De novo DNA methylation is dispensable for the initiation and propagation of X chromosome inactivation

Takashi Sado1,2,3,*, Masaki Okano4, En Li4 and Hiroyuki Sasaki1,5

1Division of Human Genetics, National Institute of Genetics, 1111 Yata, Mishima, 411-8540, Japan
2Department of Biosystems Science, The Graduate School for Advanced Studies (SOKENDAI)
3PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama, Japan
4Cardiovascular Research Center, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA
5Department of Genetics, The Graduate School for Advanced Studies (SOKENDAI)

*Author for correspondence (e-mail: tsado@lab.nig.ac.jp)

Accepted 12 November 2003

Summary

Xist (X-inactive specific transcript) plays a crucial role in X-inactivation. This non-coding RNA becomes upregulated on the X chromosome that is to be inactivated upon differentiation. Previous studies have revealed that although maintenance-type DNA methylation is not essential for X-inactivation to occur, it is required for the stable repression of Xist in differentiated cells. However, it is unknown whether differential de novo methylation at the Xist promoter, which is mediated by Dnmt3a and/or Dnmt3b, is a cause or a consequence of monoallelic expression of Xist. We show that Xist expression is appropriately regulated in the absence of Dnmt3a and Dnmt3b and that a single X chromosome undergoes proper inactivation in mutant females. Our results indicate that a mechanism(s) other than DNA methylation plays a principal role in initiating X-inactivation. We also demonstrate that delayed upregulation of Xist does not induce X-inactivation, consistent with a crucial developmental window for the chromosomal silencing.

Key words: X chromosome inactivation, De novo DNA methyltransferases, Xist

Introduction

It is well known that DNA methylation plays an important role(s) in the regulation of gene expression, maintenance of genomic integrity, parental imprinting and X chromosome inactivation (X-inactivation). It is thought that the methylation pattern of the genome is created and stably maintained by a combination of de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and maintenance-type DNA methyltransferase Dnmt1. Targeted disruption of these DNA methyltransferases revealed that the establishment and maintenance of the genomic methylation pattern is essential for normal mammalian development (Li et al., 1992; Okano et al., 1999).

The dose difference of the X-linked genes between males and females is functionally equalized by inactivating one of the two X chromosomes in female somatic cells during early development (Lyon, 1961). Although X chromosomes inherited from father and mother are both active in undifferentiated cells of preimplantation embryos, one of them becomes transcriptionally silenced upon cellular differentiation. This process is regulated by a cyogenetically defined region on the X chromosome called X chromosome inactivation center (Xic) (reviewed by Avner and Heard, 2001). Xic is essential for X-inactivation to occur in cis and involved in both ‘choice’ of X chromosome to be inactivated and subsequent ‘initiation’ of chromosomal inactivation. The inactivated state then spreads both proximally and distally from the Xic. Non-coding RNAs, Xist and its antisense Tsix, which are mapped in Xic, play a crucial role in this process (Brown et al., 1991; Brockdorff et al., 1991; Borsani et al., 1991; Penny et al., 1996; Marahrens et al., 1997; Lee et al., 1999; Lee and Lu, 1999; Lee, 2000; Sado et al., 2001). At the onset of X-inactivation, Xist is upregulated on the future inactive X chromosome and subsequently accumulates on it in cis. However, expression of Xist is repressed on the X chromosome that remains active. Tsix is a negative regulator of Xist in cis. Extinction of Tsix induces Xist accumulation on the X chromosome being subject to inactivation, whereas continued expression of Tsix prevents upregulation of Xist.

Although the mechanism how Xist mediates chromosomal silencing is not fully understood, specific association of Xist RNA along the entire region of the inactive X chromosome implies that it may recruit proteins required for heterochromatinization (Brockdorff, 2002). Recent studies showed that a polycomb group (PcG) protein complex containing Eed and Enx1 (also known as Ezh2), which harbors an activity of methylating histone H3 at lysine 9 and 27 (Cao et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002; Czermak et al., 2002), is localized to X chromosome in Xist-dependent manner in the early phase of inactivation (Mak et al., 2002; Silva et al., 2003; Plath et al., 2003; Erhardt et al., 2003).

DNA methylation has been implicated in the regulation of Xist in differentiated cells (Beard et al., 1995). The promoter
region of the transcriptionally active Xist allele on the inactive X chromosome is unmethylated, whereas that of the transcriptionally inactive Xist allele on the active X chromosome is highly methylated (Norris et al., 1994). Studies on Dnmt1-deficient ES cells and embryos suggested that although X-inactivation can occur in the absence of DNA methylation, maintenance of the methylation at the Xist promoter is necessary for its stable repression in differentiated cells (Beard et al., 1995; Panning and Jeannis, 1996). It is possible, however, that the intact de novo methyltransferases present in Dnmt1-deficient embryos generate minimal methylation patterns required for differential activation of Xist. We previously showed that the Xist locus is extensively demethylated in ES cells deficient for both Dnmt3a and Dnmt3b (Okano et al., 1999). This suggests that the locus is one of the targets for these de novo methyltransferases, which create the differential methylation pattern at the Xist promoter after the blastocyst stage (McDonald et al., 1998). It is of particular interest whether the differential methylation underlies the mechanism of monoallelic expression of Xist at the onset of X-inactivation.

We have studied X-inactivation in mouse embryos deficient for de novo methyltransferases Dnmt3a and Dnmt3b. Although the promoter of Xist is extremely hypomethylated in these embryos, expression of Xist was not affected in the majority of cells. Cytogenetic and molecular analyses indicated that one of the two X chromosomes was properly inactivated, suggesting that de novo methylation is dispensable for the initiation and propagation of X-inactivation. We further demonstrate that delayed upregulation of Xist caused by hypomethylation at the promoter does not induce X-inactivation, supporting the importance of the developmental window for the chromosomal inactivation (Wutz and Jeannis, 2000).

**Materials and methods**

**ES cells and methods**

The male ES cell line deficient for Dnmt3a and Dnmt3b is described previously (Okano et al., 1999). ES cells were induced to differentiate as embryoid bodies by suspension culture in the absence of LIF (Sado et al., 1996). The conditional alleles of Dnmt3a and Dnmt3b were introduced into mice, which harbored 2 loxP sites on both sides of the catalytic domain (M.O. and E.L., unpublished; a detailed description of these mice will appear elsewhere). These mice were crossed with CAG-cre transgenic mice to uniformly excise the catalytic domain of each methyltransferase. The null mutant alleles thus produced were functionally equivalent to the disrupted alleles previously reported (Okano et al., 1999). [Dnmt3a–/–, Dnmt3b–/–] embryos were obtained at E9.5 from intercrosses between double heterozygotes. For genotyping of the embryos, DNA was prepared from the yolk sac and all the analyses were performed on the embryos proper.

**Bisulfite genomic sequencing**

Genomic DNA prepared from the embryos proper was digested with EcoRI and treated with urea/bisulfite essentially as previously described (Paulin et al., 1998). Two round PCR was performed for amplification of both Xist and Hprt. For the first cycle, a Pr1/Pr2 pair and an Hprt-bs1/Hprt-bs3 pair were used for Xist and Hprt, respectively. For the second round, Pr2 and Pr3, and Hprt-bs2 and Hprt-bs3 were used, accordingly. Amplified products were cloned using TOPO-TA-cloning (Invitrogen). The thermal conditions for PCR was 94°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 5 minutes. Primers for Xist, Pr1, Pr2 and Pr3, are described elsewhere (McDonald et al., 1998). Primers for Hprt are as follows: Hprt-bs1, 5′-tgt att att tgt gaa tgt ttt ttg gaa aga-3′; Hprt-bs2, 5′-gta tgg ttt gta ttt tgt ttt tgt gaa-3′; and Hprt-bs3, 5′-act cta cta aaa tcc cct taa etc acc-3′.

**RNA-FISH and replication timing analysis**

RNA-FISH was performed as described previously (Sado et al., 2001). BrdU was incorporated into E9.5 embryos for 7-8 hours and cytological preparations were made in the same manner as RNA-FISH and stained with Acridine Orange.

**Immunostaining**

Embryos incubated for 3-4 hours in the presence of colcemid were trypsinized and treated with 0.075 M KCl for 8 minutes at room temperature. Cells were fixed by an addition of equal volume of 4% paraformaldehyde, which were then subjected to cyto spin for 10 minutes at 800 rpm (Cytospin 2, Shandon). The specimens thus prepared were permeabilized in KCM buffer (120 mM KCl, 10 mM NaCl, 20 mM Tris-Cl pH 7.7) containing 0.1% Triton X-100 for 10 minutes at room temperature. The first antibody against acetylated histone H4 (Upstate) and the second antibody against rabbit IgG conjugated with FITC were diluted 100-fold with KCM containing 0.1% Triton X-100.

**RT-PCR**

Total RNA (2.5 μg) was converted to cDNA using random primers. For quantitative study, real-time PCR was carried out using LightCycler-FastStart DNA Master SYBR Green I (Roche). Concentration of MgCl2 was determined at 3 mM for all primer pair sets. Standard curves for each primer pair were drawn using a series of dilution of cDNA generated from wild-type male ES cells. Expression levels of each message were determined as a value relative to the abundance of Gapd. The thermal conditions of LightCycler were as follows: for Hprt, Rps4 and Gapd, 94°C for 10 minutes, followed by 34 cycles of 94°C for 10 seconds, 55°C for 10 seconds, 72°C for 24 seconds; for G6pd and Pgk1, 94°C for 10 minutes, followed by 32 cycles of 94°C for 10 seconds, 58°C for 10 seconds, 72°C for 24 seconds. Primer sequences are as follows: Pgk1F, 5′-tgt gga gca gag atc cag ac-3′, Pgk1R, 5′-aca tgc ctg aga gca tcc ac-3′; G6pdF, 5′-atc atc tgt gag gaa ccc tct t-3′, G6pdR, 5′-ttc tcc tca aca tag agg aca gca gc-3′; Rps4F, 5′-tca tca gta tgc aca gcc cc-3′, Rps4R, 5′-gga ttt ccc ttc ctt cta gg-3′; GapdF, 5′-agt ggc gcc tgt ctt ctt acct c-3′, and GapdR, 5′-ttg gag gga gat gtc tgt gg-3′. MusHprtF and MusHprtR have been previously described (Sado et al., 1996).

Amplifications of Xist and Tsix were performed on 1/50 of the cDNA prepared from ES cells and embryoid bodies using Mx23b and Mx20 as described previously (Kay et al., 1993; Sado et al., 1996). Oct3/4 sequence was amplified using the following primers: Oct3/4F, 5′- tgt ggt tgt gat tct cga acc tg-3′; Oct3/4R, 5′-cct tct gca ggg ctt tca tcg-3′.

**Results**

**Xist is extensively hypomethylated in [Dnmt3a–/–, Dnmt3b–/–] embryos**

Expression of Xist is developmentally regulated (Panning et al., 1997; Sheardown et al., 1997). It is expressed at a low level from all active X chromosomes in undifferentiated cells in both sexes. Following differentiation, it disappears on the single X chromosome in males and the future active X chromosome in females, whereas it becomes upregulated on the future inactive X chromosome and accumulates on it in cis. This transition of Xist expression is a key event at the onset of X-inactivation and de novo methylation at the Xist promoter may prevent
X-inactivation in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos

We previously showed that the promoter of Xist is extensively demethylated in male ES cells deficient for Dnmt3a and Dnmt3b (Okano et al., 1999).

To explore the role for de novo DNA methylation in X-inactivation and also in the regulation of Xist, we analyzed mouse embryos homozygous for the disruptions of Dnmt3a and Dnmt3b. All the embryos examined were recovered at embryonic day (E) 9.5, when [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos are readily identified by gross morphology (Fig. 1A) (Okano et al., 1999). The methylation profile at the Xist promoter was analyzed in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] female embryos by bisulfite genomic sequencing. As expected, the region differentially methylated in wild-type female embryos was extremely hypomethylated in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] females (Fig. 1B). Similarly, the CpG island containing the Hprt promoter was almost completely unmethylated in these embryos, whereas in wild-type female embryos, it showed variable levels of methylation in about half of the molecules (Fig. 1B). The differential methylation at this locus is still being established at E9.5 (Lock et al., 1987).

Expression of Xist is not affected in the majority of cells in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos

It has been reported that expression of Xist is regulated by CpG methylation at the promoter in differentiated cells (Beard et al., 1994). Although a subset of cells in both male and female embryos expressed ectopic Xist (arrow heads), the majority of the cells maintained normal pattern of expression in both sexes. The chromosomes coated by Xist RNA in C and F were confirmed to be X chromosomes using a painting probe (data not shown). (G) Percentage of nuclei containing the indicated numbers of the Xist domain. Ectopic accumulation detected in males (#1 and 2) and females (#3-6) is shown in red.

**Fig. 1.** Methylation profiles of Xist in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos. (A) Gross morphology of [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryo (left) and a wild-type littermate (right) at E9.5. Scale bar: 1 mm. (B) Bisulfite sequencing of the promoter region of Xist and Hprt in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] female embryos at E9.5. Three [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] female embryos were analyzed in a comparison with wild-type females. Methylated and unmethylated CpG sites are indicated by closed and open circles, respectively. The arrows indicate the transcription start sites.

**Fig. 2.** Expression of Xist in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos. (A-F) Expression of Xist examined by RNA-FISH in [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] embryos at E9.5. Although a subset of cells in both male and female embryos expressed ectopic Xist (arrow heads), the majority of the cells maintained normal pattern of expression in both sexes. (A) Wild-type female, (B,C) [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] female, (D) wild-type male and (E,F) [Dnmt3a\textsuperscript{−/−}, Dnmt3b\textsuperscript{−/−}] male. The chromosomes coated by Xist RNA in C and F were confirmed to be X chromosomes using a painting probe (data not shown). (G) Percentage of nuclei containing the indicated numbers of the Xist domain. Ectopic accumulation detected in males (#1 and 2) and females (#3-6) is shown in red.
inactivated in [Dnmt3a –/–, Dnmt3b –/–] embryos
One of the two X chromosomes is appropriately
–/– , Dnmt3a cells, that it does not induce X-inactivation in 
[2-3.5%, data not shown] Dnmt1
–/– embryos (2-3.5%, data not shown)
Dnmt3b observed by RNA-FISH implied
Xist Dnmt1 –/– embryos (Panning and Jeanisch, 1996), we believe,
Dnmt3a –/– , in [Dnmt3a –/–, Dnmt3b –/–] double mutant embryos (see below).

One of the two X chromosomes is appropriately
inactivated in [Dnmt3a –/–, Dnmt3b –/–] embryos
The expression pattern of Xist observed by RNA-FISH implied
that X-inactivation took place normally in most cells of
[Dnmt3a –/–, Dnmt3b –/–] female embryos. Cytogenetic and
molecular analyses were, therefore, carried out to address the
activity of each X chromosome in double mutant females. As
the inactive X chromosome replicates late in S phase of the cell
cycle, we analyzed the replication timing of X chromosomes
in [Dnmt3a –/–, Dnmt3b –/–] embryos in both sexes. A single X chromosome in two [Dnmt3a –/–, Dnmt3b –/–] male embryos
replicated synchronously with the autosomes in every
metaphase spread examined (Fig. 3B). In four [Dnmt3a –/–,
Dnmt3b –/–] females, however, one of the two X chromosomes
unanimously replicated late in S phase as seen in wild-type
female embryos (Fig. 3A,B). One of the two X chromosomes
in [Dnmt3a –/–, Dnmt3b –/–] female embryos thus behaved like
an inactive X chromosome.

It is also known that acetylated histone H4 is excluded from
the inactivated X chromosome (Jeppesen and Turner, 1993),
which is a relatively late event in the X-inactivation process
(Keohane et al., 1996). Immunostaining with an antibody
against acetylated histone H4 revealed that one of chromosomes
was hypoacetylated in the mutant female embryos (n=4) as seen
in wild-type females (Fig. 3C). Chromosome painting with an
X-specific probe confirmed that the unlabeled chromosome was
indeed an X chromosome (data not shown).

Finally we performed real time PCR to compare the
expression levels of X-linked genes, Rps4, Pgk1, G6pd and
Hprt, all of which are subject to X-inactivation, in [Dnmt3a –/–,
Dnmt3b –/–] embryos. The expression level of each gene relative
to that of Gapd did not show a significant difference between
males and females (Fig. 4), suggesting that one of the two
alleles at each locus were silenced in [Dnmt3a –/–, Dnmt3b –/–]
females. It is worth mentioning that a relative expression level
of Pgk1, although appropriately compensated between males
and females, was significantly higher in double mutant than
wild-type embryos. It is likely that the regulation of basal
expression of Pgk1 has been affected by a loss of functional
de novo DNA methyltransferases.

Our cytological and molecular studies strongly suggest that
[Dnmt3a –/–, Dnmt3b –/–] female embryos properly undergo X-
inactivation in almost all cells and that a loss of functional
Dnmt3a and Dnmt3b does not impair either the initiation of X-inactivation or
the propagation of the inactivated state
along the X chromosome.

Prolonged culture of [Dnmt3a –/–,
Dnmt3b –/–] male ES cells causes
derepression of Xist
In the above experiments, however, a small proportion of cells in [Dnmt3a –/–,
Dnmt3b –/–] embryos did show ectopic
accumulation of Xist (Fig. 2A-F). As
previous studies on Dnmt1-deficient ES cells suggested that DNA methylation is required for stable repression of Xist (Panning and Jeanisch, 1996), the ectopic Xist accumulation in the [Dnmt3a–/–, Dnmt3b–/–] embryos may have arisen from delayed activation, not from failure in initial repression. To address this issue, we made use of male ES cells deficient for both Dnmt3a and Dnmt3b (Okano et al., 1999). RNA-FISH was performed on these male ES cells before and after induction of differentiation. Although the single X chromosome was never coated with Xist RNA in undifferentiated state, ectopic Xist accumulation was observed in about 3.2% and 16.8% of cells at day 2 and day 5 of differentiation, respectively (Fig. 5B; data not shown). This is in agreement with the results obtained with the E9.5 [Dnmt3a+/, Dnmt3b+/] embryos (0-17.7%). At day 12 of differentiation, however, a surprisingly high percentage (68%) of cells from [Dnmt3a+/, Dnmt3b+/] embryoid bodies showed ectopic Xist accumulation (Fig. 5A,B), suggesting progressive activation of the unmethylated Xist locus in the differentiated [Dnmt3a+/, Dnmt3b+/] cell population. In control experiments, Xist accumulation was never detected in either Dnmt3a+/-, Dnmt3b+/- or parental wild-type ES cells, but a subset of Dnmt1-/- cells showed ectopic Xist accumulation (Fig. 5B), in agreement with the previous report (Panning and Jeanisch, 1996). These observations confirm the importance of DNA methylation in stable repression of Xist (Panning and Jeanisch, 1996).

**Ectopic accumulation of Xist in differentiated [Dnmt3a+/-, Dnmt3b+/-] cells does not induce X-inactivation**

A previous study on Dnmt1-/- ES cells suggested that ectopic Xist accumulation causes aberrant X-inactivation and subsequent cell death and that this may explain why ectopic Xist accumulation was detected only in a small proportion of the mutant cells (Panning and Jeanisch, 1996). However, in our experiment, cells with ectopic Xist accumulation predominated in the differentiated [Dnmt3a+/-, Dnmt3b+/-] male ES cell population, implying that X-inactivation was not induced. If ectopic X-inactivation took place in the mutant male ES cells, these cells would die upon differentiation because of functional nullisomy for X chromosome. We addressed the issue by examining whether selective death of [Dnmt3a+/-, Dnmt3b+/-] cells occurs in a 1:1 mixture of wild-type and the mutant cells. We took advantage of the deletion introduced at the Dnmt3b locus to compare the proportion of wild-type and mutant cells, which was visualized by Southern blotting as a restriction fragment length difference. The intensities of the bands derived from the wild-type and [Dnmt3a+/-, Dnmt3b+/-] ES cells appeared almost identical before and after differentiation (up to day 12) (Fig. 6A), indicating no selective elimination of the mutant cells.

We then examined whether X-inactivation occurs in [Dnmt3a+/-, Dnmt3b+/-] male ES cells upon induction of differentiation. We amplified X-linked Pgk-1, G6pd, and Rps4 by RT-PCR and, as shown in Fig. 6B, none of them showed drastic decrease in expression over the course of differentiation, despite the fact that a significant proportion of the cells exhibited Xist accumulation with downregulation of antisense Tsix. This suggests that X-inactivation did not occur in the differentiated mutant ES cells. The absence of a late replicating X chromosome in these differentiated cells further supported this notion (n=25, data not shown). These results strongly suggest that delayed accumulation of Xist RNA ectopically expressed in [Dnmt3a+/-, Dnmt3b+/-] male ES cells would die upon differentiation because of functional nullisomy for X chromosome.
Fig. 6. Ectopic expression of Xist does not induce X-inactivation in [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] male ES cells. (A) No selective loss of [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] male ES cells upon differentiation in a 1:1 mixture of wild type and [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] male ES cells. (B) RT-PCR amplifying X-linked genes on cDNA prepared from wild-type and [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] male ES cells, and their differentiated derivatives as embryoid bodies. Tsix and Xist were amplified from a nascent and a processed transcript, respectively, with a common primer pair. Differentiation was monitored by Oct3/4. No amplification from genomic DNA was confirmed by PCR on a reaction of cDNA synthesis without reverse transcriptase (data not shown).

Discussion

We studied the role for de novo methylation in X-inactivation using mouse embryos and ES cells deficient for de novo DNA methyltransferases Dnmt3a and Dnmt3b. Our cytological and molecular studies demonstrated that one of the two X chromosomes was properly inactivated in [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] female embryos, suggesting that X-inactivation can initiate and propagate along the chromosome in the absence of de novo DNA methylation. The de novo methyltransferases thus appear to be dispensable for the conversion of X chromosome into heterochromatin. Recent studies showed that the PcG proteins, Eed and Enx1/Ezh2, are implicated in X-inactivation (Mak et al., 2002; Silva et al., 2003; Plath et al., 2003; Erhardt et al., 2003). Interestingly, Enx1/Ezh2 has a histone methyltransferase activity specific for lysine 9 and 27 in the N-terminal tail of histone H3, which is involved in heterochromatin formation and gene silencing (Cao et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002; Czermin et al., 2002). As methylation of histone H3 at lysine 9 (and probably 27) becomes evident at relatively early phase of the X-inactivation process in female ES cells (Heard et al., 2001; Mermoud et al., 2002), it is possible that histone H3 methylation at these residues plays a more pivotal role than DNA methylation in spreading of the inactive state from the Xic.

Previous studies on Dnmt1<sup>−/−</sup> embryos and ES cells showed that initiation of monoallelic Xist expression and X-inactivation can occur in the absence of maintenance-type DNA methylation (Beard et al., 1995; Panning and Jaenisch, 1996). They did not, however, address whether de novo methylation at the Xist promoter, which should occur in the mutant cells, contributes to the initiation of X-inactivation. Some residual methylation was, in fact, detected at the Xist promoter in Dnmt1<sup>−/−</sup> embryos (Sado et al., 2000), which was most probably mediated by the de novo DNA methyltransferases. In [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] female embryos, despite the lack of de novo methylation at the Xist promoter, accumulation of Xist RNA was confined to a single X chromosome in the great majority of nuclei. The present study, therefore, provides the first evidence that a mechanism(s) other than DNA methylation is responsible for causing the differential expression of Xist. It is clear, nevertheless, that DNA methylation is important for the stable repression of Xist on the active X chromosome in differentiated cells. In agreement with the previous reports on Dnmt1-deficient cells (Beard et al., 1995; Panning and Jaenisch, 1996), the present study indicated that upon differentiation, hypomethylation at the Xist promoter caused by a functional loss of de novo DNA methyltransferases resulted in progressive derepression of Xist. It should be noted, however, that our finding was at variance with the study by Panning and Jaenisch (Panning and Jaenisch, 1996) in that ectopically expressed Xist did not lead to silencing of X-linked genes. While available evidence suggests that Xist expressed in differentiated cells does not cause X-inactivation (Tinker and Brown, 1998; Wutz and Jaenisch, 2000) (this study), Hall et al. (Hall et al., 2002) recently demonstrated that a human XIST transgene could induce chromosome inactivation in postdifferentiation human HT-1080 cells. Perhaps, ectopic expression of Xist might exert its effect on silencing in some particular cell types.

Our results suggested that X-inactivation was not induced despite the cis-association of Xist on the X chromosome. Taking advantage of an inducible expression system of Xist in transgenic ES cells, Wutz and Jaenisch (Wutz and Jaenisch, 2000) previously showed that Xist can initiate chromosomal silencing only during an early phase of ES cell differentiation (up to 48 hours after induction of differentiation), which indicates that there is a crucial developmental window for X-inactivation. Ectopic expression of Xist, therefore, most probably occurs later than this window in [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] embryos and male ES cells, thereby resulting in no induction of X-inactivation.

It has been suggested that DNA methylation is important for the stable repression of genes on the inactivated X chromosome. Our previous study on Dnmt1-deficient mouse embryos suggests that substantial loss of DNA methylation leads to partial reactivation of X-linked lacZ transgenes in the embryonic lineage of E9.5 embryos (Sado et al., 2000). By contrast, hypomethylation caused by the failure of de novo DNA methylation did not appear to reactivate the silent copy of the four endogenous X-linked genes in [Dnmt3a<sup>−/−</sup>, Dnmt3b<sup>−/−</sup>] females at E9.5. This may be ascribed to different susceptibility to hypomethylation between the endogenous
genes and the transgenes. It is possible that the regulation of multicyclog enogenes sequences such as a tandem array of the lacZ transgenes is more sensitive to the loss of DNA methylation than the endogenous genes. It will be important to address whether or not the maintenance mechanism of the X-inactivated genes is affected in [Dnmt3a−/−, Dnmt3b−/−] background.

It is known that Xist is exclusively expressed from the paternal allele in each blastomere of early preimplantation embryos (Kay et al., 1993; Sheardown et al., 1997), although the promoter region of Xist shows a low level of CpG methylation on both parental alleles (McDonald et al., 1998). It seems, therefore, possible that distinctive chromatin structures inherited from the parents are responsible for the upregulation of the paternal allele and stable repression of the maternal allele in the preimplantation embryos. It is therefore likely that the modification of chromatin is capable of inducing the differential expression of Xist on its own. Methylation of histone H3 at lysine 9 and 27 and histone H4 at lysine 20 is implicated in regulation of gene expression in yeast and fruit fly (Nakayama et al., 2001; Noma et al., 2001; Cao et al., 2002; Nishioka et al., 2002a; Fang et al., 2002), both of which essentially lack DNA methylation. It is tempting to speculate that these histone modifications in combination with methylation of histone H3 at lysine 4, which is involved in transcriptional activation (Strahl et al., 1999; Noma et al., 2001; Wang et al., 2001; Nishioka et al., 2002b), primarily regulate the differential expression of Xist (Fig. 7), which would not be affected in [Dnmt3a−/−, Dnmt3b−/−] embryos. CpG methylation and hypacetylation (Gilbert and Sharp, 1999) then follow these events to fix the established epigenetic states. As Tsix appears to regulate the expression of Xist negatively at the onset of X-inactivation (Lee, 2000; Sado et al., 2001), it should also play a role in monoallelic upregulation of Xist. It should be noted that Tsix became downregulated in the absence of de novo methyltransferases in differentiating ES cells. The CpG island found in the vicinity of the major transcription start site of Tsix, which became methylated upon differentiation in male ES cells, stayed unmethylated after differentiation in [Dnmt3a−/−, Dnmt3b−/−] male ES cells, suggesting that regulation of Tsix is also independent of DNA methylation (data not shown). Further studies on the epigenetic regulation and function of Tsix may clarify the initial event that triggers X-inactivation.

We thank Minako Kanbayashi for genotyping and breeding of mice, and Chikako Suda for sequencing. This work was supported by grants from PRESTO, Japan Science and Technology Agency (JST) and Grant-in Aid for Scientific Research on Priority Area Genome Biology from the Ministry of Education, Science, Sports, and Culture of the National Institute of Health to E.L.

Fig. 7. A model that underlies the induction of differential expression of Xist at the onset of X-inactivation. Distinct chromatin structure above seems to be sufficient for inducing differential expression of Xist, which may be constructed by modification of histone tail such as methylation of histone H3 tail. DNA methylation and the exclusion of acetylated histones play a role in stabilizing the transcriptionally repressive state.