The biochemistry of differentiation of mouse trophoblast: studies on polyploidy

By PETER W. BARLOW and MICHAEL I. SHERMAN

From the Paediatric Research Unit, Guy's Hospital Medical School, London, the Sir William Dunn School of Pathology and the Department of Zoology, University of Oxford

SUMMARY

1. Trophoblast development in utero in the first half of gestation is accompanied by a rise in the ploidy levels in giant cell nuclei. Giant cells constitute up to one-third of the trophoblast population over this period.

2. The expression of polyploidy is independent of the maternal environment; the extent of polyploidy and the degree to which trophoblast develops morphologically under various conditions appear to be related.

3. The following events are not necessary for the initiation of polyploidization in trophoblast: (a) hatching from the zona pellucida, (b) oestrogen sensitization, (c) implantation, (d) postimplantation changes in topology.

4. That earlier developmental events such as the formation of the blastocoel cavity may in some way trigger polyploidization of trophoblast is not inconsistent with the studies described.

INTRODUCTION

The mouse blastocyst consists of a single cell layer, the presumptive trophoblast, surrounding the blastocoel cavity and a group of internal cells, the inner cell mass. It is generally accepted that following implantation, the presumptive trophoblast gives rise to the ectoplacental cone and the trophoblast layer of the placenta, while the cells of the inner cell mass are the precursors of the embryo proper. A number of experiments have been carried out to investigate the lability of early embryonic cells and the effect of position upon their developmental fate. These experiments provide information about the phenomenon of determination, and have been recently reviewed by Graham (1971a). Little, however, is known about the timing of, or the events governing, the onset of differentiation of trophoblast or other embryonic cell types.

To study trophoblast differentiation at the molecular level it is necessary to show that mature trophoblast has biochemical properties not shared by the embryo; the appearance of each new property serves as a marker of the course

1 Author's address: Unit of Developmental Botany, University of Cambridge, 181A Huntingdon Road, CB3 0DY, Cambridge, U.K.

2 Author's address: Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110, U.S.A.
of differentiation. A number of trophoblast enzymes have now been described and their bearing on the question of trophoblast differentiation will be discussed elsewhere (Sherman, 1972a, b). That the nuclei of rodent trophoblast are different from those of the embryo has been known since the early observations of Duval (1891). Using quantitative cytospectrophotometric techniques Zybinna and co-workers (1961, 1963; Zybinna & Mos'yan, 1967) have shown that the trophoblast of the rat has giant nuclei which contain hundreds of times the diploid DNA content. Indeed, she suggests that all trophoblast cells are polyploid by the second half of gestation (Zybina, 1970). (For the sake of convenience, we use the term ‘polyploid’ here only to denote the content in nuclei of DNA greater than 4 times the haploid amount. We do not wish to imply that within a giant trophoblast nucleus all chromosomes have necessarily been replicated the same number of times.) Zybinna has proposed that this phenomenon occurs by a process of endoreduplication, i.e. replication of the nuclear material without subsequent mitosis and cell division (Zybina, 1960, 1963; Zybina & Mos'yan, 1967), resulting in the formation of polytene chromosomes (Zybina, 1970) similar to those found in the salivary glands and other tissues of dipteran flies (Beermann, 1962) and in some plant tissues (Nagl, 1963; Tschermak-Woess, 1967).

Giant nuclei are also seen in the trophoblast (but not in other embryonic cell types) of the mouse. We felt that the detection of both the onset, and the subsequent time course, of polyploidization would provide us with information useful in the study of trophoblast differentiation and complementary to the biochemical studies described elsewhere (Sherman, 1972a, b). We have carried out measurements of the DNA content of trophoblast nuclei between the 5th and 11th days of pregnancy in the mouse (for DNA studies on earlier embryos, see Barlow, Owen & Graham, 1972). The results establish, and give a quantitative estimate of, the polyploid nature of mouse trophoblast. We have asked when polyploidy first arises and have considered possible mechanisms for its induction. In so doing, we have used blastocyst cultures to explore the extent to which the development of polyploidy is dependent upon the maternal environment; we conclude that polyploidy in trophoblast is at least to some degree an intrinsic phenomenon of mouse embryogenesis.

METHODS AND MATERIALS

Preparation of animals

Embryos obtained from matings within a closed Swiss albino stock (strain PO) or from a PO × A2G cross were a source of trophoblast nuclei. No strain-specific differences were noted. Trophoblast from 6th to 11th day embryos was obtained from spontaneously mated females, while preimplantation embryos were collected from females superovulated (Runner & Palm, 1953) before mating. The day of observation of the sperm plug is considered the first day of pregnancy. For kidney capsule grafts, blastocysts were removed from the uterus on
the 4th day of gestation and implanted under the kidney capsule of mature hosts (Fawcett, 1950; Kirby, 1960). The resulting haemorrhagic growths were dissected out 10 days later.

**Collection of material**

Blastocysts were obtained on the 4th day of pregnancy by flushing uteri with phosphate-buffered saline (PBS). Two-cell embryos were obtained by teasing apart oviducts in Whitten’s medium (1971) on the morning of the second day of pregnancy. Postimplantation trophoblast was obtained up to the 11th day of pregnancy as described elsewhere (Sherman, 1972b).

**Culture conditions**

Two-cell embryos were cultured to the blastocyst stage in the medium of Whitten (1971). Blastocysts removed from uteri on the 4th day of gestation were allowed to swell and enlarge by overnight culture in White’s medium (Knowland & Graham, 1972). Further development of blastocysts in vitro was effected by culture in Eagle’s medium as modified by Vogt & Dulbecco (1963), made up with four times glass-distilled water and supplemented with 10% foetal calf serum. The percentage of blastocysts ‘implanting’ (sticking to the culture dish and spreading out along it) was well over 90% when multiply-distilled water and exhaustively dialysed serum were used. ‘Unhatched’ blastocysts, i.e. those failing to rupture their zona pellucida after 4-7 days in culture without undergoing any apparent degree of degeneracy, were obtained in small numbers from these cultures.

**Preparation of materials**

Trophoblast on the 7th to 9th days of development was dissected free of embryo, yolk sac and decidua in a drop of PBS directly on a microscope slide. The trophoblast cells were then teased apart with forceps, and the material was fixed by dipping the slide into a dish of absolute ethanol/glacial acetic acid (3/1, v/v). This dipping procedure appeared to remove most of the blood cells. The slide was left in fixative for 10 min. For 6th day embryos, trophoblast could not be satisfactorily separated from the embryo proper, and thus the cells on the slide were from the two tissues. This general procedure was unsatisfactory for preparations of 10th and 11th day materials as a great many cells were present and the nuclei overlapped. To obtain giant nuclei on these days, trophoblast was dissected away from the decidua. In the process, a number of giant cells were disrupted and their nuclei spilled out of the cells and sank to the bottom of the dissecting dish. Individual nuclei were then sucked up in a drawn-out capillary pipette, placed on a slide in a small drop of PBS and then fixed. Alternatively, where indicated, nuclei were teased out of intact cells following dissection of trophoblast, and fixed on slides as above. Nuclei from kidney capsule grafts were obtained in the same way. Blastocyst cultures grown on coverslips were
removed from the medium, washed in PBS, fixed for 10 min and mounted on slides. Preimplantation and unimplanted embryos were prepared as described by Barlow et al. (1972).

**Staining and measurements**

The slides with nuclei for DNA measurements were hydrolysed in 5N-HCl for 30 min at room temperature and then stained with Schiff's reagent for 1–2 h. The preparations were washed with three changes of freshly prepared SO₂ water, 10 min each change, then dehydrated and mounted in Euparal. Measurements of nuclear DNA contents were made with an integrating microdensitometer (Barr and Stroud, Glasgow, model GN 2) at 565 nm. All readings were corrected for background absorption. A slide of liver imprints was used to calibrate each set of measurements. Liver nuclei are either diploid, tetraploid, or octaploid and so contain 2C, 4C and 8C units of DNA respectively (Fig. 1): C is the haploid amount of DNA. Nuclear contents were converted to values of C and are here presented as histograms using a log₂ scale. Nuclear areas were calculated by the formula $A = \pi \frac{1}{2} d_1 d_2$, where $d_1$ and $d_2$ are the major and minor diameters of the nucleus in micrometres (μm).

**RESULTS**

**Ploidy measurements on isolated trophoblast nuclei**

As noted by Zybina in the rat (1960), giant trophoblast nuclei in the mouse stain with Feulgen as intensely as small nuclei (Fig. 2). DNA measurements on the largest of these nuclei indicate that they contain enormous amounts of DNA, up to 850 times the haploid value (Fig. 3). In Fig. 3(a) are DNA values for nuclei which were released from cells of 11th day trophoblast during dissection, while Fig. 3(b) illustrates measurements of trophoblast nuclei teased from cells which have survived the dissection procedure intact. It may be seen that although
Polyploidy in mouse trophoblast

Fig. 2. Nuclei of 11th day mouse trophoblast. Trophoblast tissue was smeared on a slide and stained with Schiff's reagent. Nuclei from both normal and giant cells are present. Scale line = 50 μm.

nuclei reach the same high ploidy values in both cases, Fig. 3(a) shows clustering of the DNA contents around euploid values whereas Fig. 3(b) does not. Measurements on the trophoblast nuclei in Fig. 3(a) indicate that DNA content is roughly proportional to nuclear size (Fig. 4). The largest nuclei have diameters as great as 140 μm (compared with a 2C value of about 10), although this value is overestimated due to flattening of the nuclei on to the slide during fixation.

The nuclei of trophoblast dissected from several placentas on the 6th to 11th days of pregnancy were scanned, and the largest ones measured. The results are shown in Table 1. By the sixth day of pregnancy, nuclei were found containing between 32 and 64 times the haploid amount of DNA. The DNA content of this largest class of trophoblast nuclei roughly doubled on each day of development. The highest DNA content of a nucleus from 11th day trophoblast contained about 850 times the haploid amount of DNA.

To obtain some idea of the relative proportions of cells with different ploidies in the total trophoblast population, trophoblast from single embryos on the 7th to 11th days of gestation were dissected out and smeared on slides. DNA measurements were made on a random selection of the nuclei; at the same time efforts were made to avoid measuring contaminating immature red blood nuclei which
Fig. 3. The DNA content of giant-cell nuclei in 11th day trophoblast. In (a), free nuclei were collected from the bottom of a dissecting dish with a capillary tube after trophoblast tissue was pulled apart from the decidua. The nuclei were then fixed on slides, stained with Schiff's reagent and measured as indicated in Methods. In (b), trophoblast tissue was separated from those nuclei freed by the dissection procedure, and then disrupted with watchmaker's forceps to free further nuclei. These were measured for DNA content as in (a).

are distinguishable from diploid trophoblast nuclei on the basis of morphology. The results are shown in Fig. 5. On the 7th day (Fig. 5a) 8% of the total number of nuclei have DNA contents greater than 4C; of the remaining population, one-third have a DNA value greater than 2C but no more than 4C, while the other two-thirds are 2C nuclei. By the 8th and 9th days of development (Fig. 5b, c) 30–40% of the total nuclear population measured have DNA values greater than 4C; the distribution of DNA values within the diploid population is about the same as that of the 7th day sample. In the course of these studies, however, we suspected that the proportion of diploid cells in the total population was being constantly underestimated, as many clumps of overlapping small cells were present on the slides, and their DNA contents could not be measured. The problem of overlapping nuclei in smears of 10th and 11th day trophoblast was so marked that the measurements of a random sample was not feasible. Because of the apparent relationship between nuclear area and DNA content, at least for larger nuclei (Fig. 4), we attempted to rescore the slides visually to assess the population of polyploid nuclei. In this way, clumps of overlapping diploid cells
Polyploidy in mouse trophoblast

Fig. 4. The relationship between DNA content and area of giant trophoblast nuclei. Areas of the nuclei measured in Fig. 3 (a) were determined as indicated in Methods. The area of each nucleus is plotted on a log_{10} scale against its DNA content.

Table 1. Highest ploidy reached by trophoblast of different gestation age

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>32–64</td>
</tr>
<tr>
<td>7</td>
<td>64–128</td>
</tr>
<tr>
<td>8</td>
<td>64–128</td>
</tr>
<tr>
<td>9</td>
<td>128–256</td>
</tr>
<tr>
<td>10</td>
<td>256–512</td>
</tr>
<tr>
<td>11</td>
<td>512–850</td>
</tr>
</tbody>
</table>

Nuclei from trophoblast from a number of embryos of the indicated ages were prepared for DNA measurements as described in Methods, and the largest nuclei were selected for microspectrophotometry. DNA values are expressed in terms of C, the haploid content for mouse.

which could not be measured spectrophotometrically could be taken into account. Mitotic index was also scored. All the mitoses were diploid as judged by microdensitometry or chromosome counts (Barlow & Sherman, unpublished); consequently, the mitotic index is expressed as the percentage of diploid cells in division. The results of the visual observations are presented in Table 2. By these estimates, between 10 and 15 % of the cells appear to be polyploid on the 8th to 11th days of gestation. We thus propose on the basis of the data in Fig. 5 and Table 2 that no more than one-third of trophoblast cells are polyploid between the 7th and 11th days of gestation. Furthermore, because of the paucity of nuclei with DNA contents between 2C and 4C (Fig. 5), and the very low mitotic
Fig. 5. DNA contents in trophoblast nuclei on the 7th to 9th days of gestation. Trophoblast tissue from single conceptuses was dissected out and smeared on slides. After staining with Schiff's reagent the DNA content of a random sample of nuclei was determined. The animals were sacrificed on the evening of the (a) 7th, (b) 8th and (c) 9th days of gestation. Each histogram represents results pooled from at least two slides.

index (Table 2), it would appear as though most of the population of diploid cells on the 7th to 9th days of gestation are in the $G_1$ and $G_2$ phases of the cell cycle.
Polyploidy in mouse trophoblast

Table 2. Percentages of diploid and polyploid cells and cells in mitosis at different stages of trophoblast development

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Total no. of cells</th>
<th>No. of polyploid cells</th>
<th>Polyploid (%)</th>
<th>No. of diploid cells</th>
<th>No. of diploid mitoses</th>
<th>Mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 L</td>
<td>1008</td>
<td>12</td>
<td>1-2</td>
<td>996</td>
<td>3</td>
<td>0-3</td>
</tr>
<tr>
<td>8 E</td>
<td>2598</td>
<td>243</td>
<td>9-4</td>
<td>2355</td>
<td>2</td>
<td>0-09</td>
</tr>
<tr>
<td>8 L</td>
<td>4775</td>
<td>541</td>
<td>11-3</td>
<td>4234</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9 L</td>
<td>3964</td>
<td>516</td>
<td>13-0</td>
<td>3448</td>
<td>2</td>
<td>0-06</td>
</tr>
<tr>
<td>10 L</td>
<td>6258</td>
<td>809</td>
<td>12-9</td>
<td>5449</td>
<td>76</td>
<td>1-4</td>
</tr>
<tr>
<td>11 L</td>
<td>10920</td>
<td>1163</td>
<td>10-6</td>
<td>9757</td>
<td>190</td>
<td>1-9</td>
</tr>
</tbody>
</table>

Trophoblast dissected from placentas in the morning (E) or evening (L) of the indicated day of pregnancy were smeared on slides and stained with Schiff's reagent. Nuclei were then scored as polyploid visually if they were equal to or greater than the size of an average 8C liver nucleus. A number of individual trophoblasts were so treated at each of the gestation ages, and the results have been pooled.

Nuclear morphology

In view of the claim by Zybina (1970) that the giant nuclei of rat trophoblast contain polytene chromosomes with a distinct chromomere banding pattern, we paid particular attention to the morphology of giant nuclei in the mouse. In preparations of giant nuclei dissected from the trophoblast and stained with Feulgen, Giemsa or fluorochromes we have been unable to find any evidence of polytene chromosomes. Some nuclei possess chromatin that has a fibrillar appearance, but for the most part these fibrils show no particular orientation within the nucleus, nor is there any apparent lateral attraction to form a single polytene chromosomal unit. Unlike Zybina (1970) we have not seen any nucleus that could be interpreted as an endomitotic nucleus (in sensu Geitler, 1953). These observations will be elaborated more fully elsewhere (in preparation).

Dependence upon the maternal environment for the expression of polyploidy

Experiments were designed to test whether polyploidy could develop and, if so, to what extent, under conditions independent of the maternal environment. In the first experiment blastocysts were removed from the uterus on the 4th day of pregnancy and transplanted under the kidney capsule of a non-pregnant adult female. After 10 days incubation, the haemorrhagic growths were removed and the cells teased apart. As in the case with mature uterine trophoblast, many large nuclei fall to the bottom of the dissecting dish during this procedure. These nuclei are highly polyploid (Fig. 6), the largest being equivalent in DNA content to the maximum values found in uterine trophoblast on the 11th day of development (cf. Fig. 3). This result confirms the finding of Hunt & Avery (1971) that trophoblast nuclei from kidney capsule implants contain large amounts of DNA, and illustrates that implantation in the uterus is not required for the expression of polyploidy.
Fig. 6. DNA content in giant nuclei from an ectopic pregnancy. Ten days after implanting a blastocyst under a kidney capsule of an adult mouse the haemorrhagic growth was removed and disrupted with watchmaker's forceps. Following this, giant nuclei were collected, fixed on slides, stained, and subjected to DNA determination.

Fig. 7. DNA content of nuclei in cultured blastocysts. Blastocysts were obtained on the middle of the 4th day of pregnancy and cultured overnight in White's medium (Knowland & Graham, 1972). They were then transferred in groups of about ten to drops of modified and supplemented Eagle's medium (see Methods) maintained in place on glass coverslips with paraffin oil. After 24 h (Day 1) a coverslip was removed, rinsed in PBS and fixed in acetic acid/ethanol (1/3, v/v) for 10 min. After staining with Schiff's reagent the nuclear DNA content was measured where possible in those blastocysts which had outgrown on the coverslip. This was repeated on coverslips removed from the dish after 48 h (Day 2), 72 h (Day 3), etc., in Eagle's medium.

To determine the extent to which contact with tissue is responsible for the onset of polyploidy, blastocysts were removed on the 4th day of pregnancy and placed in culture on coverslips, first overnight in White's medium (Knowland & Graham, 1972) and then into modified Eagle's medium (Vogt & Dulbecco, 1963). Cole & Paul (1965) and Gwatkin & Meckley (1965) have already shown that giant cells appear in such cultures. Feulgen-stained nuclei in these blastocyst cultures were measured and the results are shown in Fig. 7. After one day in Eagle's medium, nuclei as large as 16C were recorded; the ploidy reached 64C after 5 days in this medium. Higher ploidy values were not observed with further culture. This could well have been due to suboptimal culture conditions, as many of the nuclei appeared to lift off the coverslip after 5 or 6 days in culture. Some of the larger nuclei also appeared to be breaking up into smaller fragments.
Polyploidy in mouse trophoblast

Table 3. Proportion of polyploid cells in blastocyst cultures

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Days of culture in Eagle's medium</th>
<th>Total no. of nuclei measured</th>
<th>No. of nuclei with DNA content ( \leq 4C )</th>
<th>% of nuclei with DNA content ( \leq 4C )</th>
<th>No. of nuclei with DNA content ( &gt; 4C )</th>
<th>% of nuclei with DNA content ( &gt; 4C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>222</td>
<td>137</td>
<td>62</td>
<td>85</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>254</td>
<td>138</td>
<td>54</td>
<td>116</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100</td>
<td>45</td>
<td>45</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>91</td>
<td>23</td>
<td>25</td>
<td>68</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>111</td>
<td>7</td>
<td>6</td>
<td>104</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100</td>
<td>20</td>
<td>20</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>33</td>
<td>11</td>
<td>33</td>
<td>22</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>70</td>
<td>13</td>
<td>18</td>
<td>57</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>103</td>
<td>60</td>
<td>58</td>
<td>43</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>103</td>
<td>49</td>
<td>48</td>
<td>54</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>103</td>
<td>32</td>
<td>31</td>
<td>71</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100</td>
<td>14</td>
<td>14</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>30</td>
<td>1</td>
<td>3</td>
<td>29</td>
<td>97</td>
</tr>
</tbody>
</table>

The data in Exp. 1 are taken from Fig. 7. The data in Exp. 2 are taken from Fig. 8.

Most of the smaller cells had already detached from the coverslips, as evidenced by the striking reduction in the proportion of diploid cells after the 4th day of culture in Eagle’s medium (Table 3, Exp. 1). In fact, the proportion of the diploid cell population up to the 5th day is under-represented in these histograms because many of the smaller (presumably inner cell mass) cells remain as a clump in the centre of the culture and are not easily measured. By the 6th day, however, these clumps have lifted off, leaving only the outgrowing polyploid cells. Consequently, while it would appear that embryonic precursor cells do not fare well under these culture conditions, giant trophoblast cells survive somewhat more successfully, and reach high levels of ploidy before they begin to degenerate.

While these results demonstrate that contact with tissue is not required for the expression of polyploidy, it might still be argued that the onset of polyploidy inevitably resulted from a stimulus received by blastocysts while they were still in the uterus. To investigate this, eggs were removed from oviducts at the 2-cell stage, maintained in culture (Whitten, 1971) until they were mature blastocysts (approximately 72 h), and then transferred to Eagle’s medium. Nuclei were measured for DNA content in these cultures, and as Fig. 8 and Table 3, Exp. 2 show, a pattern emerges very similar to that seen in Fig. 7. After 6 days in Eagle’s medium, nuclei with DNA contents in the 64–128C class were present. Once again, very few diploid cells remained after 6 days of culture (Table 3). We conclude from these experiments that trophoblast cells will undergo polyploidization independently of the maternal environment, at least from the two-cell stage.
Day

10

5

°C

|-

r

4 4 4 4 +

4 4 4 *

|-

n n

2 4 8 16 32 64 128

C

Fig. 8. DNA content in blastocyst cultures derived from two-cell embryos. Embryos at the 2-cell stage were removed from oviducts and cultured in Whitten's medium (1971) until they reached mid to late blastocyst stage. They were then grown on coverslips as described in Fig. 7 for 2, 4, 5, 6, or 8 days. DNA contents were then determined as in Fig. 7.

Other factors involved in the onset of polyploidy

The above experiments have demonstrated that neither a uterine signal nor contact with foreign cells is responsible for the onset of polyploidy in trophoblast. We have considered the possibility that in blastocyst cultures, contact with the culture dish and the concomitant spreading of trophoblast along the surface would be sufficiently analogous to events during implantation in utero to provide the necessary stimulus for the onset of polyploidy. We have thus examined the DNA content of blastocyst nuclei under conditions that preclude attachment to the culture dish. Barlow et al. (1972) were unable to detect blastocyst cells containing more than 4C amounts of DNA up to the fifth day of development. On the fifth day, blastocysts which have not yet implanted or which are just in the process of implantation can be flushed out of the uterus. We have examined the DNA contents of cells from such blastocysts, and the results may be seen in Fig. 9. Of the 336 nuclei measured, a very small number, 6, had DNA contents significantly greater than 4C, between 5C and 7C. Furthermore, while some or most of the population of nuclei at 4C may be in the G2 phase of the diploid cell cycle, others may be held in readiness for polyploidization, i.e. in G1 of the first endoreduplication cycle. These results confirm similar studies by Graham (1971b); the timing of the onset of polyploidization is described in Barlow et al. (1972).

When carefully prepared Eagle's medium is used for blastocyst culture (see Methods), over 80% of the blastocysts will shed their zona pellucidae, stick to,
and grow out along the surface of the dish or coverslip within 2 days of culture. The majority of the blastocysts which do not behave in this way will have failed to hatch from their zona pellucida. These do not, however, appear to be degenerate (Fig. 10). We do not yet know the reason behind the failure to hatch, but spreads of these unhatched blastocysts after 4 days in culture reveal that they contain from 64 to 250 cells (average of 12 samples = 130). All have giant cells, and over 80% of them have cells in mitosis. They are able to grow out along the culture dish after enzymic removal of the zona. Finally, they appear to have the same life-span of normal blastocyst cultures, i.e. they degenerate after 8–10 days.

We have measured DNA contents in nuclei of unhatched blastocysts after 4 and 7 days in culture. As seen in Fig. 11, there are a substantial number of polyploid cells, even after 4 days in culture. Indeed, the patterns are strikingly similar to those seen in normal blastocyst cultures (cf. Figs. 7, 8). The exception, however, is that there are proportionately more diploid cells in unhatched blastocysts although the proportion of diploids falls significantly between 4 and 7 days in culture. These results confirm that implantation, contact with any surface, and indeed, even rupture of the zona pellucida are not essential for the onset of polyploidy.

The ploidy of the trophoblast stem cell

Zybina has indicated that in the rat, by the 15th day of pregnancy, all of the nuclei in the trophoblast are polyploid (Zybina, 1970; Zybina & Grischenko,
Fig. 10. Unhatched blastocysts. Photomicrograph of blastocysts removed from the uterus on the 4th day of development and cultured in modified, supplemented Eagle's medium for 4 days. Scale line = 25 μm.

Fig. 11. DNA contents in nuclei from unhatched blastocysts. Unhatched blastocysts after (a) 4 or (b) 7 days in culture were treated as were uncultured blastocysts (Fig. 9).
Polyploidy in mouse trophoblast

No. of nuclei

- 5
- 10
- 15
- 20

(a)

(b)

Fig. 12. DNA content of small nuclei in 10th and 11th day trophoblast. Trophoblast from (a) 10th or (b) 11th day embryos was dissected free of other tissues and smeared on slides. DNA determinations were carried out on a random sample of Feulgen-stained nuclei. The histogram includes only those nuclei with DNA contents equal to or less than 8C.

1970). It is generally assumed that the ectoplacental cone is the stem cell region of the trophoblast and that it derives from the presumptive trophoblast layer in the blastocyst. If this is so, and if all the cells of this layer become polyploid before implantation, then the stem cells for the trophoblast could not be diploid. But Fig. 5 and Table 2 show that diploid cells are present in excess of polyploids between the 7th and 11th days of gestation. Fig. 12 shows a study of the small cells in trophoblast preparations on the 10th and 11th days of gestation. Clearly many cells with DNA values of 4C or less are present. If these diploid cells are in fact stem cells, their disappearance in the rat near the end of pregnancy (Zybina, 1970; Zybina & Grischenko, 1970) would be consistent with the termination of proliferation of trophoblast.

Fig. 12(a) shows that the proportion of 4C to 2C nuclei on the 10th day of pregnancy is greater than on any of the other days studied (cf. Figs. 5 and 12(b)). Table 2 shows that the mitotic index falls to a minimum value on the 8th and 9th days and then rises markedly on the 10th and 11th days. It may be that the
large number of 4C nuclei seen on the 10th day represents a population of nuclei that are just about to divide, so contributing to the increasing proportion of mitotic figures on this and the subsequent day.

DISCUSSION

In this paper we have demonstrated that in the mouse, as is the case in the rat (Zybina, 1960, 1963), giant trophoblast cells reach very high levels of ploidy. Indeed, the levels reached are comparable with those found in the rat, i.e. between 512 and 1024 times the haploid amount of DNA. As Table 1 illustrates, the 'cell cycle' time for the largest class of trophoblast nuclei is of the order of 24 h between the 6th and 11th days of pregnancy. The mechanism by which giant trophoblast nuclei accumulate DNA is still open to some question; although the presence of discrete nuclear size classes (Fig. 3a) would be consistent with Zybina's (1960) proposal that endoreduplication is responsible, this histogram represents a population of selectively chosen cells (i.e. those whose nuclei rupture upon separating trophoblast from the decidua, possibly establishing these as cells in direct contact with the maternal part of the placenta). Cells outside of this population, as well as more randomly chosen samples, do not show this same type of distribution (e.g. Fig. 3b). Because the type of distribution illustrated in Fig. 3b might occur as a result of specific amplification of only some parts of the genome, we have been carrying out biochemical studies designed to distinguish between DNA accumulation by endoreduplication and specific gene amplification. Preliminary results (M. I. Sherman, A. McLaren and P. M. B. Walker, in preparation) are consistent with the endoreduplication hypothesis.

The possibility that polyploidy arises by engulfment of maternal cells following implantation (Galassi, 1967) is unlikely because trophoblast can proceed to relatively high ploidy values in the absence of any foreign cells. Our data do not exclude, however, some contribution of trophoblast nuclear fusion to the formation of giant nuclei (Avery & Hunt, 1969).

There is no evidence from our studies that the maternal environment is directly responsible or even required for the onset of polyploidy. Polyploidization is evidenced in trophoblast in ectopic pregnancies and under a number of culture conditions. Especially pertinent are the findings that many polyploid cells are present in embryos cultured from the two-cell stage (Fig. 8) and in unhatched blastocysts (Fig. 11). In the former case the embryos, removed from the oviducts, had not been exposed to any specific uterine stimuli such as oestrogen sensitization (see Shelesnyak & Marcus, 1971). In the latter case, neither release from the zona pellucida nor contact with any surface had occurred.

Although trophoblast nuclei in culture reach ploidy values as high as 128C, they do not achieve the levels of nuclei in vivo or in ectopic pregnancies. Neither are trophoblast esterase and alkaline phosphatase expressed to the full extent in
Polyploidy in mouse trophoblast

blastocyst cultures (Sherman, 1972a, b). These results suggest that although trophoblast can begin to differentiate in culture it does not reach its full developmental potential, possibly because blastocyst cultures have a restricted life-span or because of the diminution of some influence supplied by the inner cell mass, which, as we have shown, degenerates in culture prior to the giant cells (Figs. 7, 8).

In so far as the onset of polyploidy is independent of the implantation event and indeed appears to precede it (Fig. 9; Graham, 1971a; Barlow et al. 1972), we have considered other developmental events which might have provided the stimulus for polyploidization. We find the formation of the blastocoel cavity a particularly attractive candidate. The blastocoel may confer upon the presumptive trophoblast cells surrounding it special properties not shared by the other cells of the embryo, as these cells are the only ones not in contact with the inner cell mass, and they may also be uniquely exposed to specific physical stresses involved in the cavitation process. Although cells adjacent to the blastocoel could thus be specifically stimulated to undergo polyploidization, that part of the presumptive trophoblast layer in apposition to the inner cell mass may be protected from the stimulus. By remaining diploid, these cells could then serve as precursors of the ectoplacental cone. This is consistent with the observation by Dickson (1963) that presumptive trophoblast cells surrounding the blastocoel appear enlarged during implantation while those adjacent to the inner cell mass do not. Furthermore, in sections of recently implanted blastocysts, Amoroso (1952) has noted that giant cells with enlarged nuclei are first found at the abembryonic pole.

The data in Table 2 suggest that the trophoblast is again exposed to a stimulus later in development. The mitotic index of trophoblast before the 10th day of gestation is extremely low. On the 10th day there appears to be an increase in the percentage of cells with a 4C amount of DNA (Fig. 12a), accompanied by a marked increase in the mitotic index (Table 2). This activation of the diploid population could be related to the formation of the labyrinth and spongy layers of the trophoblast which are first evidenced at about this time in development (Amoroso, 1952). Because the proportion of diploid cells in our cultures is always substantially lower than that found in vivo (cf. Tables 2 and 3), it is likely that the activation does not occur under our in vitro conditions.

This work was done, in part, in the Paediatric Research Unit, Guy's Hospital, London, and later completed in the laboratory of Dr C. F. Graham, Zoology Department, Oxford. We wish to thank both Professor P. E. Polani and Dr Graham for their hospitality in the respective laboratories. We also thank Dr Graham for his suggestions and for his comments on the manuscript. We are grateful to the Medical Research Council and the Medical Research Council of Canada for support. We also wish to thank Dr P. Macdonald for help with computer programming, Professor Henry Harris for reading the manuscript, and Miss Z. White for excellent technical assistance.
REFERENCES


Polyploidy in mouse trophoblast


(Manuscript received 8 September 1971)