Timing of X-chromosome inactivation in postimplantation mouse embryos

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SUMMARY

The onset of X-chromosome inactivation was investigated cytologically in postimplantation female mouse embryos of age 5½, 6½, and 7½ days post-coitum (d.p.c.) and in the isolated epiblasts of 6 d.p.c. embryos before primitive streak formation using a heat/hypotonic technique to reveal the inactive X chromosome by differentially dark staining with Giemsa. The results indicate that X inactivation has taken place in all the cells of the so-called 'undifferentiated' epiblast by 6 d.p.c. before primitive streak formation. Further evidence is presented to suggest that X inactivation is complete in all cells of the mouse embryo by 5½ d.p.c.

INTRODUCTION

According to the X-inactivation hypothesis (Lyon, 1961) differentiation of the two X chromosomes in cells of female mammals takes place early in embryogenesis. There is now considerable cytological and biochemical evidence that both X chromosomes of the female are in the active or potentially active state in cleavage embryos, and that the initial differentiative event is inactivation of a previously active X chromosome (Austin, 1966; Ohno, 1967; Takagi, 1974; Adler, West & Chapman, 1977; Chapman, West & Adler, 1978; Monk & Harper, 1979; Epstein, Smith, Travis & Tucker, 1978).

However, X inactivation may not occur in all cells of the embryo at the same time. It has been known for many years that sex chromatin and late replication of a X chromosome appear earlier in the trophoblast than in the embryo proper (Park, 1957; Plotnick, Klinger & Kusseff, 1971) and it has therefore been suggested that X inactivation takes place earlier in the cells of the trophoeoderm than in the cells of the inner cell mass (ICM). Were this contention correct it would reconcile the biochemical dosage evidence (Monk, 1978; Epstein et al. 1978; Monk & Kathuria, 1977) and the cytological evidence (Takagi, 1974) that X-chromosome inactivation has occurred in most cells of the blastocyst with the genetic evidence, based on injection of single ICM cells into host

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blastocysts, that both X chromosomes are still active in the ICM of 3½ and 4½ d.p.c. blastocysts (Gardner & Lyon, 1971).

Because trophectoderm is the first tissue to differentiate in the mouse embryo, and as there is evidence for activity of both X chromosomes in female tetra
carcinoma stem cells and only one active X chromosome in their differentiated
products (Martin et al. 1978) it has been suggested that onset of X inactivation
is linked to cellular differentiation. Monk (1978) has further suggested a stem-
cell model of X inactivation in the mouse embryo with X-chromosome inactiva-
tion occurring at different times in different cell populations as they differentiate
from a pluripotent stem-line. This hypothesis predicts the presence of two active
X chromosomes in pluripotent epiblast or embryonic ectoderm cells which
give rise to all the tissues of the embryo proper (Gardner & Papiannou, 1975)
in the early postimplantation embryo prior to primitive streak formation, but
only one active X in the corresponding extraembryonic ectoderm which is a
trophectoderm-derived tissue. In support of this prediction Monk & Harper
(1979) found a bimodal distribution of HGPRT activities for isolated epiblasts
of preprimitive streak unsexed 6 d.p.c. embryos and a unimodal distribution
for both the extraembryonic ectoderm and the endoderm regions. However
Kozak and Quinn (1975) assayed the activity of another X-linked enzyme,
PGK, in whole 6 d.p.c. mouse embryos and found a unimodal distribution,
suggesting that X chromosome inactivation is complete by this time.

In view of the disadvantage of the biochemical gene dosage approach due to
developmental asynchrony of embryos, unknown enzyme synthesis and turn-
over rate and unknown RNA longevity, a cytogenetic approach was adopted
using the modified Kanda technique (Kanda, 1973), which reveals the inactive
X chromosome in female mouse embryonic tissue by differentially dark staining
with Giesma after heat/hypotonic treatment (Rastan, Kaufman, Handyside &
Lyon, 1980). Strong evidence that the dark-staining chromosome is the inactive
X chromosome is that it is seen in over 70 % of adult XX female cells but not
in XO female cells nor male cells (Rastan 1981; Rastan et al. 1980). A dark-
staining chromosome was also present in over 75 % of cells from a bone marrow
preparation of an adult XX male mouse carrying Sxr, the autosomal gene for
sex reversal (Rastan, 1981; Rastan et al. 1980) but not in XX LT teratocarcinoma
stem cells (Rastan, unpublished) in which both X's are known to be active.
The advantage of using such an approach to determine when X inactivation is
complete in all cells of the mouse embryo is that one can determine directly
whether or not X-chromosome inactivation has occurred in individual cells.
The modified Kanda technique was used on isolated epiblasts from 6 d.p.c.
embryos in a cytological experiment designed to be comparable with the bio-
chemical one of Monk & Harper (1979). An attempt was made at a similar
dissection of 5½ d.p.c. embryos but was unsuccessful due to the very small size
of the embryo at this stage. Instead X inactivation in whole 5½ d.p.c. embryos
was studied by the modified Kanda technique and the results compared with
X-inactivation in mouse embryos

6 d.p.c. embryos

Embryos were obtained from female F₁ hybrids between two inbred strains, C3H/HeH and C57BL/Go-a₁, which were maintained under a day/night reversed regime of 15 h light and 9 h dark. The dark period was from 10.00 a.m. to 7.00 p.m. and the light period from 7.00 p.m. to 10.00 a.m. Ovulation was assumed to occur at the midpoint of the dark period which was at 2.30 p.m. Spontaneously ovulating females were placed with fertile males of the same F₁ strain at 12.00 noon and removed at 3.00 on the same day. Mating was ascertained by the presence of a copulation plug.

Pregnant females were killed by cervical dislocation exactly 6 days later at 2.00 p.m., uteri were removed and embryos isolated in medium 199 (Flow). Reichert’s membrane, the trophectoderm and ectoplacental cone were removed and discarded and the 6 d.p.c. preprimitive streak egg cylinders (see Fig. 1) were placed in medium 199 to which colchicine had been added (4 µg ml⁻¹ final concentration) for 2 h at room temperature to accumulate cells at metaphase. The possible effects of small variation between females in conception time was compensated for by using embryos from several litters.

Fig. 1. 6 d.p.c. egg cylinder embryo after removal of Reichert’s membrane, trophectoderm and ectoplacental cone, showing position of lateral cut made in order to isolate epiblast.

those from whole 6 ½ and 7½ d.p.c. embryos (known to be postinactivation stages) treated by the modified Kanda method.
Table 1. Results of modified Kanda method for individual 6 d.p.c. epiblasts versus rest of embryo

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Tissue</th>
<th>Total metaphases scored</th>
<th>Metaphases with a dark X chromosome (%) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epiblast</td>
<td>9</td>
<td>8 (88.9 ± 10.5)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>15</td>
<td>12 (80.0 ± 10.3)</td>
</tr>
<tr>
<td>2</td>
<td>Epiblast</td>
<td>7</td>
<td>6 (85.7 ± 13.2)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>24</td>
<td>22 (91.7 ± 5.6)</td>
</tr>
<tr>
<td>3</td>
<td>Epiblast</td>
<td>8</td>
<td>7 (87.5 ± 11.7)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>15</td>
<td>12 (80.0 ± 10.3)</td>
</tr>
<tr>
<td>4</td>
<td>Epiblast</td>
<td>7</td>
<td>7 (100.0 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>12</td>
<td>11 (91.7 ± 8.0)</td>
</tr>
<tr>
<td>5</td>
<td>Epiblast</td>
<td>11</td>
<td>10 (90.9 ± 8.7)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>13</td>
<td>11 (84.6 ± 10.0)</td>
</tr>
<tr>
<td>6</td>
<td>Epiblast</td>
<td>13</td>
<td>12 (92.3 ± 7.4)</td>
</tr>
<tr>
<td></td>
<td>Extraembryonic ectoderm + primary endoderm</td>
<td>19</td>
<td>17 (89.5 ± 7.0)</td>
</tr>
</tbody>
</table>

Isolation of 6 d.p.c. epiblasts

After the period in colchicine the embryos were dissected in the following way. First a lateral cut was made with fine-glass needles at the level of the junction between the epiblast and the extraembryonic ectoderm (Fig. 1), thus dividing the embryo into two parts containing the embryonic and extraembryonic regions respectively. The epiblast and overlying primary endoderm were then separated by placing the embryonic half of the embryo in a solution of 0.5% trypsin (w/v) and 2.5% pancreatin (w/v) made up in Ca²⁺⁻⁻⁻ and Mg²⁺⁻⁻⁻-free Tyrode solution at 4 °C for 10 min. Towards the end of the 10 min period in cold enzyme solution the embryonic region was gently pipetted in and out of a flame-polished glass micropipette whose internal diameter was marginally less than that of the cut surface of the embryo. After one or two passages in and out of the micropipette the two cell layers (primary endoderm and epiblast) usually separated spontaneously. The enzyme reaction was then immediately stopped by transferring the tissues to fresh medium containing 10% foetal calf serum followed by washing in fresh medium 199. The extraembryonic part of the egg cylinder plus the primary endoderm were processed separately from the epiblast and thus served as a control in each case for the corresponding epiblast.
Fig. 2. (a) Metaphase chromosomes from epiblast cell from a 6 d.p.c. female embryo after treatment by the modified Kanda technique showing a clear dark-staining, inactive X chromosome (arrow). (b) Metaphase chromosomes from a cell from the rest of the same 6 d.p.c. embryo (primary endoderm or extraembryonic ectoderm) after treatment by the modified Kanda technique with a dark-staining inactive X chromosome (arrow).

Fig. 3. (a) Early prophase cell from epiblast of 6 d.p.c. female embryo showing a prematurely condensed, heteropycnotic inactive X chromosome in comparison with (b). Early prophase cell from the rest of the same 6 d.p.c. female embryo with a prematurely condensed, heteropycnotic inactive X chromosome.

5½, 6½ and 7½ d.p.c. embryos

Embryos were obtained from female mice of the 3H1 strain (F1 hybrids between the two inbred strains C3H/HeH and 101/H) under a normal light regime by synchronizing ovulation with an injection of 5 i.u. Pregnant mare's serum (PMS) followed 44 h later by 5 i.u. human chorionic gonadotrophin (HCG) and caging with fertile males of the same strain overnight. Mating was ascertained by the presence of a copulation plug the next morning and the day of finding the plug designated day ½.
Table 2. Results of modified Kanda method for 5½ d.p.c. female embryos

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Total metaphases scored</th>
<th>Metaphases with a dark X chromosome (% ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15</td>
<td>13 (86.7 ± 8.8)</td>
</tr>
<tr>
<td>B</td>
<td>31</td>
<td>27 (87.1 ± 6.0)</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>13 (86.7 ± 8.8)</td>
</tr>
<tr>
<td>D</td>
<td>27</td>
<td>24 (88.9 ± 6.0)</td>
</tr>
<tr>
<td>E</td>
<td>41</td>
<td>38 (92.7 ± 4.1)</td>
</tr>
<tr>
<td>F</td>
<td>42</td>
<td>35 (83.3 ± 5.8)</td>
</tr>
<tr>
<td>G</td>
<td>22</td>
<td>19 (86.4 ± 7.3)</td>
</tr>
<tr>
<td>H</td>
<td>28</td>
<td>23 (82.1 ± 7.2)</td>
</tr>
<tr>
<td>Total</td>
<td>221</td>
<td>192 (86.9 ± 2.3)</td>
</tr>
</tbody>
</table>

Table 3. Results of modified Kanda method for 6½ d.p.c. female embryos

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Total metaphases scored</th>
<th>Metaphases with a dark X chromosome (% ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>133</td>
<td>115 (86.5 ± 2.9)</td>
</tr>
<tr>
<td>B</td>
<td>143</td>
<td>124 (86.7 ± 2.8)</td>
</tr>
<tr>
<td>C</td>
<td>47</td>
<td>41 (87.2 ± 4.9)</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>31 (87.1 ± 5.8)</td>
</tr>
<tr>
<td>E</td>
<td>21</td>
<td>19 (90.5 ± 6.4)</td>
</tr>
<tr>
<td>F</td>
<td>85</td>
<td>71 (83.5 ± 4.0)</td>
</tr>
<tr>
<td>G</td>
<td>118</td>
<td>98 (83.1 ± 3.5)</td>
</tr>
<tr>
<td>H</td>
<td>44</td>
<td>38 (83.4 ± 5.2)</td>
</tr>
<tr>
<td>I</td>
<td>70</td>
<td>63 (90.0 ± 3.6)</td>
</tr>
<tr>
<td>J</td>
<td>60</td>
<td>53 (88.3 ± 4.1)</td>
</tr>
<tr>
<td>K</td>
<td>161</td>
<td>139 (86.3 ± 2.7)</td>
</tr>
<tr>
<td>L</td>
<td>201</td>
<td>169 (84.1 ± 2.6)</td>
</tr>
<tr>
<td>M</td>
<td>69</td>
<td>58 (84.1 ± 4.4)</td>
</tr>
<tr>
<td>Totals</td>
<td>1188</td>
<td>1019 (85.8 ± 1.0)</td>
</tr>
</tbody>
</table>

Pregnant females were killed by cervical dislocation 5, 6 and 7 days after finding the copulation plug and the embryos recovered in medium 199. Reichert's membrane, the trophectoderm and ectoplacental cone were removed and discarded to ensure that there was no contamination by maternal cells. Again the possible effects of small variation between females in conception time was compensated for by using embryos from several litters for each stage. The embryos were then placed in medium 199 with added colchicine (4 µg ml⁻¹ final concentration) for 2 h at room temperature to accumulate metaphases.
Table 4. Results of modified Kanda method for 7½ d.p.c. female embryos

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Total metaphases scored</th>
<th>Metaphases with a dark X chromosome (%±s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>37</td>
<td>28 (75.7±7.1)</td>
</tr>
<tr>
<td>B</td>
<td>33</td>
<td>26 (78.8±7.1)</td>
</tr>
<tr>
<td>C</td>
<td>90</td>
<td>72 (80.0±4.2)</td>
</tr>
<tr>
<td>D</td>
<td>109</td>
<td>90 (82.6±3.6)</td>
</tr>
<tr>
<td>E</td>
<td>133</td>
<td>115 (86.5±3.0)</td>
</tr>
<tr>
<td>F</td>
<td>143</td>
<td>124 (86.7±2.8)</td>
</tr>
<tr>
<td>Totals</td>
<td>545</td>
<td>455 (83.5±1.6)</td>
</tr>
</tbody>
</table>

Chromosome preparation by the modified Kanda method

After the period in colchicine whole 5½, 6½ and 7½ d.p.c. embryos and isolated epiblasts from 6 d.p.c. embryos versus the rest of the embryo were treated by the modified Kanda method as previously described (Rastan et al. 1980). Briefly, the embryos were transferred to hypotonic 0.5% potassium chloride solution at room temperature and incubated in a waterbath at 50 °C for 15 min, fixed in 3:1 absolute ethanol/acetic acid solution, disaggregated in 60% acetic acid and slides made on a hotplate at about 40 °C by allowing small drops of the acetic acid mixture to evaporate off leaving the metaphase chromosomes spread on the slide (Meredith, 1969). All slides were stained in 2% Giemsa buffered at pH 6.8 for 20 min.

Analysis

Slides were randomly coded, by a colleague, before being scored blind by the author. They were sexed cytologically by determining the presence or absence of a Y chromosome. This task was facilitated by the fact that the Y chromosome is the darkest staining element in over 70% of cells from postimplantation male mouse embryos treated by the modified Kanda method (Rastan, 1981). Slides of cells without a Y chromosome were assumed to be from female embryos and metaphase cells were scored for the presence or absence of a dark-staining inactive X chromosome. Only intact, well-spread metaphase cells containing 40 chromosomes were scored.

RESULTS

6 d.p.c. epiblast versus extraembryonic ectoderm plus primary endoderm

A total of fourteen 6 d.p.c. preprimitive streak embryos from four mothers had their epiblasts successfully isolated and were treated individually by the modified Kanda method. Six of these embryos proved to be female on cytological analysis. Table 1 shows the results for individual embryos. Contrary to
the prediction of the stem-cell model in every case a clear dark-staining inactive X chromosome was seen in over 85% of epiblast cells of female embryos at metaphase (Fig. 2a). In addition, a prematurely condensed heteropycnotic inactive X chromosome was visible in early prophase cells from 6 d.p.c. epiblasts (Fig. 3a). X inactivation had also occurred, as expected, in the extraembryonic part of the embryo and the primary endoderm, as revealed by a dark-staining X chromosome at metaphase in over 80% of cells (Table 1 and Fig. 2b) and by a prematurely condensed heteropycnotic X in early prophase cells (Fig. 3b).

The individual results in Table 1 were tested for heterogeneity by an extension of Fisher's exact test and found to be sufficiently homogeneous to justify pooling the results. In total 50 out of 55 metaphase cells from 6 d.p.c. epiblast (i.e. 90.9%) exhibited an inactive X chromosome compared with 85 out of 98 metaphase cells (i.e. 86.7%) from the extraembryonic ectoderm plus primary
endoderm. There is no statistically significant difference between the frequency of finding a dark-staining inactive X chromosome between the cells of the epiblast and the rest of the embryo ($\chi^2 = 0.591$). The results indicate that by 6 d.p.c. X-inactivation has taken place in all tissues of the embryo including the epiblast.

**Whole $5\frac{1}{2}$, $6\frac{1}{2}$ and $7\frac{1}{2}$ d.p.c. embryos**

Tables 2, 3 and 4 show the results for individual embryos at $5\frac{1}{2}$, $6\frac{1}{2}$ and $7\frac{1}{2}$ d.p.c. respectively. The results from single embryos at each age were statistically tested for heterogeneity by a test for the equality of binomial proportions and found to be sufficiently homogeneous to be pooled.

A comparison of pooled results is therefore permissible. On average 86.9% of metaphase cells from $5\frac{1}{2}$ d.p.c. female embryos showed a dark-staining inactive X chromosome (Fig. 4a) compared with 85.8% from $6\frac{1}{2}$ d.p.c. embryos (Fig. 4b) and 83.5% from $7\frac{1}{2}$ d.p.c. embryos (Fig. 4c). There is no statistically significant difference in the proportion of cells which show a dark-staining inactive X chromosome in embryos of any age ($\chi^2 = 4.189$ with 3 degrees of freedom). As $6\frac{1}{2}$ and $7\frac{1}{2}$ d.p.c. embryos are known to be postinactivation stages in which X inactivation is complete in all cells (Monk & Harper, 1979) this suggests that X inactivation is also complete in all cells of the $5\frac{1}{2}$ d.p.c. embryo.

**DISCUSSION**

The results presented in this paper clearly indicate that by day 6 of mouse embryogenesis the cells of the epiblast or embryonic ectoderm are equivalent to the cells of the rest of the embryo as far as X inactivation is concerned. The clear, dark-staining, inactive X chromosome seen in metaphase cells from 6 d.p.c. epiblast, combined with the prematurely condensed, heteropycnotic inactive X chromosome in epiblast cells at prophase, constitutes compelling evidence that X inactivation has occurred in all tissues of the embryo by 6 d.p.c. In addition, the very high proportion of epiblast cells which showed a dark-staining, inactive X chromosome (90.9%) strongly suggests that X inactivation has taken place in all cells of the epiblast by this time.

This result is supported by the fact that as early as $5\frac{1}{2}$ d.p.c. a dark-staining inactive X chromosome is seen, on average, in over 86% of metaphase cells, and there is no statistically significant difference between the frequency of finding a dark-staining, inactive X chromosome in metaphase cells from $5\frac{1}{2}$ d.p.c. embryos and $6\frac{1}{2}$ and $7\frac{1}{2}$ d.p.c., of which the latter two are known to be postinactivation stages.

The system for producing embryos of age 6 d.p.c. used in this experiment from the mating of day-/night-reversed mice was specifically chosen to produce embryos directly equivalent in age to those used by Monk & Harper (1979) in the HGPRT dosage experiment, therefore slight differences in age of the embryo
used in this experiment compared with Monk and Harper's experiment can be excluded. The possibility that strain differences could be responsible for the discrepancy in the results (MFI Swiss mice were used by Monk and Harper) is considered to be unlikely. Differences in the timing of early preimplantation development in the mouse between inbred strains have been shown (McLaren & Bowman, 1973). These differences were slight, however, and were shown to be due to the time at which cleavage began. In both the present experiment and Monk and Harper's experiment F₁ hybrids and outbred mice respectively were used rather than inbred strains, and any differences in the rate of development between the two types of mice are unlikely to be of such a magnitude as to be able to cause a difference in timing of X-inactivation of up to 1 day.

The chance of contamination of epiblast cells by primary endoderm cells in the present work is also very remote, as the tissues were well washed after separation and processed separately. In addition, only tissues which on pipetting fell apart spontaneously into two discrete pieces were used.

Given the discrepancy in the results presented here and the findings of Monk and Harper, more weight must be attached to the cytogenetic evidence that suggests X inactivation has occurred in day-6 epiblast cells than to the biochemical enzyme-dosage evidence, for the following reasons. Firstly, the modified Kanda technique provides direct visual evidence of whether or not individual cells of individual embryos of known sex contain an inactive X chromosome, whereas the biochemical dosage approach is indirect and rests on the assumption that the activity of the HGPRT enzyme reflects the activity status of the X chromosome. Secondly, there are several reasons why the biochemical gene-dosage approach could be unreliable; (i) although activity of HGPRT in individual embryos is analysed the conclusions with respect to X inactivation depend on the distribution of activities within a population of embryos, hence developmental variability among embryos could affect the bimodality or unimodality of a distribution; (ii) the disappearance of a dosage difference between male and female embryos will depend on both the relative rates of synthesis of the enzyme after X inactivation occurs and the turnover rate of the previously synthesized message and enzyme protein. Without some knowledge of these parameters, conclusions about the relative timing of X inactivation and the change in enzyme activity seem to be unjustified. The most likely explanation for the discrepancy between the results of Monk and Harper and those presented in this paper is that the bimodality of the distribution of HGPRT activities in 6 d.p.c. epiblasts reflects residual enzyme or mRNA activity.

One way of reconciling the cytogenetic evidence presented here that X inactivation has occurred in 6 d.p.c. epiblast cells before primitive streak formation and, indeed, is probably complete in all cells of the embryo by 5½ d.p.c., with the biochemical evidence that X inactivation has not yet occurred in epiblast cells by day 6, is the possibility that the differential staining property of the inactive X chromosome revealed by the Kanda technique precedes genetic
inactivation of X-linked genes. This explanation is considered to be unlikely as the differential staining property of the inactive X chromosome, first seen in 5½ d.p.c. embryos, persists unchanged in cells of older embryos and adults (Rastan, 1981) and therefore presumably represents the stable physicochemical manifestation of genetic X-chromosome inactivation. Support for this contention is provided by the fact that a prematurely condensed, heteropycnotic inactive X chromosome is also visible in prophase cells by this stage.

The results presented in the paper show that X inactivation has taken place in the so-called 'undifferentiated' epiblast cells of the 6 d.p.c. mouse embryo and is in fact complete in all cells of the embryo by 5½ d.p.c. This does not, of course necessarily mean that the idea of X inactivation linked to cellular differentiation is incorrect. There is very good evidence that X-inactivation does occur earlier in the trophoderm than in the ICM. The confusion with the stem-cell model of Monk may arise by the use of the term 'undifferentiated'. It is known that by 4½ d.p.c. in mouse embryogenesis a layer of primitive endoderm cells has delaminated on the blastocoelic surface of the ICM (Dickson, 1966; Snell & Stevens, 1966). Single primitive endoderm cells from 4½ d.p.c. embryos in cell lineage studies contribute only to the endoderm layer of the yolk sac and no other tissue: single embryonic ectoderm (epiblast) cells from 4½ d.p.c. embryos, on the other hand, are able to contribute to the mesoderm layer of the yolk sac and/or foetus, but never to the yolk sac endoderm as well (Gardner & Rossant, 1979). However, the fact that epiblast cells are multipotential does not necessarily mean that they are also undifferentiated. Gardner & Rossant (1979) showed that as early as 4½ days primitive ectoderm and primitive endoderm cells are stably differentiated with respect to properties that ensure their precise partitioning in host embryos.

As epiblast or extraembryonic ectoderm cells can thus be considered to be differentiated by 4½ d.p.c. it is possible that X inactivation is indeed linked with cellular differentiation and occurs at different times in trophoderm, primary endoderm and epiblast but is complete by 5½ d.p.c. The HGPRT dosage difference found by Monk and Harper between epiblast and the other tissues of the 6 d.p.c. embryos may thus reflect a difference in the timing of onset of X inactivation in the various tissues that occurred earlier, caused by residual enzyme or mRNA activity.

The analogy between mouse teratocarcinoma stem cells and the cells of the normal early embryo is well documented (reviewed by Martin, 1975, 1978; Graham, 1977). Martin et al. (1978) have used differentiation of a teratocarcinoma stem-cell line as an in vitro model of X chromosome differentiation in mouse embryogenesis. Both X chromosomes were shown to be active in clonal cultures of undifferentiated female mouse teratocarcinoma stem cells derived from a spontaneous ovarian tumour as measured by the ratio of three X-linked enzymes, 6GPD, HGPRT and α-galactosidase in an XX cell line to an XO cell line. As the cell cultures were allowed to differentiate in vitro, however, there
was concomitant inactivation of one of the two X chromosomes present in the XX line. There is some conflicting evidence, however. In an earlier study of a similar nature, McBurney & Adamson (1976) could find no difference between the activities of several X-linked enzymes in six different teratocarcinoma stem-cell lines containing either one or two X chromosomes. They concluded that only one chromosome was active in undifferentiated female teratocarcinoma stem-cell lines. This conclusion was supported by an equal recovery of azaguanine-resistant mutants from cell lines containing one of two X chromosomes. A further confusing observation was that a late-replicating X chromosome was seen in one teratocarcinoma stem-cell line with two X chromosomes, but not in another.

Martin has suggested that the presence of only a single active X chromosome in McBurney and Adamson's stem-cell lines could be due to their culture procedures which may have resulted in a change of activity state of one of the X chromosomes, or could be explained by the fact that the cells examined were isolated from a tumour obtained from a 6.5 d.p.c. embryo and may thus have been derived from a later (postinactivation) stage of development. However, there is evidence that at least some teratocarcinoma stem cells derived from postinactivation embryos can have two active X chromosomes. McBurney & Strutt (1980) found activity of both X chromosomes in a teratocarcinoma stem-cell line derived from a 7.5 d.p.c. female embryo heterozygous for two electrophoretic variants of the X-linked enzyme PGK. As all the evidence points to X-chromosome inactivation being complete in all cells of the female mouse embryo by 7.5 d.p.c. the activity of both X chromosomes in a teratocarcinoma stem-cell line derived from such an embryo must have arisen by reactivation of the inactive X chromosome in the cell line, or loss of the inactive chromosome and duplication of the active chromosome.

One must conclude, therefore, that although there are analogies between teratocarcinoma stem cells and the cells of the early mouse embryo, the major one being that both are pluripotent, the use of such a system as an in vitro model of X inactivation in embryogenesis may be misleading.

One further indication that the scheme of the stem-cell model of X-chromosome differentiation may be suspect is that the model predicts that female germ cells remain on the 'undifferentiated' stem-cell line and thus escape X inactivation altogether. However, although clearly 'totipotential' germ cells must be regarded as very highly specialized cells, programmed to migrate to the gonads, colonize the gonads, proliferate and enter into meiosis at a specific time, and there is now ample evidence, both biochemical and cytological, that X inactivation occurs in primordial germ cells and that the two active X chromosomes seen in oocytes result from reactivation of the inactive X chromosome at, or shortly before, the time of meiosis (Ohno, 1964; Gartler Andina & Gant, 1975; Andina, 1975; Johnston, 1979; Gartler Rivest & Cole, 1980).
REFERENCES


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