinsed with PBS containing 0.02% NP-40 twice and stained in 0.5 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% deoxycholate, 0.02% NP-40 at 37°C overnight. Those stained embryos were postfixed in 4% paraformaldehyde in PBS.

Histology

Embryos stained with X-gal were dehydrated, embedded in paraffin, sectioned at 7 μm, and counterstained with nuclear fast red.

Methylation Analysis

DNA from the embryo proper and yolk sac was isolated according to Beard *et al.* (1995). Following digestion with restriction enzymes, DNA was run on a 1% agarose gel, blotted onto nylon membrane, and probed with following fragments: *Xist*, a *Hpa*I–*Sac*II genomic fragment; and *Msg1*, a PCR fragment amplified from the promoter region using primers 5′-att tcc cag cgt cgt cgc at-3′ and 5′-atg gtc act tct cgc cca gt-3′.

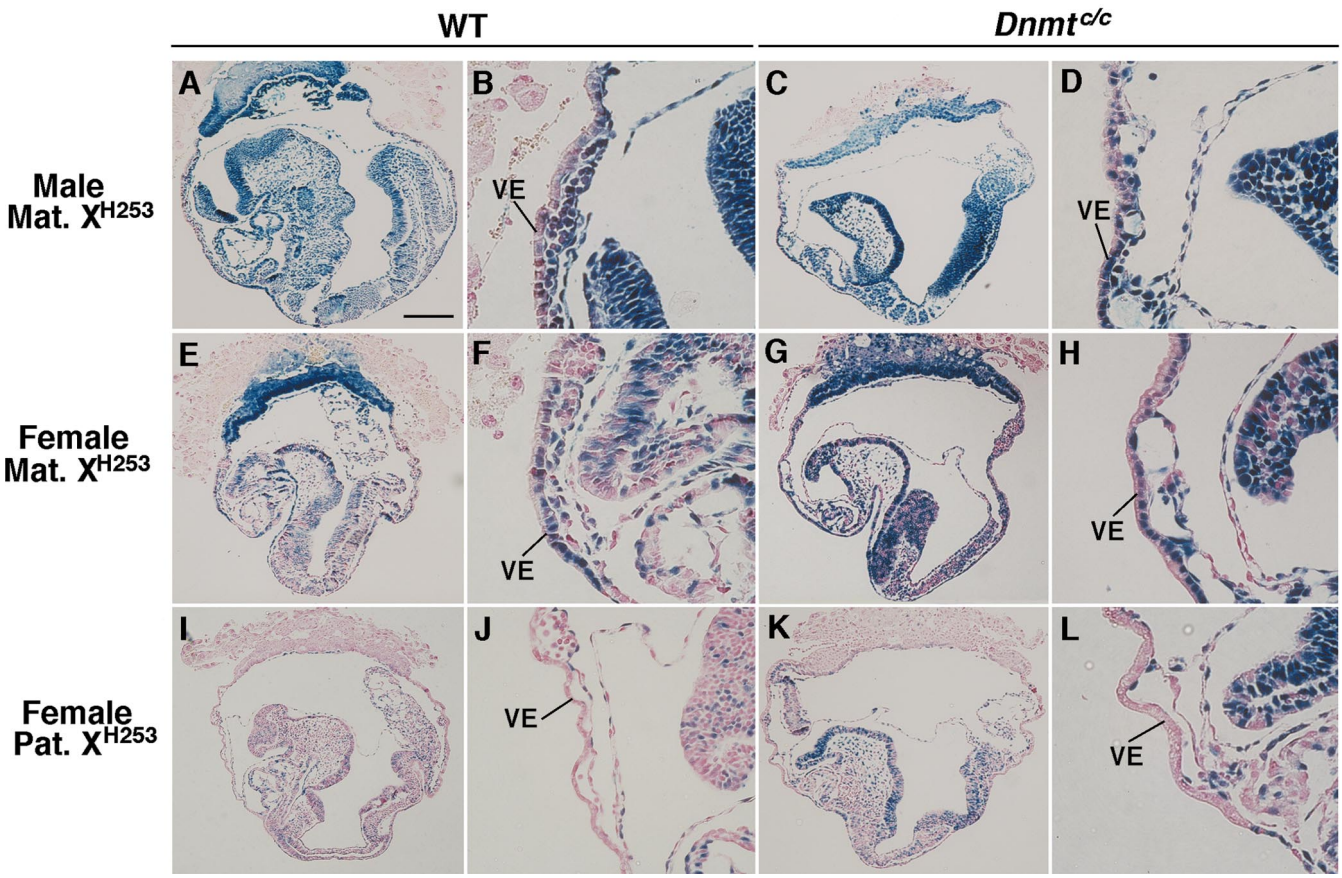


FIG. 2. Activity of the *lacZ* transgene on X^{H253} derived from either parent in the visceral endoderm of wild-type and $Dnmt^{c/c}$ embryos. Embryos recovered at E8.5 were stained with X-gal and sectioned. Whole views of each section (A, C, E, I, and K) and blow-ups of the yolk sac (B, D, F, H, and L). (A, B) A wild-type male embryo with maternally derived X^{H253}; (C, D) a $Dnmt^{c/c}$ male embryo with maternally derived X^{H253}; (E, F) a wild-type female embryo with maternally derived X^{H253}; (G, H) a $Dnmt^{c/c}$ female embryo with maternally derived X^{H253}; (I, J) a wild-type female embryo with paternally derived X^{H253}; (K, L) a $Dnmt^{c/c}$ female embryo with paternally derived X^{H253}. Female $Dnmt^{c/c}$ embryos showed expression pattern of *lacZ* essentially the same as wild-type female. VE, visceral endoderm. Scale bar, 0.2 mm in A, C, E, G, I, and K.

RESULTS

We previously reported that methylation levels of the genome were extremely low in $Dnmt^{c/c}$ embryos as only a trace level of methylation was detected in the endogenous retroviral sequences and minor satellite sequences (Lei *et al.*, 1996). Here we showed that genomic methylation in the mutant yolk sacs was as low as that in the embryo proper (Fig. 1). Overall hypomethylation in the yolk sac indicated that both the visceral endoderm and the mesoderm were substantially demethylated. Base on these observations, we attempted to elucidate effects of demethylation on imprinted X inactivation in the extraembryonic lineages as well as random X inactivation in the embryonic lineage.

Imprinted X Inactivation in $Dnmt^{c/c}$ Female Embryos Carrying X^{H253}

The transgenic mouse line, H253, carries the *lacZ* transgene on the X chromosome (X^{H253}) under the control of the 5′ regulatory element of the mouse 3-hydroxy-3-methylglutaryl-coenzyme A reductase with SV40 nuclear localization signal (Tam and Tan, 1992), in which the transgene is ubiquitously expressed and subject to X inactivation during embryogenesis (Tan *et al.*, 1993; Tam *et al.*, 1994). In female embryos, expression of *lacZ* in the epiblast-derived tissues, such as the embryo proper, allantois, amnion, and yolk sac mesoderm lining the visceral endoderm, was basically mosaic irrespective of the parental origin of X^{H253}, manifesting random X inactivation occurring in the embryonic lineage (Figs. 2E, 2F, 2I, and 2J). In the visceral endoderm, however, paternal transmission of X^{H253} resulted in transcriptional repression of the transgene, whereas maternal inheritance kept it active in almost every cell (Figs. 2E, 2F, 2I, and 2J). Expression of *lacZ* appears to mirror imprinted preferential inactivation of the paternal X chromosome in the extraembryonic lineages. It is, therefore, reasonable to assume that expression of the transgene reflects activity of the X chromosome bearing it. Accordingly, we introduced X^{H253} into the $Dnmt^{c}$ background to

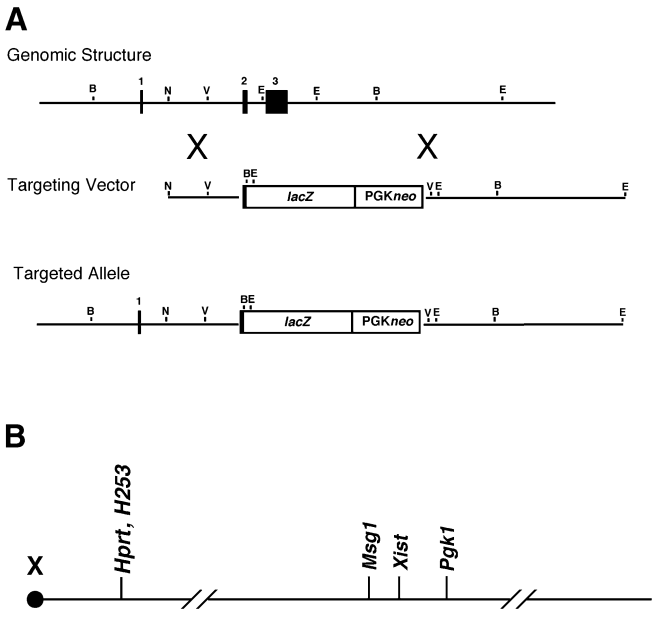


FIG. 3. Structure of the *Msg1*^{lacZ} allele and a linkage map of the X chromosome. (A) The entire sequence coding for Msg1 protein was deleted and replaced with a *lacZ-Pgk*neo cassette. The *lacZ* gene is driven by the endogenous promoter of *Msg1*. (B) A linkage map of *H253*, *Msg1*, and *Xist* on the X chromosome. *H253* transgene is located near *Hprt*, while *Msg1* is located between *Zfx* and *Xist*.

study effects of demethylation on imprinted X-inactivation in the extraembryonic tissues. The X^{H253} mouse heterozygous for *Dnmt*^{c} was reciprocally crossed with *Dnmt*^{c} heterozygote and *lacZ* expression was analyzed in the visceral endoderm of E8.5 embryos homozygous for *Dnmt*^{c}. Upon maternal transmission, both male and female homozygotes expressed the transgene in every cell of the visceral endoderm (Figs. 2C, 2D, 2G, and 2H). In contrast, the paternally derived transgene was unanimously inactivated in the visceral endoderm of female *Dnmt*$^{c/c}$ embryos although mosaic expression was evident in the embryonic tissues (Figs. 2K and 2L). Similar results were obtained when E9.5 embryos were analyzed (data not shown). These results indicate that hypomethylation caused by the null mutation of *Dnmt1* does not affect expression pattern of the transgene in the visceral endoderm, which is totally dependent on its parental origin. It seems likely that imprinted X inactivation is properly maintained in the extraembryonic lineages of *Dnmt1*-deficient female embryos.

Imprinted X Inactivation in Dnmt$^{c/c}$ *Female Embryos Carrying Msg1*^{lacZ}

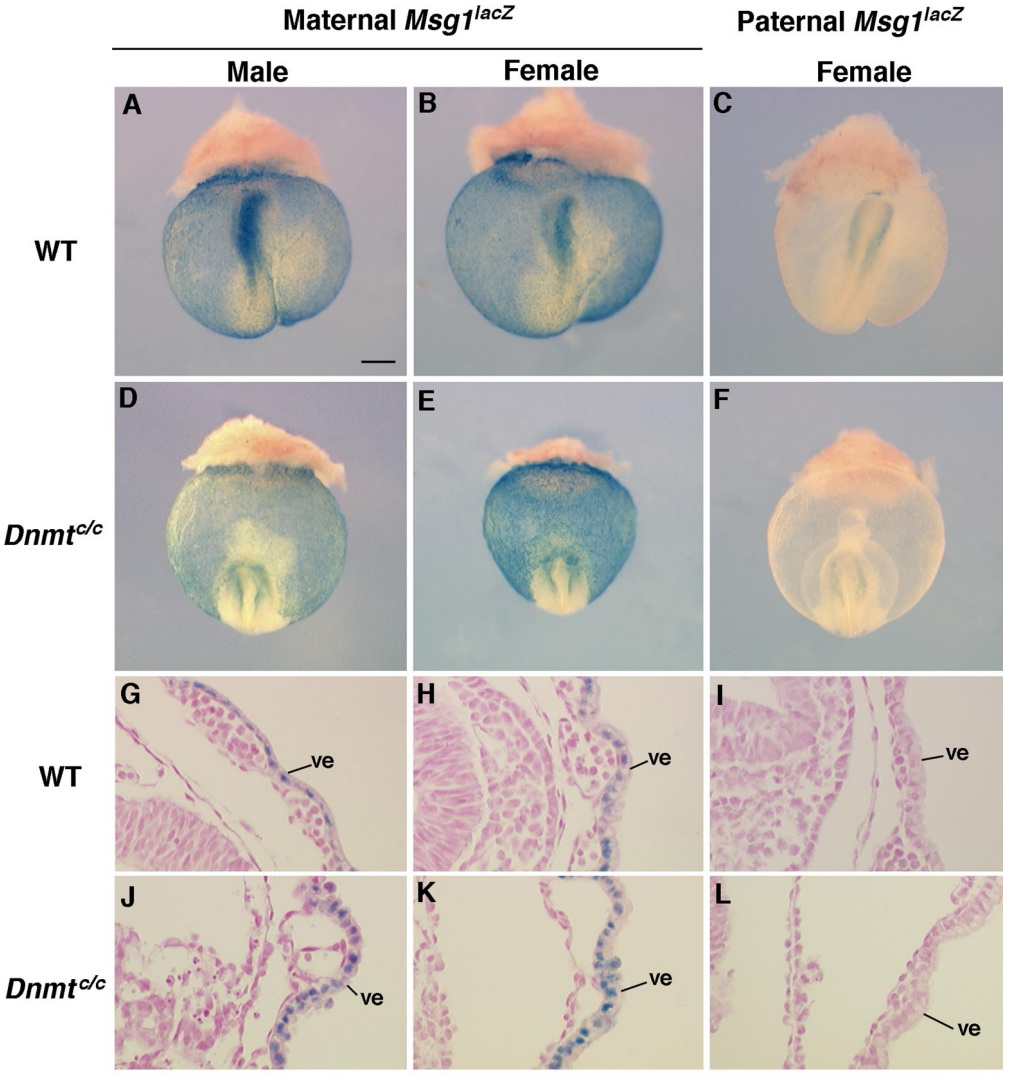
We then took advantage of the second X-linked *lacZ* marker to evaluate the above finding. It was a targeted allele of the *Msg1* (or *Cited1*) gene, termed *Msg1*^{lacZ}, which was generated by inserting and replacing the entire protein coding sequence with the *lacZ-Pgk*neo cassette leaving the promoter intact (Fig. 3A). The mouse *Msg1* gene is located in the region syntenic to Xq13 on the human X chromosome (Fenner *et al.*, 1998), proximal to the *Xist* gene (Fig. 3B). The heterozygous and homozygous female and hemizygous male for *Msg1*^{lacZ} appeared to be normal and fertile (detailed analysis will be described elsewhere). In the mutant mice, expression of *lacZ* was expected to recapitulate that of the endogenous *Msg1* gene. Since the visceral endoderm is one of the tissues that predominantly express *Msg1* in developing mouse embryos (Dunwoodie *et al.*, 1998), *Msg1*^{lacZ} would be an ideal marker, if subject to X inactivation, to analyze imprinted X inactivation.

Expression of *lacZ* from the targeted allele was examined in the visceral endoderm of E8.5 female embryos carrying either paternally or maternally derived *Msg1*^{lacZ}. The maternally inherited *Msg1*^{lacZ} was expressed in the visceral endoderm of both male and female embryos, whereas *lacZ* was silenced following paternal transmission (Figs. 4A, 4B, and 4C). As was the case of X^{H253}, expression of *Msg1*^{lacZ} seemed to reflect imprinted X inactivation in the visceral endoderm. In addition, histological analysis revealed that *lacZ*-positive cells were uniformly distributed in the tail bud in male embryos, where endogenous expression of *Msg1* is detected at E8.5 (Dunwoodie *et al.*, 1998), but only a subset of cells (approximately 50%) expressed *lacZ* in the equivalent region of female (Figs. 5A and 5B), suggesting that random inactivation of the *Msg1*^{lacZ} allele has occurred in the embryonic lineage. Taken together, it is most probable that mouse *Msg1* gene is subject to X inactivation and only the maternal allele is transcribed in the visceral endoderm.

Subsequently, expression from the *Msg1*^{lacZ} allele was studied in the visceral endoderm lacking functional *Dnmt1* in the same way as X^{H253}. *Msg1*^{lacZ}, if inherited from the mother, was active in both male and female embryos (Figs. 4D and 4E), while its expression was not detected when transmitted from the father (Fig. 4F). The expression pattern of *Msg1*^{lacZ} in wild-type and *Dnmt1*-deficient embryos was further confirmed by histological examination (Figs. 4G–4L) and was shown to be essentially identical. Similar results were obtained when E9.5 embryos were analyzed (data not shown). The expression pattern of *Msg1*^{lacZ} implies that imprinted X inactivation is maintained despite extensive demethylation of genomic DNA in the visceral endoderm tissue.

Methylation Status of the Xist Locus in the Dnmt$^{c/c}$ *Embryo and Yolk Sac*

Imprinted X inactivation in the visceral endoderm seemed appropriately maintained even in *Dnmt*$^{c/c}$ female embryos. This observation prompted us to examine methylation status of the *Xist* locus in the yolk sac of E9.5 mutant embryos, because previous studies suggested that methylation regulates *Xist* expression in differentiated cells (Beard *et al.*, 1995). DNA isolated from the embryo proper and yolk sac was digested with *Sac*II in combination with



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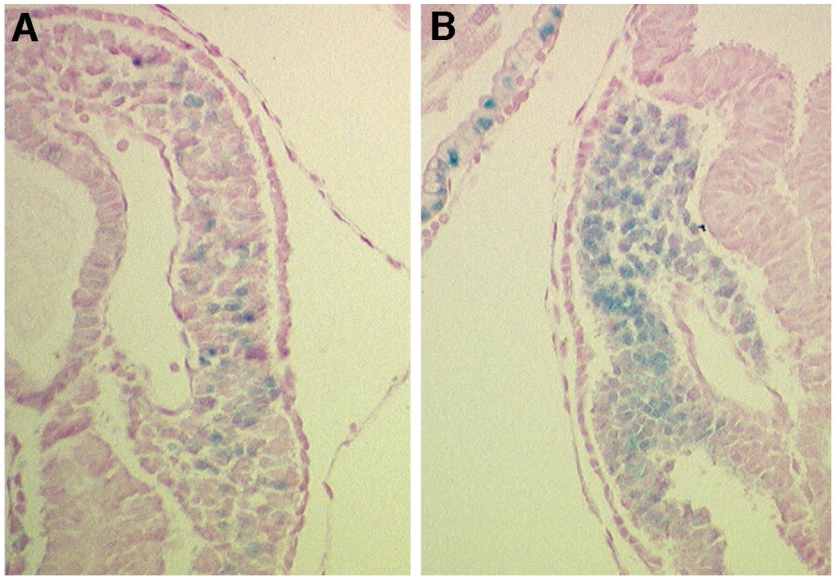

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*Eco*RV, and the blot was probed with a *Hpa*I–*Sac*II genomic fragment (Fig. 6A). The *Sac*II site was chosen because it had been proposed to be one of possible gametic methylation imprints responsible for exclusive expression of the paternal *Xist* allele during early preimplantation development (Zuccotti and Monk, 1995). This site was completely methylated in the wild-type male embryo and yolk sac as shown by the presence of the single 4.2-kb band but partially methylated in both tissues of wild-type female due to differential methylation between active and inactive X chromosomes (Norris *et al.*, 1994). In $Dnmt^{c/c}$ mutants, however, the *Sac*II site was substantially demethylated in tissues of both male and female embryos although a small fraction still retains some methylation. Complete digestion was confirmed by reprobing the blot with a genomic fragment containing a CpG island at the *Htf9* (*Hpa*II tiny fragment 9) locus (data not shown), which had been shown to be methylation free (Lavia *et al.*, 1987). Another presumptive gametic imprinting site, a *Hea*II site, neighboring the *Sac*II site (Zuccotti and Monk, 1995), was similarly demethylated (data not shown).

Methylation Status of the Msg1 Locus in the $Dnmt^{c/c}$ Embryo and Yolk Sac

Methylation status of the *Msg1* locus was also analyzed in E9.5 wild-type and $Dnmt^{c/c}$ mutant embryo proper and yolk sacs by Southern blot hybridization (Fig. 6B). Genomic DNA was digested with *Eco*RV and *Hpa*II (or *Msp*I as a control) and probed with a genomic fragment located in the promoter region of *Msg1* containing CpG-rich sequences (Fenner *et al.*, 1998). Those *Hpa*II sites in the promoter region were not methylated in the embryo proper and yolk sac of either wild-type or $Dnmt^{c/c}$ males at E9.5, implying that the *Msg1* locus was hypomethylated on an active X chromosome. In wild-type females at E9.5, however, those sites were characterized by partial methylation in the embryo proper, suggesting differential methylation between active and inactive X chromosomes, like many other X-linked genes. As expected, they were not methylated in females homozygous for $Dnmt^{c}$. Interestingly, no methylation was detected even in the wild-type yolk sac at E9.5 despite the above finding that, based on $Msg1^{lacZ}$ expression, *Msg1* had been inactivated by X inactivation by E8.5 in the visceral endoderm. A similar result was obtained when several *Hha*I sites were examined. Apparently, DNA methylation of the CpG-rich sequence is not essential to render *Msg1* inactive in response to X inactivation in the visceral endoderm.

Effect of Demethylation on Random X Inactivation in the Embryonic Tissues

It has been shown that, by E8.5, *lacZ*-positive cells in female embryos hemizygous for X^{H253} represent half of the population in most tissues due to random X inactivation, except for the notochord, heart, cranial mesenchyme, and hindgut, in which inactivation apparently is completed around E10.5–11.5 (Tan *et al.*, 1993). To assess effects of demethylation on random X inactivation in the embryonic lineage, *Dnmt1* mutant female embryos with the paternally derived transgene were carefully examined for *lacZ* expression in the embryonic tissues. The proportion of cells positive for *lacZ* was roughly 50% in E8.5 $Dnmt^{c/c}$ (n = 14), which was comparable to the wild type (Figs. 7A–7D). However, the distribution of *lacZ*-positive cells in mutant embryos recovered at E9.5 (n = 7) was quite distinct from those at E8.5. A considerable increase in the number of *lacZ*-positive cells (>80%) was evident in the embryonic tissues, while the transgene was still strictly repressed in the visceral endoderm (Figs. 7E and 7F). This dramatic increase in *lacZ*-positive cells in $Dnmt^{c/c}$ female embryos suggests that the *lacZ* transgene that has been inactivated initially by X inactivation becomes reactivated as a result of demethylation.

DISCUSSION

In this study, we used two X-linked *lacZ* transgenes that are subject to X inactivation to monitor the activity of the X chromosome in methylation-deficient mouse embryos. We observed that in female embryos homozygous for a *Dnmt1* null mutation, the *lacZ* transgene from the hemizygous X^{H253} locus was expressed in the visceral endoderm upon only maternal and not paternal transmission. The other *lacZ* marker, $Msg1^{lacZ}$, which is under the control of the endogenous promoter of *Msg1*, produced essentially the same result, i.e., only the paternally derived $Msg1^{lacZ}$ was inactivated in the visceral

FIG. 4. Activity of $Msg1^{lacZ}$ in the visceral endoderm of wild-type and $Dnmt^{c/c}$ embryos at E8.5 visualized by X-gal staining. Gross views of embryos (A–F) and sections of the yolk sacs (G–L). (A) A wild-type male embryo with maternally derived $Msg1^{lacZ}$; (B) a wild-type female embryo with maternally derived $Msg1^{lacZ}$; (C) a wild-type female embryo with paternally derived $Msg1^{lacZ}$; (D) a $Dnmt^{c/c}$ male embryo with maternally derived $Msg1^{lacZ}$; (E) a $Dnmt^{c/c}$ female embryo with maternally derived $Msg1^{lacZ}$; (F) a $Dnmt^{c/c}$ female embryo with paternally derived $Msg1^{lacZ}$. (G–L) Histological analyses of A–F, respectively. In wild-type female embryos expression of $Msg1^{lacZ}$ in the visceral endoderm depends totally on its parental origin, in that the paternal allele was invariably silenced. This was also the case in $Dnmt^{c/c}$ female embryos. VE, visceral endoderm. Scale bar, 0.2 mm in A–F.

FIG. 5. Expression of $Msg1^{lacZ}$ in the embryonic tissues. (A) Female and (B) male. $Msg1^{lacZ}$ was uniformly expressed in a certain part of tail bud in male, while its expression was mosaic in female.

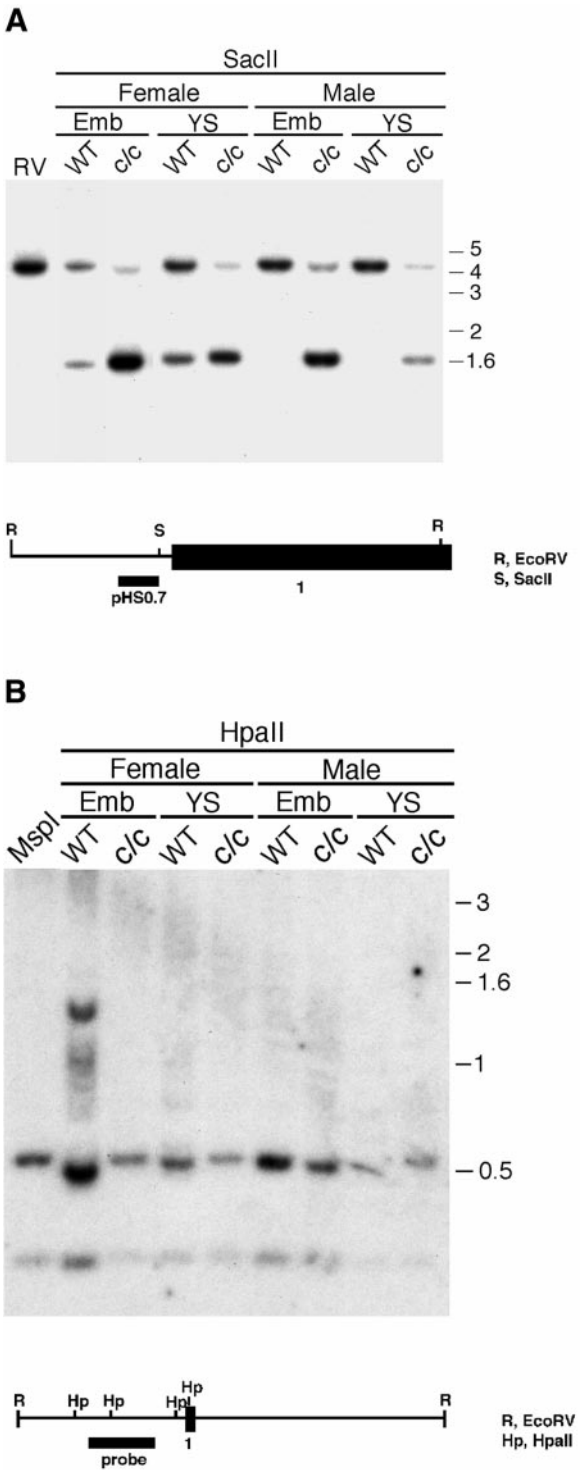

FIG. 6. Methylation analyses of the *Xist* locus (A) and *Msg1* locus (B). (A) DNAs isolated from the embryo proper and yolk sac at E9.5 were digested with *Eco*RV (lane 1) or *Eco*RV/*Sac*II (lanes 2–9), and Southern hybridization was carried out with a probe as shown. (B) Similarly DNA was digested with *Eco*RV/*Msp*I (lane 1) or *Eco*RV/*Hpa*II (lanes 2–9) and probed with a genomic fragment as shown. WT, wild type; c/c, $Dnmt^{c/c}$.

endoderm. It seems, therefore, likely that imprinted X inactivation in extraembryonic tissues is not disrupted in *Dnmt1*-deficient embryos by the time other imprinted genes are affected, which allows the paternal copy of both *lacZ* transgenes to remain silent.

Xist is imprinted in the extraembryonic lineages with the expression of only the paternal allele, which seems to be responsible for preferential inactivation of the paternal X chromosome (Kay *et al.*, 1993, 1994). Methylation analysis of the *Xist* locus in the *Dnmt1*-deficient embryos and yolk sac revealed that the region showing differential methylation between active and inactive X chromosomes was substantially demethylated although low levels of methylation were still detectable (Beard *et al.*, 1995; Fig. 6A). It has been demonstrated that loss of methylation causes ectopic expression of *Xist* in ES cells and embryos, which appears to cause aberrant X inactivation (Beard *et al.*, 1995; Panning and Jaenisch, 1996). Given the apparent tolerance of imprinted X inactivation to demethylation, the visceral endoderm may be able to sustain only the paternal X chromosome inactivation even though both copies of *Xist* may have become transcriptionally active. Alternatively, demethylation may not trigger expression of *Xist* on the maternal X chromosome in the visceral endoderm.

The finding that imprinted X inactivation is not compromised by extensive loss of genomic methylation in the visceral endoderm contrasts with the previous finding that DNA methylation is essential to maintain appropriate imprinting during embryogenesis (Li *et al.*, 1993). However, Caspery *et al.* (1998) made an interesting observation that imprinting of *Mash2*, which is specifically expressed in the spongiotrophoblast of placenta (Guillemot *et al.*, 1994, 1995), an extraembryonic ectoderm-derived tissue, is unaffected by null mutation of *Dnmt1* despite the fact that imprinting of *H19*, $p57^{kip2}$, and *Kvlqt1* is impaired in the ectoplacental cone of *Dnmt1* null mutant by E9.5. It may be possible that there are parallel mechanisms for imprinting that differ by their dependence on DNA methylation. The methylation-independent mechanism might be specifically associated with genomic imprinting in the extraembryonic lineage. It appears that the mouse extraembryonic tissues are less dependent on DNA methylation in normal development as they are characterized by lower methylation than the embryonic lineage (Chapman *et al.*, 1984), and genes on the inactive X chromosome from the yolk sac endoderm are efficiently expressed following DNA-mediated gene transfer compared to the ones from the embryo proper (Kartzer *et al.*, 1983). Maintenance of X inactivation in extraembryonic tissues may involve histone deacetylases which have been shown to play a universal role in gene silencing in organisms with or without methylation. It may also involve *Xist* RNA acting *in cis*, considering the possibility that, like *Mash2*, the maintenance of imprinted expression of *Xist* in extraembryonic lineage may be unaffected by demethylation.

It is well known that extraembryonic tissues of mouse embryos and somatic tissues of marsupials share some features in terms of DNA methylation and X inactivation. They

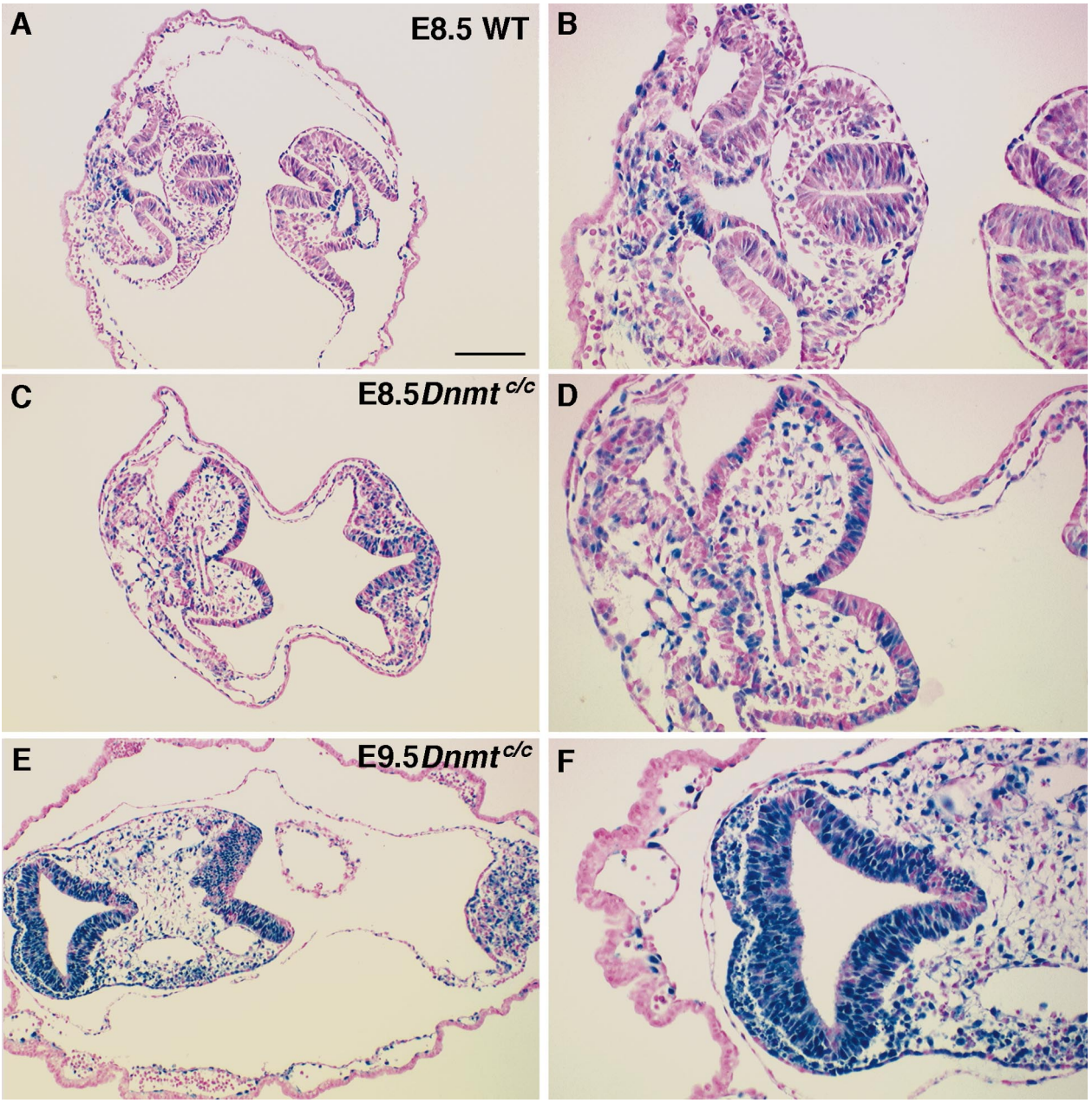

FIG. 7. Expression of the paternally derived *lacZ* transgene on X^{H253} in the embryonic tissues of female embryos. (A) A wild-type embryo at E8.5; (C) a $Dnmt^{c/c}$ embryo at E8.5; (E) a $Dnmt^{c/c}$ embryo at E9.5; (B, D, and F) blow-ups of A, C, and E, respectively. A striking increase of *lacZ*-expressing cells was evident in $Dnmt^{c/c}$ embryos at E9.5. Scale bar, 0.2 mm in A, C, and E.

are characterized by: (1) relatively low levels of genomic methylation, (2) preferential inactivation of the paternal X chromosome, and (3) less stable X inactivation compared to random X inactivation in eutherians (Cooper *et al.*, 1983; Loebel and Johnston, 1996; Samollow *et al.*, 1995). Apparently those tissues do not rely on DNA methylation very much to regulate gene expression. In fact, inactivation of *Msg1* in the visceral endoderm seems to be achieved without *Msg1* being methylated. It is also worth mentioning that we occasionally observed some cells expressing $Msg1^{lacZ}$ in the visceral endoderm or the X^{H253} *lacZ* transgene in the chorionic ectoderm upon paternal transmission (data not shown), which might indicate that an inactivated X chromosome had been reactivated in those rare cases. It is tempting to speculate that the methylation-independent imprinting emerged first, since it is found in marsupials and the extraembryonic lineage of the

mouse. Methylation-dependent imprinting might have evolved later and become fixed in eutherians as an essential part of imprinting in somatic tissues (Vandeberg *et al.*, 1987).

Random X inactivation in the embryo proper appears to be normal in *Dnmt1*-deficient embryos during early development up to the early somite stage, but the inactive X (or at least the X^{H253} *lac*Z) undergoes reactivation when the mutant embryo develop further. This result suggests that DNA methylation does play a crucial role in maintenance of X inactivation in the embryonic tissues. Analysis of replication timing of each X chromosome in *Dnmt1*-deficient embryos would help us understand whether reactivation is a chromosome-wide event or is confined to the transgene locus. While maintenance of stable X inactivation may also involve other silencing mechanisms such as histone deacetylation, it apparently does not require *Xist*. It has been reported that deletion of the *XIST* locus has no impact on the activity of the inactive human X chromosome in somatic cell hybrids (Brown and Willard, 1994). Similarly, conditional knockout of *Xist* in mouse primary embryonic fibroblasts did not perturb maintenance of X inactivation though deposition of histone macroH2A1.2 to the inactive X chromosome was disrupted (Csankovszki *et al.*, 1999).

Since the mature oocyte contains a large amount of maternal Dnmt1 protein (Carlson *et al.*, 1992), it is likely that even $Dnmt1^{c/c}$ mutant embryos can keep relatively normal levels of methylation during early development. Most probably, methylation levels in the mutant embryos gradually decrease as they consume the stock of maternal Dnmt1 protein, which could eventually induce expression of X-linked genes hitherto being repressed on the inactive X chromosome. It has been reported that demethylation caused by null mutation of *Dnmt1* can induce inappropriate X inactivation through ectopic *Xist* expression (Panning and Jaenisch, 1996). This observation seems to contradict our result that demethylation does not affect X inactivation during early development. One possible explanation is that a small proportion of cells in *Dnmt1* mutant embryos may undergo initiation of X inactivation on both X chromosomes, which could be detected by FISH analysis as an accumulation of *Xist* RNA on them, but not by our method. Although aberrant *Xist* expression was detected in male $Dnmt1^{c/c}$ embryos and from both X chromosomes in female mutants (Beard *et al.*, 1995; Panning and Jaenisch, 1996), it might not be activated at the stage early enough to trigger X inactivation in all cells. Perhaps, ectopic *Xist* expression can induce X inactivation only in cells about to differentiate, but not any more after cells have undergone differentiation. In fact, *Xist*/*XIST* expression from the active X chromosome induced by treatment with a DNA demethylating agent, even if the transcripts are associated with the active X chromosome, does not accompany repression of X-linked genes in somatic cells or somatic cell hybrids (Tinker and Brown, 1998; Clemson *et al.*, 1998). This suggests that stable expression and localization of *Xist* are not sufficient for X inactivation.

While DNA methylation appears to be essential for the maintenance of stable X inactivation in somatic tissues, its role in initiation of X inactivation and spreading of the inactive signals along the X chromosome remains largely unknown. During X inactivation, the 5′ region of *Xist* on the active X and CpG islands on the inactive X are methylated *de novo* probably by two other methyltransferases, Dnmt3a and Dnmt3b (Okano *et al.*, 1998, 1999). The presence of *de novo* methyltransferases and maternal Dnmt1 protein in *Dnmt1* null mutant embryos during early development may allow X inactivation to initiate normally. On the other hand, the failure of X inactivation following ectopic expression of *Xist* in somatic cells might be due to the absence of *de novo* DNA methylation activities in those differentiated cells. This would not allow the methyl moiety to propagate along the X chromosome in response to the cue of X inactivation. Further studies of X inactivation in mouse embryos lacking *de novo* methyltransferases Dnmt3a and Dnmt3b may shed lights on whether DNA methylation plays a role in regulation of the early events of X inactivation.

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