Development 127, 4137-4145 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4328

X-chromosome inactivation in XX androgenetic mouse embryos surviving implantation

Ikuhiro Okamoto[1], Seong-Seng Tan[3] and Nobuo Takagi[1,2,*]

[1]Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 0600810, Japan
[2]Research Center for Molecular Genetics, Hokkaido University, Sapporo 0600810, Japan
[3]Developmental Biology Laboratory, Howard Florey Institute, The University of Melbourne, Parkville, Victoria, Australia
*Author for correspondence (e-mail: ntakagi@ees.hokudai.ac.jp)

Accepted 20 July; published on WWW 7 September 2000

SUMMARY

Using genetic and cytogenetic markers, we assessed early development and X-chromosome inactivation (X-inactivation) in XX mouse androgenones produced by pronuclear transfer. Contrary to the current view, XX androgenones are capable of surviving to embryonic day 7.5, achieving basically random X-inactivation in all tissues including those derived from the trophectoderm and primitive endoderm that are characterized by paternal X-activation in fertilized embryos. This finding supports the hypothesis that in fertilized female embryos, the maternal X chromosome remains active until the blastocyst stage because of a rigid imprint that prevents inactivation, whereas the paternal X chromosome is preferentially inactivated in extra-embryonic tissues owing to lack of such imprint. In spite of random X-inactivation in XX androgenones, FISH analyses revealed expression of stable *Xist* RNA from every X chromosome in XX and XY androgenonetic embryos from the four-cell to morula stage. Although the occurrence of inappropriate X-inactivation was further suggested by the finding that *Xist* continues ectopic expression in a proportion of cells from XX and XY androgenones at the blastocyst and the early egg cylinder stage, a replication banding study failed to provide positive evidence for inappropriate X-inactivation at E6.5.

Key words: Androgenetic mouse embryos, X-chromosome inactivation, Genomic imprinting, Nuclear transplantation, *lacZ* gene, FISH, *Xist* gene

INTRODUCTION

X-chromosome inactivation (X-inactivation) (Lyon, 1961) in female mouse embryos first occurs in the trophectoderm of expanded blastocysts and the primitive endoderm of implanting blastocysts (Sugawara et al., 1985). In these cells the paternally derived X chromosome (X^P) is preferentially inactivated (Takagi and Sasaki, 1975; West et al., 1977). In the embryo proper, X-inactivation occurs randomly before gastrulation (Takagi et al., 1982; Rastan, 1982) with equal probability of either the maternal X (X^M) or X^P being inactivated. Lyon and Rastan (1984) hypothesized that X^M is imprinted to remain active, and that this imprinting is erased in early postimplantation development, allowing random X-inactivation to occur in cells of the embryo proper. Monk and McLaren (1981), however, proposed that the X^P chromosome is prone to inactivation in early development because it retains memory that it was previously inactive during spermatogenesis.

The *XIST/Xist* gene, which is mapped in the region of X-chromosome inactivation center and is exclusively expressed from the inactivated X chromosome of differentiated female somatic cells (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991), is essential for X-inactivation in vivo and in vitro (Penny et al., 1996; Marahrens et al., 1997, 1998; Lee et al., 1996, 1999a; Lee and Jaenich, 1997; Herzing et al., 1997; Heard et al., 1999a,b; Wutz and Jaenisch, 2000). RT-PCR and RNA FISH (fluorescent in situ hybridization) analyses revealed two different levels of *Xist* gene expression in mice. Low expression corresponding to the spot or pinpoint signal detected in ES and early embryonic cells of both sexes, and high expression corresponding to the large paint signal found in female somatic cells and differentiated embryonic cells after X-inactivation (Panning et al., 1997; Sheardown et al., 1997). Prior to X-inactivation, unstable *Xist* RNA is expressed from all X chromosomes and accumulates only at the site of transcription; stabilization and spread of *Xist* RNA in *cis* from the inactivation center along the entire length correlate with genetic silencing of the X chromosome (Panning et al., 1997; Sheardown et al., 1997). Johnston et al. (1998) favor the promoter switch as a mechanism for the change in the stability of *Xist* RNA, but Lee et al. (1999b) speculate that *Tsix* RNA antisense to *Xist* RNA is involved in destabilization of the latter. The recent study by Warshawsky et al. (1999) does not uphold the promoter switch hypothesis.

Previous work has shown that early development of androgenones (two paternal sets of chromosomes) with two X chromosomes are more severely affected than that of XY androgenones. Kaufman et al. (1989) failed to find any XX embryo among 12 egg-cylinder-stage androgenones.

Circumstantial evidence led Kay et al. (1994), and Latham and Rambhatla (1995) to speculate that XX androgenones were dead by the blastocyst stage. Two diverse causes are proposed for the early death of XX androgenones: two active X chromosomes, owing to the failure of X-inactivation (Kay et al., 1994); and no active X chromosome, owing to inactivation of both X chromosomes (Kaufman et al., 1989; Latham, 1996). So far as we are aware, no direct information is yet available that allow one to evaluate these possibilities. The present study was initiated to obtain more-relevant information from androgenetic mouse embryos produced by pronuclear transplantation. We made use of male mice carrying a Robertsonian-type translocation involving the X chromosome or exclusively autosomes, and an X-chromosome linked *lacZ* transgene that is subject to X-inactivation (Tan et al., 1993) as markers for verifying the androgenetic origin of each embryo.

The present histological and cytogenetic study provides compelling evidence that, contrary to the widely accepted view (Kaufman et al., 1989; Kay et al., 1994; Latham and Rambhatla, 1995), XX androgenones survive on embryonic (E) day 7.5, achieving basically random X-inactivation in all tissues including those derived from the trophectoderm and primitive endoderm. This finding supports the hypothesis that X^M carries a rigid imprint that prevents inactivation until implantation, whereas X^P is free of such imprint. Furthermore, *Xist* RNA FISH analysis suggested the occurrence of inappropriate X-inactivation resulting in a proportion of cells functionally nullisomic for X in XX and XY androgenetic embryos. A chromosome study, however, failed to provide positive evidence for it.

MATERIALS AND METHODS

Mice

Male mice from the transgenic H253 (Tan et al., 1993) and Robertsonian translocation stocks, Rb(X.2)2Ad (abbreviated to Rb2) (Adler et al., 1989) and Rb(X.9)6H (abbreviated to RX9) (Tease and Fisher, 1991) were mated with wild-type females (C57BL/6J×CBA/J) F1 (abbreviated to F1). The transgenic H253 stock carries *E. coli lacZ* gene with the promoter of a mouse housekeeping gene, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA) and a SV40 T antigen nuclear localization signal sequence on the X chromosome (Tan et al., 1993). The *HMG-lacZ* transgene is subject to X inactivation in certain tissues, but residual β-galactosidase (β-gal) activity may persist for a little while after X-inactivation (Tan et al., 1993; Lebon et al., 1995). In addition to Rb2 and RX9 males, we mated F1 females with Rb(10;11)8Bnr (abbreviated to Rb8) (Gropp et al., 1972) and Rb(11;13)6Lub (abbreviated to Rb6) (Gropp et al., 1975) males to obtain fertilized eggs for generating androgenetic embryos used for the *Xist* RNA FISH analysis. Metacentric translocation chromosomes are easily identified in metaphase cells as all remaining chromosomes are acrocentric.

Production of androgenones

Female F1 mice were superovulated by injections of pregnant mare's serum gonadotropin (PMSG, 10 IU) and human chorionic gonadotropin (hCG, 10 IU) 48 hours apart before mating. Fertilized eggs at the pronuclear stage were recovered from oviducts in M2 medium 18-19 hours after hCG injection and cultured in M16 medium (Whittingham, 1971). The female pronucleus was removed from recipient eggs and replaced with male pronucleus from donor eggs to yield F1+H253, H253+Rb2, H253+RX9, Rb2+RX9 and Rb6+Rb8 androgenetic eggs as previously described (McGrath and Solter, 1983; Barton et al., 1987). Fusion of the karyoplast with the egg was induced with inactivated Sendai virus (2700 hemagglutination U/ml). After cytoplasmic fusion, embryos were transferred to oviducts of pseudopregnant F1 foster mice. Some embryos used for FISH analysis were cultured in M16 medium under paraffin oil at 37°C in 5% CO_2 in air up to the four- or 8-16-cell stage.

Histological analysis of X-inactivation

Embryos were recovered at E7.5 from foster mothers and fixed with 4% paraformaldehyde (5-10 minutes) in 0.1 M phosphate buffer (pH 7.2). They were washed briefly and stained in 0.1% 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal), 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Nonidet P-40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.6H_2O$ at 37°C overnight. Embryos were dehydrated with ethanol, embedded in JB-4 resin (Electron Microscopy Sciences, Ft Washington, PA) and sectioned at 2 μm. Sections were counterstained with Eosin or Nuclear Fast Red (Tan et al., 1993).

Chromosomal analysis of X-inactivation

Embryos recovered at E6.5-E7.5 were incubated in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 150 μg/ml 5-bromo-2-deoxyuridine (BrdU) at 37°C in 5% CO_2 in air for 7.5 hours including the last hour of incubation in the presence of 1 μg/ml Colcemid. Chromosome slides were prepared according to the method described previously (Takagi et al., 1982). Cells were analyzed for the karyotype and chromosome replication pattern after staining with freshly prepared Acridine Orange solution.

Cytological preparation for FISH

Most androgenetic embryos recovered at E3.5 were at the morula stage and developed into fully expanded blastocysts after culture in M16 medium for 12 hours. Cytogenetic preparations were made from preimplantation embryos according to the methods described by Takagi et al. (1982) with minor modifications. Briefly, after hypotonic treatment with 1% sodium citrate for 10 minutes at room temperature, embryos were fixed with 3:1 mixture of methanol: glacial acetic acid on ice. Each embryo, together with a small volume of the fixative, was placed on a clean glass slide. A small drop of 3:1 mixture of glacial acetic acid and 25% lactic acid was applied immediately to the embryo to spread cells on the slide. Lactic acid was removed from the preparation by repeated application of the fixative. Preparations for RNA FISH were made from E6.5 embryos as mentioned above except for the omission of BrdU incorporation.

RNA FISH Analysis

Xist RNA was detected with the use of pBluescript-based plasmid clones, pR97E1, pR95B, pR91E and pR53E1 encompassing exon 1 to 6 (Sado et al., 1996). An equimolar mixture of these plasmids was labeled by nick translation with Cy3-dCTP (Amersham Pharmacia, Little Chalfort, UK). After addition of salmon sperm DNA and yeast tRNA, labeled probe was precipitated with ethanol, resuspended in deionized formamide, and denatured at 70°C for 10 minutes. About 250 ng of probe DNA was applied per slide in 10 μl of hybridization mixture (2×SSC, 1 mg/ml BSA, 20% dextran sulphate), incubated at 42°C overnight in a moist chamber. After hybridization, slides were washed twice in 50% formamide/2×SSC for 5 minutes at 42°C, and twice in 2×SSC/ 0.05% Tween-20 for 5 minutes at 42°C. Slides were mounted with antifading solution containing DAPI. Preparations used for RNA FISH were painted with X- and Y-specific probes to determine the sex chromosome constitution and to verify signals produced by *Xist* RNA. After removal of the cover slip, slides were washed three times in 2×SSC/0.05% Tween-20 for 10 minutes at room temperature and refixed with 3:1 methanol: glacial acetic acid twice for 10 minutes on ice and dried at room temperature. Preparations were hardened for 24 hours at 65°C. Biotin-labeled mouse X and Cy3-

labeled mouse Y chromosome paint probes (Cambio, Cambridge, UK) were denatured for 10 minutes at 65°C and incubated for 1 hour at 37°C. Chromosome preparations were denatured in 70% formamide/ 2×SSC for 5 minutes at 70°C and dehydrated with ice-cold ethanol series. A mixture of 10 μl each of X and Y chromosome paint probe was applied onto the slide and hybridization was performed at 42°C overnight in a moist chamber. Slides were washed twice in 50% formamide in 2×SSC, and twice in 0.05% Tween-20 for 5 minutes at 42°C. Hybridization was detected with streptavidin-fluorescein isothiocyanate (Gibco BRL, Life Technologies, Rockville, MD). After incubation, slides were washed three times in 4×SSC/0.05% Tween-20 for 5 minutes at 42°C and mounted with antifading solution containing DAPI. Androgenetic origin of each embryo was ascertained by the presence of two Robertsonian translocation chromosomes. Slides were examined with an OLYMPUS fluorescence microscope, and images were captured with a Photometrics CCD camera coupled to IPLab software (Signal Analytics, Vienna, VA). Color channels were merged in Adobe PhotoShop.

RESULTS

Viability and X-inactivation in XX androgenones

To determine whether XX androgenones have a chance of surviving to the egg-cylinder stage, we first studied the genotype of androgenones at E7.5. Androgenetic embryos were produced by pronuclear transplantation between fertilized wild-type embryos and those carrying the *lacZ* transgene (Tan et al., 1993) on the X^P. Seventeen out of 52 reconstituted androgenetic embryos transferred to pseudopregnant females were recovered at E7.5. They showed growth retardation with slightly underdeveloped embryonic region (Fig. 1) consistent with their androgenetic origin (Barton et al., 1984; Kaufman et al., 1989).

A part of the embryonic region cut with a fine glass needle was used for karyotyping, and the remaining part was subjected to X-gal staining of β-gal activity. Five embryos were XX, whereas 12 embryos were XY. In every XX embryo, all tissues including the chorion and yolk-sac endoderm were mosaic of X-gal-positive and X-gal-negative cells. This staining pattern has never been observed in fertilized female embryos hemizygous for *HMG-lacZ* substantiating androgenetic origin of these embryos. Although tissues of embryonic ectoderm origin are mosaic in every female hemizygous for *HMG-lacZ*, the extra-embryonic ectoderm is uniformly β-gal negative when the *HMG-lacZ* is inherited from father, and it is uniformly β-gal positive when the transgene is inherited from mother (Tam et al., 1994). We suggest that the mosaic pattern is the result of random X-inactivation, with the implication that XX androgenones can survive beyond early postimplantation stages and their X chromosomes are capable of undergoing X-inactivation. An example of XX androgenones thus verified is shown in Fig. 1C. Three of XY embryos were uniformly positive and remaining nine were negative for β-gal. Androgenetic origin is evident in β-gal-positive embryos, but it may not always be true in β-gal-negative embryos.

X-inactivation mosaicism in XX androgenones

Since the *HMG-lac*Z transgene alone is not enough to prove the androgenetic origin of manipulated embryos, we employed a Robertsonian translocation involving the X chromosome, Rb2 (Adler et al., 1989) or RX9 (Tease and Fisher, 1991) as an additional marker (Table 1). XX androgenetic embryos generated by pronuclear transplantation should have a metacentric translocation X chromosome and carry the *lacZ* transgene on the other X. A total of 47 presumptive H253+Rb androgenetic embryos were recovered from 57 implantation sites. Although most embryos were retarded for their age (Barton et al., 1984), 10 embryos were large and comparable in size and morphology to normal E7.5 embryos. The ectoplacental cone tissue from each embryo was assayed for β-gal activity to assess X^{lacZ} transmission, and remaining tissues were used for analyzing karyotype and X chromosome replication patterns after they were divided into two (extra-embryonic and embryonic) or three (chorionic, yolk-sac and embryonic) parts depending on their sizes. Classification of all embryos obtained at E7.5 according to the sex chromosome and β-gal expression is shown in Table 1. As predicted from exceptionally good growth, contribution of both the maternal

Table 1. Growth of cytogenetically and histochemically verified E7.5 androgenones produced by pronuclear transplantation between F1♀ × H253♂ and F1♀ × Rb*♂ eggs

Sex chromosome	β-gal expression	Judgement	Growth	Number of embryos
Experimentals				
$X^{Rb}X^N$	+(Mosaic)	Rb♂+H253♂	Abnormal or slight to severe retardation	12
X^NY	+(Homogeneous)	Rb♂+H253♂	Abnormal or slight to severe retardation	8
$X^{Rb}Y$	–	Rb♂+H253♂	Abnormal or slight to severe retardation	11
X^NX^N	–	F1♀+H253♂	Normal	1
X^NY	–	F1♀+H253♂	Normal	9
?‡	+(Mosaic or homogeneous)		Grossly abnormal	13
Controls				
X^NX^N	+(Mosaic)	F1♀+H253♂	Normal	10
X^NY	–	F1♀+H253♂ or F1♀+F1♂	Normal	10

*Rb2 or RX9.
‡Too small to be determined.

and the paternal genome was disclosed in 10 large embryos, suggesting technical errors during the micromanipulatory procedure in this series of experiments. Apparently the error was due to the fact that relatively low reproductive potential of Rb2 and RX9 males forced us to use eggs in which the parental origin of the pronucleus was not clearly defined. Out of 31 karyotypically verified androgenetic embryos 12 were XX and remaining 19 were XY consistent with the expected ratio of 1(XX): 2(XY): 1(YY). YY androgenones should have been lost before implantation as reported previously (Kaufman et al., 1989).

In these XX androgenetic embryos, either the morphologically normal X (X^N) or the X chromosome arm of the Rb2 or RX9 translocation chromosome was replicating asynchronously in informative metaphase cells (Fig. 2). Although the metacentric X chromosome was inactivated more often than the X^N (Table 2), the mean proportion of these two types of cell was not statistically different in comparison to the embryonic and the extra-embryonic regions by Cochran's approximation of Behrens-Fisher test (t'=0.44, $t_{0.05}$=2.45; 0.6<P<0.7). These results

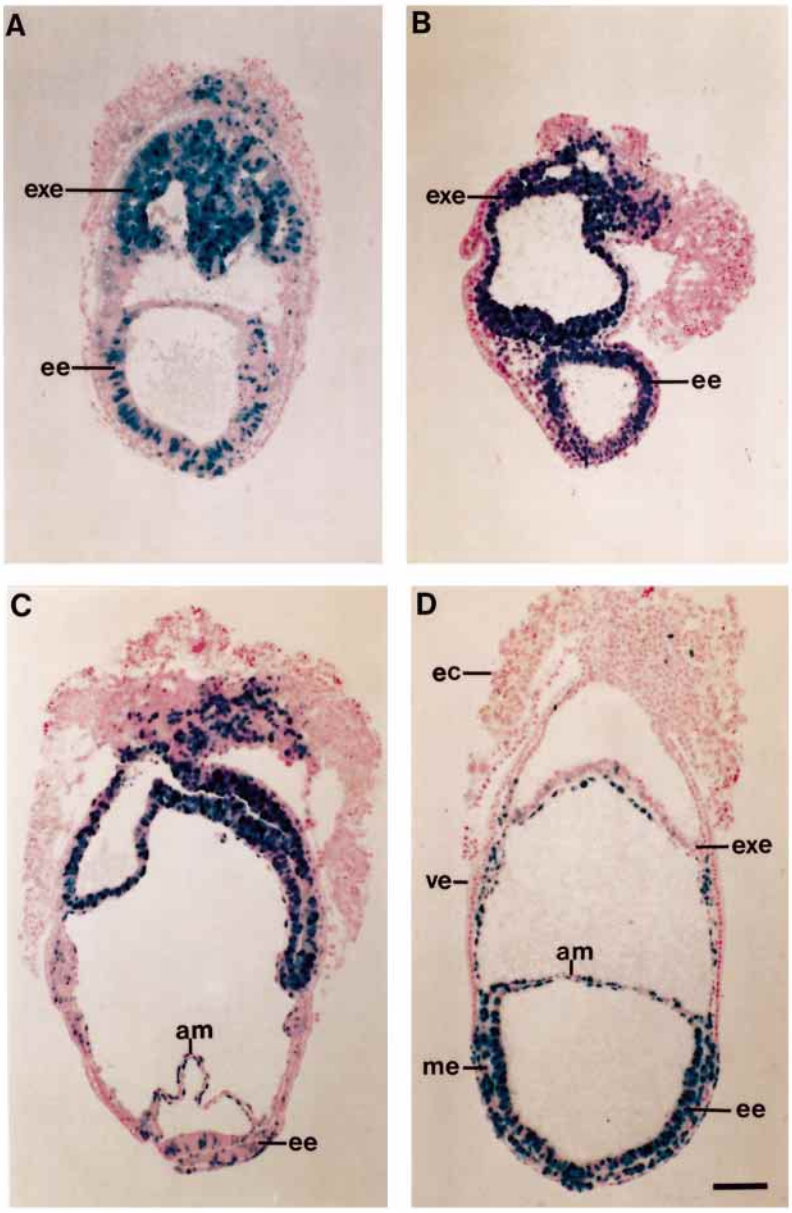

Fig. 1. (A) Mosaic expression of β-gal in E7.5 androgenetic embryos hemizygous for the X-linked *lacZ* transgene. Both embryonic and extra-embryonic ectoderm, together with the ectoplacental cone, display a mosaic staining pattern caused by random X-inactivation. In this particular embryo, β-gal-positive cells were rarely found in the mesoderm. (B) A presumptive $X^{P\text{-}lacZ}Y$ embryo showing uniform β-gal staining in both embryonic and extra-embryonic ectoderm. The β-gal activity is generally low in visceral endoderm both in control as well as in androgenetic embryos. (C) An example of $X^PX^{P\text{-}lacZ}$ embryos verified by karyotyping of the embryonic ectoderm. Mosaic staining is evident in the embryonic ectoderm and in the amnion, yolk-sac mesoderm, extra-embryonic ectoderm and ectoplacental cone. There appears to be a higher proportion of β-gal-positive cells in the extra-embryonic ectoderm. (D) A control E7.5 embryo of $X^MX^{P\text{-}lacZ}$ genotype that is comparable in developmental stage with E7.5 androgenetic embryos shown in C. The control embryo clearly displays mosaicism in the embryonic ectoderm and mesoderm, as well as in extra-embryonic mesoderm. am, amnion; ee, embryonic ectoderm; epc, ectoplacental cone; exe, extra-embryonic ectoderm; me, mesoderm; ve, visceral endoderm. Scale bar, 100 μm.

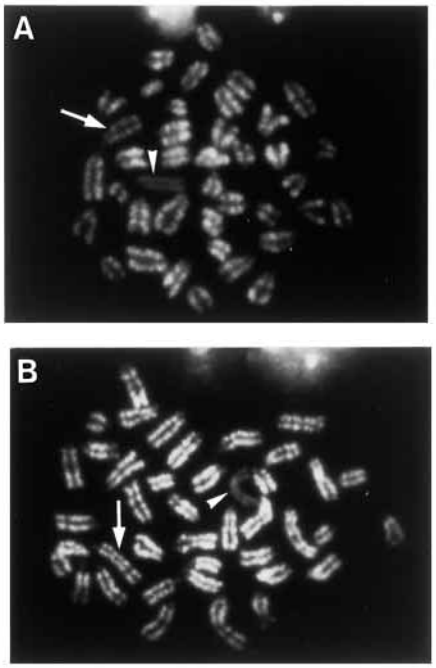

Fig. 2. Cytogenetic evidence of X-inactivation in androgenetic embryos carrying a normal X chromosome marked with the *HMG-lacZ* transgene and translocated Rb(X.2)2Ad. Cells were subjected to continuous incorporation of BrdU followed by staining with Acridine Orange. The normal X chromosome is late replicating in A, whereas the translocated chromosome is late-replicating in B. Synchronously and asynchronously replicating X chromosomes are shown by arrows and arrowheads, respectively.

Table 2. The frequency (%) of metaphase cells that inactivated X^{Rb} chromosome in the embryonic and extra-embryonic region of individual $X^{lacZ}X^{Rb}$ androgenones and $X^{P\text{-}Rb}X^{M}$ controls

	Androgenones		Controls	
Embryo number	Ex. emb.	Emb.	Ex. emb.	Emb.
1	63.4 (101)	75.0 (164)	94.2 (104)	52.4 (246)
2	62.2 (37)	70.1 (67)	95.7 (70)	51.7 (89)
3	78.0 (82)	74.7 (75)	98.6 (145)	53.4 (277)
4	69.4 (85)	73.7 (19)	97.8 (92)	55.3 (85)
5	78.7 (47)	69.0 (58)	98.0 (50)	63.4 (142)
6	67.7 (31)	75.0 (28)	97.7 (86)	66.4 (264)
7	34.3 (67)	63.6 (11)	97.4 (39)	62.1 (124)
8	–	–	98.4 (64)	58.8 (165)
Mean	64.8	71.6	97.2	57.5

Ex. emb., extra-embryonic region; Emb., embryonic region.
The number of cells is in parentheses.

strongly suggest lack of nonrandom inactivation, which occurred in extra-embryonic tissues from fertilized female embryos (t'=6.86, $t_{0.05}$=2.37; P<0.001).

The level of X-inactivation mosaicism was further studied by determining the proportion of β-gal positive cells in the chorionic ectoderm and embryonic ectoderm in nine XX androgenones that showed the androgenone-specific staining pattern (Table 3). Cochran's approximation of Behrens-Fisher test again showed that the mean proportion of X-gal positive cells was not significantly different (t'=0.64, $t_{0.05}$=2.37; 0.5<P<0.6) in these tissues. Taken together, our results point to the conclusion of random X-inactivation in XX androgenones, although further studies are required for clarifying the occasional deviation from apparent random inactivation in the extra-embryonic ectoderm (Table 3).

Table 3. The frequency (%) of β-gal positive cells in the chorionic ectoderm and the embryonic ectoderm of individual $X^{lacZ}X^{Rb}$ androgenones

Embryo	Chorionic ectoderm	Embryonic ectoderm
1	92.5	30.5
2	58.1	46.0
3	83.2	58.9
4	43.2	43.3
5	50.0	52.3
6	43.8	41.3
7	93.2	54.3
8	67.2	57.8
9	43.4	48.6
Mean	63.8	48.1

X-inactivation patterns revealed by Xist RNA FISH

Data obtained so far were consistent with no X-inactivation in XY and random inactivation of a single X chromosome in XX androgenones. However, they did not exclude the possibility that cells with aberrant inactivation patterns had occurred and were subjected to cell selection earlier, as suggested previously (Kay et al., 1994; Latham, 1996). To test this possibility and to elucidate the disagreement among the present and earlier works, we carried out RNA FISH experiments with *Xist* DNA probes on androgenetic embryos at preimplantation stages and at a postimplantation stage of E6.5.

Contrary to normally fertilized embryos, one and two strong *Xist* paint signals were observed in all nuclei of XY and XX putative androgenones, respectively, from the four-cell to the 16-cell stage (Fig. 3A,B,G,H). The sex chromosome constitution was determined by FISH with sex-chromosome-specific painting probes. As expected, YY embryos surviving at these stages did not show any *Xist* RNA signal (Fig. 3C). We believe that the unusual *Xist* RNA FISH pattern observed here proves the androgenetic origin of embryos under study, and we suggest that the paternal *Xist* allele is expressed in these embryos irrespective of the number of X chromosome in a cell. A single paint signal was still observed in about 10% of cells from XY androgenetic blastocysts (Fig. 3I; Table 4).

Table 4. Results of *Xist* RNA FISH in androgenetic and control embryos

Stage		Karyotype	Number of embryos examined	Mean cell number in an embryo	Number of cells with following count of *Xist* paint signal		
					2	1	0
Four cell	Androgenetic	X^PX^P	6	4	24 (100)	0	0
		X^PY	11	4	0	44 (100)	0
		YY	5	4	0	0	20 (100)
	Control	X^MX^P	7	4	0	27 (96.4)	1(3.6)
		X^MY	8	4	0	0	32 (100)
8-16 cell	Androgenetic	X^PX^P	14	10.8	146 (96.7)	5 (3.3)	0
		X^PY	23	11.8	0	261 (96.3)	10 (3.7)
	Control	X^MX^P	10	11.6	0	106 (91.2)	10 (8.6)
		X^MY	11	11.8	0	0	130 (100)
Blastocyst	Androgenetic	X^PX^P	5	84.0	85 (20.2)	211 (50.2)	124 (29.5)
		X^PY	7	86.4	0	58 (9.6)	547 (90.4)
	Control	X^MX^P	5	89.2	0	325 (72.9)	121 (27.1)
		X^MY	6	90.5	0	0	543 (100)
E6.5	Androgenetic	X^PX^P*	3	–	24 (6.9)	305 (88.1)	17 (4.9)
		X^PY*	3	–	0	10 (7.0)	135 (93.1)
	Control	X^MX^P	4	–	0	252 (96.6)	9 (3.4)
		X^MY	3	–	0	0	161 (100)

*Slightly larger in size with fertilized embryos at embryonic day 5.5.

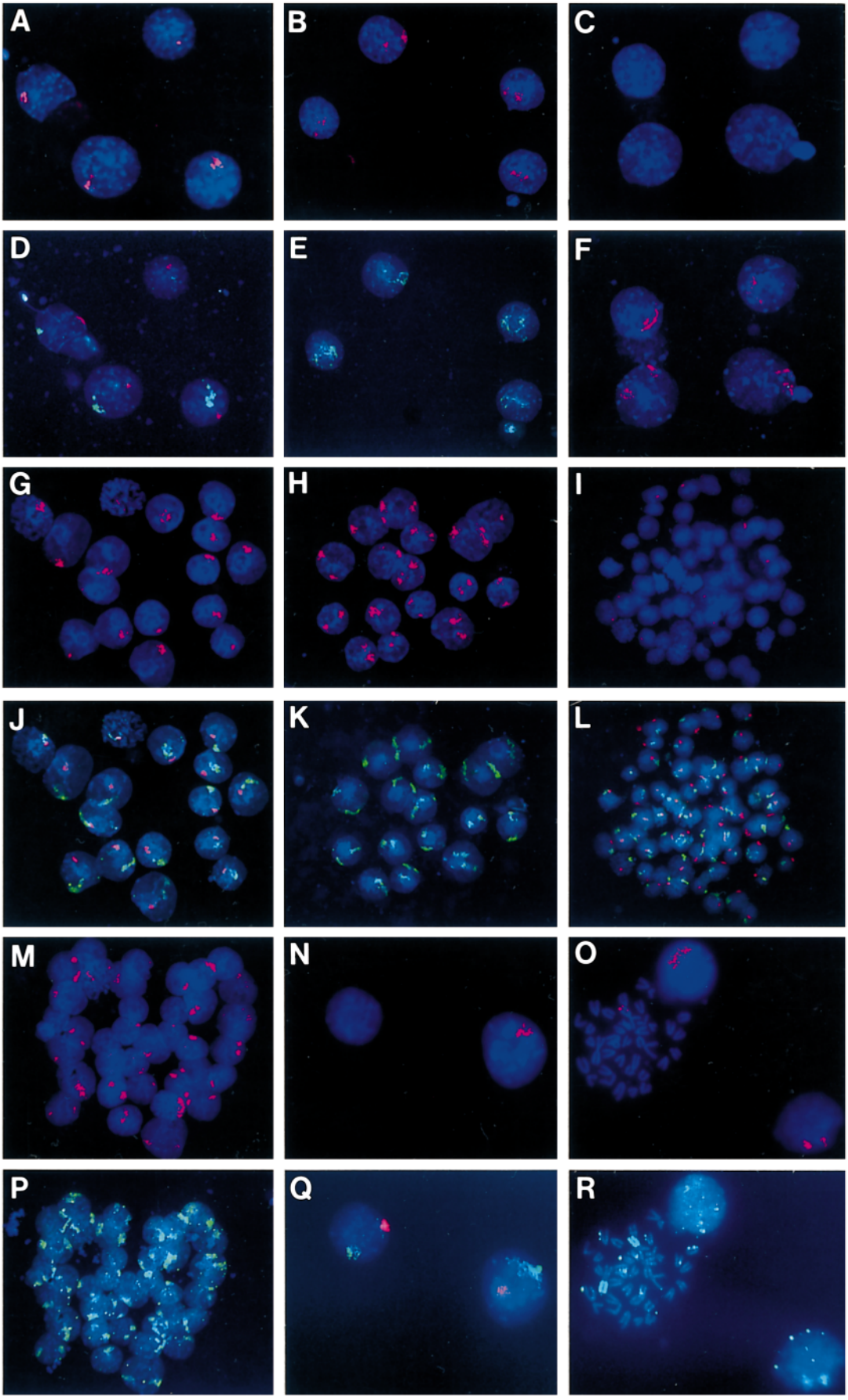

Fig. 3. *Xist* expression in androgenetic embryos revealed by *Xist* RNA FISH. Sex chromosomes were differentially painted in situ with the X-specific (green or blue) and the Y-specific (pink) painting probe to determine the sex chromosome constitution of each embryo subjected to RNA FISH, and to verify obtained signals. A single *Xist* paint signal (pink) is present in nearly all cells from XY embryos from the four-cell (A,D) to 16-cell stage (G,J), whereas two such signals are consistently detected in XX embryos at comparable stages (B,E and H,K). *Xist* signal is never found in YY embryos surviving at the four-cell stage (C,F). One paint signal is found in a proportion of cells from an androgenetic XY blastocysts (I,L), while two such signals are frequently found in XX blastocysts (M,P). XY cells with one paint signal (N,Q) and XX cells with two *Xist* paint signals (O,R) are still present in androgenetic embryos at E 6.5. X chromosome arms involved in Robertsonian translocation are specifically painted in R proving the specificity of the painting probe.

Remaining cells showed a pinpoint signal or no signal at all. In XX androgenetic blastocysts, about 20% of cells had two *Xist* paint signals, 50% had a single paint signal and the remaining 30% had no such signal (Fig. 3M; Table 4). It was recently shown that transcription of stable *Xist* RNA for X-inactivation has not started in epiblast cell lineage of fully expanded blastocysts (Goto and Takagi, 2000). Thirty percent of cells from androgenetic XX blastocysts showing no *Xist* signal may correspond, therefore, to epiblast cells before X-inactivation. Cells having an ectopic paint signal were also found in E6.5 androgenetic embryos, though much less frequently than in blastocysts (Table 4). Androgenetic embryos at this stage were slightly larger than fertilized embryos at E5.5. X-inactivation is completed in normal female embryos by E5.5 to E6.0 (Rastan, 1982; Takagi et al., 1982). It is likely, therefore, that those cells with an ectopic paint signal are functionally nullisomic for the X chromosome, owing to inactivation of every X^P chromosome, yet they still manage to survive in the embryos. A replication banding study of chromosomes in 20 E6.5 androgenones, however, failed to detect any cell that suggests inappropriate X-inactivation. A total of 132 XX and 108 XY androgenetic metaphase cells that incorporated suitable amount of BrdU for chromosome banding had one and no asynchronously replicating X chromosome, respectively. Androgenetic origin of the embryos older than blastocyst was verified by the presence of two Robertsonian metacentric chromosomes in cells at metaphase (Fig. 3R).

DISCUSSION

Several points of interest have emerged from this study. The most remarkable would be that, contrary to the earlier report (Kaufman et al., 1989), XX androgenones like XY counterparts, survive beyond implantation. During preparation of this paper, Obata et al. (2000) reported that mouse XX androgenones produced by in vitro fertilization of enucleated oocytes develop to E9.5. Data presented here and those reported by Obata et al. (2000) suggest that the failure by Kaufman et al. (1989) to find the XX androgenone at E7.5 was most probably a chance observation that was due to a small number of embryos they examined rather than factors such as differences in the genetic background of mice used for experiments. We obtained XX as well as XY androgenetic embryos from combinations of five different mouse stocks. The reported absence of XX androgenones had been corroborated by two opposing observations on *Xist* gene expression, both of which imply involvement of abnormal X-inactivation as a main cause. Kay et al. (1994) reported that *Xist* transcription that beginning at the four-cell stage is turned off by the morula-blastocyst stage resulting in failure of X-inactivation, whereas Latham and Rambhatla (1995) found that *Xist* transcription is maintained uninterruptedly culminating in inactivation of both X chromosomes.

Functional nullisomy and disomy for the X chromosome are extremely harmful to early embryonic development leading to preimplantation lethality or severely unbalanced growth after implantation (Morris, 1968; Takagi and Abe, 1990; Goto and Takagi, 1998). XX androgenones with well-defined embryonic and extra-embryonic structures, notwithstanding growth retardation, therefore predict that X-inactivation occurred successfully at least in a proportion of cells in trophectoderm and primitive endoderm as well as epiblast lineages. This prediction was fully substantiated by identification of an asynchronously replicating X chromosome in most informative cells from E7.5 androgenones. In addition to the embryonic tissues, X-inactivation was random in extra-embryonic tissues that are characterized by imprinted inactivation of the paternal X chromosome. However, the mode of *Xist* expression cast doubts on the consistent randomness of X-inactivation in XX androgenones.

In agreement with data provided by RT-PCR (Latham and Lambhatla, 1995), the present study revealed two paint signals in XX, and one paint signal in XY androgenetic embryos from the four-cell stage onward and they were still present at a considerable frequency in mature blastocysts and implanted embryos at E6.5. In normally fertilized embryos, Sheardown et al. (1997) consistently detected a single *Xist* paint signal in female E5.5 embryos, and never found any *Xist* paint signal in male embryos at the same stage. It is thus likely that X-inactivation or its initiation has finished in every cell of normal female embryos by E5.5 in agreement with earlier cytogenetic data (Rastan, 1982; Takagi et al., 1982). The ectopic *Xist* paint signal in E6.5 androgenetic embryos that are slightly larger than normal embryos at E5.5 probably indicates the occurrence of ectopic X-inactivation, unless it is delayed in androgenetic embryos. Correlation between high *Xist* expression and X-inactivation is suggested in mice with disrupted DNA methyltransferase (*Dnmt1*) genes (Beard et al., 1995; Panning and Jaenisch, 1996). In spite of our extensive effort, we could not find any XX androgenetic cell with two asynchronously replicating X chromosomes, nor any XY cell with a single asynchronously replicating X chromosome at E6.5. However, we can not rule out the possibility that such cells are present but mitotically inactive because of functional nullisomy for the X chromosome. Another intriguing possibility would be that those cells with an ectopic *Xist* paint signal at E6.5 correspond to cells in the reversible step of X-inactivation proposed by Wutz and Jaenisch (2000), and hence the late-replicating X chromosome has yet to be identified.

In fertilized embryos, extra-embryonic cells of trophectoderm origin do not inactivate the X^M, indicating that the imprint on X^M does not allow inactivation (Lyon and Rastan, 1984). Stringency of the imprinting was recently highlighted in mice carrying an *Xist* deletion (Marahrens et al., 1997). Female embryos that inherit the deleted *Xist* allele on the X^M grow normally inactivating X^P selectively, whereas those inherit the mutated *Xist* allele on the X^P die soon after implantation. The defective conceptuses are characterized by poorly developed extra-embryonic tissues, as reported in embryos carrying two X^M chromosomes (Shao and Takagi, 1990; Goto and Takagi, 1998) most probably owing to failure of inactivation. Our recent data (Tada et al., 2000) suggest that such imprint is established during the growth phase of oocytes. In view of the ectopic *Xist* paint signal in XY as well as in XX androgenones, however, it is difficult to rule out the possibility that X^P is inactivated in extra-embryonic tissues of fertilized female embryos because it carries specific imprint that promotes *Xist* expression (Monk and McLaren, 1981).

Available data allow us to propose a sequence of events culminating in accomplishing X-inactivation in androgenetic

embryos. Every X^P chromosome, irrespective of the number in a diploid cell, transcribes stable *Xist* RNA in all cells from the four-cell to the 16-cell stage. Hence, it is likely that the initial *Xist* expression is turned on by a stage-specific cue to which only X^P but not X^M responds. Apparently, counting the number of X chromosomes in a cell is not involved in this process, because stable *Xist* RNA is transcribed even in XY androgenones. Decrease in the frequency of cells showing ectopic *Xist* expression by the blastocyst stage may be resulted from random extinction of *Xist* transcription based on counting the number of the X chromosome in both XX and XY androgenones. Probably, failure in terminating ectopic *Xist* expression may result in inappropriate X-inactivation and eventual cell selection. In fertilized embryos, another stage-specific cue may be necessary for erasing imprint to certify random inactivation in the epiblast lineage of the inner cell mass origin.

It is tempting to postulate that random inactivation in the androgenetic trophectoderm cell is the consequence of choosing an X chromosome that discontinues transcription of stable *Xist* RNA. Models for random X-inactivation proposed for embryonic tissues (Panning et al., 1997; Sheardown et al., 1997; Lee et al., 1999b) also postulate selection of a single X chromosome that turns off *Xist* transcription or destabilize *Xist* RNA. It may be reasonable to assume that the choice is made in a similar manner in both occasions of random inactivation. A series of recent studies has suggested critical roles played by sequences 3′ to *Xist* (Clerc and Avner, 1998) including *Tsix* gene antisense to *Xist* (Lee et al., 1999b) and *DXPas34* locus (Debrand et al., 1999) in the control of *Xist* gene activity, hence X-inactivation. However, we are still ignorant of the roles of various players in the control of X-inactivation. Further analysis of the specific imprint imposed on X^M may contribute to achieve a breakthrough in this interesting problem.

We thank Drs Tomohiro Kono and Yuji Goto for their invaluable technical advice, and Drs Ilse-Dore Adler and Charles Tease for their generous supply of Rb(X.2)2Ad and the Rb(X.9)6H mice. C57BL/6J and CBA/J were kindly supplied by Dr Toshihiko Shiroishi. Mice used for this study were bred at the Center for Experimental Plants and Animals, Hokkaido University. I. Okamoto is a Research Fellow of the Japan Society for the promotion of Science. This study was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. Seong-Seng Tan is funded by a NH & MRC block grant to the Howard Florey Institute, and by a project grant from the Bushell Foundation. We are grateful to anonymous reviewers for helpful suggestions to improve our paper.

REFERENCES

Adler, I.-D., Johannison, R. and Winking, H. (1989). The influence of the Robertsonian translocation Rb(X.2)2Ad on anaphase non-disjunction in male laboratory mice. *Genet. Res*. **53,** 77-86.

Barton, S. C., Surani, M. A. H. and Norris, M. L. (1984). Role of paternal and maternal genomes in mouse development. *Nature* **311,** 374-376.

Barton, S. C., Norris, M. L. and Surani, M. A. H. (1987). Nuclear transplantation in fertilized and parthenogenetically activated eggs. In *Mammalian Development. A Practical Approach.* (ed. M. Monk), pp. 235-253. Oxford: IRL Press.

Beard, C., Li, E. and Jaeniscch, R. (1995). Loss of methylation activates *Xist* in somatic but not in embryonic cells. *Genes Dev*. **9,** 2325-2334.

Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. *Nature* **351,** 325-329.

Brockdorff, N., Ashworth, A., Kay, G. F., Cooper, P., Smith, S., McCabe, V. M., Norris, D. P., Penny, G. D., Patel, D. and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. *Nature* **351,** 329-331.

Brown, C. J., Ballabio, A., Rupert, J. L., Lafreniere, R. G., Grompe, M., Tonlorenzi, R and Willard, H. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* **349,** 38-44.

Clerc, P. and Avner, P. (1998). Role of the region 3′ to *Xist* exon 6 in the counting process of X-chromosome inactivation. *Nature Genet.* **19,** 249-253.

Debrand, E., Chureau, C., Arnaud, D., Avner, P. and Heard, E. (1999). Functinal analysis of the *DXPas34* locus, a 3′ regulator of *Xist* expression. *Mol. Cell. Biol.* **19,** 8513-8525.

Goto, Y. and Takagi, N. (1998). Tetraploid embryos rescue embryonic lethality caused by an additional maternally inherited X chromosome in the mouse. *Development* **125,** 3353-3363.

Goto, Y. and Takagi, N. (2000). Maternally inherited X chromosome is not inactivated in mouse blastocysts due to parental imprinting. *Chromosome. Res.* **8.** 101-109.

Gropp, A., Winking, H., Zech, L. and Muller, H. (1972). Robertsonian chromosomal variation and identification of metacentric chromosomes in feral mice. *Chromosoma* **39,** 265-288.

Gropp, A., Kolbus, U. and Giers, D. (1975) Systematic approach to the study of trisomy in the mouse. II. *Cytogenet. Cell Genet.* **14,** 42-62.

Heard, E., Mongelard, B., Arnaud, D. and Avner, P. (1999a). *Xist* yeast artificial chromosome transgenes function as X-inactivation centers only in multicopy arrays and not as single copies. *Mol. Cell. Biol.* **19,** 3156-3166.

Heard, E., Mongelard, B., Arnaud, D., Chureau, C., Vourc'h, C. and Avner, P. (1999b). Human *XIST* yeast artificial chromosome transgenes show partial X inactivation center function in mouse embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **96,** 6841-6846.

Herzing, L. B. K., Romer, J. T., Horn, J. M. and Ashworth, A. (1997). *Xist* has properties of the X chromosome inactivation center. *Nature* **386,** 272-275.

Johnston, C. M., Nesterova, T. B., Formstone, E. J., Newall, A. E. T., Duthie, S. M., Sheardown, S. A. and Brockdorff, N. (1998). Developmentally regulated *Xist* promoter switch mediates initiation of X inactivation. *Cell* **94,** 809-817.

Kaufman, M. H., Lee, K. K. and Speirs, S. (1989). Post-implantation development and cytogenetic analysis of diandric heterozygous diploid mouse embryos. *Cytogenet. Cell Genet.* **52,**15-18.

Kay, G. F., Barton, S. C., Surani, M. A. and Rastan, S. (1994). Imprinting and X chromosome counting mechanisms determine *Xist* expression in early mouse development. *Cell* **77,** 639-650.

Latham, K. E. (1996). X chromosome imprinting and inactivation in the early mammalian embryos. *Trends Genet*. **12,** 134-138.

Latham, K. E. and Rambhatla, L. (1995). Expression of X-linked genes in androgenetic, gynogenetic and normal mouse preimplantation embryos. *Dev. Genet.* **17,** 212-222.

Lebon, J. M., Tam, P. P. L., Singer-Sam, J., Riggs, A. D. and Tan, S. S. (1995). Mouse endogenous X-linked genes do not show lineage-specific delayed inactivation during development. *Genet. Res*. **65,** 223-227.

Lee, J. T., Strauss, W. M., Dausman, J. A. and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X-inactivation center. *Cell* **86,** 83-94.

Lee, J. T. and Jaenisch, R. (1997). Long-range cis effects of ectopic X-inactivation centres on a mouse autosome. *Nature* **386,** 275-278.

Lee, J. T., Lu, N. F. and Han, Y. (1999a). Genetic analysis of the mouse X-inactivation center reveals an 80kb multifunction domain. *Proc. Natl. Acad. Sci. USA* **96,** 3836-3841.

Lee, J. T., Davidow, L. S. and Warshawsky, D. (1999b). *Tsix,* a gene antisense to *Xist* at the X-inactivation center. *Nature Genet.* **21,** 400-404.

Lyon, M. F. (1961). Gene action in the X chromosome of the mouse (*Mus musculus* L.). *Nature* **190,** 372-373.

Lyon, M. F. and Rastan, S. (1984). Paternal source of chromosome imprinting and its relevance for X chromosome inactivation. *Differentiation* **26,** 63-67.

Marahrens, Y., Panning, B., Dausman, J., Strauss, W. and Jaenisch, R. (1997). *Xist*-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev*. **11,** 156-166.

Marahrens, Y., Loring, J. and Jaenisch, R. (1998). Role of the *Xist* gene in X chromosome choosing. *Cell* **92,** 657-664.

McGrath, J. and Solter, D. (1983). Nuclear transplantation in the mouse by microsurgery and cell fusion. *Science* **220,** 1300-1302.
Monk, M. and McLaren, A. (1981). X-chromosome activity in foetal germ cells of the mouse. *J. Embryol. Exp. Morph.* **63,** 75-84.
Morris, T. (1968). The X0 and 0Y chromosome constitution in the mouse. *Genet. Res.* **12,** 125-137.
Obata, Y., Ono, Y., Akuzawa, H., Kwon, O. Y., Yoshizawa, M. and Kono, T. (2000). Post-implantation development of mouse androgenetic embryos produced by in-vitro fertilization of enucleated oocytes. *Hum. Reprod.* **15,** 874-880.
Panning, B. and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. *Genes Dev.* **10,** 1991-2002.
Panning, B., Dausman, J. and Jaenisch, R. (1997). X chromosome inactivation is mediated by *Xist* RNA stabilization. *Cell* **90,** 907-916.
Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S. and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. *Nature* **379,** 131-137.
Rastan, S. (1982). Timing of X-chromosome inactivation in postimplantation mouse embryos. *J. Embryol. Exp. Morph.* **41,** 11-24.
Sado, T., Tada, T. and Takagi, N. (1996). Mosaic methylation of *Xist* gene before chromosome inactivation in undifferentiated female mouse embryonic stem and embryonic germ cells. *Dev. Dyn.* **205,** 421-434.
Shao, C. and Takagi, N. (1990). An extra maternally derived X chromosome is deleterious to early mouse development. *Development* **110,** 969-976.
Sheardown, S. A., Duthie, S. M., Johnston, C. M., Newall, A. E. T., Formstone, E. J., Arkell, R. M., Nesterova, T. B., Alghisi, G. C., Rastan, S. and Brockdorff, N. (1997). Stabilization of *Xist* RNA mediated initiation of X chromosome inactivation. *Cell* **86,** 83-94.
Sugawara, O., Takagi, N. and Sasaki, M. (1985). Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos. *Cytogenet. Cell Genet.* **39,** 210-219.
Tada, T., Obata, T., Tada, M., Goto, Y., Nakatsuji, N., Tan, S. S., Kono, T. and Takagi, N. (2000). Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. *Development* **127,** 3101-3105.
Takagi, N. and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* **256,** 640-642.
Takagi, N., Sugawara, O. and Sasaki, M. (1982). Regional and temporal changes in the pattern of X-chromosome replication during the early postimplantation development of the female mouse. *Chromosoma* **85,** 275-286.
Takagi, N. and Abe, K. (1990). Detrimental effects of two active X chromosomes on early mouse development. *Development* **109,** 189-201.
Tam, P. P. L., Williams, E. A. and Tan, S. S. (1994). Expression of an X-linked *HMG-lacZ* transgene in mouse embryos: implication of chromosomal imprinting and lineage-specific X-chromosome activity. *Dev. Genet.* **15,** 491-503.
Tan, S. S., Williams, E. A. and Tam, P. P. L. (1993). X chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. *Nat. Genet.* **3,** 170-174.
Tease, C. and Fisher, G. (1991). Two new X-autosome Robertsonian translocations in the mouse. I. Meiotic chromosome segregation in male hemizygotes and female heterozygotes. *Genet. Res.* **58,** 115-121.
Warshawsky, D., Stavropoulos, N. and Lee, J. T. (1999). Further examination of the *Xist* promoter-switch hypothesis in X inactivation; Evidence against the existence and function of P_0 promoter. *Proc. Natl. Acad. Sci. USA* **96,** 14424-14429.
West, J. D., Frels, W. I., Chapman, V. M. and Papaioannou, V. E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell* **12,** 873-882.
Whittingham, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert.* **14,** 7-14.
Wutz, A. and Jaenisch, R. (2000). A shift from reversible to irreversible X chromosome inactivation is triggered during ES cell differentiation. *Mol.Cell* **5,** 695-705.