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Tsix defective in splicing is competent to establish *Xist* silencing

Takashi Sado[1,2,3,*], Yuko Hoki[1,3] and Hiroyuki Sasaki[1,2]

Dosage differences of X-linked genes between male and female mammals are compensated for by a mechanism known as X-inactivation, and the noncoding *Xist* gene plays a crucial role in this process. The expression of *Xist* is regulated in cis by its noncoding antisense gene, *Tsix*, whose transcripts (though a fraction of them stay unspliced), are processed like common protein-coding RNAs. It has been suggested that certain classes of sense–antisense pairs of RNA are causally involved in not only gene regulation but also higher order chromatin structure in various organisms. In fact, recent studies demonstrated that *Tsix* modulates *Xist* expression through modification of the chromatin structure. It is still unknown, however, whether the RNA product is important for the function of *Tsix* or whether the antisense transcription is sufficient. To obtain insight into this issue, we eliminated the splicing products of *Tsix* in the mouse and explored the effects of this elimination on *Tsix*-mediated *Xist* silencing. To our surprise, the *Xist* locus was stably repressed on the X carrying the splicing-defective *Tsix* allele. Moreover, the repressive chromatin configuration was properly established at the *Xist* locus. These unexpected results indicate that the splicing products are dispensable for *Tsix*-mediated *Xist* silencing.

KEY WORDS: X-inactivation, Antisense regulation, *Xist*, *Tsix*

INTRODUCTION

Recent progress in transcriptome analysis has revealed that a large proportion of the genome can be transcribed from both strands, producing sense and antisense transcripts (Carninci et al., 2005; Kiyosawa et al., 2003; Yelin et al., 2003). The available evidence suggests that certain classes of RNA are causally involved in not only gene regulation but also higher order chromatin structure in various organisms (Bernstein and Allis, 2005). In addition, antisense transcription has often been implicated in either transcriptional or post-transcriptional regulation of the sense partner or the neighboring genes, although the exact molecular mechanisms have yet to be determined (Katayama et al., 2005).

It is known that X-inactivation in mammals, which ensures dosage equivalence of X-linked genes between males and females (Lyon, 1961), is regulated by the noncoding *Xist* (X-inactive specific transcript) gene (Brockdorff et al., 1992; Brown et al., 1992) and its antisense non-coding *Tsix* gene (Lee et al., 1999) mapped in the X chromosome inactivation center (Xic), a cytogenetically identified master regulatory region of X-inactivation (Avner and Heard, 2001). *Xist* is essential for the initiation of X-inactivation to occur in cis (Marahrens et al., 1997; Penny et al., 1996), and its expression is regulated by *Tsix* on the same chromosome in a negative fashion (Lee, 2000; Lee and Lu, 1999; Sado et al., 2001). The *Xist/Tsix* locus is probably one of the best-studied loci harboring a sense–antisense pair of transcripts, and study of this locus should allow us to obtain further insight into the molecular mechanisms underlying antisense-mediated gene regulation, which appears to be rather common in a variety of systems.

[1]Division of Human Genetics, National Institute of Genetics, Research Organization of Information and Systems, 1111 Yata, Mishima, 411-8540, Japan. [2]Department of Genetics, The Graduate University for Advanced Studies (Sokendai), 1111 Yata, Mishima, 411-8540, Japan. [3]PRESTO, Japan Science and Technology Agency (JST), 4-1-8, Honcho, Kawaguchi, Saitama, 332-0012, Japan.

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

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The paternal X chromosome is selectively inactivated by imprinting in the extra-embryonic tissues, which give rise to the placenta and part of the extra-embryonic membranes (Takagi and Sasaki, 1975), where the paternal copy of *Xist* is always activated and the maternal one is never expressed (Kay et al., 1993). Targeted disruption of *Tsix*, when maternally inherited, induces ectopic activation of the normally silent copy of *Xist* on the same X chromosome in these tissues (Lee, 2000; Sado et al., 2001). Subsequent inactivation of the mutated maternal X results in functional nullisomy of the X chromosome in both males and females, and eventual embryonic death at early postimplantation stages. Although the targeted disruption of *Tsix* revealed that *Tsix* is a negative regulator of *Xist* effective in cis, until recently little was known about the molecular mechanisms underlying the antisense regulation at the *Xist* locus. We recently showed that in the absence of *Tsix*, the *Xist* locus fails to establish the repressive chromatin configuration, and instead manifests the active modification in embryos, suggesting that *Tsix* mediates *Xist*-silencing through modification of the chromatin structure (Sado et al., 2005). Others also demonstrated *Tsix*-mediated chromatin effects using undifferentiated and differentiating embryonic stem (ES) cells as an in vitro model system (Navarro et al., 2005; Sun et al., 2006).

Tsix transcripts are subject to splicing and polyadenylation, like common protein-coding RNAs (Sado et al., 2001; Shibata and Lee, 2003). The major products of spliced *Tsix* RNA in undifferentiated ES cells, which are about 2.7 kb and 4.3 kb in length on northern blots, appear to be produced by differential usage of several polyA signals found in exon 4 of *Tsix* (Sado et al., 2001). RT-PCR-based methods also revealed the presence of some variants produced by alternative splicing (Sado et al., 2001; Shibata and Lee, 2003). In addition, *Tsix* RNA exists in spliced and unspliced forms, although the functional relevance of splicing remains unknown. Because a significant fraction of the RNA is processed, and the splicing variants identified so far all share exon 4, which is the only exon possessing complementarity with the *Xist* sequence, it had been postulated that the splicing products (especially the sequence in exon

4) play an important role in the function of *Tsix* by masking the A-repeats in *Xist*, a region known to be required for *Xist*-mediated silencing (Wutz et al., 2002), through base pairing (Shibata and Lee, 2003).

To obtain further insight into this issue, we studied the functional significance of spliced *Tsix* RNA produced by antiparallel transcription through the endogenous *Xist* locus. Here we eliminated the splicing products of *Tsix* in embryos by disturbing the splicing between proximal exons and exon 4. Loss of spliced *Tsix* RNA, however, compromised neither the *Tsix*-mediated *Xist* silencing nor the establishment of the proper chromatin configuration in the *Xist* promoter region. Our findings suggest that chromatin modulation at the *Xist* locus is not mediated by spliced *Tsix* RNA but rather nascent/unspliced *Tsix* RNA or the antisense transcription per se.

MATERIALS AND METHODS

Gene targeting

A plasmid containing a 4.4-kb *Eco*RI fragment and a 6.5-kb *Xba*I fragment from the *Xist* genomic locus (Sado et al., 2005) was digested with *Pma*CI and *Sma*I to release a fragment harboring the splicing acceptor site of *Tsix* exon 4, which was then replaced with a fragment containing an IRES-EGFP and a puromycin resistance gene similarly to how a targeting vector for the $Xist^{2lox}$ allele was produced (Sado et al., 2005). The targeting vector thus produced was electroporated into R1 ES cells (Nagy et al., 1993), and chimeras were produced as described previously (Sado et al., 2005). Briefly, out of 96 colonies, three harbored the expected homologous recombination ($Tsix^{\Delta SA2lox}$), and they were serially injected into blastocysts to generate chimeras. In this particular case, two female chimeras crossed with C57Bl/6 males happened to transmit the $Tsix^{\Delta SA2lox}$ allele to both male and female pups. Those females carrying $Tsix^{\Delta SA2lox}$ were crossed with males that ubiquitously expressed cre recombinase to derive animals carrying $Tsix^{\Delta SA}$.

RT-PCR and northern blotting

RT-PCR was carried out on cDNA prepared from 1 μg of total RNA using either random primer or strand-specific primers. For strand-specific RT-PCR, cDNA was synthesized using R1910J as a primer for *Tsix* and Xist7(–)20 as a primer for *Xist*. Subsequent amplification of a common sequence in *Tsix*/*Xist* was performed using primers R700P2 and Xist6(–)20. A *Gapd*-specific primer, GapdR, was also included in the cDNA reaction to amplify a *Gapd* sequence using GapdF and GapdR2 for normalization in real-time PCR analysis. Real-time PCR was performed on cDNA thus produced using SYBR Premix Ex Taq (Takara) and a LightCycler (Roche) according to the manufacturers' instructions. The expression levels of each sequence were the value normalized by *Gapd* based on a mean value of the respective sequence obtained from two or three independent experiments. Primer sequences used in this study were as described previously (Sado et al., 2005; Sado et al., 2001) except for the following: 8111F, ctg cca cct gct ggt tta tt; 8420R, cca cat gaa aga gat cag ac; EGFP1, ctt ctt caa gga cga cgg ca; EGFP2, ttg tac agc tcg tcc atg cc; Tsix2F, caa tct cgc aag atc cgg tga; Tsix2R, agt gga tgc agg act caa gat.

RNA extracted from ES cells harboring the respective mutation was subjected to northern hybridization as described previously (Sado et al., 2001). An RNA probe was prepared from p10 and p10R for detection of *Tsix* and *Xist*, respectively (Sado et al., 2001). For Gapd, a cDNA fragment was used as a probe.

Methylation analysis and DNaseI hypersensitive site assay

For methylation analysis of the *Xist* promoter region, Southern hybridization was performed in exactly the same manner as described previously (Sado et al., 2005).

The DNaseI hypersensitive site assay was carried out as previously described (Sado et al., 2005), with the exception of a 0.4 kb *Afl*III genomic fragment (Af0.4) being used as a probe.

RESULTS

Generation of a new *Tsix* mutant allele defective in splicing

In a previous study, we created a new *Xist* mutant allele, $Xist^{1lox}$, by replacing a part of exon 1 with a promoter-less IRES-EGFP cassette (Sado et al., 2005). This allele was created so as not to delete the splicing acceptor site for exon 4 of the overlapping antisense *Tsix* transcript (Fig. 1C). Although the *Xist* gene was disrupted by the replacement, primary *Tsix* transcripts were still appropriately processed to yield the major 2.7-kb and 4.3-kb RNAs with a polyA tail (Fig. 2D). We demonstrated that the function of *Tsix* was not impaired by this alteration, and the repressive chromatin configuration was properly established at the *Xist* promoter on the active mutated X chromosome (Sado et al., 2005). To elucidate the functional significance of spliced *Tsix* RNA in the proposed *Tsix*-mediated chromatin regulation at the *Xist* promoter, we attempted to disturb the splicing event by deleting the splicing acceptor site for exon 4 of *Tsix*. Accordingly, another targeting vector, which was very similar to the one used to derive the $Xist^{1lox}$ allele, was created (Fig. 1A). The difference between these vectors was that the 5′ arm either did or did not contain a 0.6-kb *Pma*CI-*Eco*RI fragment harboring the splicing acceptor site. Upon a correct targeting event, the splicing acceptor site was successfully deleted on the single X chromosome in male ES cells by replacement with the IRES-EGFP cassette ($Tsix^{\Delta SA2lox}$) (Fig. 1B,C). A puromycin resistance gene was subsequently removed by transient expression of cre recombinase in ES cells to generate the $Tsix^{\Delta SA}$ allele (data not shown).

Splicing variants are not produced from the $Tsix^{\Delta SA}$ allele in ES cells

We examined whether or not spliced *Tsix* RNA was eliminated in $Tsix^{\Delta SA}$/Y ES cells by performing RT-PCR using cDNA prepared by random priming. Unlike in XY and $Xist^{1lox}$/Y ES cells, a primer set spanning the introns (Xist1175F and 21b80F) failed to amplify the product derived from spliced *Tsix* RNA in $Tsix^{\Delta SA}$/Y as well as in $X^{dc}Y$ ES cells, which carry the $Xist^{1lox}$ allele on a *Tsix*-deficient X chromosome (Sado et al., 2005) (Fig. 2A,B). The presence of antisense transcripts was, however, evident in XY, $Xist^{1lox}$/Y, and $Tsix^{\Delta SA}$/Y ES cells when another primer set (8111F and 8420R) located in exon 4 was used, suggesting that the antisense transcription itself was not disrupted. The relative abundance of the antisense transcripts in each ES cell line was further analyzed by real-time PCR using two primer sets designed to hybridize within either exon 2 (Tsix2F and Tsix2R) or exon 4 (8111F and 8420R) (Fig. 2C). In $X^{dc}Y$, antisense expression was detected in exon 2 at a level comparable to that in XY but was barely detected in exon 4, which is consistent with the fact that *Tsix* is truncated on X^{dc} (Sado et al., 2005). On the other hand, the level of antisense transcripts in $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y turned out to be about twice as high as that in wild-type XY ES cells in both regions. It is unclear whether the transcription is upregulated or the stability of the antisense transcripts is increased by the alteration introduced on the mutated X in $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y. The absence of the spliced forms in $Tsix^{\Delta SA}$/Y ES cells was further confirmed by northern blotting using polyA RNA (Fig. 2D). Although an RNA probe specific to *Tsix* detected 2.7-kb and 4.3-kb bands in XY and $Xist^{1lox}$/Y ES cells, neither of these bands was detected in $Tsix^{\Delta SA}$/Y or $X^{dc}Y$ ES cells. The hybridization signal observed in the high molecular weight region in XY, $Xist^{1lox}$/Y, and $Tsix^{\Delta SA}$/Y, which was missing in $X^{dc}Y$, probably represented unspliced *Tsix* RNA and/or perhaps an incompletely processed

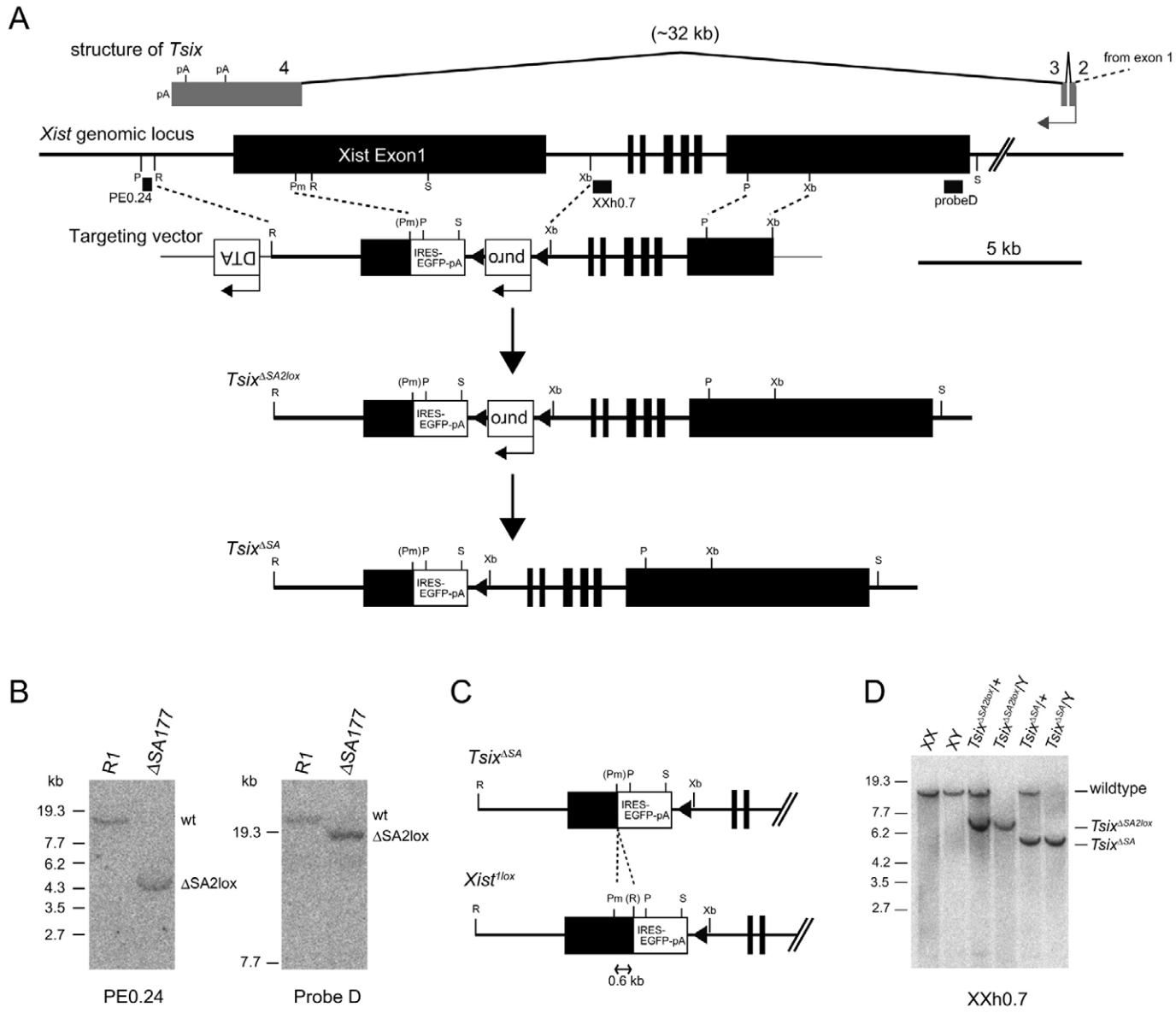

Fig. 1. Generation of the splicing-defective *Tsix* allele by gene targeting. (**A**) Scheme for generating the $Tsix^{\Delta SA}$ allele. Genomic structure of the *Xist* locus and the targeting vector are shown. *Tsix* exons (gray boxes) are also aligned in parallel with *Xist* exons (black boxes). Differential polyadenylation of the spliced *Tsix* transcripts occurs by recognizing one of the polyA signals (pA) shown in the distal region of exon 4. Positions of the probes used for Southern blotting in B and D are also indicated. P, *Pst*I; Pm, *Pma*CI; R, *Eco*RI; S, *Sac*I; Xb, *Xba*I. The *Pma*CI site destroyed in the cloning process is indicated as (Pm). (**B**) Homologous recombination was confirmed by Southern blotting. ΔSA177 is one of the three ES lines harboring the correct targeting event. Genomic DNA digested with *Pst*I (left) and *Sac*I (right) was probed with PE0.24 and probe D (Sado et al., 2001), respectively. (**C**) Comparison of the $Tsix^{\Delta SA}$ allele and the $Xist^{1lox}$ allele. The splicing acceptor site for exon 4 of *Tsix*, which is present in a 0.6-kb *Pma*CI-*Eco*RI fragment, is deleted in the $Tsix^{\Delta SA}$ allele, but left intact in the $Xist^{1lox}$ allele. (**D**) The presence of the respective mutation in the mouse was confirmed by Southern blotting. Tail DNA digested with *Pst*I was probed with XXh0.7.

one that contains the third intron. These results confirmed that the $Tsix^{\Delta SA}$ allele was defective in splicing between proximal exons and exon 4 as expected, with the antisense expression activity being essentially unaffected.

We observed unexpected activation of the *Xist* locus on the mutated X chromosome in undifferentiated ES cells. As shown in Fig. 2D, an RNA probe specific to *Xist* detected the chimeric transcripts of 4.5 kb in $X^{dc}Y$ and $Xist^{1lox}$/Y and those of 4.0 kb in $Tsix^{\Delta SA}$/Y. Despite the activation of the *Xist* locus, neither of the DNaseI hypersensitive sites specifically found in the 5′ region of the transcriptionally active *Xist* allele in somatic cells (see Fig. 6) was observed on the mutated X in undifferentiated ES cells (Fig. 2E). While the ectopic activation of the *Xist* locus in $X^{dc}Y$ was sustained even after differentiation, expression of the chimeric transcripts in $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y became downregulated upon differentiation as embryoid bodies for 12 days (Fig. 2F), suggesting that developmental regulation of the *Xist* gene was compromised on the X^{dc} chromosome, as previously described (Sado et al., 2005) but not on the mutated X in $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y. It is likely that the repressive action of *Tsix* occurs after differentiation.

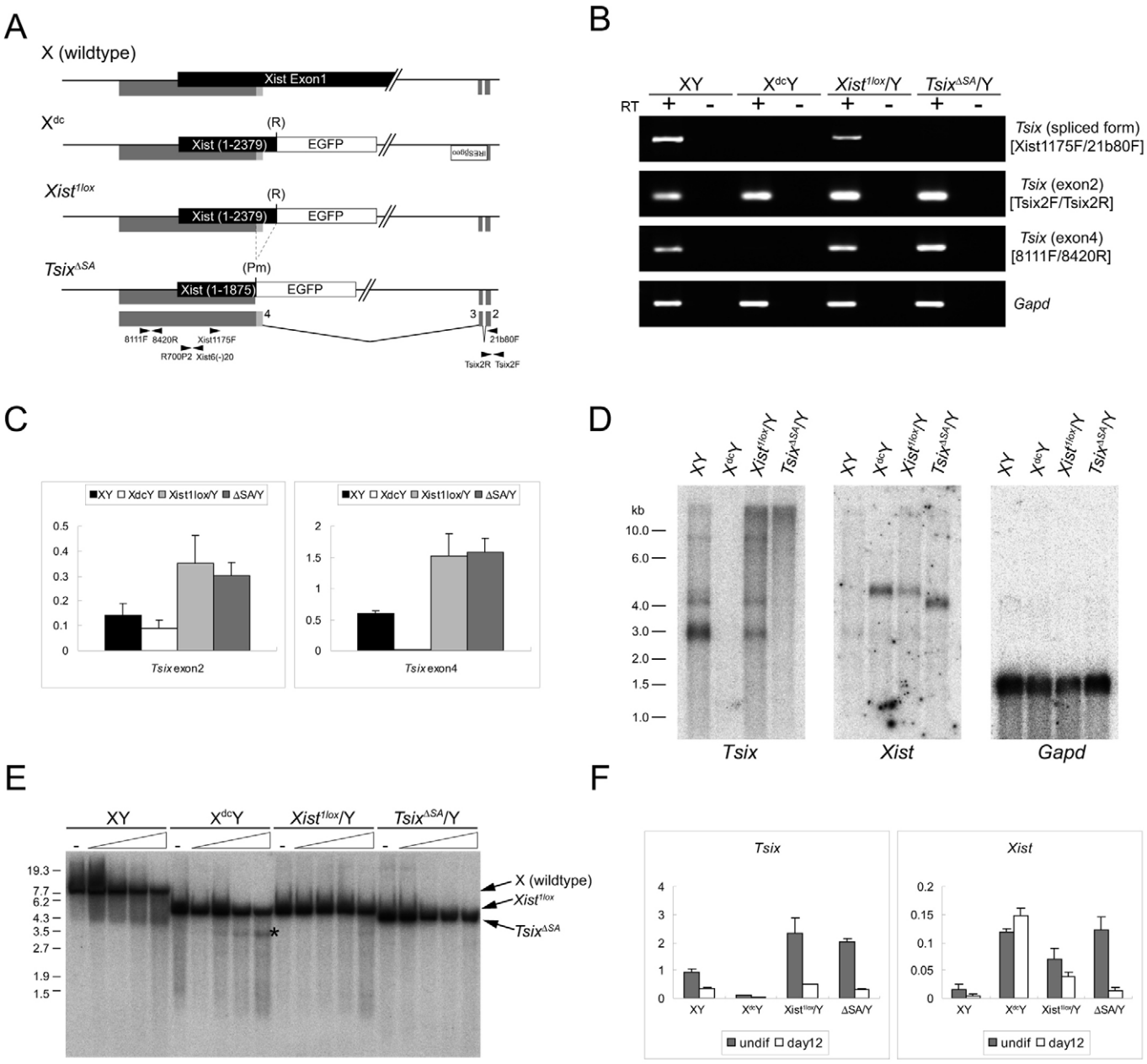

Fig. 2. Splicing products of *Tsix* are eliminated in ES cells. (**A**) The structure of the respective mutant allele is delineated with respect to the position of *Tsix* exons shown in gray. A proximal part of *Tsix* exon 4 shown in light gray indicates the region missing in the $Tsix^{\Delta SA}$ allele. *Tsix* on X^{dc} chromosome is truncated by the insertion of IRESβgeo (Sado et al., 2005). Positions of primers used for PCR and real-time PCR are also shown below the exons of *Tsix*. (R) and (Pm) are the *Eco*RI and *Pma*CI sites destroyed in the cloning process. (**B**) The absence of the splicing products of *Tsix* was confirmed by RT-PCR using cDNA prepared by random priming. The spliced form was detected using primer pair Xist1175F and 21b80F. The presence of the antisense transcription was monitored using primer pairs Tsix2F/Tsix2R and 8111F/8420R located in exon 2 and 4, respectively. (**C**) Antisense activity of *Tsix* was analyzed in each undifferentiated ES cell line by real-time PCR using primer sets Tsix2F/Tsix2R (exon 2) and 8111F/8420R (exon 4). (**D**) Northern blot analysis of polyA RNA isolated from ES cells carrying the respective mutation. Hybridization was serially performed using an RNA probe specific to *Tsix* (left) and *Xist* (middle), respectively, and a cDNA fragment of *Gapd* (right). The absence of the major splicing products is evident in $Tsix^{\Delta SA}$/Y ES cells. (**E**) DNaseI hypersensitive site assay using nuclei isolated from each ES cell line. Purified DNA was digested with *Hin*dIII and probed with Af0.4 (see Fig. 6). Neither of the known DNaseI hypersensitive sites (HS1 and HS5) found on the transcriptionally active *Xist* allele in somatic cells was observed in ES cells regardless of whether they harbor the mutation or not. A DNaseI hypersensitive site HS3 shown by an asterisk, which is known to be common to both the active and inactive X in somatic cells, was more prominent in X^{dc}Y than others. (**F**) Real-time PCR analysis of the transcripts from the *Tsix/Xist* locus in undifferentiated ES cells and 12-day embryoid bodies using primers R700P2 and Xist6(–)20 on strand-specifically prepared cDNA.

Splicing variants of *Tsix* are eliminated in the mouse

Females heterozygous for $Tsix^{\Delta SA2lox}$ were produced through germline transmission from chimeric mice and subsequently crossed with males expressing cre recombinase to derive animals carrying $Tsix^{\Delta SA}$ by excision of the puromycin resistance gene (see Materials and methods) (Fig. 1D). Since the *Xist* gene on the mutated X was consequently destroyed, males failed to transmit the $Tsix^{\Delta SA}$ allele to female offspring when crossed with wild-type females, as previously described (Marahrens et al., 1997; Sado et al., 2005). The mutant allele was, however, transmitted to both male and female pups from the mother at the expected ratio (data not shown).

Taking advantage of embryos harboring maternally derived $Tsix^{\Delta SA}$, we studied the role of spliced *Tsix* RNA in *Tsix*-mediated *Xist* silencing. We first asked whether the splicing products had been eliminated in mice as in $Tsix^{\Delta SA}$/Y ES cells. It is known that *Tsix* is imprinted to be expressed from the maternal allele in the extraembryonic tissues such as the placenta (Lee, 2000; Sado et al., 2001). RNA isolated from the placenta at embryonic day (E) 13.5 was subjected to RT-PCR. While the presence of the spliced products was evident in both wild-type males and females and those carrying maternal $Xist^{1lox}$ with primers spanning exon 2 and exon 4 (Xist1175F and 21b80F), such products were not observed in $Tsix^{\Delta SA}$/+ females or $Tsix^{\Delta SA}$/Y males (Fig. 3). In contrast, the expected fragment was amplified in all cases with a primer pair (8111F and 8420R) located in exon 4. These results suggest that $Tsix^{\Delta SA}$ impaired the splicing event but not the antisense transcription per se during embryonic development.

Xist is properly repressed in the absence of spliced *Tsix* RNA

In previous studies, loss of function mutation of *Tsix* caused activation of *Xist* in cis (Lee, 2000; Sado et al., 2001), which was attributed to the failure to establish the repressive chromatin configuration at the *Xist* promoter (Sado et al., 2005; Sun et al., 2006). We first examined if the elimination of spliced *Tsix* RNA abrogated the proper regulation of *Xist* on the mutated X chromosome carrying the $Tsix^{\Delta SA}$ allele. In female embryos heterozygous for the respective mutation, as shown in Fig. 4, the mutated X stays active in every cell because of *Xist*-deficiency on it. In our assay system, therefore, the expression of chimeric RNA from the mutated active X would demonstrate defects in the mechanism of silencing of *Xist*. As previously shown, $X^{dc}X$ female embryos expressed chimeric RNA due to functional deficiency of *Tsix* (Fig. 4). Such ectopic activation of the *Xist* promoter on the mutated X was not, however, observed in either $Xist^{1lox}$/+ or $Tsix^{\Delta SA}$/+ embryos (Fig. 4), suggesting that the *Xist* promoter was appropriately repressed on the mutated X chromosome carrying $Tsix^{\Delta SA}$. Similarly, the expression of chimeric RNA was detected in $X^{dc}Y$ but not in $Tsix^{\Delta SA}$/Y. These results demonstrated that the *Xist* locus was properly silenced even in the absence of spliced *Tsix* on the mutated X.

Appropriate chromatin configuration was established at the *Xist* promoter on the X carrying $Tsix^{\Delta SA}$

It is known that the *Xist* promoter is differentially methylated in female somatic cells, with the active allele on the inactive X being unmethylated and the inactive allele on the active X being methylated (Norris et al., 1994). Our previous study demonstrated that functional loss of *Tsix* impairs establishment of complete methylation at CpG sites in the *Xist* promoter in cis. To examine whether CpG methylation was affected by loss of the *Tsix* splicing products, we carried out Southern blot analysis. Genomic DNA isolated from both male and female embryos carrying $Tsix^{\Delta SA}$ at E13.5 was digested with methylation-sensitive *Hha*I or *Sac*II in combination with methylation-insensitive *Bcl*I. As shown in Fig. 5, the *Xist* promoter was completely methylated on the mutated active X carrying $Tsix^{\Delta SA}$ at a level comparable to that on the active X carrying $Xist^{1lox}$ as well as the wild-type allele in the embryonic and extra-embryonic tissues in both sexes. This result suggests that CpG methylation is established even in the absence of spliced *Tsix* RNA.

We went on to study the chromatin structure at the *Xist* locus by DNaseI hypersensitive site assays. Several DNaseI hypersensitive sites have been found in the *Xist* promoter region (Sado et al., 2005; Sheardown et al., 1997). It is known that HS1 and HS5 are specific to the inactive X, and HS3 is common to both the active and inactive X. In a previous study, we showed that functional deficiency of *Tsix* results in the failure to establish the closed chromatin structure in the *Xist* promoter region. As shown in Fig. 6, neither of the ectopic DNaseI hypersensitive sites seen on the X^{dc} was detected on the X carrying $Tsix^{\Delta SA}$ in either male or female embryos at E13.5. This result indicates that the closed chromatin structure is established at the *Xist* promoter even in the absence of spliced *Tsix* RNA.

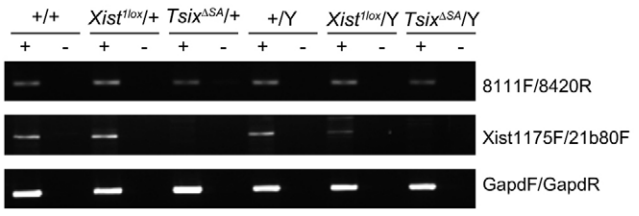

Fig. 3. Spliced *Tsix* RNA is eliminated in the placenta. The absence of the splicing products of *Tsix* was confirmed in the E13.5 placenta carrying $Tsix^{\Delta SA}$ in the same manner as in ES cells shown in Fig. 2A and B. It is evident that the splicing products are not detected in the placenta of $Tsix^{\Delta SA}$/+ and $Tsix^{\Delta SA}$/Y embryos despite the presence of the antisense transcription.

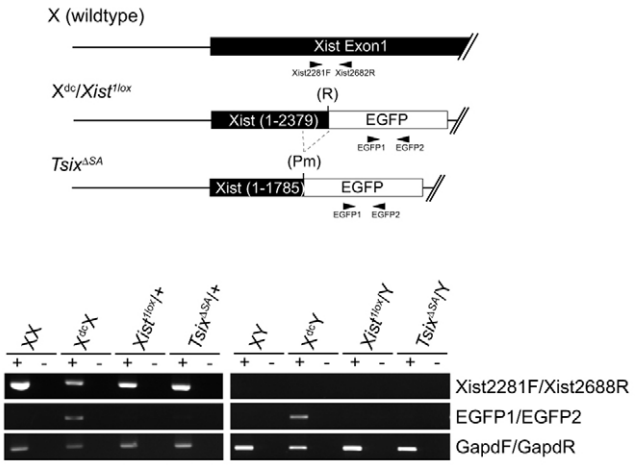

Fig. 4. *Xist* is stably repressed in the absence of spliced *Tsix* RNA. Transcriptional activity of the *Xist* locus was analyzed by RT-PCR using RNA isolated from E13.5 male and female embryos carrying the respective mutation. Diagrams show the structure of each mutant allele and positions of primers used for PCR. Transcription of the *Xist* locus on the mutated X was examined using a primer set specific for EGFP.

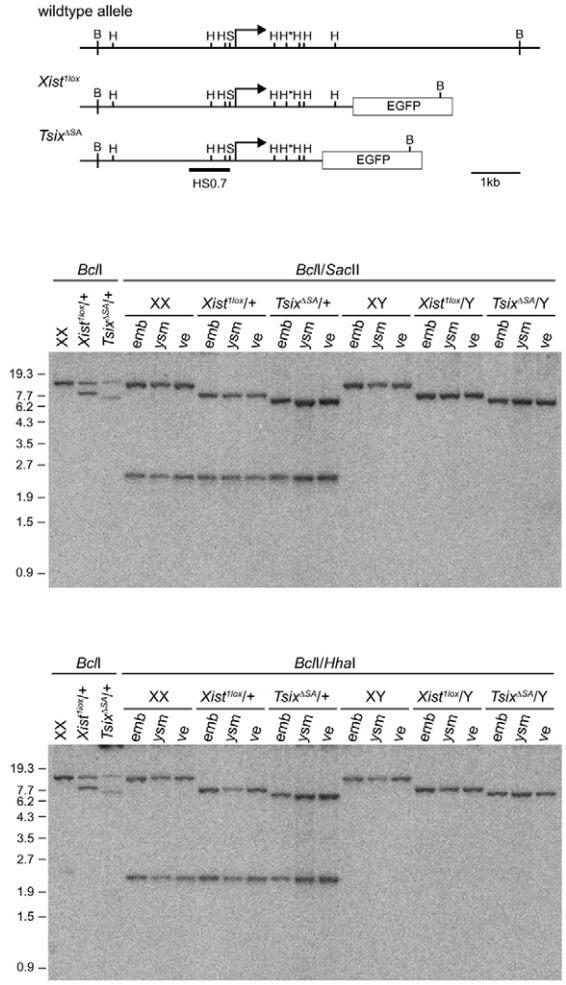

Fig. 5. Elimination of spliced *Tsix* RNA does not affect the methylation of the *Xist* promoter region. CpG methylation in the 5′ region of *Xist* was analyzed in E13.5 embryos by Southern blotting. Diagrams of the promoter region of *Xist* show relevant restriction enzyme sites and the position of the probe used for this assay. CpG methylation was appropriately established even in the absence of spliced *Tsix* RNA in both sexes. B, *Bcl*I; H, *Hha*I; S, *Sac*II.

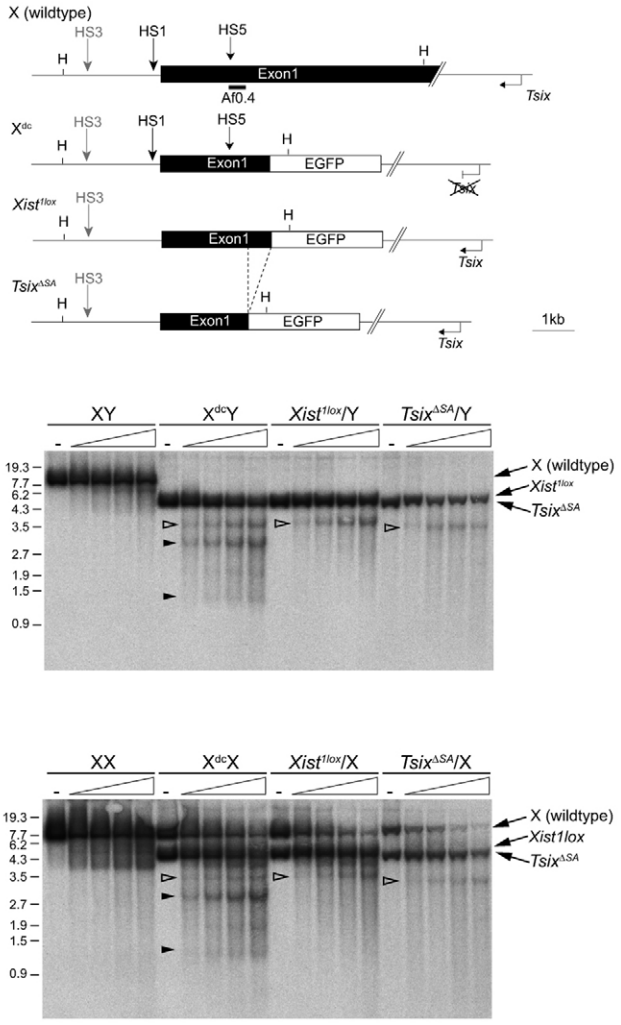

Fig. 6. Elimination of spliced *Tsix* RNA does not affect the establishment of closed chromatin structure. Chromatin structure in the 5′ region of *Xist* was analyzed by the DNaseI hypersensitive site assay. The diagrams show the structure of the *Xist*/*Tsix* locus, and the positions of DNaseI hypersensitive sites identified on the respective mutated X chromosome as well as those of *Hin*dIII sites used for the digestion of purified DNA. The position of the probe (Af0.4) used for Southern hybridization is also indicated. Ectopic DNaseI hypersensitive sites detected on X^{dc} are not found in E13.5 embryos carrying $Tsix^{\Delta SA}$. White arrowheads indicate the fragment derived from HS3 on the mutated X; black arrowheads indicate those from HS1 and HS5 on the mutated X. H, *Hin*dIII.

DISCUSSION

This study was carried out to further elucidate the molecular aspects of the *Tsix*-mediated *Xist* silencing described in our previous report (Sado et al., 2005). *Tsix* transcripts are peculiar in that although a fraction of them remain unspliced, various patterns of splicing all remove most of the region complementary to *Xist* RNA from the 40-kb primary transcript, except for a 1.9-kb region present in the proximal end of *Xist*, to yield the major processed products of length 2.7 kb and 4.3 kb (Sado et al., 2001; Shibata and Lee, 2003). In addition, the distal part of the processed *Tsix* RNA thus produced covers the region up to 1.8-kb upstream of the *Xist* transcription start site, where no transcription has been detected in the sense orientation. These features of the antisense transcripts at the *Xist* locus imply that the 1.9-kb region common to *Tsix* RNA and *Xist* RNA is relevant to the effect of *Tsix* on the antisense transcription. It has been shown that the function of *Tsix* is impaired by truncation of *Tsix* in the downstream region of *Xist* without deleting any genomic elements (Luikenhuis et al., 2001; Shibata and Lee, 2004). In addition, Shibata and Lee failed to rescue the loss-of-function phenotype of *Tsix* caused by the truncation even though a *Tsix* minigene was introduced immediately downstream of *Xist* so as to be transcribed in the same orientation as *Xist* under the control of an exogenous constitutively active promoter (Shibata and Lee, 2004). These studies suggest that *Tsix* action is not merely derived from the processed transcripts but requires concurrent antiparallel transcription through *Xist*.

In this study, we attempted to further clarify the significance of processed *Tsix* RNA produced by antiparallel transcription through the endogenous *Xist* gene. In contrast to targeted disruption (Lee, 2000; Lee and Lu, 1999; Sado et al., 2001) or transcriptional attenuation of *Tsix* (Luikenhuis et al., 2001; Shibata and Lee, 2004), loss of spliced *Tsix* RNA compromised neither the *Tsix*-mediated *Xist* silencing nor the establishment of proper chromatin configuration in

the *Xist* promoter region. These results indicate that the splicing products of *Tsix* RNA are unexpectedly dispensable for the chromatin modulation in the *Xist* promoter region. It is possible that unspliced *Tsix* RNA on its own is sufficient for mediating the establishment of the repressive chromatin configuration in the *Xist* promoter region. Alternatively, the RNA products are not required and the antisense transcription per se, especially across the *Xist* promoter region, is responsible for the bona fide effect mediated by *Tsix*. Another *Tsix* mutant allele, which prematurely terminates the antisense transcription in exon 4 of *Tsix* before it runs across the *Xist* promoter, appears to be defective in *Tsix* function despite the fact that more than 90% of the entire *Tsix* transcription unit is still transcribed to produce truncated RNA (T. Ohhata, Y.H., H.S. and T.S., unpublished). The antisense transcription across the *Xist* promoter region, therefore, seems to be crucial for *Tsix*-mediated *Xist* silencing. Considering all these findings taken together, we currently speculate that the antisense transcription is more important for the *Tsix*-mediated regulation than the RNA products. It is still possible, however, that the distal region of unspliced *Tsix* RNA covering the *Xist* promoter region is involved in the establishment of the appropriate chromatin conformation, perhaps through the formation of a secondary structure. Further study will help to clarify the role of RNA-antisense transcription in the antisense regulation by *Tsix* at the *Xist* locus.

Another unexpected observation in this study was the activation of the *Xist* locus on the mutated X chromosome in undifferentiated $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y ES cells. Unlike in $X^{dc}Y$ ES cells, however, *Xist* becomes downregulated upon differentiation in these mutant cells. This is consistent with the finding in the mutant embryos that *Tsix*'s function is compromised on X^{dc} but not affected on the mutated X chromosome carrying $Xist^{1lox}$ and $Tsix^{\Delta SA}$. Nonetheless, the activation of the *Xist* locus in the undifferentiated state in $Xist^{1lox}$/Y and $Tsix^{\Delta SA}$/Y as well as in $X^{dc}Y$ ES cells suggests that in these mutant cells, the genetic alteration introduced at the *Xist* locus impaired the silencing mechanism at the *Xist* locus, which would be independent of *Tsix* function, in undifferentiated ES cells. It is possible that the IRES-EGFP fragment introduced at the mutated locus somehow induces the *Xist* promoter, although the chromatin structure as revealed by the DNaseI hypersensitive site assay showed no difference between the wild-type and mutated alleles. Alternatively, the deleted region in these mutant alleles may contain a cis regulatory element required for *Xist*-silencing in undifferentiated cells. It has been shown that *Xist* that has been expressed from the paternal allele since the 2-4 cell stage, becomes downregulated in a cell that has contributed to the inner cell mass (ICM) at the blastocyst stage (Mak et al., 2004; Okamoto et al., 2004). *Tsix* is expressed only from the maternal allele, most probably in the trophectoderm, at this stage (Lee, 2000; Sado et al., 2001) and, therefore, repression of the paternal *Xist* in the ICM should be mediated by a *Tsix*-independent mechanism. It is tempting to speculate that the putative cis element mentioned above, if any, might be involved in the silencing of the hitherto active *Xist* in undifferentiated ICM cells. It will be of interest to see if the *Xist* locus on the paternally derived mutated X chromosome carrying either $Xist^{1lox}$ or $Tsix^{\Delta SA}$ is activated at the 2-4 cell stage and subsequently downregulated in the ICM cells at the blastocyst stage.

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