Control of Xist expression for imprinted and random X chromosome inactivation in mice

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Applying RNA fluorescence in situ hybridization to parthenogenetic embryos with two maternally derived X (X*M) chromosomes and embryos with X chromosome aneuploidy such as X*0 (X<P, paternally derived X chromosome), X*MX.P and X*MX.Y, we studied the control of Xist/Tsix expression for silencing the entire X chromosome in mice. The data show that the paternally derived Xist allele is highly expressed in every cell of the embryo from the 4-cell stage onward, irrespective of the number of X chromosomes in a diploid cell. The high level of Xist transcription is maintained in non-epiblast cells culminating in X*0-inactivation, whereas in X*0 embryos it is terminated by the blastocyst stage, probably as a result of counting the number of X chromosomes in a cell occurring at the morula/blastocyst stage. Xist is also down-regulated in epiblast cells of X*0 and X*MX.Y embryos to make X-inactivation random. In epiblast cells, Xist seems to be up-regulated after counting and random choice of the future inactive X chromosome(s). Although the maternal Xist allele is never activated in fertilized embryos before implantation, some parthenogenetic embryos show Xist up-regulation in a proportion of cells. These and other data reported earlier suggest that imprinted X-inactivation in non-epiblast tissues of rodents had been derived from the random X-inactivation system.

INTRODUCTION

Genetic and biochemical studies together with cell lineage analysis in mouse development (1) revealed that X-inactivation (2) occurs in at least three waves in mice: firstly, in the trophoderm of expanding blastocysts at embryonic day (E)3.5; secondly, in the primitive endoderm differentiating at the surface of the inner cell mass (ICM) at E4.5; and finally, in epiblast cells, including germ cells, at E5.5–6.5. A small mammalian X chromosomal region called the X chromosome inactivation center (XIC) is indispensable for achieving X chromosome inactivation in cis (reviewed in ref. 3). It has been postulated that XIC is essential for: (i) initiation of X inactivation by counting the number of X chromosomes in a cell and choosing one X chromosome to be (in)activated; (ii) spreading inactivation signal in cis from the XIC in both directions along the X chromosome; and (iii) maintaining the inactive state stably once it has been established (4). Available evidence shows that the Xist gene localized in the XIC region (5) is mainly responsible for silencing the X chromosome (6,7). Xist transgenes, for example, may induce inactivation of the autosomal carrying them (8–13), and the X chromosome carrying a targeted Xist gene fails to undergo inactivation although silencing of the normal counterpart is not affected (14,15).

Accumulation of Xist transcripts on the X chromosome in cis has been causally correlated with the initiation of X-inactivation (16). Xist expression has been extensively studied in early mouse embryos in vivo and differentiating embryonic stem (ES) cells in vitro (reviewed in ref. 17). Transcription of the Xist gene is regulated at three different levels; high, low and off (18–20). Xist RNA is transcribed at a low level from every Xist allele in undifferentiated female and male ES cells, detected as a small punctate or pinpoint signal by Xist RNA fluorescence in situ hybridization (FISH). When female ES cells are induced to differentiate, transcriptional activity becomes high in one Xist allele defined by a robust or paint signal, whereas the other allele eventually terminates transcription. Subsequent studies showed that an almost identical process of Xist expression occurs in vivo and that the high and low expression thus detected does not indicate the different levels of transcription but different stability of Xist transcripts (19,20). In this paper, up- and down-regulation will be used to indicate high and low stability.

Involvement of genomic imprinting in X-inactivation in mice was suggested by the fact that X*X* is inactivated in every cell of the trophoderm and primitive endoderm (21,22), whereas random inactivation characterizes the epiblast. Results of recent Xist knockout experiments have suggested critical roles of sequences 3’ to Xist (23), including the Tsix gene, which is antisense to Xist (24,25), with the DXPas34 locus (26), the principal initiation site for Tsix transcription, in controlling Xist expression and hence choice of the future inactive X chromosome. TsixDXPas34 is considered to block Xist accumulation on the X chromosome, but detailed mechanisms remain to be defined (25–27). Producing knockout mice, Lee (28) found that the maternally expressed Tsix gene inhibits up-regulation of the maternal Xist expression in extra-embryonic tissues, and showed that the lack of active Tsix transcript induces ectopic X-inactivation in both male and female embryos, though final proof is lacking.

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PCR and FISH studies in vivo showed that the paternal Xist allele becomes active by the 4-cell stage or even earlier and every blastomere is positive for a strong Xist paint signal at this time (20,29). Hence, it has been hypothesized that this Xist up-regulation heralds preferential inactivation of X in extra-embryonic tissues (30). If this is the case, expression of the paternal Xist allele should be down-regulated in the epiblast lineage of ICM for random inactivation to occur. Inspite of remarkable advances in our knowledge of the Xist/Tsix gene, not much is known about the control of its transcription at the initiation of X-inactivation both in human and mouse. The challenge now is to define the control of Xist expression culminating in silencing the X chromosome in different tissues. In this study we examined Xist expression by RNA FISH in mouse embryos carrying sex chromosome aneuploidy and parthenogenetic embryos to analyze the control of Xist/Tsix expression at the initiation of X-inactivation, taking advantage of the unique imprinted X-inactivation occurring in mice. Our data show that the paternal Xist allele is expressed from the 4-cell stage onward, but the transcription cannot be maintained in the absence of an additional X chromosome(s) in a cell, probably as a result of counting the X chromosome copy number. In the epiblast lineage, however, counting seems to precede up-regulation of Xist expression. Unlike fertilized embryos, the maternal Xist allele is activated in parthenogenetic trophectoderm and possibly also in primitive endoderm, though limited to a small proportion of cells. These and other findings gave us important insights into the relationship between random and imprinted X-inactivation in eutherian mammals, including man.

RESULTS

Sensitivity of the FISH method used

First, we carried out a Xist RNA FISH study in normal early mouse embryos to confirm previous results and to appraise our cytological techniques involving fixation with 3:1 methanol/glacial acetic acid and treatment with lactic acid (Materials and Methods). A single large signal was detected in a majority of cells from female X^XY embryos at the 4- to 16-cell stage, whereas no such paint signal was found in any cell of male X^YY embryos at the same stage, as reported by Sheardown et al. (20) and Debrand et al. (26). In fact, all blastomeres carried a single paint signal on the X chromosome domain in 22 out of 37, and 24 out of 33 female embryos at the 8- and 16-cell stages, respectively (Figs 1A and B). Most probably a single Xist allele is regularly up-regulated in all blastomeres at these stages, and a small number of cells without an overt paint signal would be technical artifact. The proportion of cells having no paint signal increased gradually thereafter (Fig. 3C). The tendency was almost the same in two groups of XX embryos; one from In(X)1H/+ and Rb2/RX9 females, whereas it stayed more or less constant in XMXP embryos (A and B). In parthenogenetic embryos, the frequency of paint signal-positive cells was getting gradually higher but was extremely variable (D).

Figure 1. Frequency of cells with a single Xist paint signal in early X^XY (A and B), X^0 (C) and parthenogenetic (D) mouse embryos. The size of each circle is proportional to the number of embryos at each point. The smallest circle represents one embryo and the largest represents 20 embryos. The center of each circle indicates the stage of embryos and the percentage of cells with a Xist paint signal in individual embryos. The proportion of cells lacking the Xist paint signal increased rapidly in growing pre-implantation X^0 embryos from both In(X)1H/+ and Rb2/RX9 females, whereas it stayed more or less constant in X^XY embryos (A and B). In parthenogenetic embryos, the frequency of paint signal-positive cells was getting gradually higher but was extremely variable (D).

from earlier results (20,26) was that we found up to 10% XX cells without any signal and very low frequency of cells with two pinpoint signals throughout pre-implantation stages (Fig. 3C). Thus, the present method should be reasonably sensitive for detecting high level Xist/Tsix expression, though
it might not be perfect for studying the low level Xist expression in early mouse embryos. Contrary to the report by Duthie et al. (34), we did not obtain better results in embryos fixed with 4% paraformaldehyde.

Inspite of apparently perfect co-localization of the Xist paint signal, if present, to the entire X chromosome domain at interphase throughout pre-implantation stages, the signal was restricted to a small area of one X chromosome at metaphase (Table 1). In total, partial Xist paint was found over one X chromosome in ∼25% of metaphase cells, whereas no Xist signal was detected in the remaining metaphase cells. Sometimes the signal was dispersed around an X chromosome. In contrast, Xist signal was detected over nearly the entire length of an X chromosome in 190 out of 283 (67.2%) metaphase cells from XX embryos at E7.5 prepared by an air-drying method involving treatment with lactic acid/acetic acid mixture, as in the case of pre-implantation embryos. This finding suggests that the association between Xist RNA and the X chromosome is weaker in cells of pre-implantation than post-implantation embryos, and the X chromosome lost most
Table 1. Frequency of cells derived from pre-implantation mouse XX embryos showing partial retention of Xist RNA by an X chromosome at metaphase

<table>
<thead>
<tr>
<th>Stage of embryos</th>
<th>n ≤ 16</th>
<th>16 &lt; n ≤ 32</th>
<th>32 &lt; n</th>
</tr>
</thead>
<tbody>
<tr>
<td>In(X)1H-derived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial paint signal</td>
<td>4 (17.4%)</td>
<td>17 (58.6%)</td>
<td>21 (26.3%)</td>
</tr>
<tr>
<td>No signal</td>
<td>19</td>
<td>12</td>
<td>59</td>
</tr>
<tr>
<td>MBH-derived ²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial paint signal</td>
<td>1 (7.7%)</td>
<td>13 (18.5%)</td>
<td>25 (21.0%)</td>
</tr>
<tr>
<td>No signal</td>
<td>12</td>
<td>41</td>
<td>94</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial paint signal</td>
<td>5 (13.9%)</td>
<td>30 (36.1%)</td>
<td>46 (23.1%)</td>
</tr>
<tr>
<td>No signal</td>
<td>31</td>
<td>53</td>
<td>153</td>
</tr>
</tbody>
</table>

n, cell number.
²Female mice doubly heterozygous for Rh2 and RX9.

Xist RNA during fixation and slide preparation. In humans, however, Xist RNA is stripped from the inactive X chromosome during mitosis (16).

Xist expression in X0 embryos

To further characterize possible epigenetic differences between Xm and X0 in the initiation of X-inactivation, we examined X0 embryos from female mice heterozygous for In(X)1H, a large paracentric inversion of the X chromosome. About 23% of female offspring from heterozygous females are X0, because a recombination in the inverted segment results in the formation of a dicentric and an acentric X chromosome (35) which are ultimately lost from the oocyte. As mentioned above, normal male embryos were uniformly negative for the paint signal. Notwithstanding a single X chromosome in a cell, a distinct paint signal was present in every cell of X0 embryos at the 8-cell stage (Figs 1C and 2E and F), as in X0X0 embryos. This is in agreement with the recent finding by Okamoto et al. (36) that all blastomeres were positive for a single and a double Xist paint signal in XY and XX androgenetic embryos, respectively, at the 4- and 8-cell stage. Taken together, these findings substantiate the view that the initial expression of stable Xist RNA in XX embryos depends solely on the paternal imprinting and not on the number of X chromosomes in a cell.

Almost all cells were still positive for a single paint signal in certain X0 embryos at the 16-cell stage, whereas the frequency decreased to 20–40% in fully expanded blastocysts (Fig. 1C). It would be reasonable to postulate that Xist transcription was terminated in the trophectoderm by the late blastocyst stage as a result of sensing only one X chromosome in the cell during the period between the morula and the early blastocyst stage. In normal female blastocysts the paint signal was detected in 60–90% cells as shown above. In such embryos, counting two X chromosomes in a cell should have allowed the paternal Xist allele to keep expression high in non-epiblast cell lineages for the inactivation of X0. Thus, individual cells respond promptly to counting by turning off Xist transcription or keeping it on. It is very likely that counting of X chromosome copy number is an integral component of imprinted as well as random X-inactivation and it temporarily links closely to the advent of the cytologically identifiable inactive X chromosome both in the trophectoderm and embryonic ectoderm. In the epiblast cell lineage, on the other hand, Xist transcription should be down-regulated in preparation for random inactivation. Alternatively, active Xist transcription may be switched from the paternal to the maternal allele in about half of epiblast cells. However, 10–30% of cells without a paint signal in fully expanded XX blastocysts seem to support the former possibility.

Xist expression in parthenogenetic embryos

After ethanol activation and diploidization with cytochalasin B, parthenogenetic embryos were grown in culture. Embryos were at the 8- to 10-cell stage ~3.5 days after activation. A large paint signal was not found in any cell at this stage, in agreement with the earlier PCR data (25) and the finding obtained by RNA FISH in X0X0 embryos (37). Six to 12 h later, a large paint signal was detected at variable frequencies even in embryos consisting of only 10–12 cells (Fig. 1D). It is noteworthy that some embryos consisting of about 30 cells were uniformly negative for the paint signal, whereas 20–80% of cells had a paint signal in other embryos at comparable stages (Fig. 2M–P). The proportion of cells with a paint signal was 5–50% in blastocysts of more than 50 cells. These unpredictable results, summarized in Figure 1D, may be peculiar to parthenogenetic embryos. In addition, a small proportion of cells from parthenogenetic embryos had two paint signals. One might suspect that in vitro growth is responsible for the unusual Xist transcription patterns in these embryos. However, we did not find any abnormality in fertilized female embryos kept in culture (data not shown). Thus, it may be concluded that parthenogenetic embryos are characterized by up-regulation of the maternal Xist allele only in a limited proportion of cells, and lack of consistency regarding the stage of development at which stable Xist expression occurs.

X-inactivation occurs almost normally in the epiblast cell lineages of parthenogenetic embryos (38–40). A small proportion of cells with a single paint signal in parthenogenetic blastocysts may correspond to ICM cells initiating X-inactivation. However, comparison between intact blastocysts and ICM isolated by immunosurgery showed that this is not necessarily the case. In control XX embryos, the frequencies of three classes of cells, one having a single paint signal, the second having a single pinpoint signal and the last having no Xist RNA signal, were almost identical in intact blastocysts and isolated ICMs. Table 2 shows that the tendency is similar in parthenogenetic embryos. About 20% of ICM cells were positive for a Xist paint signal, whereas ~15% of cells from whole blastocysts were positive for it. The most noticeable difference was the high incidence of cells with a single pinpoint signal in the isolated ICM. The significance of this observation remains unknown. Tsx RNA that is solely transcribed from the maternal allele (28) may elucidate it. In order to examine the situation in trophectoderm cells, we constructed giant blastocysts by aggregating six 8-cell stage embryos. About 35% of trophectoderm cells mechanically separated from giant parthenogenetic blastocysts had a Xist paint signal, whereas the percentage was 83% in trophectoderm from comparable giant blastocysts derived from fertilized embryos. Probably, Xist
of X M in parthenogenetic embryos. About 13% of embryos from various pre-implantation stages obtained from female mice were DsXM. Previously, Goto and Takagi (37) studied Xist expression in fertilized blastocysts disomic for X M (DsXM). They found only one Xist paint signal in XMXMY embryos, but no such signal in XMXMXP embryos. We examined DsXM embryos again at various pre-implantation stages obtained from female mice doubly heterozygous for Rh2 and RX9 to evaluate the behavior of X M in parthenogenetic embryos. About 13% of embryos from such females were DsXM. Although data are still meager, the Xist expression pattern was considerably more consistent in DsXM blastocysts than in parthenogenetic embryos (Figs 2K and L and 3D and E). The frequency of cells with a paint signal was at most 10% in XMXMY blastocysts consisting of more than 50 cells. Similarly, the one paint signal, most probably derived from X P, was present in >70% of cells in XMXMXP blastocysts. None of the XMXMXP cells at this stage had two paint signals, in agreement with the genetic evidence that the X-inactivation has not yet initiated in the epiblast at this stage (41).

**DISCUSSION**

Xist RNA FISH discriminates between large (paint) signals and small punctate (pinpoint) ones with relative ease in undifferentiated and fully differentiated female ES cells. However, there could be an intermediate stage between them. Debrand et al. (26) identified a small proportion of differentiating ES cells an immature Xist RNA signal with or without a small signal from the closely linked DXPas34 locus which might be involved in the X chromosome choice and imprinting. Large ‘paint’ signals, co-localized with Xist in cleaving mouse embryos detected in this study, may correspond more closely to this immature Xist signal than the mature signal found in adult or embryonic female cells. This assumption is supported by the observation that the Xist accumulation is still reversible at least in some cells at the morula stage, and that Xist transcripts stick to the X chromosome less tightly in pre-implantation embryos than in post-implantation embryos. Thus, we never found the X chromosome at metaphase coated with Xist RNA along its entire length during pre-implantation development of mouse embryos, in contrast to nearly complete coating in adult or fetal female cells (16). Incidentally, Costanzi et al. (42) detected first substantial localization of macroH2A1 to a single X chromosome in female morulae. A likely explanation is that macroH2A1 helps to facilitate stable association between the X chromosome and Xist RNA as suggested by Csankowski et al. (43).

Imprinted Xist expression is considered the exclusive cause of non-random inactivation of X p in the trophectoderm (29). The preferential inactivation of X p is due either to the resistance of X M to inactivation or a predisposition of X p to inactivation. A recent finding made by Tada et al. (44) favors the former possibility. They showed that genomic modification occurring during the growth phase of the oocyte has made XM unresponsive to the inactivation cue in the extra-embryonic tissue. Contrary to X chromosomes from mature oocytes, those from the newborn mouse oocytes were readily inactivated in trophectoderm cells of embryos derived by combining maternal genomes from a fully grown oocyte and from a non-growing 1 day post partum oocyte (45). These findings are consistent with the view that X M carries imprint that prevents it from inactivation in non-epiblast cells, whereas X p is free of such imprint. An obvious possibility would be differential methylation of Tsix/DXPas34, but Prissette et al. (46) detected no parent-of-origin specific methylation in DXPas34 and associated downstream CpG island.

Only the paternal X chromosome is inactivated and two maternal X chromosomes remain active in trophectoderm and primitive endoderm cell lineages of XMXMXP embryos (47). These findings suggest either that counting does not participate in the imprinted X-inactivation or that the result of counting is ignored by the X M chromosome (28,48). Involvement of counting in imprinted inactivation was demonstrated by Okamoto et al. (36). Their cytogenetic and RNA FISH study in XX androgenetic embryos consistently detected two Xist paint signals in all blastomeres at the 4- to 16-cell stage, whereas only one X chromosome was inactivated in almost all embryonic as well as extra-embryonic cells at E6.5 and E7.5. Therefore, adjustment of Xist expression must have been made sometime after the morula stage. Findings in X0 embryos (Fig. 3) suggest that counting takes place at the morula/early blastocyst stage. Counting and choice are important events occurring at the initiation of random X-inactivation. Unlike trophectoderm cells, Xist up-regulation seems to follow counting and choice

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**Table 2. Xist expression patterns in the whole blastocyst, isolated inner cell mass and trophectoderm of normal female and parthenogenetic embryos**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells (%) with the following signal pattern</th>
<th>A paint signal</th>
<th>A pinpoint signal</th>
<th>No signal</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal female embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>1619 (74.7)</td>
<td>93 (4.3)</td>
<td>439 (20.3)</td>
<td>15 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Inner cell mass</td>
<td>283 (72.6)</td>
<td>14 (3.6)</td>
<td>93 (23.8)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Trophectoderm</td>
<td>453 (83.1)</td>
<td>–</td>
<td>92 (16.9)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Parthenogenetic embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>733 (15.0)</td>
<td>419 (8.5)</td>
<td>3717 (75.8)</td>
<td>32 (0.7)</td>
<td></td>
</tr>
<tr>
<td>Inner cell mass</td>
<td>126 (19.7)</td>
<td>224 (35.0)</td>
<td>264 (41.3)</td>
<td>26 (4.1)</td>
<td></td>
</tr>
<tr>
<td>Trophectoderm</td>
<td>471 (35.2)</td>
<td>–</td>
<td>868 (64.8)</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>
in epiblast cell lineages, probably immediately after implanta-
tion. This is supported by the observation that up-regulation in
one or the other \textit{Xist} allele occurs in differentiating XX ES
cells at the advent of a late replicating X chromosome (19,49),
and that the future inactive X chromosome is not determined in
E4.5 epiblast cells (41).

An overwhelming majority of parthenogenetic embryos
cannot survive implantation (40,50), although the cause of the
everal lethality is not clear. Most parthenogenones surviving
implantation suffer from severely underdeveloped extra-
embryonic structures, probably due to lack of X-inactivation in
a large proportion of extra-embryonic cells (40) predicted by
generally low frequency of cells up-regulating \textit{Xist} expression
before implantation. Only exceptional parthenogenetic
embryos survive by mid-gestation with poorly developed
extra-embryonic tissues (51). In view of the unpredictably
variable frequency of cells up-regulating \textit{Xist} expression in
parthenogenetic pre-implantation embryos, it is not surprising
that a few such embryos manage to undergo X-inactivation in
non-epiblast cells barely enough for organizing extra-embryonic
tissues and survive by mid-gestation. Contrary to partheno-
genetic embryos, activation of the maternal \textit{Xist} allele has
scarce been found in fertilized embryos with X\textsuperscript{M}X\textsuperscript{M}Y and
X\textsuperscript{M}X\textsuperscript{M}X\textsuperscript{P} sex chromosome constitution before implantation
(37). A major difference between these embryos and parthe-
nogenones is that the latter have no paternal genome. It is thus
tempting to postulate that the paternal genome helps to keep
the maternal \textit{Xist} allele inactive during preimplantation stages.
The 'n–1' rule, maintained strictly in humans, does not always
hold true in mice and probably throughout rodents, mainly
because the potent imprint exempts X\textsuperscript{M} from inactivation in
non-epiblast cell lineages. Furthermore, random inactivation in
XX androgenones and, probably to a much limited extent, in
parthenogenetic embryos, suggests that X\textsuperscript{P} inactivation in
mural extra-embryonic tissues is a modified form of random
X inactivation, not the ancestral one. A similar hypothesis was
advanced recently by Ohlsson \textit{et al.} (52).

Data available so far allow us to outline a sequence of events
culminating in X-inactivation in female mouse embryos. Every
X\textsuperscript{P} chromosome begins to transcribe stable \textit{Xist} RNA in all

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Changes in the frequency of cells showing different \textit{Xist} expression patterns in fertilized mouse embryos with X\textsuperscript{M}X\textsuperscript{M}Y, X\textsuperscript{M}X\textsuperscript{P}, X\textsuperscript{M}X\textsuperscript{M}X\textsuperscript{P} and X\textsuperscript{M}X\textsuperscript{M}X\textsuperscript{P} sex chromosome constitutions, and parthenogenetic embryos from the 4-cell to the blastocyst stage.}
\end{figure}
cells from the 4- to 16-cell stage. The functional significance of Xist transcript at this stage remains obscure. Counting the number of X chromosomes in a cell is not involved in this process. Subsequent counting occurring at the morula/early blastocyst stage is a key to the fate of the X chromosome. It is irreversibly inactivated by maintaining Xist expression if the trophectoderm precursor cells have at least two X chromosomes, but it reverts to the initial active state by down-regulating Xist transcription if there is no other X chromosome in the cell. We have no data as to the timing of counting in the primitive endoderm cell lineage. In the epiblast cell lineage, Xist is up-regulated after counting and choice of the future inactive X. To ensure the choice is random the parent-of-origin-specific imprint should have been erased from the XIC. Further scrutiny into the differences between XM and XP may help clarify the mechanism and evolution of the interesting system of counting X chromosome copy number and choosing one future inactive X chromosome.

MATERIALS AND METHODS

Mice

X0 embryos were obtained from female mice heterozygous for In(X)1H (31), a large paracentric inversion for the X chromosome, mated with (C57BL/6J × CBA/J)F1 (abbreviated to F1) males. Female mice heterozygous for two Robertsonian X-auto-some translocations, Rh(X.2)2Ad (abbreviated to Rh2) (32) and Rb(X.9)6H (abbreviated to Rb2) (33), were mated with F1 males to obtain X0, X0XMY and X0XMYXP embryos as reported by Goto and Takagi (47). Unfertilized oocytes from F1 females were used for generating parthenogenetic embryos.

Parthenogenetic activation

Oocytes were collected from oviducts of superovulated F1 females 17–18 h after hCG injection. Cumulus masses recovered were treated with 1% (w/v) hyaluronidase (Sigma, St. Louis, MO) in M2 medium (53) for 5 min to remove cumulus cells (54). Eggs were washed with M2 medium twice, treated with 7% ethanol in M2 medium for 6 min at room temperature and washed again with M2 medium twice (55). Extrusion of the second polar body was suppressed by incubation of oocytes in medium M16 (56) containing 5 µg/ml cytochalasin B (Sigma) for 4–5 h at 37°C (57). Parthenogenetically activated embryos were cultured in M16 medium under paraffin oil at 37°C in 5% CO2 in air up to the 4-cell to blastocyst stage.

Production of giant blastocysts

Giant chimeric blastocysts were produced by aggregating zona-free 8-cell embryos recovered from females mated with males or parthenogenetically activated embryos. After the removal of zona pellucida in acidic Tyrode solution (58), six embryos were transferred to a small hole (∼0.5 mm in diameter) on the bottom of a plastic culture dish to facilitate aggregation (59). Chimeric embryos were incubated in M16 medium at 37°C in an atmosphere of 5% CO2 in air.

ICMs were isolated from fully expanded blastocysts by a standard method (60). In brief, zona-free blastocysts were incubated with rabbit anti-mouse thymocyte serum (Cedar Lane Laboratory, Hornby, Ontario) at a dilution of 1:2 at 37°C for 10 min and washed thoroughly with Dulbecco’s modified Eagle’s medium supplemented with HEPES and heat-inactivated fetal bovine serum. Blastocysts were then incubated with guinea pig complement serum (Sigma) at a dilution of 1:5 at 37°C for 30 min to lyse the outer trophectoderm layer.

Cytological preparation for FISH

Cytological preparations were made from pre-implantation embryos according to the methods described by Takagi et al. (61) with minor modifications. Briefly, after hypotonetic treatment with 1% sodium citrate for 10 min at room temperature, embryos were fixed with a 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 the 3:1 mixture of glacial acetic acid and 25% lactic acid was applied onto the embryo to help spread cells on the slide. Up to the morula stage lactic acid was applied when embryos were completely dry, whereas in the case of blastocyst it was applied slightly before complete evaporation of the fixative. Lactic acid was removed from the preparation by repeated application of the fixative. We adopted the conventional methanol/acetic acid fixation for preserving morphology and cell number, although Dutchie et al. (34) reported it does not adequately conserve the integrity of the RNA signal.

FISH analysis

Xist RNA was detected with the use of pBluescript-based plasmid clones, pR53E1 encompassing exons 1–6 (62), according to the method described earlier (36,37) with minor modifications. Briefly, ~70 ng of probe DNA was applied per slide in 5 µl of hybridization mixture for Xist RNA FISH. Fixation of the slide was omitted after RNA FISH for painting sex chromosomes with biotin-labeled mouse X and Cy3-labeled mouse Y chromosome probes (Cambio, Cambridge, UK). Biotin was detected with fluorescein isothiocyanate-conjugated anti-biotin antibody (Calbiochem, La Jolla, CA). As reported earlier, slides were examined with an OLYMPUS fluorescence microscope and images were captured with a Photometrics CCD camera coupled to IPLab software (Scanalytics, Fairfax, VA). Color channels were merged in Adobe PhotoShop.

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