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 IRES/geos 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 embryonic (epiblast) lineage is random, 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. While the choice of 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
probability of which X chromosome in female cells is to be inactivated (Cattanach et al., 1972), is segregated from the Xist gene and located 3’ to Xist (Simmler et al., 1993). It is, therefore, less likely that the Xist gene is solely responsible for all the function of Xic. Moreover, Clerc and Avner have recently shown that deletion of a 65 kb genomic region 3’ to Xist resulted in aberrant expression of Xist and subsequent X-inactivation in differentiating ES cells (Clerc and Avner, 1998), suggesting the presence of an element in the 3’ region, which is involved in regulation of Xist expression.

Several lines of evidence suggest that in the extra-embryonic tissues, the maternal X chromosome (X_m) is imprinted to resist the inactivation cue rather than X_p being imprinted to become inactivated (Lyon and Rastan, 1984; Shao and Takagi, 1990; Goto and Takagi; Okamoto et al., 2000). In this study, we attempted to identify an element in Xic region responsible for the imprinting laid on X_m. We report the isolation of a processed antisense RNA at the Xist locus and effects of its targeted mutagenesis on X-inactivation and embryonic development in mice.

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 tac tta cct; GSP2, cgg ggc tgt gtt gga aat; GSP3, gag tta tgt cac tct gaa. For the isolation of clone21b and 1b: GSP1, tgc gct ggc cta gta aca aca a; GSP2, tgc ggg att cgc ctt gat tt; GSP3, gcc aag gag tgc tcg tat ta. For the isolation of clone10: GSP1: cag gta tgt cca aca ctc a; GSP2, tga aaa ggc agg aag tta gta tca c. For the isolation of clone4:GSP1: cag gta tgt cca aca ctc a; GSP2, aca tga cgc gtt aat tct aat. For the isolation of clone12/3: GSP1: cag gta tgt cca aca ctc 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 agc ggc gca cac tt; 21b352R, gga ggc cgc atg ctt gca at; 1b36F, gcc agc acg tgt gct agc tt. Thermal condition for PCR consisted of 30 minutes denature 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 cct; a primer for Xist-specific cDNA synthesis (F1063AS), gca cca ccc cgc cta ta; for PCR (700P2), cgg ggc tgt gtt gat gga aat; AS1634F, ggc taa tgt cgt 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-Sacl fragment was cloned into plBluescriptII SK (Stratagene), from which all of the internal four Nhel-Nhel fragments across exon 1 were removed and replaced with a Pgs-geo cassette, so as to be transcribed in an opposite direction to Tsix. For the construction of pAA2Δ1.7, a SpeI-Apal fragment was cloned into plBluescriptII SK, from which a 1.7 kb Smal-Sfi fragment that contained exons 2 and 3 was removed and an IRES-geo 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 by 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 Axioplan microscope, and images were captured with a Photometrics 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
Fig. 1. (A) Genomic structure of Tsix. The location of each exon relative to Xist (Brockdorff et al., 1992; Hong et al., 1999) and Tsx (Simmler et al., 1996) are shown under the map. A 65 kb region deleted by Clerc and Avner (Clerc and Avner, 1998) is also shown on the top of the map. Clones obtained by RACE and RT-PCR are aligned under the map. Clones, 10, 4/4, and 1/23 were isolated by 3′RACE (open boxes) and 24, 21b and 1b were by 5′RACE (filled boxes). (A)n represents a poly(A) tail found in 3′RACE clones. The exon 2′ is an isoform of exon 2, whose splicing donor site appears 17 bp upstream from the end of exon2, which was found in rt34 and 49. Primers used for RT-PCR were shown on the right of each clone and their positions are indicated below the Tsix exons. (B) Northern analysis of Tsix expression in male and female ES cells. 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.

(Moore et al., 1997; Wutz et al., 1997; Rougeulle et al., 1998), we surmised that there might be transcription occurring in an opposite direction to Xist in the extra-embryonic tissues, where the paternally derived X chromosome is preferentially inactivated. Accordingly, we examined this possibility by performing 5′RACE on RNA prepared from the placenta at embryonic day 12.5 (E12.5) with primers annealing to sequences complementary to the 5′ region of Xist. This attempt allowed us to isolate a fragment extended along Xist (clone24), whose 5′ end was at 1109 of the Xist cDNA sequence reported by Brockdorff et al. (Brockdorff et al., 1992; GenBank Accession Number L04961; Fig. 1A). Further 5′RACE was carried out using another set of GSPs designed according to the sequence of clone24 and two additional clones were subsequently obtained (clone21b and 1b). Sequencing of clone21b and 1b revealed that they contained not only a 919 bp sequence common to Xist, which corresponds to a region 1020-1938 in Xist relative to the transcript start site, but different extra sequences of 220 bp and 177 bp, respectively, both of which were not present in Xist. Homology search with a 220 bp sequence in clone21b and a 177 bp sequence in 1b revealed the presence of identical sequences in a 94 kb region located 3′ to Xist (Fig. 1A), which had been fully sequenced by Simmler et al. (Simmler et al., 1996; GenBank Accession Number X99946). The position of 220 bp and 177 bp sequences were 77705-77924 and 61493-61669, respectively, according to X99946, strongly suggesting that they coded for alternative exons of the antisense transcript of Xist. To confirm this, reverse transcription polymerase chain reaction (RT-PCR) was conducted with a primer specific to either the 220 bp sequence in 21b (21b80F) or the 177 bp sequence in 1b (1b36F) in combination with one designed in a sequence shared with 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, both parental alleles were expressed in the embryo proper, only the maternal transcript was detected in the placenta in either cross (Fig. 2A). By E11.5, 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 not expressed in eight-cell embryos. Control was a reverse transcriptase minus reaction with a Tsix-specific primer. (C) Allelic expression profile of Tsix and Xist in pooled blastocysts. RT-PCR products amplified from the strand-specifically primed cDNA were sequenced. While Xist was paternally expressed (Kay et al., 1993), Tsix was transcribed from only the maternal allele in blastocysts.

Targeted disruption of the Tsix gene

Based on the positions of each exon, we created two different
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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 (XSS1Δ2.7*XSS1Δ2.7) were apparently healthy and fertile, and no discernible phenotypic abnormality was found. In contrast, females
heterozygous for $X^{AA2\Delta1.7}$ (XX$^{AA2\Delta1.7}$) (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 $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y 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 $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y littersmates. Although variation among $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y embryos was still observed, all of them were invariably smaller than wild-type egg-cylinder stage embryos. The variations found among $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y 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 Tsix$^{AA2\Delta1.7}$ had no deleterious effect on embryonic development.

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

![Fig. 4](image_url)

**Fig. 4.** Phenotypic comparison between $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y 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 Tsix$^{AA2\Delta1.7}$. Histology of (D) E6.5 wild-type embryo, (E,F) E6.5 presumptive $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y embryos, (G) E7.5 wild-type embryo, (H,I) E7.5 presumptive $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y embryos, (J,K) E8.5 presumptive $X^{AA2\Delta1.7}$X/$X^{AA2\Delta1.7}$Y embryos. All were photographed at the same magnification. a, aminotic 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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<tr>
<th>Table 1. No. of pups born to the chimeric male or females heterozygous for Tsix$^{AA2\Delta1.7}$</th>
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<td>B6 × chimera</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>w/X$^{AA2\Delta1.7}$</td>
</tr>
<tr>
<td>Total</td>
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<td>(19 litters)</td>
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* XO female.
‡ XX$^{AA2\Delta1.7}$Y male.
§ All pups but one were embryonic stem cell-derived.
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cone, extra-embryonic ectoderm, visceral endoderm and embryonic ectoderm were formed (Fig. 4E,F). At E7.5, XAA2Δ1.7X/XAA2Δ1.7Y 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 XAA2Δ1.7X/XAA2Δ1.7Y 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 XAA2Δ1.7 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 of Tsix mutations. Tail fibroblasts from these females doubly heterozygous for one of Tsix mutation and XH253 were stained for β-galactosidase activity. (B) Proportion of fibroblasts positive and negative for lacZ expression. (C) Upper panel, gross morphology of male (left; XAA2Δ1.7Y) and female (two middle; XAA2Δ1.7XH253) mutant embryos and a wild-type littermate (right); lower panel, histology of three mutant female embryos stained for β-galactosidase activity. The embryonic tissues were uniformly positive for β-galactosidase. Note that a subset of cells in the extra-embryonic tissues were also β-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>
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<tr>
<th>Sex Chr.</th>
<th>lacZ⁺</th>
<th>lacZ⁻</th>
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<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>XAA2Δ1.7</td>
<td>498 (95.8%)</td>
<td>22 (4.2%)</td>
</tr>
<tr>
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<td>400 (100%)</td>
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<tr>
<td>XH253 xAS1Δ2.7</td>
<td>316 (49.1%)</td>
<td>327 (50.9%)</td>
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Fig. 5. Impaired random X-inactivation in female fibroblasts and embryos heterozygous for XAA2Δ1.7. (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 β-galactosidase activity.
of fibroblasts derived from an X\(^{H253}\) female were positive for \(\beta\)-galactosidase, indicating that random \(X\)-inactivation was not affected by this mutation. In contrast, almost every X\(^{H253}\) \(X_{AA2}\) fibroblast was \(\beta\)-galactosidase-positive as seen in X\(^{H253}\) Y, which demonstrated that \(X_{AA2}\) had been inactivated in every cell. No \(\beta\)-galactosidase-staining was detected in \(XX_{AA2}\) cells, although X\(^{AA2}\) carried the IRES\(\beta\)geo cassette, presumably reflecting almost no expression of \(Tsix\) in adult somatic cells (data not shown).

Embryos recovered at E7.5 from \(XX_{AA2}\) females crossed with X\(^{H253}\) Y males were stained for \(\beta\)-galactosidase activity. None of X\(^{AA2}\) Y embryos were positive for \(\beta\)-galactosidase, indicating that \(\beta\)-galactosidase expression in females came from X\(^{H253}\) rather than from IRES\(\beta\)geo on X\(^{AA2}\). Fig. 5C shows gross morphology of male and female mutant embryos carrying X\(^{AA2}\) and histology of three female mutant embryos, in which most of cells consisting of the embryonic ectoderm and mesoderm were positive for \(\beta\)-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 \(Tsix^{ACG}\) (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 X\(^{AA2}\)X\(^{H253}\) were positive for \(\beta\)-galactosidase (Fig. 5C), indicative of an active paternal X\(^{H253}\) and an inactive maternal X\(^{AA2}\), 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 \(XX_{AA2}\) and \(X_{AA2}\) Y embryos

To elucidate the possible role of \(Tsix\) in \(X\)-inactivation, RNA FISH was carried out to analyze the expression of \(Xist\) in \(XX_{AA2}\) and \(X_{AA2}\) Y 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 \(XX_{AA2}\) Y 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 \(XX_{AA2}\) 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 \(X\) chromosome 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 \(X_{AA2}\) 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 \(XX_{AA2}\) Y/\(XX_{AA2}\) Y 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 \(X_{AA2}\) may lead to aberrant \(X\)-inactivation in both male and female embryos.

### Genetic complementation between the maternal \(XX_{AA2}\) and the paternal \(X\) \(\times\) \(Xist\)

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 $X^{AA2a1.7}$, 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 $X^{AA2a1.7}$ 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 $X^{AA2a1.7X}$ mice with $X^{AXistY}$ 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 $X^{AA2a1.7X}$ and $X^{AXist}$ mice were found. This result suggested that not all $X^{AA2a1.7X^{AXist}}$ 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 $X^{AA2a1.7X^{AXist}}$ female embryos, seven were significantly smaller than their wild-type male littersmates 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 $X^{AA2a1.7X^{AXist}}$ 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 $X^{XX^{AXist}}$ embryos were recovered from these litters, indicating that $X^{XX^{AXist}}$ embryos died at earlier stages than $X^{AA2a1.7X^{AXist}}$ embryos. These results indicate that most $X^{AA2a1.7X^{AXist}}$ mice are partially rescued when compared with their littersmates $X^{AA2a1.7X}$ and $X^{AXist}$, 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 ($Tsix^{SS1a2.7}$) did not appear to have discernible effect on X-
inactivation and embryonic development, disruption of transcripts from both promoters by insertion of a promoterless IRESβgeo cassette in exon 2 (Tsix\textsuperscript{A2Δ1.7}) 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 X\textsuperscript{A2Δ1.7} resulted in embryonic lethality in both sexes, while paternal transmission of X\textsuperscript{A2Δ1.7} had no deleterious effect on embryonic development. As Tsix is exclusively expressed from the maternal allele in blastocysts and the placenta, our result provides direct evidence that the expression of the maternal Tsix allele is essential for embryonic development. The loss of Tsix expression from X\textsubscript{m} in the extra-embryonic lineages appeared to have caused ectopic accumulation of Xist RNA in a subset of cells in X\textsuperscript{A2Δ1.7}\textsubscript{X}/X\textsuperscript{A2Δ1.7}\textsubscript{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 Tsix and 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 Tsix in the extra-embryonic lineages renders X\textsubscript{m} 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 X\textsubscript{m} carrying Tsix\textsuperscript{A2Δ1.7} in X\textsuperscript{A2Δ1.7}\textsubscript{X}/X\textsuperscript{A2Δ1.7}\textsubscript{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 X\textsuperscript{A2Δ1.7}\textsubscript{X} embryos suggests that imprinted paternal X-inactivation should have occurred at the appropriate time in X\textsuperscript{A2Δ1.7}\textsubscript{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 Xist and Tsix in the epiblast. Both alleles of X\textsubscript{m} and X\textsubscript{p} may express basal levels of expression in the epiblast as seen in undifferentiated ES cells (Lee et al., 1999; Debran et al., 1999). It is possible that once multipotent epiblast cells begin to differentiate, one of the two Tsix alleles is repressed in a random fashion, perhaps, by de novo methylation, which would then allow accumulation of stable Xist RNA in cis, eventually leading to X-inactivation. On the other X chromosome, as in differentiating ES cells (Lee et al., 1999; Debran et al., 1999), expression of Tsix is sustained for a while after differentiation to suppress the production of stable Xist RNA. We have shown that elimination of Tsix RNA without perturbation of the counting mechanism causes nonrandom X-inactivation, rendering the X\textsuperscript{A2Δ1.7} chromosome uniformly inactive in female embryos (Fig. 5). Once X-inactivation is established by the epigenetic modification such as DNA methylation and histone deacetylation, Tsix may not be required anymore for repression of Xist in cis and eventually disappears. In other words, Tsix may be important only for the period in which differential expression of Xist is established during the reversible phase of X-inactivation (Wutz and Jaenisch, 2000).

Genetic complementation between mutations of the two reciprocally imprinted genes, Xist and Tsix, supports the model that coordinated expression of Xist and 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\textsuperscript{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 X\textsuperscript{A2Δ1.7}\textsubscript{X} embryos, allowing them to survive longer than XX\textsuperscript{Xist} embryos. It is probable that X\textsubscript{p} is inactivated normally in X\textsuperscript{A2Δ1.7}\textsubscript{X} embryos, and that subsequent activation of the maternal Xist gene leads to delayed inactivation of X\textsubscript{m}. In rare cases, the maternal Xist may be activated in blastocysts before Xp undergoing inactivation, allowing Xp to respond to counting and to reverse imprinted X-inactivation (see below). Importantly, most X\textsuperscript{A2Δ1.7}\textsubscript{X}\textsuperscript{Xist} embryos were partially rescued to the early somite stage (some developed to adult). As ectopic inactivation of X\textsubscript{m} takes place later than imprinted inactivation of Xp, the successful rescue of females carrying the maternal X\textsuperscript{A2Δ1.7} and paternal X\textsuperscript{Xist} would depend on the proportion of extra-embryonic cells carrying the inactivated X\textsubscript{m} before the detrimental effect of two active X chromosomes becomes severe.

Lee has recently described the effect of Tsix deletion (Tsix\textsuperscript{ΔCpG}) on X-inactivation and embryonic development (Lee, 2000). She showed that maternal transmission of Tsix\textsuperscript{ΔCpG} 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 Tsix allele and the severity of its effect on embryonic development. Lee found that about 18% of mice with maternal transmission of Tsix\textsuperscript{ΔCpG} survived to term. She proposed that imprinting of X-inactivation was not absolute, which allowed (though less frequent), inactivation of X\textsubscript{m}, leaving Xp active in the extra-embryonic lineages (which would explain the infrequent survivors carrying the maternally derived X\textsubscript{m}). However, this assumption does not accommodate with complete lethality of female embryos with paternally derived X\textsuperscript{Xist} (Marahrens et al., 1997). In contrast, we recovered only two pups who carried maternally transmitted X\textsuperscript{A2Δ1.7} among 102 wild-type littermates (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 Tsix\textsuperscript{ΔCpG} and Tsix\textsuperscript{A2Δ1.7} to offspring. Frequency of survivors with each mutation may represent the efficiency to perturb the production of such minor transcripts.

Unlike X\textsuperscript{A2Δ1.7}\textsubscript{Y} embryos, which die during early development, X\textsubscript{p}O 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 X\textsuperscript{A2Δ1.7}\textsubscript{Y} and X\textsubscript{p}O embryos, despite the common absence of Tsix expression, we propose that Xp, but not 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 Xp to keep expressing Xist to initiate X-inactivation (Fig. 8). In X\textsubscript{p}O embryos, it senses
Table: Preimplantation Embryos (Before Blastocyst Formation) vs. Extraembryonic Lineages

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| X
| Inactivated         | Active                         |
| O                  | Active                          |                              |
| X                   | Inactivated                     | Active                       |
| O                  | Inactivated                     | Active                       |
| X
| No Inactivation     | Active                         |
| O                  | Inactivated                     | Active                       |
| X                   | No Inactivation                 | Active                       |
| O                  | No Inactivation                 | Active                       |
| X
| No Inactivation     | Active                         |
| O                  | No Inactivation                 | Active                       |
| X                   | No Inactivation                 | Active                       |
| O                  | No Inactivation                 | Active                       |

Fig. 8. A model of Tsix-mediated regulation of Xist silencing in cis and imprinted X-inactivation in the extra-embryonic lineages. Xist is expressed from the Xp by default in preimplantation embryos. Paternal expression of Xist from counting-sensitive Xp can be turned off according to the number of X chromosome which an individual cell senses. Elimination of Tsix RNA on Xm allows ectopic expression of Xist in cis after blastocyst formation. As Xm is insensitive to the counting, even though Xp has undergone X-inactivation, Xm is unable to shut off ectopic expression of maternal Xist, leading to delayed X-inactivation in the extra-embryonic lineages.

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 XAA2Δ1.7Y, because Xm 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 XAA2Δ1.7X (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 Xp, 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 XpXp/XpY androgenes (Okamoto et al., 2000), supporting the notion that Xp is responsive to the counting mechanism that is operative in the extra-embryonic lineages. Conversely, the failure of Xm to be inactivated in the extra-embryonic tissues of the embryos with supernumerary Xm (Shao and Takagi, 1990; Goto and Takagi, 1998) or with Xist-deficient Xp (Marahrens et al., 1997) is not due to the lack of counting but to the differential sensitivity between Xm and Xp 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 trophectoderm. The epigenetic mechanism underlying the differences between Xm and Xp 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 TsixAA2Δ1.7 allele. The deletion in TsixAA2Δ1.7 did not appear to block Tsix transcription per se as the IRESgeo 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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