Regulation of imprinted X-chromosome inactivation in mice by Tsix

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SUMMARY

In mammals, X-chromosome inactivation is imprinted in the extra-embryonic lineages with paternal X chromosome being preferentially inactivated. In this study, we investigate the role of Tsix, the antisense transcript from the Xist locus, in regulation of Xist expression and X-inactivation. We show that Tsix is transcribed from two putative promoters and its transcripts are processed. Expression of Tsix is first detected in blastocysts and is imprinted with only the maternal allele transcribed. The imprinted expression of Tsix persists in the extra-embryonic tissues after implantation, but is erased in embryonic tissues. To investigate the function of Tsix in X-inactivation, we disrupted Tsix by insertion of an IRESgeo cassette in the second exon, which blocked transcripts from both promoters. While disruption of the paternal Tsix allele has no adverse effects on embryonic development, inheritance of a disrupted maternal allele results in ectopic Xist expression and early embryonic lethality, owing to inactivation of both X chromosomes in females and single X chromosome in males. Further, early developmental defects of female embryos with maternal transmission of Tsix mutation can be rescued by paternal inheritance of the Xist deletion. These results provide genetic evidence that Tsix plays a crucial role in maintaining Xist silencing in cis and in regulation of imprinted X-inactivation in the extra-embryonic tissues.

Key words: X-inactivation, Dosage compensation, Xist, Tsix, Genomic imprinting, Mouse

INTRODUCTION

During early embryogenesis of female mammals, one of the two X chromosomes becomes transcriptionally silenced (Lyon, 1961). While the choice of which X chromosome to be inactivated in the embryonic (epiblast) lineage is random, the paternally derived X chromosome (Xp) is preferentially inactivated in the extra-embryonic lineages in mouse (Takagi and Sasaki, 1975). The mechanism that controls this complex chromosome-wide epigenetic process is believed to consist of multiple steps, including (1) counting of the number of X chromosomes, (2) choosing which X chromosome to be inactivated, (3) initiation of inactivation, (4) spreading of the inactive state along the X chromosome and (5) its maintenance.

It is known that a cytogenetically identified region, X-chromosome inactivation center (Xic), is essential for X-inactivation, from which X-inactivation initiates and propagates in both directions along the chromosome (Russell and Montgomery, 1965). The Xist gene, mapped in the Xic region, is exclusively expressed from the inactive X chromosome in female somatic cells (Brown et al., 1991; Borsani et al., 1991; Brockdorff et al., 1991), and is thought to play a role as a functional RNA. Xist is paternally expressed from four-cell stage onwards, and is thought to be responsible for imprinted X-inactivation in the extra-embryonic lineages that takes place afterwards, while it is randomly expressed from either allele in the embryonic lineage, reflecting random X-inactivation (Kay et al., 1993; Kay et al., 1994). Targeted mutagenesis of the Xist gene demonstrated that Xist is essential for X-inactivation to occur in cis (Penny et al., 1996; Marahrens et al., 1997). Fluorescent in situ hybridization (FISH) analyses detecting Xist RNA revealed that there are two distinct patterns for Xist expression (Lee et al., 1996; Panning and Jaenisch, 1996; Panning et al., 1997; Sheardown et al., 1997). One form is a pinpoint signal that represents a nascent unstable RNA characteristic of the transcriptionally active X chromosome in undifferentiated male and female embryonic stem (ES) cells and epiblast cells. The other form is an accumulated signal occupying a relatively large domain in a nucleus, which is a processed stable RNA that coats the inactivated X chromosome in female somatic cells and differentiated female ES cells.

It has been shown that YAC and cosmid-derived transgenes containing the Xist gene appear to have the properties of Xic (Lee and Jaenisch, 1997; Herzing et al., 1997; Heard et al., 1999). However, Simmler et al. have suggested that another genetically defined locus lying in Xic, X chromosome controlling element (Xce), which is known to influence the
MATERIALS AND METHODS

RACE, RT-PCR and sequencing

5' and 3' RACE systems (Life Technologies) were used. Total RNA was prepared as described (Chomczynski and Sacchi, 1987). 5' and 3' RACE were performed on RNA prepared from the placenta of E12.5 male embryos and female ES cells, respectively, according to manufacturer’s instruction with following gene-specific primers (GSPs). For the isolation of clone24: GSP1, cgg gtt ttt gga tca tct; GSP2, cgg ggc tgg tgt gaa aat; GSP3, gag tta tct cac tga gaa. For the isolation of clone21b and 1b: GSP1, tgc gct gcg cta gct aaa; GSP2, cgg ggc ttg gat gga aat; GSP3, gag tta ttg cac tac ctg gaa. For the isolation of clone10: GSP1: cag gta tgt cca taa tca aca a; GSP2, tga aaa ggc agg taa gta tca a. For the isolation of clone4/4: GSP1: cag gta tgt cca taa tca aca a; GSP2, aca tgc ggg tgt gaa aat tct atc a. For the isolation of clone1/23: GSP1: cag gta tgt cca taa tca aca a; GSP2, cca cat gaa gat cag ac. The RACE products were subsequently cloned into pCRII vector (Invitrogen) for sequencing.

For the isolation of rt-clones, RT-PCR was carried out on cDNA converted from RNA prepared from female ES cells with random hexamer (Sado et al., 1996), and the amplified products were cloned into pCRII vector (Invitrogen) for sequencing. Primers used were as follows: 21b80F, cct gca agg gct aca cac tt; 21b352R, gga ggc cgg agt ctt gca at; 1b36F, gcc gac acg tgt tgt gct tt. Thermal condition for PCR consisted of a 5 minutes denaturation at 94°C, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 1 minute at 72°C, followed by a 5 minutes extension at 72°C.

Sequencing was performed with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Allelic expression assay of Tsix

By comparing genomic sequences of each exon between C57Bl/6 and JF1, a single nucleotide polymorphism was identified in exon 4. Total RNA were prepared from eight-cell embryos, blastocysts, the embryo proper and placenta at E11.5 derived from crosses between C57Bl/6 and JF1, which were converted into cDNA at 50°C using Superscript II (Life Technologies) and a strand-specific primer for either Tsix or Xist, as described by the manufacturer. Total RNA (2.5 µg) of the embryo proper and placenta was used for each cDNA synthesis. Total RNA isolated from 30 eight-cell embryos or 20 blastocysts were divided into three aliquots for Tsix-specific, Xist-specific cDNA synthesis, with an RT-minus control in each attempt. Following PCR on thus generated cDNA, the amplified products were subjected to direct sequencing. Thermal conditions were as already described above with 40 cycles instead of 35. Primer sequences used are as follows: a primer for Tsix-specific cDNA synthesis (R1910J), cat cgg ggc tgt gga tac ct; a primer for Xist-specific cDNA synthesis (F1063AS), gca caa ccc cggaa tgtc ta; for PCR (700P2), cgg ggc tgt ggt gat gaa aat; AS1634F, ggc taa tct gct cga gaa ta.

Targeted disruption

On the basis of a 94 kb sequence (GenBank Accession Number X99946) reported by Simmler et al. (Simmler et al., 1996), restriction maps of the genomic regions containing exons 1, 2, and 3 were produced. For the isolation of relevant genomic clones, a 129/Sv BAC library (Genome Systems) was screened using either exon 1 sequence or exon 2 sequence as a probe. For the construction of pSS1Δ2.7, a SacI-SacI fragment was cloned into pBluescriptII SK (Stratagene), from which all of the internal four Nhel-Nhel fragments across exon 1 were removed and replaced with a Pek-neo cassette, so as to be transcribed in an opposite direction to Tsix. For the construction of pA2AΔ1.7, a SpeI-Apal fragment was cloned into pBluescriptII SK, from which a 1.7 kb Smal-Solf fragment that contained exons 2 and 3 was removed and an IRES/Neo cassette (Mountford et al., 1994) was placed in the same direction as the transcription of the antisense RNA. The linearized targeting constructs thus produced were electroporated into J1 male ES cells, which were subsequently selected in the presence of G418 as described (Li et al., 1992). Appropriate targeting events were verified by Southern hybridization. Chimeric males were generated following injection of each type of targeted ES cells into blastocysts.

Northern hybridization

Poly(A)+RNA was isolated from male and female ES cells using Oligotex-dT30 (Qiagen). About 5 µg of highly enriched poly(A)+RNA were run on a 1% agarose gel containing formaldehyde and blotted onto HybondN+ (Amersham Pharmacia). Hybridization was performed in standard hybridization buffer at 65°C in the presence of a radiolabeled RNA probe prepared by in vitro transcription using T7 RNA polymerase (Promega). Washing was carried out according to manufacturer’s instruction.

Histology and X-gal staining

Embryos were fixed in Bouin’s fixative without taking out from the decidium at E6.5, E7.5 and E8.5, followed by dehydration and embedding in paraffin. Each specimen was serially sectioned at 6 µm, dewaxed and stained with Hematoxylin and Eosin. Fibroblasts grown on culture dishes or embryos were fixed and stained for β-galactosidase activity as described (Sado et al., 2000).

RNA FISH

Preparations for FISH on E7.5 embryos were made according to the methods previously described (Takagi et al., 1982). A probe was prepared by nick translation with Cy3-dUTP (Amersham Pharmacia) from an equimolar mixture of a series of Xist cDNA clones encompassing exons 1-7. Hybridization and following washes were carried out as described (Lawrence et al., 1989) with minor modifications. Following examination of Xist RNA signal, preparations used for RNA FISH were re-probed with either X- or Y-specific painting probes (Cambio), as described in the manufacturer’s instructions for determining the sex of embryos. Slides were examined with a Zeiss Axiosplan microscope, and images were captured with a Photometics CCD camera coupled to IPLab software (Signal Analytics).

RESULTS

Identification of a processed antisense RNA from the Xist locus

As antisense RNA is often associated with imprinted genes
both of which were not present in different extra sequences of 220 bp and 177 bp, respectively, located 3¢ revealed the presence of identical sequences in a 94 kb region.

A northern blot containing 5 μg of poly(A)±RNA was serially hybridized with a RNA probe prepared from either clone rt50 or 4/4. β-actin was a loading control.

A northern blot containing poly(A)±RNA isolated from male and female ES cells was hybridized with a clone rt50-derived RNA probe specific to the antisense transcript but not Xist, which displayed that the major transcripts were about 4.3 and 2.7 kb in length (Fig. 1B). Probing with the opposite strand did not yield a specific hybridization signal (data not shown; also see Fig. 3B). To determine the transcription unit of the antisense RNA, we employed 3′RACE on RNA prepared from the placenta at embryonic day 12.5 (E12.5) with primers annealing to extra-embryonic tissues, Xist shared with Xist (1b36F) in combination with one designed in a sequence common to Xist (21b352R; Fig. 1). Both RT-PCR produced multiple bands including one with an expected size on RNA prepared from male and female ES cells as well as the placenta (data not shown). Sequencing of the amplified products (clone rt34, rt35, rt37, rt49, and rt50) verified that they were indeed splicing variants transcribed from the Xist locus, which also allowed us to identify various splicing events and an additional exon (Fig. 1A). Exon-intron boundaries in these 5′RACE and RT-PCR products precisely followed the GT-AG rule in all instances. None of these splicing variants contained a long open reading frame, suggesting that they did not encode a protein. Additional 5′RACE from 177 bp segment in 1b did not extend the exon by more than 8 bp.

A northern blot containing poly(A)±RNA isolated from male and female ES cells was hybridized with a clone rt50-derived RNA probe specific to the antisense transcript but not Xist, which displayed that the major transcripts were about 4.3 and 2.7 kb in length (Fig. 1B). Probing with the opposite strand did not yield a specific hybridization signal (data not shown; also see Fig. 3B). To determine the transcription unit of the antisense RNA, we employed 3′RACE on RNA prepared from female ES cells and identified three different 3′ ends, all of which were followed by a poly(A) tail (clone10, 4/4, and 1/23 in Fig. 1A). The position of each 3′ end relative to the transcription start site of Xist were –171, –1375 and –1738, respectively. Therefore, the transcription unit of the antisense RNA spanned about 50 kb covering the entire Xist gene. An RNA probe generated from clone4/4 failed to detect the lower 2.7 kb band on the northern blot (Fig. 1B), indicating that it represented RNA terminated at the 3′ end identified in clone10.
The presence of antisense RNA at the Xist locus has been shown previously (Lee et al., 1999), which is termed Tsix. Although Lee et al. claimed that Tsix is a 40 kb RNA without introns, our results unequivocally demonstrate that Tsix was, at least in part, subject to processing. The functional significance of the processing of both Xist and Tsix transcripts remains to be determined.

Imprinted expression of Tsix in blastocysts and extra-embryonic tissues

To elucidate whether Tsix is involved in imprinted X-inactivation in the extra-embryonic lineages, allelic expression profile was studied in the embryo proper and placenta. We took advantage of a single nucleotide polymorphism found in exon 4 between C57Bl/6 (M. m. domesticus) and JF1 (M. m. molossinus) (Fig. 2A). Following the reciprocal crosses between C57Bl/6 and JF1, total RNA was prepared from the embryo proper and placenta at E11.5. Although expression levels of Tsix appeared much lower in the embryo proper than in the placenta (data not shown), an expected fragment was successfully amplified from both RNA by RT-PCR with extended cycles. Direct sequencing demonstrated that in male, Tsix expressed in the embryo proper and placenta was certainly derived from the single maternal X chromosome (Fig. 2A). In female, however, although both parental alleles were expressed in the embryo proper, only the maternal transcript was detected in the placenta in either cross (Fig. 2A). By E15.5, Tsix expression in the embryo proper and placenta became downregulated and barely detectable (data not shown). The allelic expression pattern was further analyzed in eight-cell embryos and blastocysts. With a common primer set across the polymorphism, RT-PCR was performed to amplify each of Tsix and Xist on the strand-specifically primed cDNA. Tsix was detected as previously shown, but Tsix did not amplify the Tsix sequence (Fig. 2B). No amplification occurred even after the second round of PCR with another 40 cycles (data not shown), indicating Tsix expression had not yet been started at the eight-cell stage. On the other hand, the expected fragments were successfully amplified from pooled blastocysts from both templates. Direct sequencing revealed that Xist was derived from the paternal allele, while Tsix was transcribed from only the maternal allele in blastocysts. It seemed that Tsix expression began around the time of blastocyst formation, when preferential paternal X-inactivation first occurs in the trophectoderm that has just differentiated. This suggests that Tsix play a role in trophectoderm formation and/or in imprinted X-inactivation occurring in this tissue.

Targeted disruption of the Tsix gene

Based on the positions of each exon, we created two different
Role for Tsix in X-inactivation

Targeting constructs to disrupt the Tsix gene in two different ways. In one construct (pSS1Δ2.7), putative exon 1 and its 5' region, which is located about 28 kb away from the recently revised 3' end of Xist (Hong et al., 1999), was deleted and replaced with a Pgk-neo cassette (Fig. 3A). We expected that removal of these sequences would abolish transcription itself at the Tsix locus. In the other construct (pAA2Δ1.7), an IRESβgeo cassette (Mountford et al., 1994) was placed in exon 2 so as not to disturb the transcription from the promoters but eliminate Tsix RNA (Fig. 3A). Making use of these two constructs, targeted disruption was carried out in J1 male ES cells (Li et al., 1992) and multiple targeted clones were isolated for each targeting constructs.

Following confirmation of the accurate targeting events (Fig. 3A), we examined expression of Tsix in the knockout cell lines by northern blotting with an RNA probe prepared from clone 10 (see Fig. 1A), which specifically detects Tsix (Fig. 3B). Unexpectedly, #218 cell line involving pSS1Δ2.7 (TsixSS1Δ2.7) still retained Tsix expression at a level comparable to the parental J1 cells, while expression of Tsix was not detected in cell lines #6, involving pAA2Δ1.7 (TsixAA2Δ1.7). These were confirmed in multiple cell lines in both cases by RT-PCR as well as northern analysis (data not shown). It seemed, therefore, likely that there were, at least, two distinct promoters for Tsix transcription and a major one would be located in the vicinity of exon 2. Deletion of exon 1 and the putative first promoter probably did not affect this second promoter, allowing Tsix expression from the TsixSS1Δ2.7 allele. A strong splicing acceptor site present in pAA2Δ1.7, however, would trap transcripts originated from both promoters, resulting in essentially no mature Tsix RNA produced from the TsixAA2Δ1.7 allele. Interestingly, when the northern blot was reprobed with an RNA probe specific to Xist, significant expression of Xist was detected in #6 cell lines but not in #218 and the parental J1 cells (Fig. 3B). FISH on multiple ES cell lines carrying the TsixAA2Δ1.7 allele demonstrated that although the majority of nuclei were negative for Xist expression, a small proportion of cells showed accumulation of Xist RNA as seen in female somatic cells (detailed analysis will appear elsewhere). This implied that elimination of Tsix RNA somehow impaired the regulatory mechanism of Xist expression.

Maternal transmission of the Tsix-disrupted XAA2Δ1.7 is embryonic lethal

The knockout ES cell lines, #218 and #6, were injected into blastocysts to create germline chimeras, and the mutated X-chromosome derived from either #218 (XSS1Δ2.7) or #6 (XAA2Δ1.7) was transmitted to female offspring from the chimeric males. Females heterozygous for XSS1Δ2.7 transmitted it to both male and female offspring, from which females homozygous for XSS1Δ2.7 were produced. Both hemizygous male (XSS1Δ2.7Y) and homozygous female (XSS1Δ2.7XSS1Δ2.7) were apparently healthy and fertile, and no discernible phenotypic abnormality was found. In contrast, females...
heterozygous for \(X^{AA2\Delta1.7}\) (the maternally derived \(X\) chromosome is written first and the paternally derived one the second) mated with wild-type males delivered totally 59 males and 45 females, out of which only one male and one female had \(X^{AA2\Delta1.7}\) transmitted (Table 1). This raised the possibility that maternal transmission of \(X^{AA2\Delta1.7}\) caused predominant lethality during embryogenesis.

PCR genotyping of 42 embryos recovered from five litters at E6.5 and E7.5 revealed that \(X^{AA2\Delta1.7}\) had been reasonably transmitted from \(XX^{AA2\Delta1.7}\) mothers to both male (14 out of 20) and female (8 out of 22) embryos. Gross morphology of \(X^{AA2\Delta1.7}\) embryos at E6.5 showed some variations, that is, some appeared developmentally retarded and others were indistinguishable from wild-type littersmates (Fig. 4A). By E7.5, the differences in gross morphology became quite obvious between wild-type embryos and \(X^{AA2\Delta1.7}\) embryos. Although variation among \(X^{AA2\Delta1.7}\) embryos was still observed, all of them were invariably smaller than wild-type egg-cylinder stage embryos. The variations found among \(X^{AA2\Delta1.7}\) embryos at E8.5 appeared developmentally arrested at or prior to E7.5, while others developed an empty yolk sac with little or no embryonic tissues (Fig. 4C). In contrast, female embryos that inherited \(X^{AA2\Delta1.7}\) from the chimeric father were indistinguishable from wild-type male littersmates at E8.5 (data not shown), suggesting that the paternally derived \(Tsx^{AA2\Delta1.7}\) had no deleterious effect on embryonic development.

To further characterize the embryonic defects, we performed histology on embryos recovered from \(XX^{AA2\Delta1.7}\) females at E6.5, E7.5 and E8.5. At E6.5, the presumptive \(X^{AA2\Delta1.7}\) embryos were developmentally retarded and failed to form a well organized egg-cylinder though all the primitive tissues, including the ectoplacental...

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**Table 1. No. of pups born to the chimeric male or females**

<table>
<thead>
<tr>
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<th>(B6\times chi)</th>
<th>(XXAA2\Delta1.7\times B6)</th>
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<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Wild type</td>
<td>1  *</td>
<td>86</td>
</tr>
<tr>
<td>w/(XXAA2\Delta1.7)</td>
<td>87</td>
<td>1‡</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>87</td>
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*\(XO\) female.
‡\(XXAA2\Delta1.7\) male.
§All pups but one were embryonic stem cell-derived.

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**Fig. 4.** Phenotypic comparison between \(X^{AA2\Delta1.7}\) and wild-type embryos. Gross morphology of (A) E6.5, (B) E7.5 and (C) E8.5 embryos upon maternal transmission of \(X^{AA2\Delta1.7}\). Embryos marked with an asterisk were wild type and remaining all carried maternally derived \(Tsx^{AA2\Delta1.7}\). Histology of (D) E6.5 wild-type embryo, (E,F) E6.5 presumptive \(X^{AA2\Delta1.7}\) embryo, (G) E7.5 wild-type embryo, (H,I) E7.5 presumptive \(X^{AA2\Delta1.7}\) embryo, (J,K) E8.5 presumptive \(X^{AA2\Delta1.7}\) embryo. All were photographed at the same magnification. a, amniotic cavity; al, allantois; ch, chorion; ec, ectoplacental cavity; ee, embryonic ectoderm; epc, ectoplacental cone; exe, extra-embryonic ectoderm; m, mesoderm; ve, visceral endoderm. Scale bars: 1 mm in A–C; 0.1 mm in D–K.
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cone, extra-embryonic ectoderm, visceral endoderm and embryonic ectoderm were formed (Fig. 4E,F). At E7.5, XAA2\Delta1.7 Y embryos were shown to contain mesodermal tissues localized predominantly at the embryonic and extra-embryonic junction (Fig. 4H,I). It appeared that cells of the ectoplacental cone failed to expand into the decidual tissue, but instead were accumulated in the proximal region of the egg cylinder. The ectoplacental cavity, exocoelom and amniotic cavity were all small, which was probably due to failure of the egg-cylinder to elongate distally. Morphology of XAA2\Delta1.7 Y embryos was variable at E8.5. Some of them retained the same morphology as the ones at E7.5 (Fig. 4J). Others grew larger and formed the yolk sac with disorganized embryonic tissues in it (Fig. 4K). The embryos lacked discernible patterning and were characterized by the presence of disorganized allantois and chorion and dead cells in the exocoelom (Fig. 4K). These results indicate that maternal transmission of XAA2\Delta1.7 leads to abnormal formation of the extra-embryonic tissues and growth arrest of the embryos prior to or during gastrulation.

Non-random X-inactivation in somatic cells deficient for Tsix

To examine whether each of these mutations affected random X-inactivation occurring in female somatic cells, we produced females carrying the X-linked lacZ transgene (XH253) in combination with either XSS1\Delta2.7 or XAA2\Delta1.7. XH253 had been extensively studied and shown to be a useful marker to monitor the activity of the X chromosome carrying the transgene (Tan et al., 1993; Tam et al., 1994; Sado et al., 2000). Fibroblasts derived from a tail tip of each female were stained for \( \beta \)-galactosidase activity. As shown in Fig. 5A,B, about 50% subset of cells in the extra-embryonic tissues were also \( \beta \)-galactosidase positive. ch, chorion; ee, embryonic ectoderm; epc, ectoplacental cone; m, mesoderm. Scale bars in C: 1 mm in the upper panel and 0.1 mm in the lower panels.

<table>
<thead>
<tr>
<th>Sex Chr.</th>
<th>lacZ+</th>
<th>lacZ−</th>
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<tbody>
<tr>
<td>XH253 X</td>
<td>232 (51.8%)</td>
<td>216 (48.2%)</td>
</tr>
<tr>
<td>XH253 Y</td>
<td>424 (97.9%)</td>
<td>9 (2.1%)</td>
</tr>
<tr>
<td>XH253XAA2\Delta1.7</td>
<td>498 (95.8%)</td>
<td>22 (4.2%)</td>
</tr>
<tr>
<td>XXAA2\Delta1.7</td>
<td>0 (0%)</td>
<td>400 (100%)</td>
</tr>
<tr>
<td>XH253XSS1\Delta2.7</td>
<td>316 (49.1%)</td>
<td>327 (50.9%)</td>
</tr>
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Fig. 5. Impaired random X-inactivation in female fibroblasts and embryos heterozygous for XAA2\Delta1.7. (A) An X chromosome carrying the lacZ transgene (XH253) had been introduced in female mice in combination with one of either Tsix mutation. Tail fibroblasts from these females doubly heterozygous for one of Tsix mutation and XH253 were stained for \( \beta \)-galactosidase activity. (B) Proportion of fibroblasts positive and negative for lacZ expression. (C) Upper panel, gross morphology of male (left; XAA2\Delta1.7 Y) and female (two middle; XAA2\Delta1.7 XH253) mutant embryos and a wild-type littermate (right); lower panel, histology of three mutant female embryos stained for \( \beta \)-galactosidase activity. The embryonic tissues were uniformly positive for \( \beta \)-galactosidase. Note that a
galactosidase-staining was detected in XX AA2 indicative of an active paternal X H253 and an inactive maternal -galactosidase expression in females came from indicating that X H253 rather than from IRES embryos. (B,D,F,H) Either X or Y chromosome painting or both. (B) Y chromosome painting on the nucleus in (A). (D) X chromosome painting on the nucleus in (C). (F) X and Y chromosome painting of the metaphase spread in (E). (H) X chromosome painting on the metaphase spread in (G).

of fibroblasts derived from an XH253X female were positive for β-galactosidase, manifesting random X-inactivation. The proportion of β-galactosidase-positive cells in XH253XSS1A2.7 fibroblasts were almost the same as XH253X, indicating that random X-inactivation was not affected by this mutation. In contrast, almost every XH253XAA2A1.7 fibroblast was β-galactosidase-positive as seen in XH253Y, which demonstrated that XAA2A1.7 had been inactivated in every cell. No β-galactosidase-staining was detected in XXAA2A1.7 cells, although XAA2A1.7 carried the IRESβgeo cassette, presumably reflecting almost no expression of Tsix in adult somatic cells (data not shown).

Embryos recovered at E7.5 from XXAA2A1.7 females crossed with XH253Y males were stained for β-galactosidase activity. None of XAA2A1.7Y embryos were positive for β-galactosidase, indicating that β-galactosidase expression in females came from XH253 rather than from IRESβgeo on XAA2A1.7. Fig. 5C shows gross morphology of male and female mutant embryos carrying XAA2A1.7 and histology of three female mutant embryos, in which most of cells consisting of the embryonic ectoderm and mesoderm were positive for β-galactosidase (>95%, more than 150 cells were counted for each embryo). No abnormal cell death was observed in the embryonic tissues, arguing against the possibility of cell selection. It is likely that elimination of Tsix RNA in the epiblast lineage disrupted the mechanism for random X-inactivation in female embryos with the mutated X chromosome being exclusively inactivated. These findings were consistent with skewed X-inactivation observed in differentiated female ES cells heterozygous for TsixACG (Lee and Lu, 1999) and the transgenic male ES cell lines carrying Xic with the DXPas34-deletion at the ectopic site (Debrand et al., 1999). It is, therefore, most probable that Tsix is involved in the choice mechanism in the epiblast lineage. It is worth mentioning that a small fraction of extra-embryonic cells in XAA2A1.7XH253 were positive for β-galactosidase (Fig. 5C), indicative of an active paternal XH253 and an inactive maternal XAA2A1.7, because two active X chromosomes are incompatible with development of the extra-embryonic lineages (Shao and Takagi, 1990; Goto and Takagi, 1998) (see Discussion).

Ectopic accumulation of Xist RNA in XAA2A1.7X/XAA2A1.7Y embryos
To elucidate the possible role of Tsix in X-inactivation, RNA FISH was carried out to analyze the expression of Xist in XAA2A1.7X/XAA2A1.7Y embryos. Fig. 6 shows FISH detecting Xist RNA on the cells of whole egg-cylinder at E7.5. It was evident that accumulation of Xist RNA occurred in a subset of interphase nuclei and the metaphase chromosome spread in XAA2A1.7Y embryos (Fig. 6A,E). The coating of the X chromosome by Xist RNA was verified by X chromosome painting (Fig. 6B,F). Similar ectopic accumulations were observed in XAA2A1.7X female embryos, in which Xist RNA specifically co-localized with both X chromosomes in a fraction of cells (Fig. 6C,D,G,H). The frequency of cells showing the aberrant coating of Xist RNA with the X chromosomes was 8-21% (the number of embryos examined were six and five for male and female, respectively). The ectopic expression of Xist was never observed in wild-type littermates in both sexes. Our results, therefore, demonstrated that the maternal inheritance of XAA2A1.7 induced the ectopic accumulation of Xist RNA irrespective of the number of X chromosomes in a subset of cells in mutant embryos. Although it was important to determine what type of cells showed such ectopic Xist accumulation, it was practically difficult to separate embryonic and extra-embryonic tissues from developmentally retarded and disorganized XAA2A1.7X/XAA2A1.7Y embryos. However, given the fact that the paternal transmission did not compromise embryonic development and Tsix is maternally expressed in the placenta, it seemed reasonable to assume that those cells showing aberrant accumulation of Xist RNA were derivatives of the extra-embryonic tissues. These results, therefore, suggest that maternal inheritance of XAA2A1.7 may lead to aberrant X-inactivation in both male and female embryos.

Genetic complementation between the maternal XAA2A1.7 and the paternal X1Xist
A possible role of Tsix transcript is to maintain stable silencing of the maternal Xist to ensure inactivation of only the paternal
X chromosome in the extra-embryonic tissues. In female embryos with maternal inheritance of \( X^{AA2\Delta1.7} \), the normally silent maternal \( Xist \) became transcriptionally active, leading to inactivation of both X chromosomes in the extra-embryonic cells and embryonic lethality. Conversely, it has been shown that paternal inheritance of a disrupted \( Xist \) gene (\( X^{AXist} \)) precludes inactivation of the paternal X, leading to two active X chromosomes in the extra-embryonic tissues and early embryonic lethality (Marahrens et al., 1997). Based on these results, we predicted that female embryos with a maternal \( X^{AA2\Delta1.7} \) and a paternal \( X^{AXist} \) might survive as they would have an active paternal X chromosome and an inactive maternal X chromosome in the extra-embryonic tissues, opposite to the imprinted X inactivation in wild-type embryos.

To test this hypothesis, we crossed \( X^{AA2\Delta1.7}X \) mice with \( X^{AXist} \) Y mice. From a total of nine litters of mice, we recovered four female mice and 22 male mice at birth (Table 2). As predicted, all male mice were found to be wild type and the four female mice were \( X^{AA2\Delta1.7}X^{AXist} \) by Southern blot analysis (data not shown). No viable \( X^{AA2\Delta1.7}X \) and \( XX^{AXist} \) mice were found. This result suggested that not all \( X^{AA2\Delta1.7}X^{AXist} \) mice were rescued and survived to birth, some probably died in utero. We then dissected four litters of embryos at E8.5 and E9.5 and found that of 13 \( X^{AA2\Delta1.7}X^{AXist} \) female embryos, seven were significantly smaller than their wild-type male littermates while the remaining six embryos were grossly normal in size and morphology (Fig. 7A,B; Table 2). However, the smaller embryos apparently underwent gastrulation normally, as they showed morphology comparable with normal E8.5 embryos (Fig. 7B). From the same litters, nine \( X^{AA2\Delta1.7}X \) embryos recovered were severely impaired, lacking discernible embryonic tissues (Fig. 7D) (except one growth retarded embryo shown in Fig. 7C). The trophoblast tissues were present, but much smaller than those seen in normal embryos (Fig. 7D). Interestingly, no \( XX^{AXist} \) embryos were recovered from these litters, indicating that \( XX^{AXist} \) embryos died at earlier stages than \( X^{AA2\Delta1.7}X \) embryos. These results indicate that most \( X^{AA2\Delta1.7}X^{AXist} \) mice are partially rescued when compared with their littermates \( X^{AA2\Delta1.7}X \) and \( XX^{AXist} \), and some are completely viable and normal.

**DISCUSSION**

In this study, we identified processed antisense transcripts from the \( Xist \) locus in placenta and ES cells, which are subject to complex alternative splicing. These transcripts coincide with the \( Tsix \) transcript identified by Lee et al. (Lee et al., 1999) by means of RNA FISH with strand-specific probes and strand-specific RT-PCR on ES cells. \( Tsix \) was described as a continuous 40 kb RNA without introns, whose transcription started at 15 kb downstream of the \( Xist \) gene (Lee et al., 1999). Our northern analysis, however, indicates that \( Tsix \) is, at least in part, subject to processing that gives rise to the major transcripts of 4.3 kb and 2.7 kb in length. We showed that \( Tsix \) expression is first detected in the blastocyst and is imprinted with the maternal allele expressed and the paternal allele silent, opposite to the imprinted expression of \( Xist \). Targeted disruption of \( Tsix \) in two different regions in this study revealed that \( Tsix \) had two putative promoters, located upstream of exons 1 and 2, and the majority of the transcripts were derived from the latter. Although deletion of exon 1 (\( Tsix^{SS1\Delta2.7} \)) did not appear to have discernible effect on X-

| Table 2. Offspring from intercrosses between \( X^{AA2\Delta1.7}X \) and \( X^{AXist} \) Y mice |
|-----------------|----------------|----------------|----------------|----------------|
| Stage           | XY            | \( X^{AA2\Delta1.7}X^{AXist} \) | \( X^{AA2\Delta1.7}Y \) | \( XX^{AXist} \) |
| P1-20           | 22            | 4(1)           | 0              | 0              |
| E9.5            | 7             | 9(5)           | 8(8)           | 0              | 6              |
| E8.5            | 2             | 4(2)           | 1(1)           | 1              | 3              |

The numbers in the parentheses indicate the number of abnormal embryos or pups. Of four \( X^{AA2\Delta1.7}X^{AXist} \) pups, one was growth retarded and died at 3 weeks of age. One of the \( X^{AA2\Delta1.7}Y \) embryos developed to the somite stage, while the others died during gastrulation. One \( XX^{AXist} \) embryo died before gastrulation. The early aborted decidual contained no embryonic tissue or yolk sac membrane, presumably accounting for the missing \( XX^{AXist} \) embryos.
inactivation and embryonic development, disruption of transcripts from both promoters by insertion of a promoterless IRES\_geo cassette in exon 2 (\textit{Tsix}^\text{AA2D1.7}) perturbed both random X-inactivation in the embryonic tissues and the imprinted X-inactivation in the extra-embryonic tissues.

The most striking finding in our study is that maternal transmission of \textit{XAA2D1.7} resulted in embryonic lethality in both sexes, while paternal transmission of \textit{XAA2D1.7} had no deleterious effect on embryonic development. As \textit{Tsix} is exclusively expressed from the maternal allele in blastocysts and the placenta, our result provides direct evidence that the expression of the maternal \textit{Tsix} allele is essential for embryonic development. The loss of \textit{Tsix} expression from \textit{Xm} in the extra-embryonic lineages appeared to have caused ectopic accumulation of \textit{Xist} RNA in a subset of cells in \textit{XAA2D1.7/XAA2D1.7\textsuperscript{Y}} embryos (Fig. 6), which probably led to inactivation of both X chromosomes in females and a single X chromosome in males after all, resulting in cell death due to functional nullisomy for the X chromosome in the extra-embryonic tissues. Since \textit{Tsix} and \textit{Xist} are reciprocally imprinted in the extra-embryonic tissues with respect to the parental origin of the X chromosome, our results suggest that imprinted expression of \textit{Tsix} in the extra-embryonic lineages renders \textit{Xm} extremely resistant to X-inactivation in these particular lineages. Although the affected embryos were severely impaired in postimplantation growth, the primitive germ layer tissues such as trophoblast and primitive endoderm, and embryonic ectoderm and mesoderm were formed. It is, therefore, unlikely that differentiation into the trophoblast in a blastocyst immediately induces the ectopic inactivation of \textit{Xm} carrying \textit{Tsix}^\text{AA2D1.7} in \textit{XAA2D1.7/XAA2D1.7\textsuperscript{Y}} embryos. As embryos that fail to inactivate all but one X chromosome barely form the extra-embryonic tissues and mesoderm (Shao and Takagi, 1990; Goto and Takagi, 1998), the presence of these tissues in \textit{XAA2D1.7\textsuperscript{X}} embryos suggests that imprinted paternal X-inactivation should have occurred at the appropriate time in \textit{XAA2D1.7\textsuperscript{X}} embryos.

In contrast to the imprinted X-inactivation in the extra-embryonic tissues, X-inactivation is random in the embryonic tissues, probably owing to imprinting of both \textit{Xist} and \textit{Tsix} in the epiblast. Both alleles of \textit{Xist} and \textit{Tsix} may exhibit basal levels of expression in the epiblast as seen in undifferentiated ES cells (Lee et al., 1999; Debrand et al., 1999). It is possible that once multipotential epiblast cells begin to differentiate, one of the two \textit{Tsix} alleles is repressed in a random fashion, perhaps, by de novo methylation, which would then allow accumulation of stable \textit{Xist} RNA in \textit{cis}, eventually leading to X-inactivation. On the other X chromosome, as in differentiating ES cells (Lee et al., 1999; Debrand et al., 1999), expression of \textit{Tsix} is sustained for a while after differentiation to suppress the production of stable \textit{Xist} RNA. We have shown that elimination of \textit{Tsix} RNA without perturbation of the counting mechanism causes nonrandom X-inactivation, rendering the \textit{XAA2D1.7} chromosome uniformly inactive in female embryos (Fig. 5). Once X-inactivation is established by the epigenetic modification such as DNA methylation and histone deacetelyation, \textit{Tsix} may not be required anymore for repression of \textit{Xist} in \textit{cis} and eventually disappears. In other words, \textit{Tsix} may be important only for the period in which differential expression of \textit{Xist} is established during the reversible phase of X-inactivation (Wutz and Jaenisch, 2000).

Genetic complementation between mutations of the two reciprocally imprinted genes, \textit{Xist} and \textit{Tsix}, supports the model that coordinated expression of \textit{Xist} and \textit{Tsix} regulates imprinted X-inactivation in the extra-embryonic tissues. Disruption of imprinted X-inactivation is detrimental to the development of the extra-embryonic tissues. XX\textit{Xist} embryos with two active X chromosomes are characterized by extremely poor formation of the extra-embryonic tissues (Marahrens et al., 1997). Interestingly, the extra-embryonic tissues and mesoderm are formed in the \textit{XAA2D1.7\textsuperscript{X}} embryos, allowing them to survive longer than XX\textit{Xist} embryos. It is probable that \textit{Xp} is inactivated normally in \textit{XAA2D1.7\textsuperscript{X}} embryos, and that subsequent activation of the maternal \textit{Xist} gene leads to delayed inactivation of \textit{Xm}. In rare cases, the maternal \textit{Xist} may be activated in blastocysts before \textit{Xp} undergoing inactivation, allowing \textit{Xp} to respond to counting and to reverse imprinted X-inactivation (see below). Importantly, most \textit{XAA2D1.7\textsuperscript{X}} embryos were partially rescued to the early somite stage (some developed to adult). As ectopic inactivation of \textit{Xm} takes place later than imprinted inactivation of \textit{Xp}, the successful rescue of females carrying the maternal \textit{XAA2D1.7} and paternal \textit{X\textsuperscript{Xist}} would depend on the proportion of extra-embryonic cells carrying the inactivated \textit{Xm} before the detrimental effect of two active X chromosomes becomes severe.

Lee has recently described the effect of \textit{Tsix} deletion (\textit{Tsix}\textsuperscript{\textit{D}\textsuperscript{CpG}}) on X-inactivation and embryonic development (Lee, 2000). She showed that maternal transmission of \textit{Tsix}\textsuperscript{\textit{D}\textsuperscript{CpG}} resulted in ectopic X-inactivation in male embryos and inactivation of both X chromosomes in female embryos, largely in agreement with our findings described in this study. The major differences between the two studies lie in the construction of the mutant \textit{Tsix} allele and the severity of its effect on embryonic development. Lee found that about 18% of mice with maternal transmission of \textit{Tsix}\textsuperscript{\textit{D}\textsuperscript{CpG}} survived to term. She proposed that imprinting of X-inactivation was not absolute, which allowed (though less frequent), inactivation of \textit{Xm}, leaving \textit{Xp} active in the extra-embryonic lineages and would explain the infrequent survivors carrying the maternally inherited \textit{Tsix}\textsuperscript{\textit{D}\textsuperscript{CpG}}. However, this assumption does not accommodate with complete lethality of female embryos with paternally derived \textit{X\textsuperscript{Xist}} (Marahrens et al., 1997). In contrast, we recovered only two pups who carried maternally transmitted \textit{XAA2D1.7} among 102 wild-type littersmates (2%). We speculate that the minor transcript derived from the upstream promoter, which is produced by alternative splicing connecting exon 1 directly to exon4 (like 5' RACE clone1b) or a similar event, might be responsible for maternal transmission of \textit{Tsix}\textsuperscript{\textit{D}\textsuperscript{CpG}} and \textit{Tsix}\textsuperscript{\textit{AA2D1.7}} to offspring. Frequency of survivors with each mutation may represent the efficiency to perturb the production of such minor transcripts.

Unlike \textit{XAA2D1.7\textsuperscript{Y}} embryos, which die during early development, \textit{Xp0} embryos are not lost during gestation in an uterine environment of XX mothers, although developmental retardation is a common feature among them (Tada et al., 1993; Jamieson et al., 1998). Based on the different behavior of the single X chromosome in \textit{XAA2D1.7\textsuperscript{Y}} and \textit{Xp0} embryos, despite the common absence of \textit{Tsix} expression, we propose that \textit{Xp} but not \textit{Xm}, is capable of responding to the counting mechanism in the extra-embryonic tissues. In normal female embryos, the counting mechanism senses the presence of two X chromosomes in a cell and allows \textit{Xp} to keep expressing \textit{Xist} to initiate X-inactivation (Fig. 8). In \textit{Xp0} embryos, it senses
the presence of only one X chromosome and extinguishes Xist expression to prevent inactivation of a single X chromosome (Fig. 8). However, when the dormant maternal Xist allele becomes transcriptionally activated in X<sup>A2Δ1.7</sup>Y, because X<sub>m</sub> is insensitive to the counting, it cannot shut off ectopic Xist expression, leading to inappropriate X-inactivation (Fig. 8). Although similar event also occurs predominantly in X<sup>A2Δ1.7</sup>X (causing inactivation of both X chromosomes; Fig. 8), if activation of the maternal Xist takes place early enough to allow the counting mechanism to switch off Xist expression from X<sub>p</sub>, such cells would manage to achieve dosage compensation and contribute to the extra-embryonic tissues as seen in Fig. 5. Okamoto et al. have recently described proper dosage compensation in the extra-embryonic tissues of X<sub>p</sub>X<sub>p</sub>/X<sub>p</sub>Y androgenones (Okamoto et al., 2000), supporting the notion that X<sub>p</sub> is responsive to the counting mechanism that is operative in the extra-embryonic lineages. Conversely, the failure of X<sub>m</sub> to be inactivated in the extra-embryonic tissues of the embryos with supernumerary X<sub>m</sub> (Shao and Takagi, 1990; Goto and Takagi, 1998) or with Xist-deficient X<sub>p</sub> (Marahrens et al., 1997) is not due to the lack of counting but to the differential sensitivity between X<sub>m</sub> and X<sub>p</sub> to it.

We have demonstrated that although Tsix has not yet been transcribed at eight-cell stage embryos, they maintain imprinted Xist expression, keeping the maternal allele silent, which suggests that Xist expression is regulated by some mechanism other than Tsix before blastocyst formation. Most probably, however, Tsix expression stabilizes imprinted expression of Xist after initiation of X-inactivation in the trophoderm. The epigenetic mechanism underlying the differences between X<sub>m</sub> and X<sub>p</sub> is not fully understood. Differential methylation of Xist 5′ region may be responsible in part for the imprinted expression of Xist. However, what controls imprinted expression of Tsix remains to be determined. We showed that exon 2 is associated with a region differentially methylated between the active and inactive X chromosomes (Courtier et al., 1995), which is partly included in the deleted region in the Tsix<sup>A2Δ1.7</sup> allele. The deletion in Tsix<sup>A2Δ1.7</sup> did not appear to block Tsix transcription per se as the IRES<sup>geo</sup> cassette was expressed in male ES cells – detected by northern analysis (data not shown) – but it did block Tsix transcripts running across the Xist gene, which is probably responsible for the loss of Tsix function. Furthermore, the role of exon 1 in regulation of Tsix expression and function is unclear. The detailed analysis of the genomic region spanning exons 1 and 2 will shed light on our understanding of still complicated mechanism of X-inactivation.

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**REFERENCES**

- Kay, G. F., Penny, G. D., Patel, D., Ashworth, A., Brockdorff, N. and


