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Tetraploid embryos rescue embryonic lethality caused by an additional maternally inherited X chromosome in the mouse

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SUMMARY

Mouse embryos with an additional maternally inherited X chromosome, i.e., disomic for X^M (DsX^M), cease to grow early in development and have a deficient extraembryonic region. We hypothesized that the underdeveloped extraembryonic region is attributed to two copies of X^M that escape inactivation due to maternal imprinting. To examine the validity of this hypothesis and throw more light on the significance of X chromosome dosage on cell differentiation, we generated DsX^M($X^MX^MX^P$ and X^MX^MY) embryos at a high frequency taking advantage of the elevated incidence of X chromosome nondisjunction in female mice heterozygous for two Robertsonian X-autosome translocations, Rb(X.2)2Ad and Rb(X.9)6H. Although two X^M chromosomes seem to remain active in both trophectoderm and primitive endoderm, detailed histological examination showed that the polar trophectoderm derivatives (ectoplacental cone and extraembryonic ectoderm) are severely affected, but the primitive endoderm derivatives (visceral and parietal endoderm) are relatively unaffected. Successful rescue of DsX^M embryos by aggregation with tetraploid embryos show that X chromosome inactivation occurred normally leaving one X active in epiblast derivatives. Thus, two copies of active X^M chromosome in cells of the polar trophectoderm cell lineage seem to be the main cause of early lethality shown by DsX^M embryos as a result of failure in formation of ectoplacental cone and extraembryonic ectoderm.

Key words: Sex-chromosome aneuploidy, X chromosome inactivation, Genomic imprinting, Mouse, *lacZ* transgene, Chimera

INTRODUCTION

Studies mainly in humans with sex chromosome abnormalities have shown that X chromosome inactivation (Lyon, 1961), as a dosage compensation mechanism, considerably alleviates the effects of X chromosome aneuploidy in mammals. Indeed, unlike most cases with autosomal duplication which result in pre- or postnatal lethality, humans with up to 5 copies of the X chromosome are viable. The parental origin of the supernumerary X chromosome has apparently no bearing upon phenotypes of patients. The supernumerary X chromosome can be either paternal (X^P) or maternal (X^M) in origin in Klinefelter's syndrome (Jacobs et al., 1988; Lorda-Sanchez et al., 1992), and about 90% of XXX females result from the nondisjunction at female meiosis (May et al., 1990).

In laboratory mice, however, there are at least three lines of evidence to suggest that the parental origin of a supernumerary X chromosome is of crucial importance for the viability of the zygote in spite of prominent phylogenetic conservation of the X chromosome as a whole and apparent universality of X chromosome inactivation systems among mammalian species (Ohno,1967). Firstly, although adult X^MX^PY males are not rare (Cattanach, 1961; Russell and Chu, 1961; Endo et al., 1991), no postnatal X^MX^MY animal has ever been found. A single putative case of an $X^MX^MX^P$ female mouse, however, has been recorded to date (Matsuda and Chapman, 1992). Secondly, zygotes carrying an additional X^M chromosome show abnormal embryonic development with deficient extraembryonic structures (Shao and Takagi, 1990; Tada et al., 1993). And thirdly, female mice doubly heterozygous for Rb(X.2)2Ad and Rb(X.9)6H which share the X chromosome arm in common [i.e., monobrachial homology (MBH) for X chromosome], give rise to a high frequency of embryos with X chromosome aneuploidy, but do not give birth to either XXY or XXX pups (Tease and Fisher, 1993).

During female mouse development, X^M and X^P chromosomes are often distinguished from each other. X chromosome inactivation in mice occurs in three waves; X^P is preferentially inactivated in the first and the second wave that take place in trophectoderm of 3.5 days post coitum (d.p.c.) blastocysts and primitive endoderm of implanting 4.5 d.p.c. embryos, respectively (Takagi and Sasaki, 1975; West et al., 1977). The third wave that occurs in embryonic ectoderm or epiblast of 5.5 d.p.c. embryos is characterized by random inactivation: X^M and X^P are made equal and either of them is inactivated at a nearly equal probability in individual epiblast

cells. Thus, it was hypothesized that X^M is imprinted to remain active during the first two waves of X chromosome inactivation, but this imprinting is erased prior to the third wave of X inactivation (Lyon and Rastan, 1984). Nonrandom inactivation of X^P is not unanimously demonstrated in the extraembryonic tissues in man (Ropers et al., 1978; Migeon et al., 1985; Harrison and Warburton, 1986; Mohandas et al., 1989; Harrison, 1989; Goto et al., 1997). It seems reasonable to postulate that differential viability in man and mouse, of zygotes carrying an additional X chromosome depends on the species-specific differences in the nature of X chromosome imprinting.

Our previous studies (Shao and Takagi, 1990; Tada et al., 1993) making use of female mice carrying a reciprocal X-autosome translocation, T(X;4)37H or a Robertsonian translocation, Rb(X.2)2Ad showed abnormal development of DsX^M embryos, but detailed analysis has not been done because of a low incidence of X chromosome nondisjunction. In this study we intended to further analyze the effects of an additional X^M chromosome on early embryonic development adopting the MBH system of Tease and Fisher (1993) which elevates the incidence of X^MX^MY and $X^MX^MX^P$ embryos considerably. A high frequency of DsX^M embryos allowed us to analyze histological feature of their embryonic development and behavior of DsX^M cells in aggregation chimeras. DsX^M embryos are characterized by severe underdevelopment of ectoplacental cone and extraembryonic ectoderm probably due to abnormal proliferation or differentiation of polar trophectoderm, but no obvious abnormality was observed in the embryonic ectoderm. In agreement with these findings, DsX^M embryos were successfully rescued by aggregation with tetraploid embryos.

MATERIALS AND METHODS

Mouse

We adopted the mating system reported by Tease and Fisher (1993) to obtain DsX^M embryos at a high incidence. This system takes advantage of the fact that nondisjunction of X chromosome occurs at a high frequency in female mice heterozygous for two different X-autosome Robertsonian translocations, Rb(X.2)2Ad (Adler et al., 1989) and Rb(X.9)6H (Tease and Fisher, 1991) which share the X chromosome arm in common as originally shown by Gropp et al. (1975) in autosomes. These females referred to as MBH mice hereafter obtained from reciprocal crosses between homozygous females and hemizygous males were mated with karyotypically normal males.

For producing aggregation chimeras, BCF1[(C57BL/6J× CBA/J)F1], CD-1 and 129-TgR(Rosa26)26Sor (Friedrich and Sariano, 1991; referred to as TgR26Sor hereafter) mice were used. 129-TgR26Sor mice used in this study were derived from an NR-2 ES cell line (kindly supplied by Dr Azim Surani) through blastocyst injection. The NR-2 ES cell line was established from a blastocyst derived by mating a male 129/Sv-TgR26Sor transgenic mouse with a wild-type 129/Sv female (Tada et al. 1998).

Recovery of embryos

Superovulation was induced by an intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin followed 46-48 hours later by an injection of 10 IU human chorionic gonadotropin (Teikoku Hormone, Tokyo). MBH females were housed with chromosomally normal males and mating was ascertained the following morning by the presence of a vaginal plug. The day when the vaginal plug was found was taken as day 0 of pregnancy. Embryos were usually recovered from decidual swellings at 10 a.m. to 3 p.m. from day 5 to day 8 of pregnancy and they were designated as 5.5-8.5 d.p.c. embryos, respectively. Recovered embryos were photographed and processed for the study of their karyotypes and/or histology.

Production of tetraploid embryos

Tetraploid embryos were produced by fusion of blastomeres at the 2-cell stage. Embryos were flushed from oviducts of superovulated CD-1 females 1.5 days after fertilization. Two blastomeres were fused according to the technique described by Cheong et al. (1991) with slight modifications, using an electric cell fusion system SSH-2 (Shimadzu, Kyoto). In practice, the cleavage plane of 2-cell embryos was first oriented in parallel with the electrodes by the application of prolonged alternating current (20 mA), then fusion of the cell membrane was induced by two 80 μsecond pulses of direct current (200 μA) 1 second apart. Embryos were removed from the fusion chamber quickly, washed in M2 medium (Quinn et al., 1982), and cultured in M16 medium (Whittingham, 1971) under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air until aggregation.

Production of aggregation chimeras

Chimeras were produced by a standard procedure involving aggregation of a pair of zona-free 8-cell stage embryos (Mintz et al.,1973). Embryos at this stage were recovered from MBH and BCF1 females mated with BCF1 males, 2.5 days after fertilization. After removal of the zona pellucida in acidic Tyrode solution (Nicolson et al., 1975), embryos were washed in M2 medium and were transferred in pairs (one each of MBH♀×BCF1♂ embryo and BCF1♀×BCF1♂ embryo or two MBH♀×BCF1♂ embryos) to a drop of M2 medium containing 1 per cent of PHA-P(Difco, Detroit) in bacteriological grade Petri dishes. After aggregation and repeated washing in M2 medium, chimeric embryos were cultured in a drop of M16 medium under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. To examine spatial distribution of DsX^M cells in chimeric embryos by X-Gal staining, MBH♀×TgR26Sor♂ embryos were aggregated with BCF1♀×BCF1♂ embryos. In the case of 2n↔4n chimeras, 4- or 8-cell stage embryos derived from MBH mice were aggregated with 4-cell-stage tetraploid CD-1 embryos. Chimeric embryos which developed into blastocysts within 2 days in culture were transferred to the uterus of 2.5-day pseudopregnant females.

Chromosome examination

Recovered postimplantation embryos 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 an atmosphere of 5% CO_2 in air. The duration of incubation was 7.5 hours including the last hour in the presence of 1 μg/ml Colcemid. After hypotonic treatment with 1% sodium citrate, embryos were fixed with 3:1 methanol: acetic acid. Chromosome slides were prepared according to a modification (Takagi et al., 1982) of the air-drying method described by Wroblewska and Dyban (1969). Slides were stained with freshly prepared acridine orange and examined under a fluorescence microscope.

Histological examination

Embryos isolated from decidual swellings were fixed with 2.5% glutaraldehyde in phosphate buffer, postfixed with 1% osmium tetroxide, dehydrated with acetone and embedded in Epon 812. Sections cut at 1-2 μm were stained with 1% toluidine blue.

Staining of β-galactosidase activity for histological examination

2n↔2n chimeric embryos were recovered from foster mother 4 days after transfer. Embryos, mostly equivalent to 6.5 d.p.c. embryos in size, were fixed with 4% paraformaldehyde in phosphate buffer. After

detection of bacterial β-galactosidase (β-gal) activity by X-Gal staining, embryos were dehydrated with ethanol, embedded in JB-4 resin (Polyscience, Warrington, PA), sectioned at 4-5 µm, and counterstained with eosin.

RESULTS

Frequency and phenotypes of DsX^M embryos on day 6 and 7 of pregnancy

We first tried to determine the frequency and phenotypes of DsX^M embryos on day 6 and 7 of pregnancy, because a preliminary study indicated that this is the earliest stage when the effect of an extra copy of X^M becomes discernible under a stereomicroscope. A total of 477 embryos were recovered from 60 MBH females killed 6.5 d.p.c. As shown in Table 1, embryos were classified into three broad phenotypic classes: normal for their developmental age (class I); normal in appearance, but retarded in development by 12-24 hours (class II); and, grossly abnormal in appearance (class III). 83.6% of embryos were class I, whereas 7.3% and 9.0% were classes II and III respectively. Results of karyotype analyses in 477 embryos are summarized in Table 1: 378 (79.2%) embryos were karyotypically balanced, whereas 60 embryos (12.6%) were DsX^M and 36 (7.5%) were X0. Two embryos were diandric triploid, and the last one was trisomic for chromosome 9. Of karyotypically balanced embryos, 15 (4.0%) were developmentally retarded and four (1.1%) were grossly abnormal. In contrast, development of nearly 80% of DsX^M embryos were either retarded or abnormal. The combined frequency of retarded or abnormal embryos was significantly higher in X0 (16.7%) than in chromosomally balanced conceptuses (3.4%).

Findings obtained from 7.5 d.p.c. embryos were roughly comparable to those obtained from 6.5 d.p.c. embryos. Of 475 embryos studied successfully, 366 (77.1%) were chromosomally balanced, 65 (13.7%) were DsX^M, and 38 (8.0%) were X0. Of the remaining six embryos four were triploid (2 digynic and two diandric), one trisomic 9 and one XXX/XX mosaic. These frequencies were not statistically different from those found in 6.5 d.p.c. embryos (χ^2=0.25, 0.5<P<0.7), suggesting that there was very few, if any, loss of DsX^M embryos between day 6 and day 7 of pregnancy. There was a tendency for the level of growth retardation and developmental abnormalities to become more severe in 7.5 d.p.c. embryos. Thus, postimplantation development of all DsX^M embryos was disturbed, and more than 80% embryos were grossly abnormal. Furthermore, about 45% of X0 conceptuses showed growth retardation. A similar tendency was evident in karyotypically balanced embryos; the combined frequency of developmentally retarded and abnormal conceptuses was 7.1% on day 7 but it was 3.4% on day 6 of pregnancy.

A majority of day 6 and day 7 DsX^M embryos had a characteristic morphology of stunted growth with complete lack or severe deficiency of the extraembryonic region as seen under a stereomicroscope (Fig. 1). The mean long axis of such embryos was 62.3% (range, 37-100%), that of normally growing littermates on day 6, whereas it was reduced to 39.5% (range, 28-55%) on day 7. Retarded and abnormal embryos having a balanced karyotype or triploid embryos were usually distinguishable from DsX^M embryos because the former embryos were either extremely small or had a distinct extraembryonic region. Thus, it may be concluded that DsX^M embryos can be identified under a stereomicroscope with considerable accuracy except for a small number of day 6 embryos.

Histological characters of DsX^M embryos on day 6 to 8 of pregnancy

A total of 312 embryos from 36 MBH females mated with chromosomally normal males were histologically examined on day 6 of pregnancy. We also determined the karyotype in 69 of 312 embryos using cells of the ectoplacental cone. Six of these 69 embryos were DsX^M, and were characterized by the lack or extreme underdevelopment of extraembryonic ectoderm. Fourteen of remaining 243 embryos whose karyotypes were not examined showed similar histological characters and they were considered DsX^M.

Table 1. Gross morphology of embryos obtained from MBH females

Gross morphology	No. of embryos				
	Normal	X0	DsX^M	Others	Total
6.5 dpc					
normal	365	30	3	1	399 (83.6%)
retarded	10	5	18	2	35 (7.3%)
abnormal	3	1	39	0	43 (9.0%)
Total	378 (79.2%)	36 (7.5%)	60 (12.6%)	3 (0.6%)	477
7.5 dpc					
normal	340	21	0	2	363 (76.4%)
retarded	15	17	8	3	(43) (9.1%)
abnormal	11	0	57	1	69 (14.5%)
Total	366 (77.1%)	38 (8.0%)	65 (13.7%)	6 (1.3%)	475

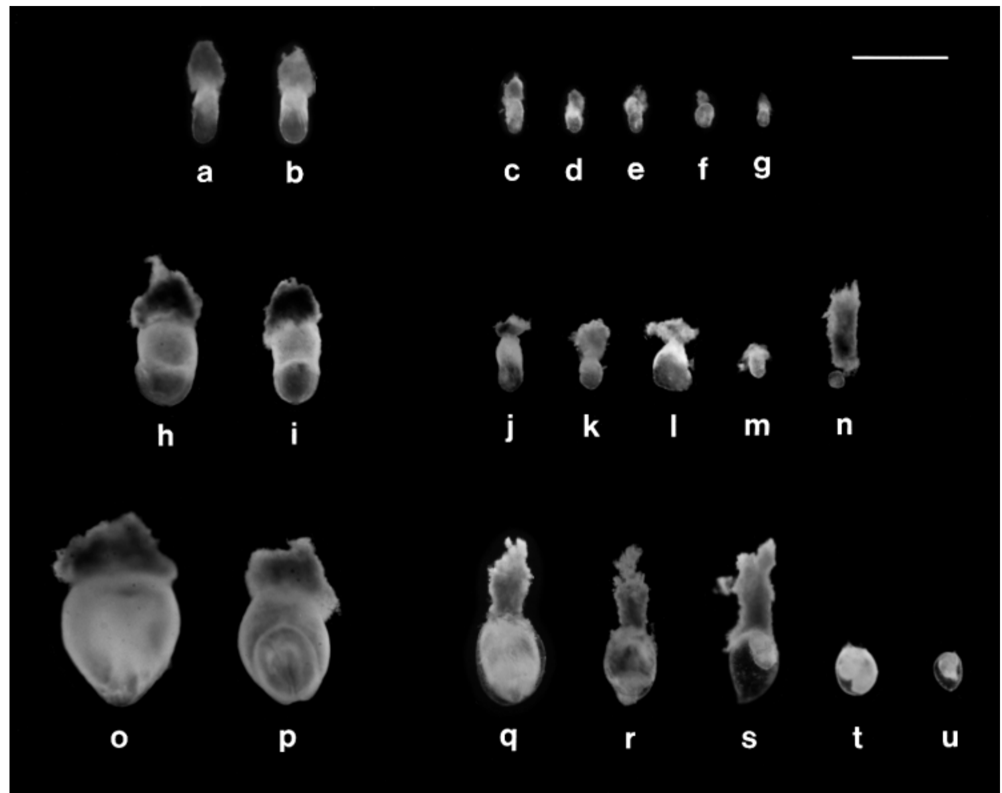

Fig. 1. Gross morphology of mouse embryos recovered at 6.5 (a-g), 7.5 (h-n), 8.5 (o-u) d.p.c. DsX^M embryos are shown on the right and normally grown embryos on the left for comparison. Main characteristics of 6.5 and 7.5 d.p.c. DsX^M embryos are growth retardation (c and j) and remarkable developmental abnormality (d-g and k-n). Scale bar,1.0 mm.

DsX^M embryos were almost equivalent in size and morphology to the embryonic region of the normally grown embryos at this stage (Fig. 2A,B). The most remarkable abnormality found at the histological level was lack of tissues originated from the polar trophectoderm (Fig. 2B). Thus, there were virtually no diploid trophoblasts in the underdeveloped ectoplacental cone, and neither were there recognizable extraembryonic ectodermal cells or extraembryonic visceral endoderm cells present at the vestigial extraembryonic region. Parietal endoderm cells were abundant on the inner surface of Reichert's membrane which was often thickened locally.

In addition to the DsX^M embryos, the histological examination revealed a high frequency of a distinct class of abnormal embryos. They were apparently normal by their gross morphology, but the embryonic ectoderm layer was disintegrated into single cells which were scattered throughout the embryonic region (Fig. 2C). The embryonic visceral endoderm layer was also affected, apparently losing connection with neighboring cells. Our preliminary study showed that this peculiar condition is not attributable to a specific chromosome aberration. These embryos will be a subject of a separate paper after further study.

The structure of five putative day 7 DsX^M embryos were basically the same as that of day 6 embryos (Fig. 3B). The

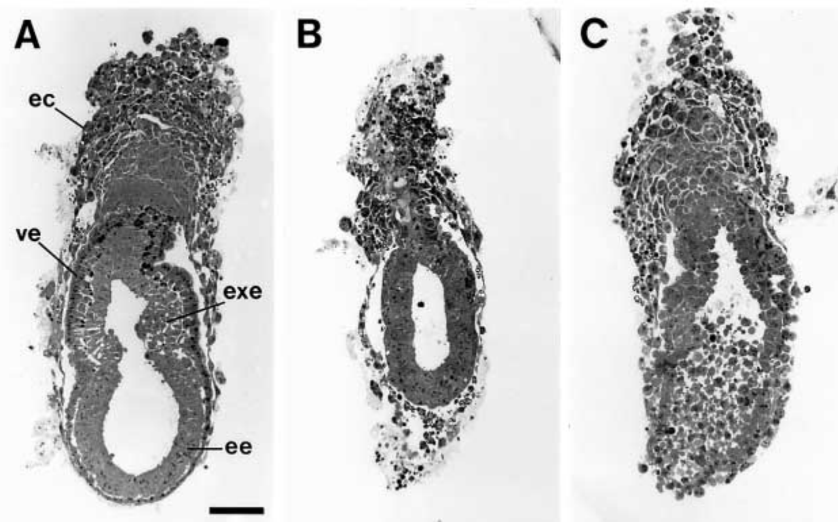

Fig. 2. Histological sections of 6.5 d.p.c. embryos recovered from MBH females mated with karyotypically normal males. (A) A normally grown embryo, (B) a putative DsX^M embryo, (C) a distinctly abnormal embryo with the embryonic region disintegrated into single cells. ec, ectoplacental cone; exe, extraembryonic ectoderm; ve, visceral endoderm; ee, embryonic ectoderm. Scale bar, 100 μm.

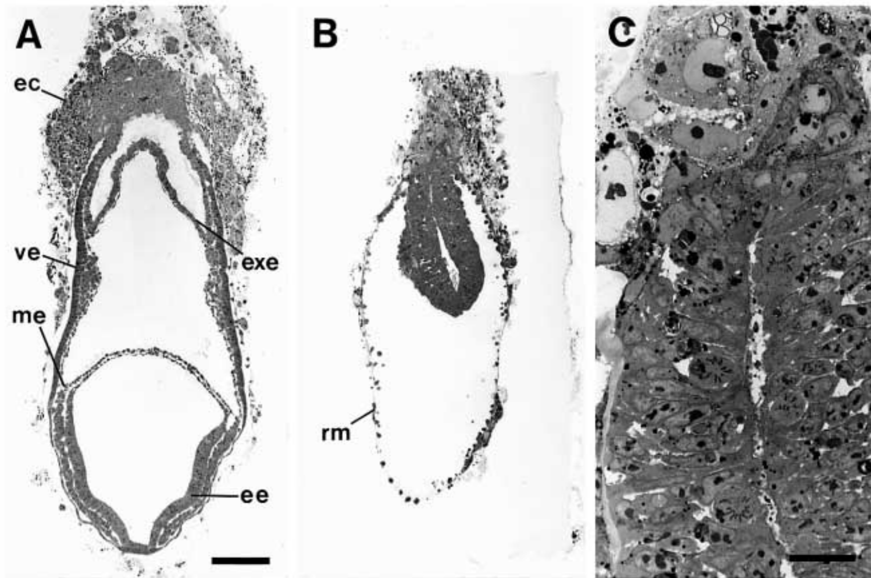

Fig. 3. Histological sections of 7.5 d.p.c. embryos recovered from MBH females mated with karyotypically normal males. (A) A normally grown embryo, (B) a putative DsX^M embryo (scale bar,100 μm), (C) higher magnification of B showing structural features typical of embryonic ectoderm, lack of extraembryonic ectoderm and deficient ectoplacental cone (scale bar,20 μm). ec, ectoplacental cone; exe, extraembryonic ectoderm; ve, visceral endoderm; ee, embryonic ectoderm; me, mesoderm; rm, Reichert's membrane.

embryonic ectoderm covered by a visceral endoderm layer was linked to underdeveloped ectoplacental cone by a tiny, if any, extraembryonic region. Although the mitotic activity was still high in embryonic ectoderm (Fig. 3C), mesoderm was not formed in any embryo. No cell debris were observed in the proamniotic cavity suggesting cell death was not occurring extensively.

Ten putative DsX^M embryos studied histologically on day 8 were divided into two categories on the basis of their sizes and histological characters. Two embryos were larger than the remaining eight embryos. Two larger ones appeared to represent the most advanced stage of development that could be attained by DsX^M embryos. As shown in Fig. 4A, the embryo had tiny extraembryonic ectoderm (chorionic ectoderm). The substantial mesoderm layer was present and a two-layered amnion was formed, though it was less well organized than in normally grown day 7 embryos as shown in Fig. 3A. The amniotic cavity lined with embryonic ectoderm was similar to that in the normal day 7 embryos, but mesoderm lined exocoelom in a disorderly fashion. Embryos of the smaller class were tiny spherical vesicles, without any extraembryonic structure, consisting of the inner layer of the embryonic ectoderm and the outer layer of the visceral endoderm enclosed in a small sac of Reichert's membrane. These embryos with cell debris in the proamniotic cavity were smaller than most day 7 embryos indicating extensive cell death (Fig. 4B).

Histological identification of 5.5 d.p.c. DsX^M embryos

To obtain insight into the initial developmental defect caused by DsX^M, 159 embryos recovered from MBH females 5.5 d.p.c. were examined histologically. Based on the above findings, we looked for embryos with abnormal or underdeveloped extraembryonic regions as candidate DsX^M embryos. Eighteen embryos (11.3%) fulfilled this requirement to varying degrees. Putative DsX^M embryos are shown in Fig. 5. One of the most consistent traits was atrophic extraembryonic visceral endoderm. The endoderm layer, if present, was discontinuous, consisting of reduced number of cells. Extraembryonic ectoderm usually lined by the extraembryonic endoderm was either absent (Fig. 5C), diminutive or normal in size. Signs of degeneration were evident even in embryos with the normal-sized extraembryonic region (Fig. 5B). Extraembryonic ectoderm in these embryos was degenerate with irregularly oriented, apparently dead or dying cells with or without vacuoles. In contrast to this region, the extreme end of ectoplacental cone still appeared healthy. Similar to older embryos, embryonic visceral endoderm was locally thickened as early as day 5 of pregnancy.

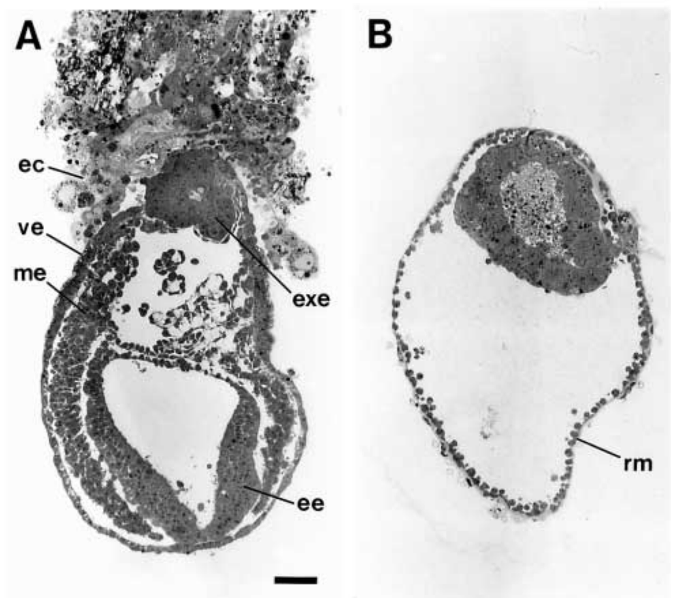

Fig. 4. Histological sections of 8.5 d.p.c. putative DsX^M embryos. (A) An exceptionally well grown DsX^M embryo similar to the one shown as Fig. 1q, (B) a tiny spherical embryo with cell debris in the central cavity. ec, ectoplacental cone; exe, extraembryonic ectoderm; ve, visceral endoderm; ee, embryonic ectoderm; me, mesoderm; rm, Reichert's membrane. Scale bar, 100 μm.

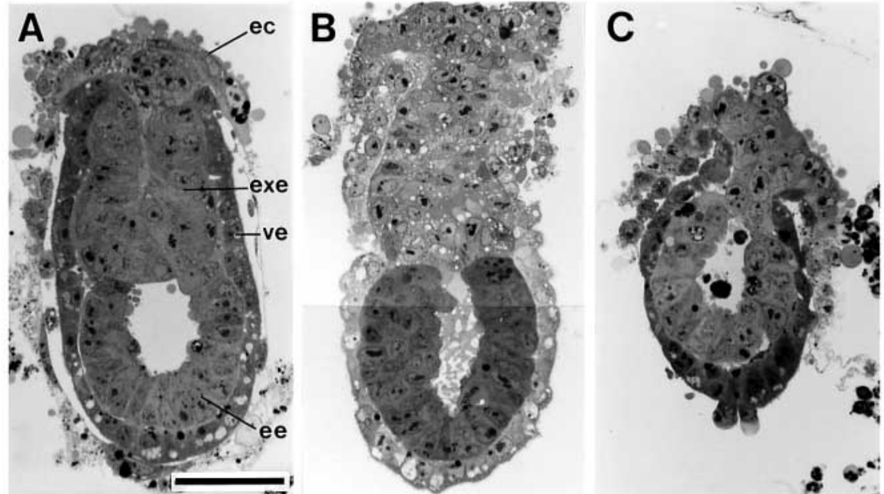

Fig. 5. Histological sections of 5.5 d.p.c. embryos recovered from MBH females. (A) A normally grown embryo with a distinct proamniotic cavity, (B) a putative DsX^M embryo characterized by degenerating extraembryonic ectoderm and visceral endoderm with cell debris in the proamniotic cavity. Ectoplacental cone cells are affected less severely than extraembryonic ectoderm cells. (C) Another putative DsX^M embryo with an extremely underdeveloped extraembryonic region. ec, ectoplacental cone; exe, extraembryonic ectoderm; ve, visceral endoderm; ee, embryonic ectoderm. Scale bar, 50 μm.

If we take 13% (i.e. 125/952 found in 6.5 and 7.5 d.p.c. embryos) as an expected frequency of DsX^M embryos in this cross, there should have been 2 or 3 DsX^M embryos that escaped detection because histological abnormality was too subtle on day 5. The number could be more, because a low proportion of chromosomally balanced or X0 embryos might have been classified as putative DsX^M embryos. Developmental abnormality must become evident within 24 hours in such embryos.

Minimal selection against DsX^M embryo by day 8 of pregnancy

The preimplantation and postimplantation losses of embryos were estimated from the difference between the number of corpora lutea and the number of implantation sites, and the number of implantation sites and the number of live embryos, respectively (Table 2). It is evident that new postimplantation loss did not occur during the period of day 6 to day 8 of pregnancy. The frequencies of $X^MX^MX^P$, X^MX^MY and X^P0 embryos was nearly equal from 6.5 to 8.5 d.p.c. (Table 2). These results again suggest that these three classes of embryos occurred at an equal frequency and most of them survived by day 8 of pregnancy. 0Y embryos expected to occur at the same frequency should have been lost before implantation (Morris, 1968).

X chromosome inactivation in DsX^M embryos

Previous studies showed that two maternally derived X chromosomes remained active in a certain proportion of cells from $X^MX^MX^P$ and X^MX^MY embryos. These data, though consistent, were still meager, hence we examined again the X chromosome replication pattern in 21 $X^MX^MX^P$ and X^MX^MY embryos in which identification of the X chromosome was easy because of the Robertsonian translocation.

Embryos were arbitrarily cut into the extraembryonic and the embryonic region immediately before slide preparation. Due to small extraembryonic structures, 'the extraembryonic regions' thus prepared should have contained considerable proportion of cells from the embryonic ectoderm. In nine $X^MX^MX^P$ embryos at 6.5 and 7.5 d.p.c., only a single X^P was asynchronously replicating in 20 of 53 (38.0%) metaphases from the extraembryonic part. Two maternally inherited X chromosomes carrying the autosomal arm were replicating synchronously in all of these cells. In the remaining cells two X chromosomes were asynchronously replicating in a random fashion. In the embryonic part, however, two of three X chromosomes replicated asynchronously in most (117 of 120) metaphases. Replication asynchrony was limited to the single X^P only in the remaining 3 cells from the embryonic part possibly representing visceral endoderm (Table 3 and Fig. 6A, B). A similar tendency was evident in 12 X^MX^MY embryos at 6.5 and 7.5 d.p.c.: no X chromosome was asynchronously replicating in 24 of 71 (34.0%) metaphases from the extraembryonic part whereas the Y chromosome was clearly late replicating. A single X chromosome was replicating asynchronously in the remaining cells from the extraembryonic part and most metaphases (131 of 136) from the embryonic part (Table 3 and Fig. 6C,D). The Y chromosome was consistently late replicating in these cells. These results strongly support the previous view that the maternally inherited X chromosome is not inactivated in the trophectoderm and possibly also in primitive endoderm lineages, and that two copies of active X chromosome in the extraembryonic structures are responsible for the abnormal development of DsX^M embryos.

Table 2. Pre- and post-implantation loss in litters produced by MBH females mated with chromosomally normal males

Stage of embryo	No. of females	No. of ovulated eggs	No. of implants	No. of embryos
5.5 day	30	244	223 (91.4%)	210 (94.2%)
6.5 day	122	1022	966 (94.5%)	926 (95.9%)
7.5 day	92	633	612 (92.6%)	583 (95.0%)
8.5 day	36	242	228 (94.2%)	223 (97.8%)

Distribution of DsX^M cells in chimeric embryos produced by embryo aggregation

Histological and cytogenetic studies revealed that the underdevelopment of extraembryonic structures is the main deleterious effect of DsX^M. The apparently uneventful growth of the embryonic ectoderm of the DsX^M embryos suggests that they might be able to develop normally, if the extraembryonic

Table 3. Asynchronously replicating X-chromosome in embryos recovered from MBH females

Karyotype	No. of embryos	Tissue	X^P		X^MX^P	X^MX^M	X^M	none	Total
			P	L					
40, X^MX^P	9	extraembryonic	173	98	–	–	18	5	294
		embryonic	34	121	–	–	168	5	328
41,$X^MX^MX^P$	9	extraembryonic	9 (38.0%)	11	28	5	0	0	53
		embryonic	0 (2.5%)	3	83	34	0	0	120
41,X^MX^MY	12	extraembryonic	–	–	–	–	47	24 (34.0%)	71
		embryonic	–	–	–	–	131	5 (3.7%)	136

P, precocious replication; L, late replication.

tissues are supplied by other embryos. To test this possibility, we set out to produce $DsX^M \leftrightarrow 2n$ chimeric embryos.

In a total of 149 $2n \leftrightarrow 2n$ chimeric embryos produced by aggregation of 8-cell embryos from MBH and BCF1 females, we never found any with the phenotype typical of DsX^M embryos under a stereomicroscope. This finding suggests rescue of DsX^M embryos by wild-type cells. To identify $DsX^M \leftrightarrow 2n$ chimeras and to estimate their chimeric composition, embryos comparable in size to those at 6.5-7.5 d.p.c. were studied cytogenetically examining the extraembryonic and the embryonic region separately. Eighteen of 106 embryos examined were $DsX^M \leftrightarrow$normal chimeras. The relative contribution of DsX^M cells in these chimeric embryos is summarized in Fig. 7. The proportion of DsX^M cells is extremely low in extraembryonic tissues of all $DsX^M \leftrightarrow 2n$ chimeras. Differences in chimeric composition between the embryonic and the extraembryonic region in individual embryos were statistically significant in all but one chimera. In the exceptional chimera, the proportion of cells disomic for X^M was low in both regions. In the control chimeric embryos, the proportion of cells contributed by an MBH-derived embryo was roughly equal to that of BCF1-derived embryos both in the extraembryonic and the embryonic tissue.

Cytogenetic examination, though, showed possible lack of DsX^M cells from the extraembryonic region, did not permit very good spatial resolution of chimerism. Consequently, we made $DsX^M \leftrightarrow 2n$ chimeras to visualize distribution of DsX^M cells in histological sections by using mice carrying bacterial *lacZ* transgenes. MBH females were mated with a TgR26Sor transgenic male mouse to obtain embryos positive for β-gal activity. In 70 of 75 chimeric embryos, β-gal positive cells were detected in both the embryonic and the extraembryonic tissues (Fig. 8A). 24 of them were $2n \leftrightarrow 2n$ chimeras without exception. In five chimeric embryos, β-gal-positive cells were uniformly absent from extraembryonic tissues (Fig. 8B). Simultaneous cytogenetic study in three of them showed that two were indeed $X^MX^MY \leftrightarrow XX$ chimeras and the remaining one was an $X^MX^MX^P \leftrightarrow XY$ chimera.

One each of $X^MX^MX^P \leftrightarrow XX$ and $X^MX^MY \leftrightarrow XY$ chimeric pups were born alive. Cytogenetic examination of tail fibroblasts showed that these chimeras contained about 40% and 43% DsX^M cells, respectively. The $X^MX^MX^P \leftrightarrow XX$ chimeric mouse was fertile, but the $X^MX^MY \leftrightarrow XY$ chimera

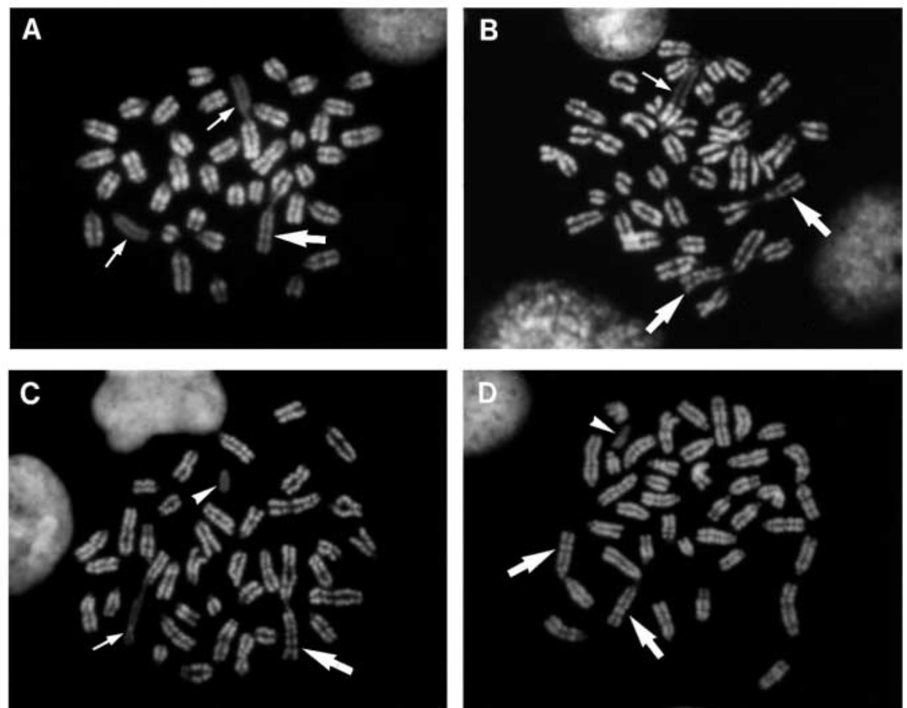

Fig. 6. Representative X chromosome replication patterns revealed in cells from DsX^M embryos by continuous incorporation of BrdU immediately before fixation followed by acridine orange staining. (A,B) Metaphase cells from the $X^MX^MX^P$ embryo. The X chromosome inherited from the mother is marked by Robertsonian centromeric fusion. One X^M in A and two X^M in B replicated synchronously. (C,D) Metaphase spreads from X^MX^MY embryos. One X^M in C and two X^M in D replicated synchronously, while the Y chromosome consistently replicated late. Synchronously and asynchronously replicating X chromosomes are indicated by large and small arrows, respectively. Arrowheads indicate Y chromosomes.

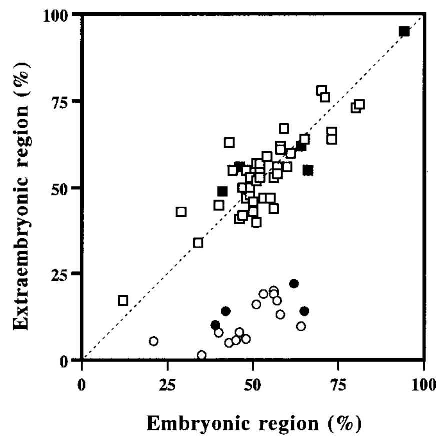

Fig. 7. A scatter diagram showing that cells disomic for X^M frequently contribute to the embryonic region but much less frequently to the extraembryonic region in chimeric embryos cytogenetically examined at stages comparable to 6.5-7.5 d.p.c. □, 2n (MBH×BCF1)↔2n (BCF1×BCF1) chimeras; ■, 2n (MBH×BCF1)↔2n (MBH×BCF1) chimeras; ○, DsX^M (MBH×BCF1)↔2n (MBH×BCF1) chimeras; ●, DsX^M (MBH×BCF1)↔2n (MBH×BCF1) chimeras.

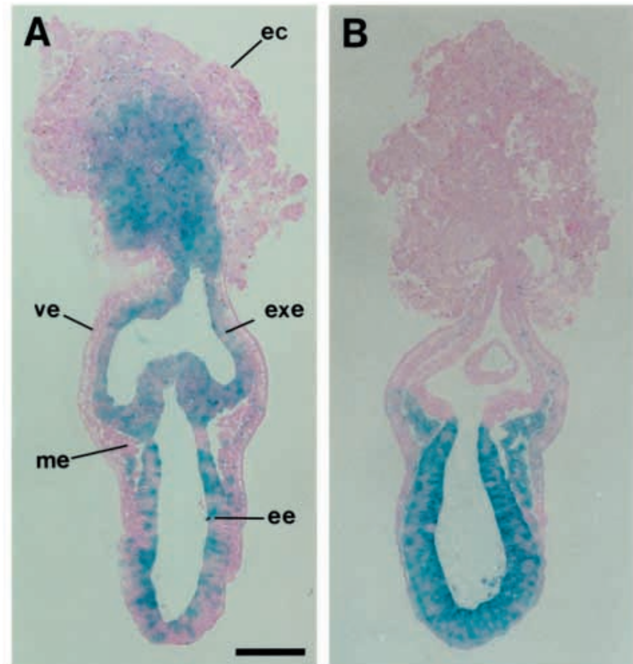

Fig. 8. Histological sections of X-Gal stained 6.5 d.p.c. chimeric embryos produced by aggregation of two 8-cell embryos, one from the cross MBH×TgR26Sor and the other from the cross BCF1×BCF1. (A) A putative karyotypically normal↔normal chimera with a substantial complement of β-gal-positive cells throughout the embryo. The visceral endoderm layer is negative for X-gal staining even in TgR26Sor embryos. (B) A putative DsX^M↔2n chimera without any contribution of β-gal-positive DsX^M cells to extraembryonic ectoderm and ectoplacental cone. Scale bar, 100 µm.

was sterile with tiny testes. These findings suggest that extraembryonic tissues but not embryonic tissues are the main target of deleterious effects of a supernumerary X^M.

Rescue of DsX^M embryos by aggregation with tetraploid embryos

To test the validity of the above findings, we provided DsX^M embryos with extraembryonic tissues by aggregating them with tetraploid embryos. Previous studies revealed that the contribution of tetraploid cells in 2n↔4n chimeric embryos is restricted to the extraembryonic tissues (Tarkowski et al., 1977; Nagy et al., 1990, 1993; Allen et al., 1994; Guillemot et al., 1994; James et al., 1995).

A total of 90 2n(MBH derived)↔4n (CD-1) chimeric embryos produced were transferred to the uteri of pseudopregnant females. Since MBH mice are agouti and CD-1 mice are albino, the contribution of tetraploid cells in chimeric mice could be readily estimated from the coat color chimerism. Thirty of 32 pups born were phenotypically agouti without any albino hair, but the remaining 2 pups had extensively chimeric coats. Chromosome examination of tail fibroblasts ascertained that one agouti pup had X^MX^MY and another agouti pup had $X^MX^MX^P$ sex chromosome constitution in every cell demonstrating that DsX^M conceptuses can survive parturition, if only they were supplemented with extraembryonic tissues. Chromosome examination was successful in one of two overt chimeras thus far. In addition to 134 diploid, 18 tetraploid $X^MX^MX^P$ cells, we indeed identified 11 tetraploid CD-1 (XXYY) cells in cultured tail tip. Further study is now under way to further clarify the contribution of tetraploid cells in 2n ↔ 4n chimeras.

DISCUSSION

Tetraploid rescue of DsX^M embryos

Previous studies showed that mouse embryos having an additional maternal X chromosome are, unlike human cases, incapable of completing embryonic development, although adult mice carrying an additional paternal X chromosome are not rare. Contrary to these findings, Matsuda and Chapman (1992) reported a single $X^MX^MX^P$ female among 200 interspecific backcross progenies between laboratory mouse (C57BL/6Ros) and *Mus spretus* [(B6×Spretus)F1×B6]. They undertook to determine parental origin of the additional X chromosome by in situ hybridization with the major satellite DNA probe and local repeated sequence DXSmh 141, and Southern hybridization with X linked genes *Pgk-1, Ags* and *Amg* (gene order: centromere–DXSmh141–*Pgk-1*–*Ags*–*Amg*). The centromeric satellite DNA and DXSmh141 sequences were uniformly of the B6 type, but the *M. spretus* allelic forms of *Ags* and *Amg* were detected with the relative intensity less than those of the B6 form. Their data are compatible with nondisjunction at meiosis II in the heterozygous female and nondisjunction at meiosis II during spermatogenesis in the B6 male. Based on the historical records, nondisjunction in the F_1 female was favored (Matsuda and Chapman, 1992), but consistent and severe growth defects, as discussed in this paper, and lack of any reliable postnatal DsX^M mouse other than this putative case strongly advocate that nondisjunction must have occurred in the B6 male.

The present study demonstrated that DsX^M embryos can be rescued by aggregation with tetraploid embryos at the 4- to 8-cell stage. Using coat color and tail fibroblasts to determine

DsX^M mice, it is apparent that they can survive after parturition without any cellular contribution from tetraploid embryos. Thus, the distinct consequence of an additional maternal X chromosome in human and mouse is more apparent than real, and a supernumerary X^M chromosome is harmful only in certain extraembryonic structure(s). Accordingly, DsX^M embryos would be invaluable tools for analyzing the role of X chromosome inactivation in mouse embryogenesis, and the nature and stability of genomic imprinting.

Developmental abnormalities in DsX^M embryos

We were able to examine development of DsX^M embryos in considerable detail for the first time because the incidence of such embryos was reasonably high among offspring of MBH mice (Tease and Fisher, 1993). It has been shown that DsX^M embryos are characterized by a deficient extraembryonic region (Shao and Takagi, 1990; Tada et al., 1993). The present histological study further demonstrated that extraembryonic ectoderm and ectoplacental cone, derivatives of polar trophectoderm, already poorly developed in most DsX^M embryos as early as day 5 of pregnancy. Polar trophectoderm maintains diploidy and proliferation potential by signals emanated from continuous inner cell mass (ICM) (Gardner et al.,1973; Copp, 1979). Trophectoderm cells not in contact with ICM cells transform into giant trophoblasts. Thus, it may be speculated that the DsX^M polar trophectoderm cells fail to respond to signals from the ICM, or they proliferate in response to the signals but fail to undergo proper cell differentiation. We found several 5.5 d.p.c. embryos whose histological features support aberrant cell differentiation, but lack of karyotypic data prevented us from drawing a definitive conclusion.

In contrast to the vestigial extraembryonic structures, the embryonic region grows well by 6.5 d.p.c. in DsX^M embryos. Developmental delay in DsX^M embryos becomes obvious on day 7, with no mesoderm formation irrespective of their sizes. In view of the fact that DsX^M embryos are rescued by tetraploid embryos without any somatic contribution from them, it would be difficult to think of any sound reason for sudden growth interruption of DsX^M embryos on day 7 of pregnancy except the shortage of nutrition. It is tempting to speculate that embryonic growth later than 6.5 d.p.c. depends largely on the nutrients supplied through the ectoplacental cone. Mesoderm differentiation occurred in only two 8.5 d.p.c. putative DsX^M embryos with a small mass of extraembryonic ectoderm cells. Absence of mesoderm formation in a majority of vesicular DsX^M embryos suggests that certain extraembryonic structures such as extraembryonic visceral endoderm and extraembryonic ectoderm are needed for the initiation of gastrulation.

Developmental effects of two copies of active X chromosome

A considerable proportion of DsX^M cells had two active X^M chromosomes (Shao and Takagi, 1990; Tada et al., 1993). Since X^P chromosome is preferentially inactivated in extraembryonic tissues of the XX embryo (Takagi and Sasaki,1975; West et al., 1977), it was suggested (Shao and Takagi, 1990; Tada et al., 1993) that two copies of the X chromosome that was forced to remain active by maternal imprinting were responsible for the abnormal development in DsX^M embryos. Another less likely possibility would be that overdosage of maternally imprinted X-linked gene(s) that escape inactivation is responsible for the abnormal growth of DsX^M embryos.

Xist gene has been shown to be required in *cis* for the occurrence of X inactivation in murine female embryonic stem cells with targeted deletion in the gene body (Penny et al., 1996). Recently reported *Xist* knockout mice (Marahrens et al., 1997) strongly support the absence of X chromosome inactivation as a cause of developmental failure in DsX^M embryos. Female embryos carrying the mutated *Xist* allele on the X^P chromosome are severely retarded in growth and die early in embryogenesis, whereas the same allele on the X^M chromosome exerts no recognizable effect on embryonic development. After paternal transmission of the mutated X chromosome, the wild-type X^M chromosome is inactivated in all cells of embryonic tissues. Presumably, X inactivation did not occur in the extraembryonic cells because of the deleted *Xist* allele on X^P together with inability to inactivate X^M due to maternal imprinting. X^M was uniformly inactivated in the embryonic tissue because of either nonrandom inactivation of X^M or post-inactivation selection against cells in which the mutated X^P did not undergo inactivation. Marahrens et al. (1998), however, provided evidence that the X^P chromosome is selectively inactivated throughout the embryo after maternal transmission of the mutant X chromosome. These abnormal embryos carrying the paternally inherited mutant *Xist* closely resemble DsX^M embryos histologically, which is attributed most likely to the occurrence of two copies of active X chromosomes in cells of extraembryonic structures. If this is so, the X^M chromosome must have been prevented from inactivation by rigid imprinting in the first and the second wave of X inactivation. Sudden shift to random inactivation silencing all but one X chromosome in the third wave, which occurs 1 or 2 days after the second wave of nonrandom inactivation raises an interesting question about its molecular biological basis. Apparent possibilities would be, erasure or tissue-specific invalidation of imprinting that inhibits X^M from inactivation or that predisposes X^P chromosome to inactivation. Results reported by Kay et al. (1994) are apparently consistent with imprinting that positively leads to X^P inactivation. Experiments using female ES cells suggests erasure of imprint (Sado et al., 1997), but further study is necessary for final conclusion.

Parthenogenones are maternally disomic for X chromosome as well as all autosomes. Although parthenogenetic inviability is ultimately determined by the possession of two sets of maternally derived autosomes (Spindle et al. 1996), development is affected more severely in XX than X0 parthenogenones (Mann and Lovell-Badge, 1988). A majority of parthenogenones cease development immediately after implantation exhibiting deficient extraembryonic structures (Tada and Takagi, 1992; Sturm et al., 1994). Severe abnormalities shown by parthenogenones at early peri-implantation stages may be elucidated by duplication of the X^M chromosome alone, rather than absence of the paternally derived genome.

Distal X chromosome segment is probably responsible for the DsX^M phenotype

The devastating effects of the partial duplication of the active X chromosome were reported in the unbalanced carrier of Searle's T(X;16)16H translocation (Takagi and Abe, 1990).

The unbalanced embryo, functionally disomic for the proximal 63% of the X chromosome and trisomic for the distal half of chromosome 16 in every cell, shows underdevelopment of the embryonic ectoderm, incessant death of embryonic ectoderm cells, and limited mesoderm formation. Although the ectoplacental cone develops poorly as in DsX^M embryos, this is not due to the paucity of diploid trophoblasts, but to their inability to migrate into the extraembryonic region in view of a large number of cells accumulated at the proximal end of the egg cylinder. In contrast to DsX^M embryos, the extraembryonic ectoderm, visceral and parietal endoderm develop well in the unbalanced carriers of T16H translocation. Phenotypic differences between these embryos and DsX^M embryos suggest that two active copies of the X chromosome segment distal to the T16H breakpoint are responsible for the deficiency of cells belonging to the polar trophectoderm lineage. Alternatively, the better extraembryonic development in the unbalanced carriers of the T16H translocation could be explained on the basis of a general reduction in the load of X chromosome activity.

The present observations in DsX^M↔2n chimeric embryos revealed that DsX^M cells are eliminated from the extraembryonic tissues or, though less likely, that cells with a single active X chromosome are selectively recruited to them. Drastic selection against cells having two active X chromosomes, if indeed it occurs, has been completed within 3 days after transfer of chimeric blastocysts to uteri of foster mothers. The current X chromosome map did not help us to predict a gene(s) responsible for the underdeveloped extraembryonic structures. Further studies are necessary to understand why two active X chromosomes are so harmful to embryonic as well as extraembryonic tissues.

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REFERENCE

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

Allen, N. D., Barton, S. C., Hilton, K., Norris, M. L., and Surani, M. A. (1994). A functional analysis of imprinting in parthenogenetic embryonic stem cells. *Development* **120**, 1473-1482.

Cattanach, B. M. (1961). XXY mice. *Genet. Res.* **2**, 156-160.

Cheong, H. T., Taniguchi, T., Hishinuma, M., Takahashi, Y. and Kanagawa, H. (1991). Effects of various electric fields on the fusion and in vitro development of mouse two-cell embryos. *Theriogenology* **36**, 875-885.

Copp, A. J. (1979). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of the polar trophectoderm. *J. Embryol. exp. Morph.* **51**, 109-120.

Endo, A., Watanabe, T. and Fujita, T. (1991). An XXY sex chromosome anomaly in the mouse. *Genome* **34**, 41-43.

Friedrich, G. and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513-1523.

Gardner, R. L., Papaioannou, V. E. and Barton, S. C. (1973). Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *J. Embryol. exp. Morph.* **30**, 561-572.

Goto, T., Wright, E. and Monk, M.(1997). Paternal X-inactivation in human trophoblast cells. *Mol. Hum. Reprod.* **3**, 77-80.

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

Guillemot, F., Nagy, A., Auerbach, A., Rossant, J., and Joyner, A. L. (1994). Essential role of *Mash-2* in extraembryonic development. *Nature* **371**, 333-336.

Harrison, K. B. and Warburton, D. (1986). Preferential X-chromosome activity in human female placental tissues. *Cytogent. Cell Genet.* **41**, 163-168.

Harrison, K. B. (1989). X-chromosome inactivation in the human cytotrophoblast. *Cytogenet. Cell Genet.* **52**, 37-41.

Jacobs, P. A., Hassold, T. J., Whittington, E., Butler, G., Collyer, S., Keston, M. and Lee, M. (1988). Klinefelter's syndrome: an analysis of the origin of the additional sex chromosome using molecular probe. *Ann. Hum. Genet.* **52**, 93-109.

James, R. M., Klerkx, A. H., Keighren, M., Flockhart, J. H., and West, J. D. (1995). Restricted distribution of tetraploid cells in mouse tetraploid ↔ diploid chimeras. *Dev. Biol.* **167**, 213-226.

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.

Lorda-Sanchez, I., Binkert, F., Maechler, M., Robinson, W. P. and Schinzel, A. A. (1992). Reduced recombination and parental age effect in Klinefelter's syndrome. *Hum. Genet.* **89**, 524-530.

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). Parental source of chromosome imprinting and its relevance to X-chromosome inactivation. *Differentiation* **26**, 63-67.

Mann, J. R. and Lovell-Badge, R. H. (1988). Two maternally derived X chromosomes contribute to parthenogenetic inviability. *Development* **104**, 129-136.

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.

Matsuda, Y. and Chapman, V. M. (1992). Analysis of sex-chromosome aneuploidy in interspecific backcross progeny between the laboratory mouse strain C57BL/6 and *Mus spretus*. *Cytogenet. Cell Genet.* **60**, 74-78.

May, K. M., Jacobs, P. A., Lee, M., Ratcliffe, S., Robinson, A., Nielsen, J. and Hassold, T. J. (1990). The parental origin of the extra X chromosome in 47, XXX females. *Am. J. Hum. Genet.* **46**, 754-761.

Migeon, B. R., Wolf, S. F., Axelman, J., Kaslow, D. C. and Schmidt, M. (1985). Incomplete X chromosome dosage compensation in chorionic villi of human placenta. *Proc.Natl. Acad. Sci. USA* **82**, 3390-3394.

Mintz, B., Gearhart, J. D. and Guymont, A. O. (1973). Phytohemagglutinin-mediated blastomere aggregation and development of allophenic mice. *Dev. Biol.* **31**, 195-199.

Mohandas, T. K., Passage, M. B., Williams, J. W. 3d, Sparkes, R. S., Yen, P. H. and Shapiro, L. J. (1989). X-chromosome inactivation in cultured cells from human chorionic villi. *Somat. Cell Mol. Genet.* **15**, 131-136.

Morris, T. (1968). The X0 and 0Y chromosome constitution in the mouse. *Genet. Res.* **12**, 125-137.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815-821.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc., Natl. Acad. Sci. USA* **90**, 8424-8428.

Nicolson, G. L., Yanagimachi, R. and Yanagimachi, H. (1975). Ultrastructural localization of lectin-binding sites on the Zonae pelluchidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263-274.

Ohno, S. (1967). *Sex Chromosomes and Sex-Linked Genes*. New York: Springer.

Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S. and Brockdorff, N. (1996). Requirement for *Xist* in the X chromosome inactivation. *Nature* **379**, 131-137.

Quinn, P., Barros, C. and Whittingham, D. G. (1982). Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.* **66**, 161-168.

Ropers, H.-H., Wolf, G. and Hitzeroth, H. W. (1978). Preferential X inactivation in human placenta membranes: Is the paternal X inactive in early embryonic development of female mammals? *Hum. Genet.* **43**, 265-273.

Russell, L. B. and Chu, E. H. Y. (1961). An XXY male in the mouse. *Proc. natl. Acad. Sci.USA* **47**, 571-575.

Sado, T., Tada, T. and Takagi, N. (1997). 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**, 969-975.

Spindle, A., Sturm, K. S., Flannery, M., Meneses, J. J., Wu, K. and Pedersen, R. A. (1996). Defective chorioallantoic fusion in mid-gestation lethality of parthenogenone ↔ tetraploid chimeras. *Dev. Biol.* **173**, 447-458.

Sturm, K. S., Flannery, M. L. and Pedersen, R. A. (1994). Abnormal development of embryonic and extraembryonic cell lineages in parthenogenetic mouse embryos. *Dev. Dyn.* **201**, 11-28.

Tada, T, Tada, M., Hilton, K., Barton, S. C., Sado, T., Takagi, N. and Surani, M. A. (1998). Epigenetic switching of imprintable loci in embryonic germ cells. *Dev. Genes Evol.* **207**, 551-561.

Tada, T. and Takagi, N. (1992) Early development and X-chromosome inactivation in mouse parthenogenetic embryos. *Mol. Rep. Dev.* **31**, 20-27.

Tada, T., Takagi, N. and Adler, I.-D. (1993). Parental imprinting on the mouse X chromosome: effects on the early development of X0, XXY and XXX embryos. *Genet. Res.* **62**, 139-148

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 post-implantation 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.

Tarkowski, A. K., Witkowska, A. and Opas, J. (1977). Development of cytochalasin B-induced tetraploid and diploid ↔ tetraploid mosaic mouse embryos. *J. Embryol. Exp. Morph.* **41**, 47-64.

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.

Tease, C. and Fisher, G. (1993). The production and fate of embryos with disomy for the maternal X chromosome. *Mouse Genome* **91**, 334-336.

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. Fertil.* **14** (Suppl.), 7-21.

Wroblewska, J. and Dyban, A. P. (1969). Chromosome preparation from embryos during early organogenesis: dissociation after fixation, followed by air drying. *Stain Technol.* **44**, 147-150.