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

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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 cytogenetically 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; Czermin 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 Jeanisch, 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 Jeanisch, 2000).

Materials and methods

ES cells and mice

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 *lox*P 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 *Eco*RI 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′-gtg att att tgg gaa ttt ttt ggg aga-3′; *Hprt*-bs2, 5′-gta tgg tta gta tta ttt ttt tta gaa-3′; and *Hprt*-bs3, 5′-act cta cta aaa tcc cct taa ctc 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 cytospin 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-HCl 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 $MgCl_2$ 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: *Pgk1*F, 5′-gtt gca gac aag atc cag ac-3′, *Pgk1*R, 5′-aca ttg ctg aga gca tcc ac-3′; *G6pd*F, 5′-atc atc gtg gag aag ccc tt-3′, *G6pd*R, 5′-ttc ttc aca tag agg aca gc-3′; *Rps4*F, 5′-tca tca gca ttg aca aga cc-3′, *Rps4*R, 5′-ggattc ctt ttc ctc tgg ga-3′; *Gapd*/F, 5′-atg gcc ttc cgt gtt cct ac-3′, and *Gapd*/R, 5′-tgt gag gga gat gct cag tg-3′. Mus*Hprt*F and Mus*Hprt*R 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 MIX20 as described previously (Kay et al., 1993; Sado et al., 1996). *Oct3/4* sequence was amplified using the following primers: *Oct3/4*F, 5′-tgg gtg gat tct cga acc tg; *Oct3/4*R, 5′-cct tct gca ggg ctt tca tg-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

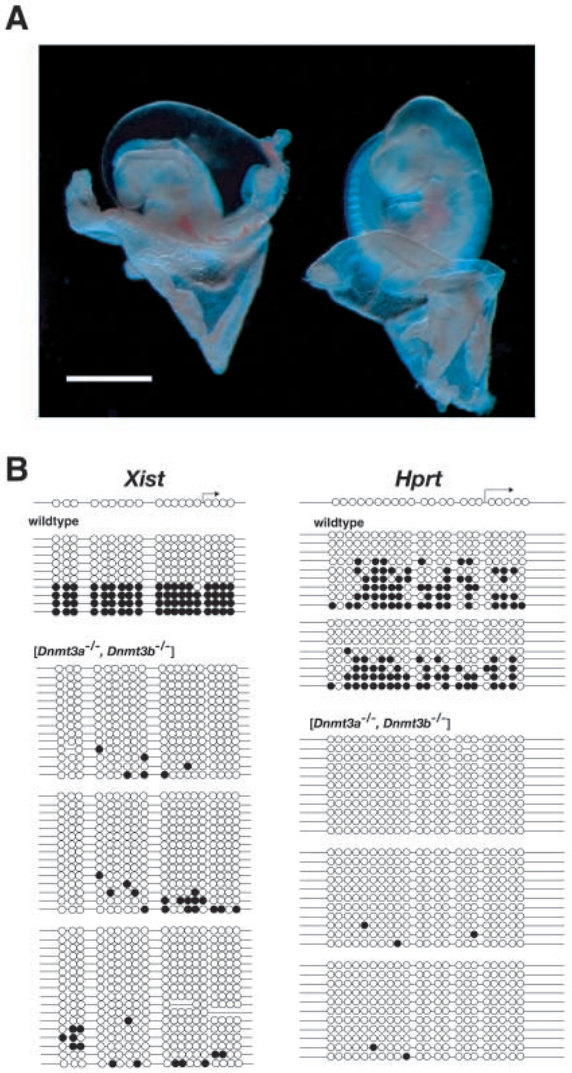


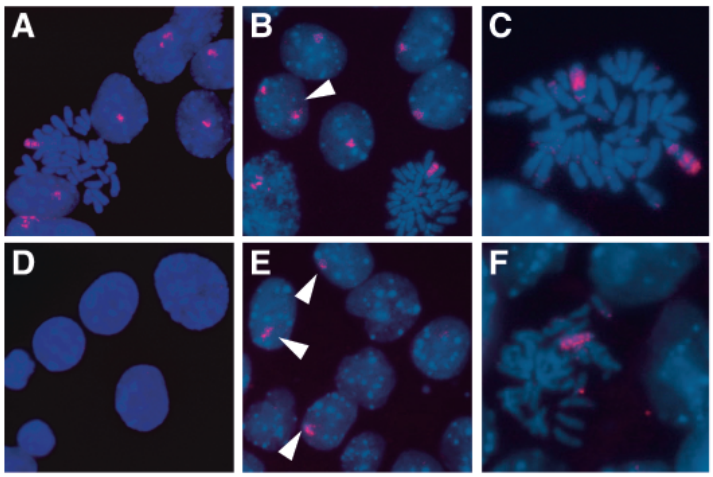
Fig. 1. Methylation profiles of *Xist* in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos. (A) Gross morphology of [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryo (left) and a wild-type littermate (right) at E9.5. Scale bar: 1 mm. (B) Bisulfite sequencing of the promoter region of *Xist* and *Hprt* in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos at E9.5. Three [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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.

upregulation of *Xist* on the X chromosome that remains active. 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* at the onset of X-inactivation, 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*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos are readily identified by gross morphology (Fig. 1A) (Okano et al., 1999). The methylation profile at the *Xist* promoter was analyzed in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos by bisulfite genomic sequencing. As expected, the region differentially methylated in wild-type female embryos was extremely hypomethylated in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos

It has been reported that expression of *Xist* is regulated by CpG methylation at the promoter in differentiated cells (Beard et al.,



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embryo	none	one	two
#1	95.1% (137/144)	4.9% (7/144)	
#2	100% (156/156)	0% (0/156)	
#3	7.8% (10/128)	88.3% (113/128)	3.9% (5/128)
#4	4.1% (6/147)	78.9% (116/147)	17.7% (25/147)
#5	8.4% (12/143)	87.4% (125/143)	4.2% (6/143)
#6	6.7% (10/149)	88.6% (132/149)	4.7% (7/149)

Fig. 2. Expression of *Xist* in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos. (A-F) Expression of *Xist* examined by RNA-FISH in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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*$^{-/-}$, *Dnmt3b*$^{-/-}$] female, (D) wild-type male and (E,F) [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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.

1995). We therefore examined the expression of *Xist* in double mutant male and female embryos. RNA-FISH revealed that ectopic accumulation of *Xist* RNA occurred on one and two X chromosomes in males and females, respectively (Fig. 2A-F). However, such accumulation was found only in a subset of the cells in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos (0-4.9% in males and 3.9-17.7% in females) and the majority of the cell population in female embryos manifested a normal pattern of *Xist* accumulation despite extensive hypomethylation at the promoter on both alleles (Fig. 2G). This implies that monoallelic expression of *Xist* can be induced in the absence of differential methylation at the promoter. It is therefore likely that methylation at the *Xist* promoter is not essential for preventing upregulation of *Xist* on the future active X chromosome at the onset of X-inactivation. It should be noted that the proportion of cells exhibiting ectopic *Xist* expression in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos was comparable with that detected in *Dnmt1*$^{-/-}$ embryos (2-3.5%, data not shown) (Panning and Jeanisch 1996). Although it has been suggested that ectopic expression of *Xist* may lead to X-inactivation in *Dnmt1*$^{-/-}$ embryos (Panning and Jeanisch, 1996), we believe, based on the study in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells, that it does not induce X-inactivation 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

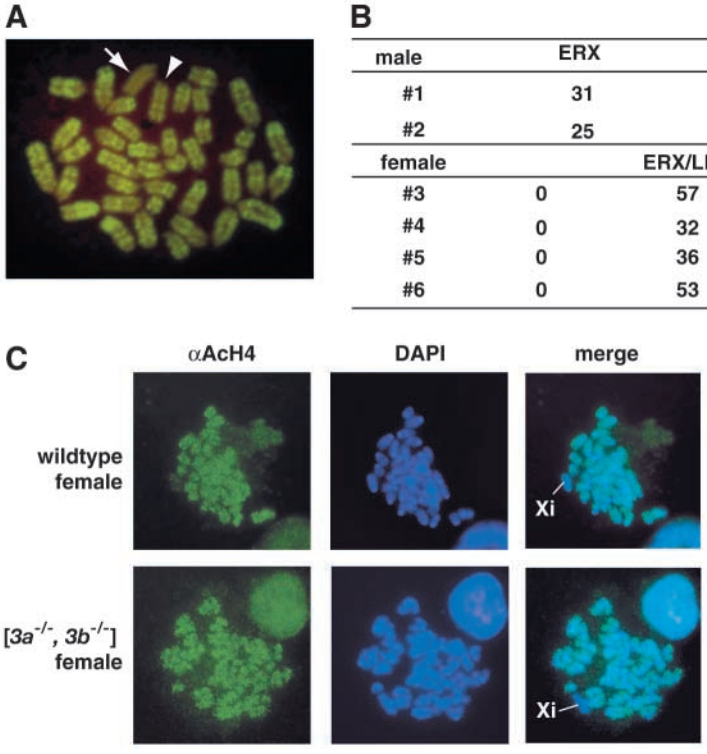

Fig. 3. Cytological evidence that one of the two X chromosomes is inactivated in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos. (A) A late replicating X chromosome found in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos at E9.5 (arrow). An active counterpart is indicated by an arrowhead. (B) The number of cells with early or late replicating X chromosomes in male (#1 and 2) and female (#3-6) embryos. ERX, early replicating X chromosome; LRX, late replicating X chromosome. (C) Immunostaining of metaphase chromosomes in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos. Metaphase chromosomes were stained with an antibody against acetylated histone H4. Acetylated histone H4 was excluded from one (Xi) of the two X chromosomes in wild-type and [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] female embryos at E9.5.

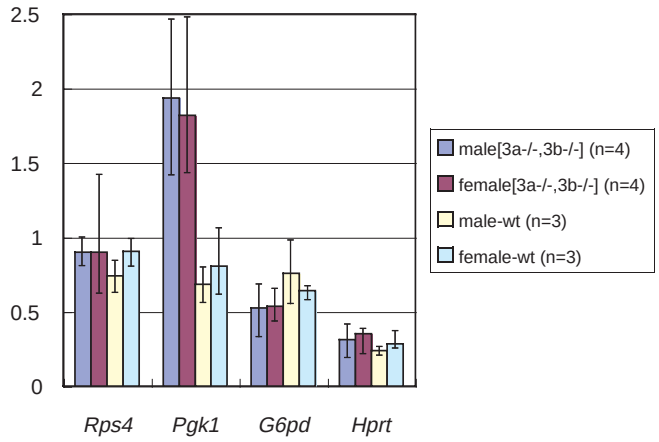

Fig. 4. Quantitative RT-PCR of X-linked genes. Expression levels of each gene (*Rps4*, *Pgk1*, *G6pd*, and *Hprt*) relative to the abundance of *Gapd* were compared between males and females in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos.

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

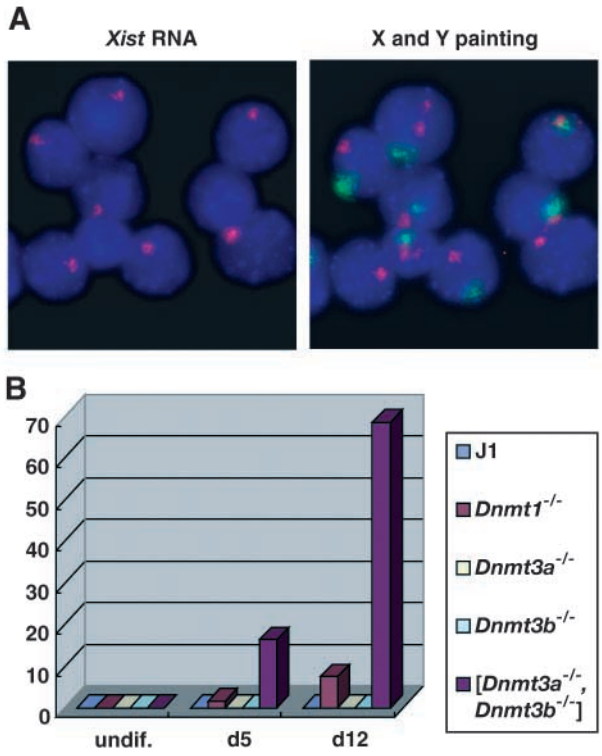

Fig. 5. [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells were induced to differentiate. (A) RNA-FISH was performed on [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells at 12 days of differentiation. Ectopic expression of *Xist* was evident, which colocalized with the X chromosome visualized by X (red) and Y (green) chromosome painting. (B) Percentage of cells with ectopic *Xist* accumulation in J1 (wild type), *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$ and [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] ES cells at 0, 5 and 12 days of differentiation. Similar results were observed in 2 or 3 different experiments.

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

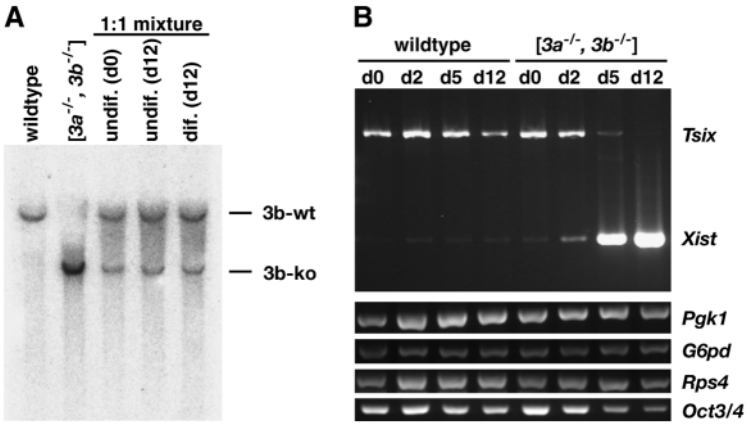

Fig. 6. Ectopic expression of *Xist* does not induce X-inactivation in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells. (A) No selective loss of [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells upon differentiation in a 1:1 mixture of wild type and [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] male ES cells. (B) RT-PCR amplifying X-linked genes on cDNA prepared from wild-type and [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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).

does not trigger aberrant X-inactivation, and this is most probably the case for [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] embryos. These findings support the previous proposal that *Xist* RNA needs to be expressed in a critical window of differentiation to initiate X-inactivation (Wutz and Jeanisch, 2000).

Discussion

We studied the role for de novo methylation in X-inactivation using mouse embryos and ES cells deficient for de novo methyltransferases Dnmt3a and Dnmt3b. Our cytological and molecular studies demonstrated that one of the two X chromosomes was properly inactivated in [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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*$^{-/-}$ 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 Jeanisch, 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*$^{-/-}$ embryos (Sado et al., 2000), which was most probably mediated by the de novo DNA methyltransferases. In [*Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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 Jeanisch, 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*$^{-/-}$, *Dnmt3b*$^{-/-}$] 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*$^{-/-}$, *Dnmt3b*$^{-/-}$] females at E9.5. This may be ascribed to different susceptibility to hypomethylation between the endogenous

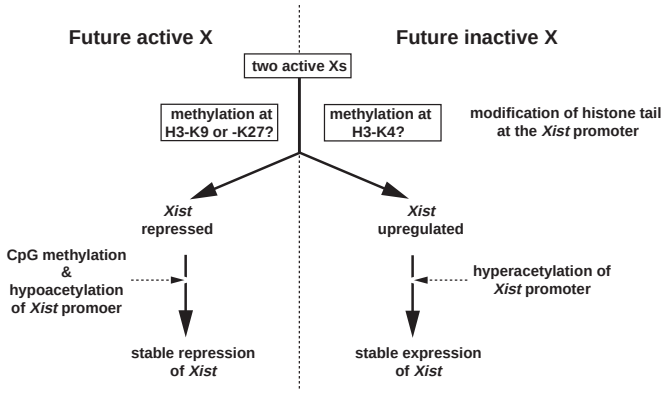

Fig. 7. A model that underlies the induction of differential expression of *Xist* at the onset of X-inactivation. Distinct chromatin structure above seems to be sufficient for inducing differential expression of *Xist*, which may be constructed by modification of histone tail such as methylation of histone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

genes and the transgenes. It is possible that the regulation of multicopy exogenous 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 repression 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 hypoacetylation (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.

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