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A proximal conserved repeat in the *Xist* gene is essential as a genomic element for X-inactivation in mouse

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X-inactivation in female mammals is triggered by the association of non-coding *Xist* RNA in cis with the X chromosome. Although it has been suggested that the A-repeat located in the proximal part of the *Xist* RNA is required for chromosomal silencing in ES cells, its role in mouse has not yet been addressed. Here, we deleted the A-repeat in mouse and studied its effects on X-inactivation during embryogenesis. The deletion, when paternally transmitted, caused a failure of imprinted X-inactivation in the extraembryonic tissues, demonstrating the essential role of the A-repeat in X-inactivation in the mouse embryo. Unexpectedly, the failure of X-inactivation was caused by a lack of *Xist* RNA rather than by a defect in the silencing function of the mutated RNA, which we expected to be expressed from the mutated X. Interestingly, the normally silent paternal copy of *Tsix*, which is an antisense negative regulator of *Xist*, was ectopically activated in the preimplantation embryo. Furthermore, CpG sites in the promoter region of paternal *Xist*, which are essentially unmethylated in the extraembryonic tissues of the wild-type female embryo, acquire a significant level of methylation on the mutated paternal X. These findings demonstrate that the DNA sequence deleted on the mutated X, most probably the A-repeat, is essential as a genomic element for the appropriate transcriptional regulation of the *Xist*/*Tsix* loci and subsequent X-inactivation in the mouse embryo.

KEY WORDS: X-inactivation, Xist, Tsix, Gene targeting, Mouse embryo

INTRODUCTION

During the early development of female mammals, one of the two X chromosomes undergoes transcriptional silencing along almost the entire region of the chromosome to achieve dosage equivalence with males of the X-linked genes, which females have twice as many of as males do (X-inactivation) (Lyon, 1961). Several lines of evidence indicate that the paternal X chromosome is preferentially inactivated in the early preimplantation stage embryo (Huynh and Lee, 2003; Mak et al., 2004; Okamoto et al., 2004). This imprinted X-inactivation is maintained throughout development of the extraembryonic tissues, such as the placenta and part of the extraembryonic membranes (Takagi and Sasaki, 1975), which originate from the trophectoderm and primitive endoderm of the blastocyst. In the epiblast lineage, a derivative of the inner cell mass of the blastocyst, the previously inactivated paternal X transiently restores transcriptional activity, and subsequently either the maternal or paternal X undergoes inactivation in a basically random fashion as cells differentiate (random X-inactivation).

It is known that the *Xist* (X-inactive specific transcript) gene located in the X inactivation center (Xic), a cytogenetically identified chromosomal region essential for X-inactivation to occur in cis, plays a crucial role in both imprinted and random X-inactivation. Targeted disruption of *Xist* renders the mutated X incompetent to undergo inactivation (Marahrens et al., 1997; Penny et al., 1996), and therefore paternal transmission of *Xist* deficiency results in the failure of imprinted paternal X-inactivation in the extraembryonic tissues and, consequently, a selective loss of female embryos soon after implantation owing to the extremely poor development of the extraembryonic tissues.

Xist encodes long non-coding transcripts as long as 17 kb in length, which are subjected to splicing and polyadenylation like common protein-coding RNAs (Brockdorff et al., 1992; Brown et al., 1992). The *Xist* RNA is peculiar in that it stays in the nucleus and associates with the X chromosome, from which it is transcribed (Brown et al., 1992; Clemson et al., 1996), where it eventually induces chromosomal silencing by unknown mechanisms. Although the overall structure of the *Xist* gene is relatively conserved among eutherian mammals, its nucleotide sequence diverges greatly (Chureau et al., 2002; Nesterova et al., 2001), which is consistent with the presumed role of the *Xist* gene product as a functional RNA. It is known, however, that several regions consisting of a series of repeats are conserved between mouse and human (Brockdorff et al., 1992; Brown et al., 1992). Wutz et al. previously showed that one of these repeats, known as the A-repeat, which is located in the proximal part of the *Xist* RNA, is crucial for the silencing function of the RNA (Wutz et al., 2002). They demonstrated that the RNA transcribed from a single copy *Xist* cDNA lacking the A-repeat driven by an inducible promoter can accumulate on the X chromosome in male ES cells upon induction but fails to initiate chromosomal silencing. The A-repeat contains 7.5 copies of a conserved direct repeat unit, which harbors two short inverted repeats that might fold into a secondary structure comprising two stem loops. These findings imply that the stem loop structures might be the modules that interact with the putative protein factors that are involved in chromosome silencing.

Although the above inducible expression assay in ES cells identified for the first time a likely functional domain in *Xist* RNA responsible for chromosomal silencing, its significance for X-inactivation taking place in the developing embryo has not yet been addressed. In this study, we introduced a mutant *Xist* allele lacking the A-repeat into the mouse and examined its effects on X-

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inactivation in the embryo. Our results clearly demonstrate that deletion of the A-repeat rendered the mutated X incompetent to undergo inactivation in embryos, which was consistent with the previous ES cell assay. However, the incompetence of the mutated X to undergo inactivation was apparently due to the lack of *Xist* expression on the mutated X chromosome. This finding suggests an unexpected essential role of the A-repeat as a genomic element for the appropriate regulation of *Xist* and subsequent X-inactivation in the mouse embryo.

MATERIALS AND METHODS

Targeted deletion of the A-repeat

Targeting vector pXBΔA was constructed so that a floxed HSV-tk and PGK-neo fragment from pflox (a gift from En Li. location?) was flanked by genomic fragments derived from Bac clone 333J22 containing the *Xist* gene as shown in Fig. 1A (the 5′ arm, nucleotides 97, 704-106, 412 in AJ421479; the 3′ arm, nucleotides 107, 228-113, 284 in AJ421479). J1 ES cells (Li et al., 1992) were electroporated with pXBΔA as previously described (Sado et al., 2005), and selection was started 24 hours later in the presence of 250 μg/ml G418. Of 254 selected colonies, four harbored the expected homologous recombination ($Xist^{\Delta A2lox}$). Chimeric males were generated and crossed with females heterozygous for a *Tsix* deficiency (Sado et al., 2001) to facilitate germ-line transmission in the same manner as previously described (Sado et al., 2005). Females heterozygous for $Xist^{\Delta A2lox}$ were crossed with CAG-cre transgenic males to derive pups carrying $Xist^{\Delta A}$. Excision of the selection marker in ES cells was performed by transient expression of Cre recombinase using pBS185 (Life Technology).

Histology

Deciduas dissected out from the uterus were fixed in Bouin's fixative. Following dehydration, deciduas were embedded in Technovit 7100 (Kulzer), sectioned at 2 μm, and stained with Hematoxylin and Eosin.

Genotyping blastocysts

Blastocysts were flushed from the uterus at E3.5 and the zona pellucida was removed by acid tyroid treatment. Each blastocyst was transferred to 10 μl of water and heated for 3 minutes at 95°C. Five microliters of this solution was used for two-round PCR with semi-nested primer sets for genotyping and sexing. The primers used for the first round amplification were R700P2 (wild-type-specific), dA1F ($Xist^{\Delta A}$-specific), *Xist*1395R, Zfy1 and Zfy2. Subsequently, the wild-type *Xist*, $Xist^{\Delta A}$ and *Zfy* sequences were individually amplified in a second round PCR using R700P2/F1063AS for wild-type *Xist*, dA1F/F1063AS for $Xist^{\Delta A}$ and Zfy1/Zfy4 for *Zfy*. Primer sequences used in this study are shown in Table 1.Total RNA was extracted from the remaining 5 μl using Trizol (Invitrogen) in the presence of 10 μg of *E. coli* tRNA.

RT-PCR

For quantitative RT-PCR analysis of undifferentiated ES cells, cDNA was prepared at 60°C using Thermoscript (Invitrogen) with *Xist* 2688R, *Tsix*2R and GapdR as primers. Real-time PCR was carried out as previously described (Sado et al., 2006), using *Xist*2281F and *Xist* 2424R as primers for *Xist*, *Tsix*2F and P422R for *Tsix*, and GapdF and GapdR2 for *Gapd*. The expression levels of *Xist* and *Tsix* were normalized to *Gapd* levels as previously described (Sado et al., 2006).

For allelic expression analysis of X-linked genes in the trophoblast and in E7.5 embryos, cDNA was synthesized from 1 μg of total RNA using an oligo-dT primer, and PCR was carried out using G6pdF4 and G6pdR4 as primers for *G6pd*, and HprtF4 and HprtR3 for *Hprt*. The amplified products of *G6pd* and *Hprt* were subsequently digested with *Dra*I and *Hin*fI, respectively (Sugimoto and Abe, 2007).

For real-time PCR of individual F1 blastocysts isolated from $X^{JF1}X^{JF1}$ females crossed with either $X^{\Delta A}Y$ or wild-type XY males, each embryo was lysed in 10 μl of distilled water by heating for 3 minutes at 95°C, and RNA was prepared using Trizol (Invitrogen) in the presence of 10 μg of *E. coli* tRNA. Following DNase I treatment, cDNA was synthesized using an oligo-dT primer using the whole RNA sample in a reaction of 20 μl. One microliter of cDNA was subjected to real-time PCR amplification of a region containing a *Sac*I polymorphism between JF1 and the laboratory strain in exon 7 of *Xist* with XistEx7F31 and XistEx7R20. Because *Xist* and *Tsix* are reciprocally imprinted in the blastocyst, *Sac*I digestion of the product allowed the confirmation of specific amplification of *Xist* and not *Tsix*.

For allelic expression analysis of *Tsix* in individual genotyped F1 blastocysts, two-round PCR was carried out using cDNA synthesized in a strand-specific manner with Tsix4R and GapdR. The first round PCR was carried out on whole cDNA produced using Tsix4F, Tsix4R, GapdF and GapdR (25 cycles). The second round PCR was carried out on one-twentieth of the first round reaction using Tsix4F and Tsix4R2 (33 cycles), or GapdF and GapdR2 (25 cycles). The amplified product of *Tsix* was subsequently digested with *Bsm*AI.

RNA-FISH

An RNA probe was prepared by in vitro transcription with Cy3-UTP (Amersham Pharmacia) and a plasmid (pBE1.5) containing a *Xist* cDNA fragment (nucleotides 9829-11,335 in NR_001463 in GenBank) using T7 RNA polymerase. Cytological preparations of blastocysts were made according to Okamoto et al. (Okamoto et al., 2000).

Following examination of RNA-FISH, X- and Y-chromosome painting was carried out according to the manufacturer's instructions (Cambio) to determine the sex chromosome constitution.

RNA half-life assay

About 2.5×10^4 undifferentiated ES cells were seeded onto each dish without feeder cells. The medium of each dish was replaced with that containing 25 μg/ml of DRB (5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole, Calbiochem) on the following day and cells were collected every three hours. One microgram of total RNA isolated at each time point was converted into cDNA using an oligo-dT primer, and the level of *Xist* and *Tsix* RNA was quantitated by real-time PCR using the primer sets XistEx7F31/XistEx7R20 and Tsix4F/Tsix4R2, respectively. While XistExF31/XistEx7R20 are located about 20-kb downstream from the 5′ end of *Xist*, Tsix4F/Tsix4R2 are located upstream of the transcription start site of *Xist*. It was confirmed that there was no *Tsix* cDNA in the reaction, which was long enough to serve as a template for XistExF31/XistEx7R20. The abundance of the respective RNAs at each time point was shown as the value relative to the abundance at 3 hours after the addition of DRB.

Bisulfite sequencing

Bisulfite treatment of genomic DNA prepared from the trophoblast at E6.5 and sperm was carried out using a Bisulfast kit (TOYOBO), and DNA was purified using an EZ kit (Zymo Research). Two-round PCR was carried out using semi-nested primers. First round PCR was carried out with 25 cycles on whole DNA prepared from the trophoblast or 300 ng of sperm DNA, and the second round PCR was done with 30 cycles on one-twentieth of the reaction from the first round PCR. Primer sequences will be provided upon request.

RESULTS

The mutated *Xist* allele lacking the A-repeat was introduced into the mouse

To study the importance of the A-repeat in X-inactivation taking place in the mouse embryo, we created a new mutant allele of *Xist* lacking the A-repeat by a gene targeting strategy (Fig. 1). Following verification by Southern blot analysis, ES cells harboring the expected homologous recombination were serially injected into blastocysts to generate chimeras. We postulated that the presence of a floxed selection marker in the targeted allele ($Xist^{\Delta A2lox}$) would functionally disrupt the *Xist* gene, and that the mutated allele, when paternally inherited, would result in a selective loss of female embryos soon after implantation because of the failure of imprinted X-inactivation in the extraembryonic lineages. We previously demonstrated, however, that this female-specific lethality is sometimes rescued by the simultaneous presence of a *Tsix* deficiency on the maternal X (Ohhata et al., 2008; Sado et al., 2005; Sado et al., 2006). Accordingly, the male chimeras were crossed with females heterozygous for the *Tsix* deficient allele (*ΔTsix*) (Sado et al., 2001) to facilitate transmission of

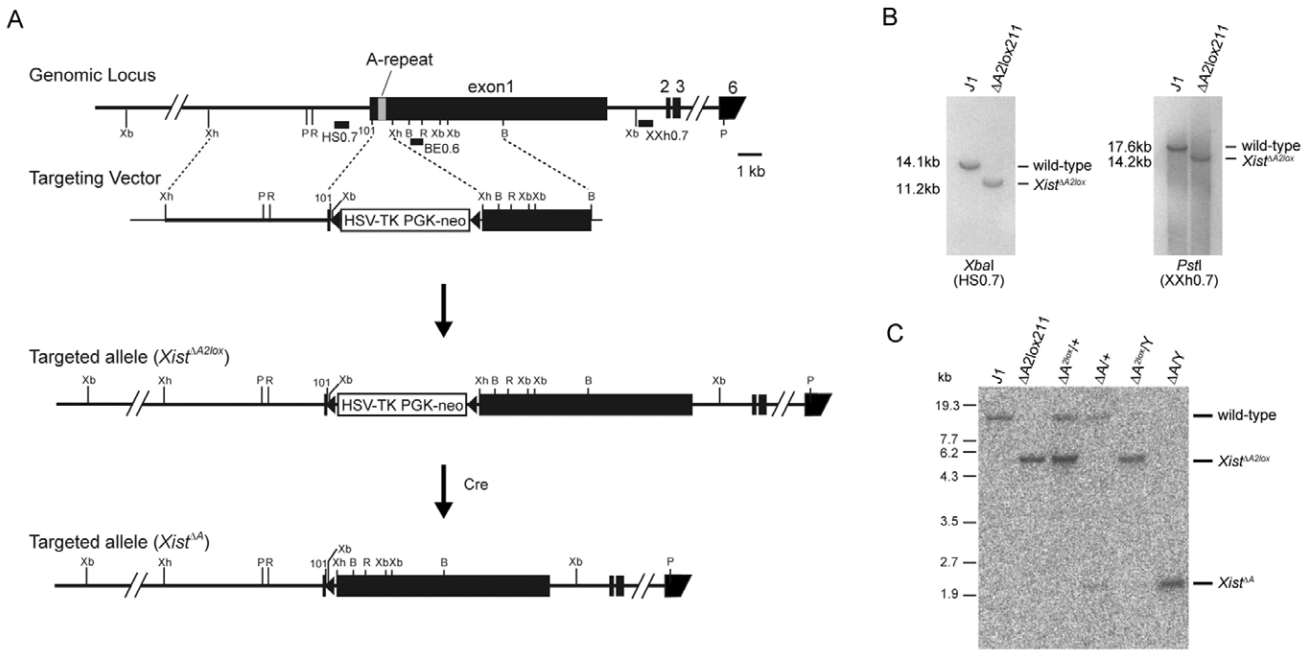

Fig. 1. Targeted deletion of the A-repeat in the *Xist* gene. (**A**) Scheme for generating the $Xist^{\Delta A}$ allele. Genomic structure of the *Xist* gene and the targeting vector are shown. The A-repeat region is shown in gray. The $Xist^{\Delta A}$ allele lacks an 812 bp fragment containing the A-repeat (nucleotides 101-912 in GenBank L04961). Positions of the probes used for Southern blotting in B and C are also indicated. B, *Bam*HI; P, *Pst*I; R, *Eco*RI; Xb, *Xba*I; Xh, *Xho*I. (**B**) Homologous recombination was confirmed by Southern blotting. ΔA2lox211 is one of the four ES lines harboring the correct targeting event. Genomic DNA digested with *Xba*I (left) and *Pst*I (right) was probed with HS0.7 (Sado et al., 2005) and XXh0.7 (Sado et al., 2006), respectively. (**C**) The presence of the respective mutation in the mouse was confirmed by Southern blotting. Tail DNA digested with *Xba*I was probed with BE0.6 (Sado et al., 2005).

the $Xist^{\Delta A2lox}$ allele from fathers to live female pups, as previously described. Consequently, females carrying the *ΔTsix* allele and $Xist^{\Delta A2lox}$ allele on the maternal and paternal X, respectively, were successfully recovered. They were subsequently crossed with males expressing Cre recombinase ubiquitously to derive $Xist^{\Delta A}$/+ females and $Xist^{\Delta A}$/Y males. These animals were apparently normal and fertile.

Basal transcription of *Xist* was not affected by deletion of the A-repeat in undifferentiated male ES cells

The targeting strategy was designed to delete the A-repeat without disrupting the endogenous *Xist* promoter. To confirm that the promoter remained functional in the $Xist^{\Delta A}$ allele, we took advantage of $Xist^{\Delta A}$/Y ES cells established by transiently expressing Cre recombinase in $Xist^{\Delta A2lox}$/Y ES cells (data not shown). It is known that the *Xist* locus is transcribed at a very low level in undifferentiated male ES cells, although this basal transcription is eventually downregulated after differentiation. We examined the basal transcription of *Xist* to determine whether it was affected by the deletion of the A-repeat in undifferentiated $Xist^{\Delta A}$/Y ES cells. Quantitative RT-PCR with strand-specifically prepared cDNA revealed that, in $Xist^{\Delta A}$/Y ES cells, the mutated $Xist^{\Delta A}$ RNA was expressed at a level comparable to wild-type *Xist* RNA in the parental male ES cells (Fig. 2A). In addition, the $Xist^{\Delta A}$ allele was downregulated in the same manner as the wild-type allele after the induction of differentiation (Fig. 2A). These results demonstrated that neither the function of the *Xist* promoter per se nor the mechanism for downregulating *Xist* on the future active X was affected by the deletion.

We further analyzed the stability of $Xist^{\Delta A}$ RNA by treating cells with DRB, an inhibitor of RNA polymerase II. Real-time PCR on cDNA prepared from a series of DRB-treated cells demonstrated that there was no significant difference in stability between wild-type and mutant *Xist* RNA (Fig. 2B), indicating that the deletion of the A-repeat did not impair the stability of the RNA.

Intriguingly, the expression level of *Tsix* was increased in the mutant male ES cells. The stability of *Tsix* RNA in the mutant ES cells was, however, comparable to that in wild-type ES cells (Fig. 2B). It seemed likely therefore that the higher expression of *Tsix* in the mutant was mediated not by an increased stability of the RNA (Fig. 2A), but by a higher level of transcription. The expression level of *Tsix*, however, declined once ES cells were induced to differentiate. This suggests that although the genetic alterations we introduced at the *Xist* locus somehow facilitated the transcription of *Tsix* on the mutated X, they did not affect the mechanism for downregulating *Tsix* upon differentiation.

Paternal transmission of $Xist^{\Delta A}$ results in a selective loss of female embryos

The functional significance of the A-repeat in embryonic development was first addressed by examining whether or not the mutated $Xist^{\Delta A}$ allele could be transmitted to female pups from the father. Of 218 pups born to wild-type females crossed with $Xist^{\Delta A}$/Y males, 216 were male and 2 were female. One of the two females turned out to be XO, where the X chromosome was maternal in origin, and the other female inherited the $Xist^{\Delta A}$ allele (Fig. 3A), suggesting that most female embryos had been lost in utero. In reciprocal crosses, the $Xist^{\Delta A}$ allele was transmitted to apparently healthy female pups from the mothers at the expected ratio (Fig. 3A). Thus, the selective loss of females upon paternal transmission of the mutation was probably due to defects in the imprinted X-inactivation in the extraembryonic lineages. When embryos were dissected out at embryonic day (E) 6.5, +/$Xist^{\Delta A}$ (the maternal allele precedes the paternal one by convention) female

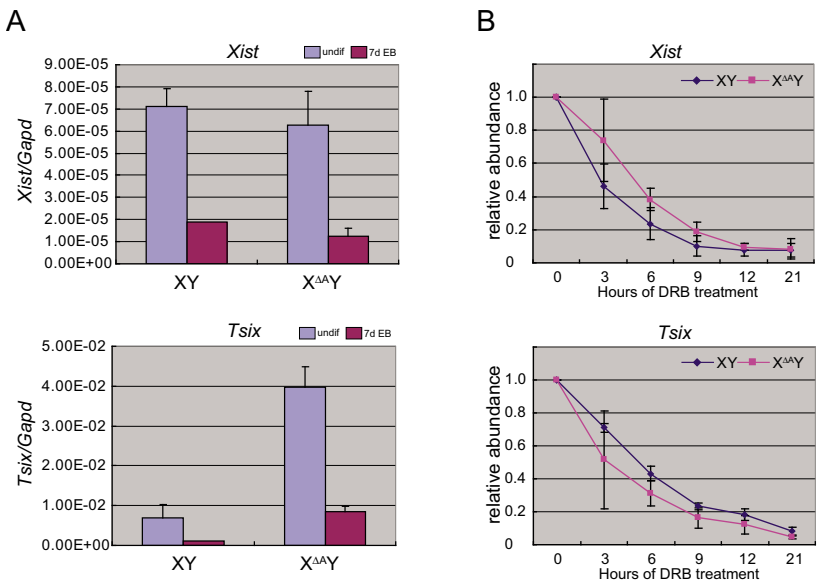

Fig. 2. Expression of *Xist* and *Tsix* in *Xist*$^{\Delta A}$/Y ES cells. (**A**) The stable level of *Xist* and *Tsix* RNA was quantitated by real-time RT-PCR. *Xist* expression in *Xist*$^{\Delta A}$/Y ES cells was comparable to that in the parental wild-type male ES cells before and after differentiation (upper panel). There was a striking difference in the expression level of *Tsix* in *Xist*$^{\Delta A}$/Y and wild-type XY ES in the undifferentiated state (lower panel). The former was about 5-fold higher than the latter, although both were downregulated upon differentiation. (**B**) The stability of *Xist* and *Tsix* RNA was analyzed by treating undifferentiated ES cells with DRB. There was no significant difference in the stability of either RNA between wild-type and *Xist*$^{\Delta A}$/Y ES cells.

embryos, although found in a reasonable number, were all stunted with an abnormal morphology that was indistinguishable from that of female embryos carrying a dysfunctional *Xist* allele derived from the father (Fig. 3B). Histological analysis revealed that the extraembryonic ectoderm was severely affected in the morphologically abnormal embryos, which were most probably females carrying the paternal *Xist*$^{\Delta A}$ allele (Fig. 3C). These embryos were reminiscent of those carrying an extra-copy of the maternal X (Goto and Takagi, 1998; Tada et al., 1993) or the paternally derived *Xist*-deficient X (Marahrens et al., 1997). These results strongly suggest that the deletion of the A-repeat severely compromised the function of the *Xist* gene to initiate X-inactivation.

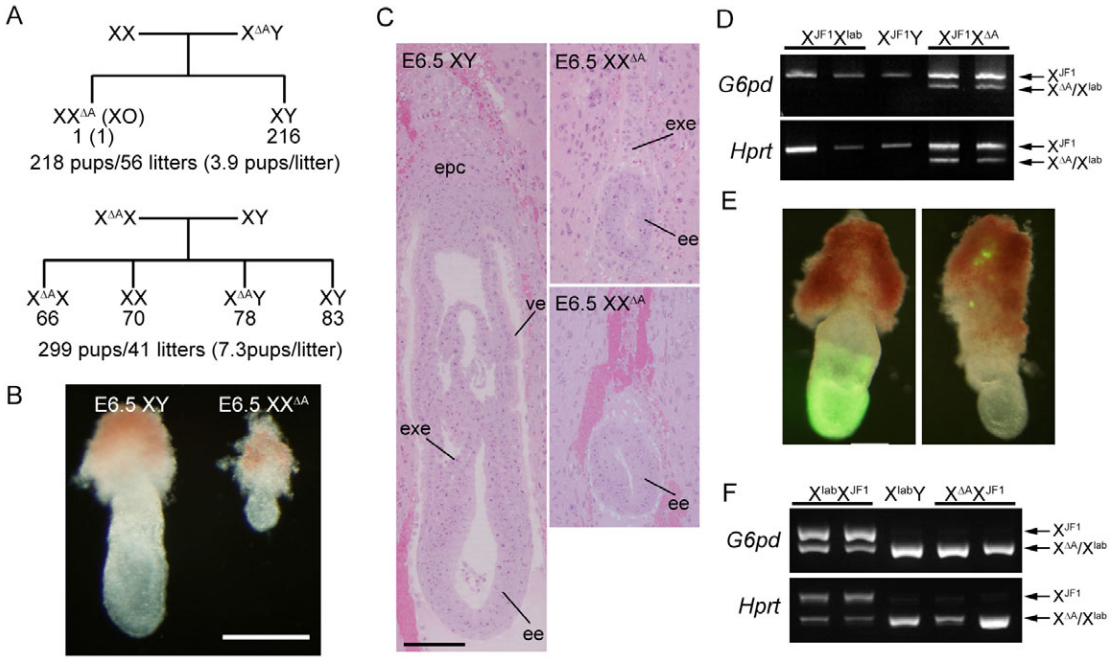

Fig. 3. *Xist*$^{\Delta A}$ fails to inactivate the mutated X chromosome. (**A**) The paternal transmission of *Xist*$^{\Delta A}$ results in an extreme bias in the sex ratio of live pups born to wild-type females crossed with X$^{\Delta A}$Y males in favor of males (upper panel). One of the two females turned out to be XO; therefore, the mutated allele was transmitted to only one female (0.46%). By contrast, the mutated allele was transmitted to both male and female pups at the expected ratio from the mothers (lower panel). (**B**) Gross morphology of an embryo typical of those that inherited the paternal X$^{\Delta A}$ (XX$^{\Delta A}$). Scale bar: 200 μm. (**C**) Histological sections of the presumptive XX$^{\Delta A}$ embryos are shown in comparison with a presumptive male litter mate at E6.5. epc, ectoplacental cone; exe, extraembryonic ectoderm; ee, embryonic ectoderm (**D**) RT-PCR analysis of allelic expression of X-linked *G6pd* and *Hprt* in the trophoblast recovered from E6.5 embryos. X^{JF1} and X$^{\Delta A}$/X^{lab} are maternal and paternal in origin, respectively. Expression of the paternal copy was evident in X^{JF1}X$^{\Delta A}$ in both cases. (**E**) Expression of the GFP transgene on the paternal X in female embryos at E7.5. In contrast to wild-type female embryos (XX^{GFP}), GFP fluorescence was uniformly negative in the embryonic tissues in X$^{\Delta A}$X^{GFP} embryos, suggesting that X$^{\Delta A}$ failed to undergo inactivation even in the embryonic tissues. (**F**) RT-PCR analysis of the expression of X-linked *G6pd* and *Hprt* in the embryonic tissues at E7.5. The maternal copies of both genes on X^{JF1} were not expressed in X$^{\Delta A}$X^{JF1} embryos, suggesting that X$^{\Delta A}$ failed to undergo inactivation.

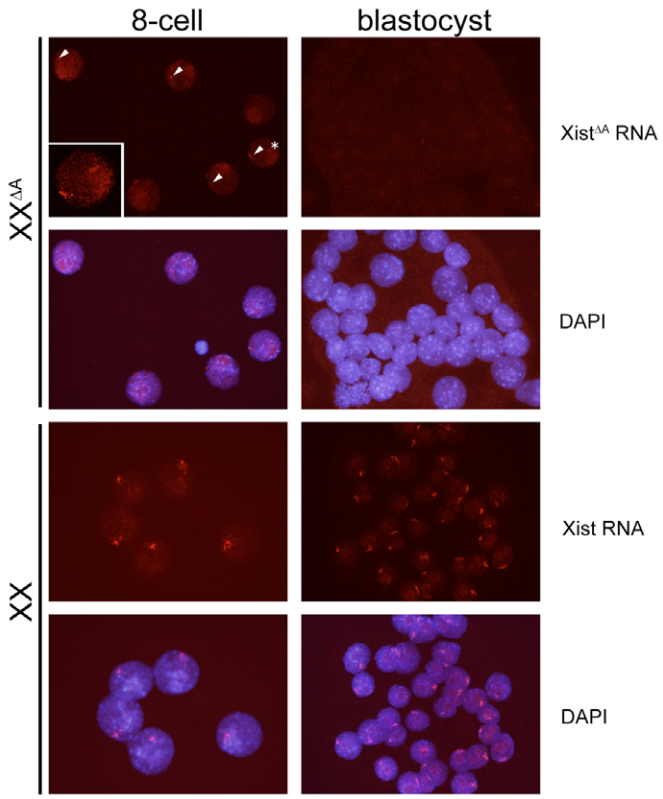

Fig. 4. Expression of *Xist*$^{\Delta A}$ RNA in preimplantation embryos. RNA-FISH was carried out in XX$^{\Delta A}$ and XX embryos at the eight-cell and blastocyst stages. At the eight-cell stage, although the accumulation of *Xist* RNA was evident in XX embryos, expression of *Xist*$^{\Delta A}$ RNA in XX$^{\Delta A}$ was very faint (arrowheads) and subsequently became undetectable by the blastocyst stage. Inset in the panel of the 8-cell XX$^{\Delta A}$ embryo is an enlargement of the nucleus showing a faint *Xist* signal (marked with an asterisk).

Genes on the mutated X are not repressed in either the embryonic or extraembryonic tissues

We went on to study the transcriptional status of the mutated paternal X (X$^{\Delta A}$) in the extraembryonic tissues. X-inactivation is imprinted in the trophoblast, a derivative of the trophectoderm in the blastocyst, in favor of the paternal X. The expression of X-linked genes was analyzed using trophoblasts isolated from E6.5 embryos. Embryos were prepared by crossing females carrying an X chromosome derived from JF1 (*Mus m. molossinus*) with X$^{\Delta A}$Y males, so that the parental origin of the X-linked gene transcripts could be addressed by the presence or absence of restriction site polymorphisms between JF1 and the laboratory strains used in this study (C57Bl/6 and 129). The regions harboring a polymorphism in the X-linked *G6pd* and *Hprt* genes (Sugimoto and Abe, 2007) were amplified by RT-PCR and the products were subsequently digested with *Dra*I and *Hin*fI, respectively. In the trophoblast of wild-type female embryos (X^{JF1}X^{lab}), the expression of *G6pd* and *Hprt* was confined to the maternal alleles, consistent with the imprinted paternal X-inactivation in this tissue (Fig. 3D). By contrast, expression of the paternal copy of these genes was evident in X^{JF1}X$^{\Delta A}$ females (Fig. 3D). This result demonstrated that genes on the paternally derived X$^{\Delta A}$ were, at least in part, misexpressed in the trophoblast. It seemed likely therefore that the paternal X$^{\Delta A}$ failed to undergo inactivation in the extraembryonic lineages, where the paternal X is programmed to be inactivated.

Subsequently, we examined whether the maternal X$^{\Delta A}$ could undergo inactivation in the embryonic lineage, where the X chromosome imprint is no longer effective, by using wild-type males carrying EGFP transgenes on the single X (X^{GFP}) (Nakanishi et al., 2002) as the father. It has been shown that the expression of the transgene reflects the activity of X^{GFP} (Ohhata et al., 2004). In wild-type female embryos recovered at E7.5, GFP fluorescence was observed in the embryonic tissue but not in the extraembryonic tissue, as expected (Fig. 3E). This substantiates that the transgene used here serves as a good reporter for addressing the activity of the X bearing it at this stage of embryo development. Female embryos heterozygous for *Xist*$^{\Delta A}$, which were morphologically indistinguishable from their wild-type littermates, were essentially negative for GFP throughout the embryo, suggesting that X^{GFP} was uniformly inactivated in the embryonic lineage, which is normally subject to random X-inactivation (Fig. 3E). Furthermore, allelic expression analysis of *G6pd* and *Hprt* revealed that both genes were expressed exclusively from X$^{\Delta A}$, and that the transcripts from X^{JF1} were barely detectable in X$^{\Delta A}$X^{JF1} heterozygotes (Fig. 3F). These results indicate that, as is the case with the X carrying the dysfunctional *Xist* allele, X$^{\Delta A}$ is incompetent to undergo inactivation in the embryonic lineage, as well as in the extraembryonic lineages.

Expression of *Xist* is diminished on the mutated X in the preimplantation embryo

The above finding demonstrates that the A-repeat plays an essential role in X-inactivation during mouse development. This is consistent with the previous report by Wutz et al. that *Xist* RNAs lacking the A-repeat fail to initiate X-inactivation in transgenic ES cells (Wutz et al., 2002). In particular, one of the mutated *Xist* RNAs tested by Wutz et al. (ΔSX), which lacks almost the same region as the *Xist*$^{\Delta A}$ RNA expressed from X$^{\Delta A}$, is defective in silencing despite its accumulation on the X chromosome. This observation predicts that *Xist*$^{\Delta A}$ RNA coats X$^{\Delta A}$ but fails to induce chromosomal silencing at the onset of X-inactivation in the embryo. To examine whether this was the case, RNA-FISH was carried out using an *Xist*-specific RNA probe in the preimplantation embryo, in which only the paternal copy of *Xist* is expressed and accumulated in cis (Kay et al., 1993; Sheardown et al., 1997). Embryos were recovered from wild-type females crossed with *Xist*$^{\Delta A}$/Y males at the eight-cell and blastocyst stages. All the female embryos should inherit the *Xist*$^{\Delta A}$ allele in this cross. The sex of each embryo was identified by painting with X- and Y-specific probes afterwards (data not shown). RNA-FISH demonstrated that, although the expression of *Xist*$^{\Delta A}$ was detected in XX$^{\Delta A}$ eight-cell embryos, the hybridization signal was very faint, essentially like a pinpoint (Fig. 4), and eventually disappeared at the blastocyst stage (Fig. 4). In control female embryos, accumulation of *Xist* RNA was detected as an intense signal at the eight-cell and blastocyst stages (Fig. 4). In agreement with these observations, real-time RT-PCR on total RNA of individual blastocysts demonstrated that the level of *Xist* RNA in XX$^{\Delta A}$ was much lower than that in XX. The level in XX$^{\Delta A}$ was, in fact, almost the same as that in XY, which was nearly below the detection limit (Fig. 5A). This excluded the possibility that *Xist*$^{\Delta A}$ RNA, although expressed in the blastocyst, failed to coat the mutated paternal X chromosome, and is in contrast to human *XIST* RNA lacking the A-repeat expressed in tumor cells by an inducible promoter, which does not coat the X chromosome (Chow et al., 2007). These results raised the unexpected possibility that the failure of X$^{\Delta A}$ to undergo inactivation was primarily due to the lack of *Xist*$^{\Delta A}$ RNA coating the mutated X.

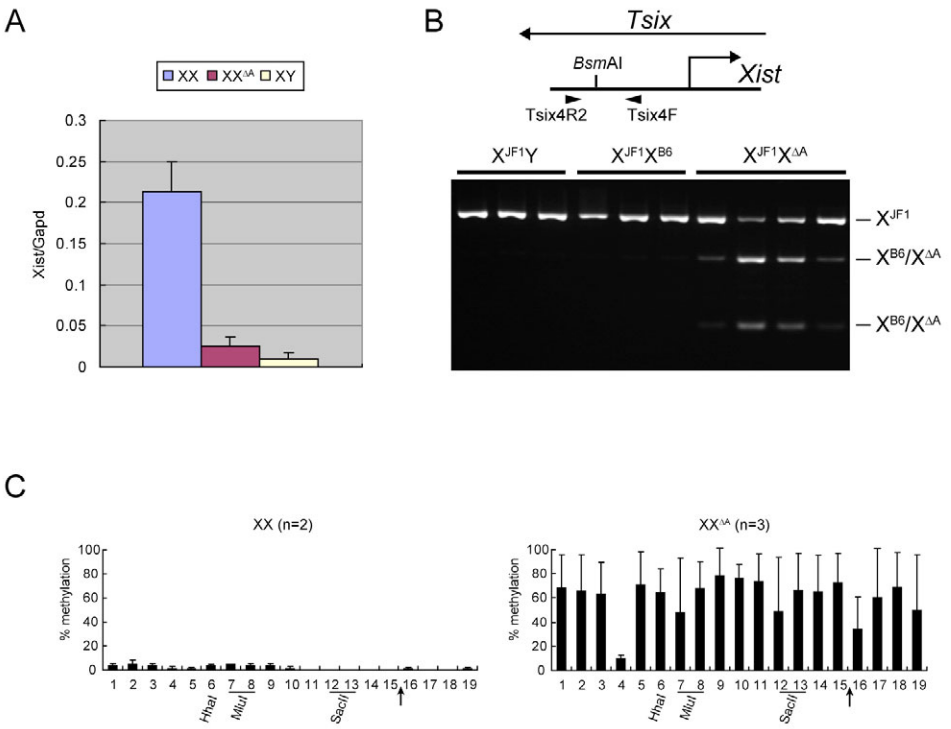

Fig. 5. *Xist* and *Tsix* are aberrantly regulated in XX$^{\Delta A}$ embryos. (**A**) Quantitative RT-PCR analysis of individual F1 blastocysts recovered from $X^{JF1}X^{JF1}$ females crossed with either XY or $X^{\Delta A}Y$ males. The level of $Xist^{\Delta A}$ RNA in $X^{JF1}X^{\Delta A}$ was much lower than that of wild-type *Xist* in XX blastocysts. (**B**) Normally, a silent copy of paternal *Tsix* was ectopically expressed in $X^{JF1}X^{\Delta A}$ blastocysts upon paternal transmission of the $Xist^{\Delta A}$ allele. Following two-round PCR on cDNA prepared from single F1 blastocysts, the amplified products were digested with *Bsm*AI, the recognition site of which is present only in the laboratory strain. (**C**) Methylation profile of the *Xist* promoter region on the paternal X in the trophoblast isolated from XX and $XX^{\Delta A}$ embryos at E6.5 was analyzed by bisulfite sequencing. It was evident that the methylation level was significantly higher in $XX^{\Delta A}$ than that in XX, suggesting that the expression of normally silent *Tsix* on the paternal $X^{\Delta A}$ induces aberrant methylation of the *Xist* promoter region in cis. An arrow indicates the position of the transcription start site of *Xist*.

Tsix is ectopically activated on the paternal X$^{\Delta A}$ in the blastocyst

Available evidence suggests that *Tsix*, the expression of which becomes detectable as early as the eight-cell to morula stage (Y.H. and T.S., unpublished) and is confined to the maternal allele in the preimplantation embryo, prevents the upregulation of *Xist* on the maternal X during the process of imprinted X-inactivation (Lee, 2000; Sado et al., 2001). Given this negative effect of *Tsix* on *Xist* expression, it was of interest to explore whether the expression of *Tsix* was affected on the paternal $X^{\Delta A}$ at the blastocyst stage. Accordingly, RNA fractions of single genotyped blastocysts recovered from $X^{JF1}X^{JF1}$ females crossed with $X^{\Delta A}Y$ males were individually converted into cDNA using gene-specific primers and subjected to two-round PCR. The parental origin of the *Tsix* transcripts was subsequently addressed by restriction digestion with *Bsm*AI, the recognition site of which is present only on the laboratory strain-derived X chromosome (Sugimoto and Abe, 2007). Intriguingly, the paternal copy of *Tsix*, which is normally silent at the blastocyst stage, was ectopically expressed from the mutated paternal $X^{\Delta A}$ in $XX^{\Delta A}$. Such ectopic activation of *Tsix* was not observed in blastocysts that inherited either the wild-type X or the X chromosome carrying another *Xist* mutant allele, $Xist^{1lox}$ (Sado et al., 2005), from the father (Fig. 5B; data not shown). This finding raised an interesting possibility: that the unexpected silencing of *Xist* in $XX^{\Delta A}$ embryos during preimplantation development might be ascribed to the ectopic activation of the normally silent paternal copy of *Tsix* on $X^{\Delta A}$. Given the proposed function of *Tsix* in the establishment of repressive chromatin in the *Xist* promoter region (Navarro et al., 2005; Sado et al., 2005; Sun et al., 2006), it is reasonable to assume that *Tsix* ectopically activated on $X^{\Delta A}$ attracts repressive chromatin modifications, which are not normally associated with the paternal allele, onto the *Xist* promoter region in cis in the tissues where X-inactivation is imprinted. Accordingly, the methylation status of 19 CpG sites in the *Xist* promoter (McDonald et al., 1998) was examined on the paternal $X^{\Delta A}$ in the trophoblast isolated from $X^{JF1}X^{\Delta A}$ embryos at E6.5. The methylation profile of the maternal JF1-type allele could not be addressed here because the trophoblast samples inevitably contained maternal tissues with two X^{JF1} chromosomes. Bisulfite sequencing revealed that whereas the paternal allele in $X^{JF1}X^{B6}$ embryos was essentially unmethylated, as previously described (McDonald et al., 1998), significant methylation was evident on the paternal $X^{\Delta A}$ in $X^{JF1}X^{\Delta A}$ (Fig. 5C). It seems possible therefore that the ectopic expression of *Tsix* facilitates CpG methylation at the *Xist* promoter on the paternal $X^{\Delta A}$ in the trophoblast.

CpG sites in the *Xist* promoter on X$^{\Delta A}$ are not methylated in sperm

The methylation profile of the *Xist* promoter was further analyzed in sperm to see whether the aberrant methylation of the paternal allele in the trophoblast was derived from sperm or acquired after fertilization. It has been shown previously that the methylation level of the *Xist* promoter is relatively low in oocytes, sperm and the preimplantation embryo (McDonald et al., 1998). Bisulfite sequencing revealed that the *Xist* promoter in sperm exhibited even lower methylation in mutant $X^{\Delta A}Y$ males than in wild-type males

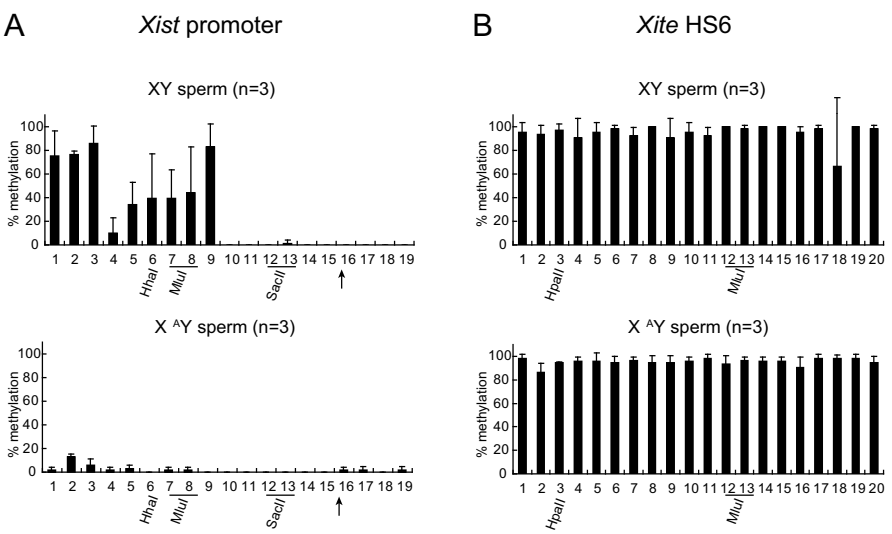

Fig. 6. Methylation profile of the *Xist* promoter region and *Xite* HS6 in sperm recovered from XY and $X^{\Delta A}Y$ males. (**A**) Although the *Xist* promoter region was partially methylated in sperm recovered from XY as previously reported (McDonald et al., 1998), it exhibited even lower methylation in the sperm of $X^{\Delta A}Y$ males. (**B**) The *Xite* HS6 region, the methylation of which in sperm was suggested to be an imprint responsible for the repression of paternal *Tsix* in the tissues or cells showing imprinted X-inactivation, was heavily methylated in sperm of both XY and $X^{\Delta A}Y$ males.

(Fig. 6A). The significance of this difference in the methylation levels is not known at present, but this result indicates that the aberrant methylation found in the *Xist* promoter on the paternal $X^{\Delta A}$ in the trophoblast arises during embryogenesis.

It has been proposed that the differentially methylated domains in *Tsix* and *Xite* found between oocytes and sperm might constitute primary marks for the imprinted expression of *Tsix* in the zygote (Boumil et al., 2006). One of these domains, HS6, in *Xite* has been relatively well characterized by bisulfite sequencing and has been shown to be heavily methylated in sperm. It was postulated that if the methylation of this region in sperm was causally involved in the repression of paternal *Tsix* in the preimplantation embryo, it might be abolished in sperm isolated from $X^{\Delta A}Y$ males. We therefore assessed the methylation status of this region in sperm of the mutant males. As shown in Fig. 6B, this region was heavily methylated on $X^{\Delta A}$ as on the wild-type X, suggesting that the methylation status of HS6 in the *Xite* region was not directly involved in the ectopic activation of *Tsix* on the paternal $X^{\Delta A}$ chromosome.

DISCUSSION

By taking advantage of an inducible expression system of a single copy *Xist* cDNA with various deletions from the single X chromosome in male ES cells, Wutz et al. showed that the silencing function of *Xist* RNA is dramatically compromised if the A-repeat is deleted, even though the mutant forms of the RNA accumulate in cis on the X chromosome (Wutz et al., 2002). This finding was further corroborated by the functional assay of *Xist* RNA lacking the A-repeat expressed from the endogenous locus by the inducible promoter in male ES cells (Wutz et al., 2002). Although that study highlighted for the first time the possible functional domain of *Xist* RNA that is crucial for inducing chromosome-wide silencing, the importance of the A-repeat for X-inactivation occurring in the mouse embryo had not been addressed until this study. The $Xist^{\Delta A}$ allele was created so that it would produce a transcript nearly the same as the one lacking the A-repeat expressed from the endogenous *Xist* locus upon induction in the study by Wutz et al. (Wutz et al., 2002). Genetic and molecular analyses of female embryos heterozygous for $Xist^{\Delta A}$ clearly demonstrated that the deletion of the A-repeat rendered the mutated X incompetent to undergo inactivation, indicating the crucial role of the A-repeat in X-inactivation during mouse development. The presence or absence of the A-repeat, however, does not seem to be directly involved in the primary choice of X-inactivation in the embryonic lineage, as different deletions in the *Xist* gene, although they retain the A-repeat, have resulted in primary non-random X-inactivation similar to that observed in this study (Marahrens et al., 1998; Sado et al., 2005).

We initially postulated that the failure of X-inactivation could be ascribed to the defect in $Xist^{\Delta A}$ RNA, which should be capable of coating the mutated X based on the inducible expression assay in ES cells (Wutz et al., 2002). Intriguingly, it was found that the level of $Xist^{\Delta A}$ RNA was greatly reduced in $XX^{\Delta A}$ preimplantation embryos compared with that of wild-type *Xist* RNA in XX embryos. This could be due to a reduction either in the stability of the mutated RNA or in the expression level per se. Quantitative RT-PCR demonstrated, however, that the stability of *Xist* RNA detected in undifferentiated male ES cells was comparable regardless of the presence or absence of the A-repeat, suggesting that the latter possibility was more favorable. It is likely therefore that the reduction in the level of *Xist* RNA in preimplantation embryos is primarily due to the

Table 1. Primer sequences used for PCR

Primer	Sequence (5′→3′)
dA1F	GCAGGTCGAGGGACCTAATA
F1063AS	GCACAACCCCGCAAATGCTA
G6pdF4	CGCCATTTTGTCCTATGCTG
G6pdR4	AGGTTGACCATGGAGTATGG
GapdF	ATGGCCTTCCGTGTTCCTAC
GapdR	TGTGAGGGAGATGCTCAGTG
GapdR2	ATAGGGCCTCTCTTGCTCAG
HprtF4	GTTGAATCTGCAAATACGAGG
HprtR3	CAACGATTTACTGAAAGTGGG
P422R	TCAAGATGCGTGGATATCTCGG
R700P2	CGGGGCTTGGTGGATGGAAAT
Tsix2F	CAATCTCGCAAGATCCGGTGA
Tsix2R	GATGCCAACGACACGTCTGA
Tsix4F	TGGGTCATTGGCATCTTAGTC
Tsix4R	TCAGCGTGAATCAACGAGAC
Tsix4R2	CCCAGGGTGTCTGATCTCTT
Xist1395R	AAGCTGACATGTGACACACAAA
Xist2281F	GATGCCAACGACACGTCTGA
Xist2424R	AAGGACTCCAAAGTAACAATTCA
Xist2688R	AGAGCATTACAATTCAAGGCTC
XistEx7F31	GGCATAGTCTCTACAAAATTTTCATT
XistEx7R20	CTCCAATTTCTGGGCTCAAG
Zfy1	GATAAGCTTACATAATCACATGGA
Zfy2	CCTATGAAATCCTTTGCTGC
Zfy4	TGCTTTTTGAGTGCTGATGG

transcriptional silencing of *Xist*. Our result demonstrates that the region encoding the A-repeat plays a crucial role as a regulatory element in the appropriate regulation of *Xist* in vivo.

Intriguingly, the lack of *Xist* RNA in the blastocyst was accompanied by an ectopic activation of the normally silent paternal copy of *Tsix* on the same X chromosome. Furthermore, the *Xist* promoter on the mutated paternal X in the trophoblast at E6.5 was aberrantly methylated at CpG sites that are normally unmethylated on the paternal X. These findings raised an interesting possibility that the transcriptional silencing of *Xist* in preimplantation embryos is triggered by ectopically expressed *Tsix*, which subsequently promotes CpG methylation in the *Xist* promoter region on the mutated paternal X. In this scenario, the deleted region in the $Xist^{\Delta A}$ allele, most probably the A-repeat, is crucial for the appropriate repression of *Tsix* on the paternal X at the onset of imprinted X-inactivation. Given the fact that the transcription of *Tsix* is initiated 40 kb downstream from the A-repeat, it is tempting to speculate that the A-repeat exerts its effect on *Tsix* through a long-range chromatin conformation. However, the opposite scenario is also possible: the upregulation of *Tsix* was somehow caused by the primary silencing of *Xist* that resulted from the loss of some crucial regulatory element located within the deleted region. Because the aberrant methylation of the *Xist* promoter appears to be established during embryogenesis, it might be expected that the mutated $Xist^{\Delta A}$ RNA would be transcribed from the paternal $X^{\Delta A}$ in the early preimplantation embryo. This was not the case, however, and the expression of paternal $Xist^{\Delta A}$ was diminished from the very early stages. This observation may favor the later scenario that the silencing of *Xist* is the primary event. These two possibilities cannot be distinguished between on the basis of current data and the further experimentation is certainly required. The simplest way to address this issue is to terminate *Tsix* on $X^{\Delta A}$ and see whether $Xist^{\Delta A}$ is expressed or not. We are currently trying to produce mice carrying a *Tsix* deficiency on $X^{\Delta A}$ through second gene targeting in ΔA2lox211 ES cells.

Although the targeted deletion of the A-repeat did not allow us to address the functional significance of the A-repeat as an element in the *Xist* RNA because of the unexpected lack of expression from the mutated X, this study clearly demonstrates that the region encoding the A-repeat is essential as a genomic element for X-inactivation in the mouse embryo. Further attempts to identify the factors that interact with the DNA sequence harboring the A-repeat should provide further insight into the molecular mechanisms of *Xist*/*Tsix* regulation and the random choice of X-inactivation.

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