Extra-uterine development of mouse blastocysts cultured in vitro from early cleavage stages

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Many experiments indicate that oviducal mouse eggs are unable to form embryonic tissues following transplantation to extra-uterine sites; they give rise only to trophoblast and extra-embryonic membranes (Fawcett, Wislocki & Waldo, 1947; Fawcett, 1950; Chester-Jones, 1951; Whitten, 1958; Kirby, 1962; Billington, 1965). Possible rare exceptions exist in the reports by Runner (1947) and Stevens (1967). In contrast, about a quarter of uterine blastocysts form morphologically normal embryos when similarly transplanted (Kirby, 1963; Billington, 1965). The conditions under which the mouse eggs reach the blastocyst stage may also have developmental consequences. Eggs cultured in vitro to the blastocyst stage (Whitten, 1956) or tube-locked blastocysts (Kirby, 1962) did not develop embryonic tissue in extra-uterine sites, but were capable of complete development following transfer to a receptive uterus.

These findings suggest that mouse eggs must be subjected to the uterine environment if they are to ‘realize totipotency’ in an extra-uterine site (Kirby, 1962, 1965). We have re-investigated this conclusion by culturing oviducal eggs in vitro to the blastocyst stage and then transferring them to the testis or kidney.

MATERIALS AND METHODS

Donor and recipient mice were from randomly breeding colonies (‘ZO’ strain, Oxford, and ‘Q’ strain, Edinburgh). Normal and superovulated pregnancies were used. For the transfers to the testis, the two-cell eggs used for culture were from superovulated animals, and were dissected from the oviduct between 48 and 50 h after the HCG injection (Runner & Palm, 1953). The four-to eight-cell eggs were dissected from the oviduct between 72 and 74 h after the HCG injection, or during the morning of the 3rd day of a normal pregnancy. For the transfers to the kidney, two-cell stages, eight-cell stages and blastocysts

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were taken in the afternoon of the 2nd, 3rd and 4th days of normal pregnancy respectively.

Culture techniques

In most cases eggs were cultured in one of two ways:

1. Brinster's method. In microdrops under liquid paraffin and 5 % CO₂ in air, using the medium described by Brinster (1965) and otherwise following the technique of Brinster (1963). For the eggs transferred to the testis, 0·02 g/l. of phenol red was added to the culture medium; for the eggs transferred to the kidney, the concentration of albumin was increased from 1 mg/ml. to 3 mg/ml.; the media preparation and culture for these two series were carried out in different laboratories.

2. Mulnard's method. In micro-tubes under liquid paraffin and air using the method and medium described by Mulnard (1965) except that rat serum was replaced by foetal bovine serum (Flow, Irvine, Scotland).

In one experiment, two-cell eggs were initially cultured in the isolated ampulla of the oviduct, on siliconized lens-paper floating in Ham F. 10 (Difco) in 5 % CO₂ in air, as described by Whittingham & Biggers (1967). From the morula stage they were cultured by Brinster's method.

These three methods are recorded separately in the results section. The time taken for eggs to reach the blastocyst stage varied from 2 to 4 days for two-cell eggs, and from 1 to 2 days for four- to eight-cell eggs.

Transfer techniques

(a) Transfer to testis. Morphologically normal blastocysts were transferred in their culture medium to a site beneath the tunica albuginea of recipient mice, using the transplantation apparatus described by Kirby (1962). In one series a single blastocyst was placed in each recipient testis, in a second series the blastocysts were transferred in pairs.

(b) Transfer to kidney. Eggs from one oviduct were transferred directly beneath the kidney capsule (controls) and those from the contralateral oviduct cultured to the blastocyst stage in Brinster's medium prior to transfer. A number of normal uterine blastocysts were also transferred to the kidney. All transfers were carried out in phosphate buffered saline with 10 % calf serum, and one egg only was transferred to each recipient kidney.

All recipient mice were killed 5–9 days following egg transfer, and the testes and kidneys examined for the haemorrhagic patches which characterize the extra-uterine development of mouse eggs. Testes and kidneys containing growths were processed for routine histological examination. Growths were classified into those containing (a) trophoblast only (Plate 1, fig. A), (b) trophoblast and other extra-embryonic material, such as Reichert's membrane and extra-embryonic endoderm cells, or abortive poorly differentiated embryonic structures (Plate 1, fig. B), or (c) trophoblast and a well-differentiated embryo (Plate 1, fig. C). Most growths fell clearly into one of these categories.
RESULTS

A summary of the results is presented in Tables 1 and 2. Brinster's medium and Mulnard's medium were equally effective for in vitro cultivation, as judged by the percentage of blastocysts developed. In the testis transfer series (Table 1) a greater proportion of four- to eight-cell stages (85%) developed into blastocysts than two-cell stages (72%). The corresponding proportions for the kidney transfer series were 46% (two-cell) and 61% (eight-cell), after 3 and 2 days' incubation respectively. There was no correlation between duration of incubation and subsequent developmental capacity.

Transfer to testis series

(a) Brinster's medium. 127 blastocysts were transferred to the testis in 90 recipient mice (53 singly, 74 in pairs). Twelve of these recipients showed successful 'takes'; this represents a success rate for blastocyst development of not less than 9% (accurate rates are not calculable since some blastocysts were transferred in pairs). Blastocysts cultured from four- to eight-cell stage eggs developed more frequently (8 out of 47) than did those cultured from two-cell stage eggs (3 out of 76). Only one blastocyst gave rise to a morphologically normal embryo.

Cultivation of two-cell eggs in the isolated ampulla of the oviduct for a period prior to normal culture in Brinster's medium produced four blastocysts, one of which developed into a small mass of trophoblast cells. The technique was discontinued.

(b) Mulnard's medium. 188 blastocysts were transferred in pairs to the right testis of 94 recipient mice; 52 of these recipients showed successful 'takes'. This

PLATE 1

Sections of growths in testis and kidney 5-9 days following transfer of mouse eggs.
Fig. A. Blastocyst cultured from two-cell egg in Brinster's medium and transferred to testis. Growth consists of trophoblast tissue only. × 50
Fig. B. Blastocyst cultured from two-cell egg in Brinster's medium and transferred to kidney. In this case, trophoblast and extra-embryonic material (e.e.m.), probably abortive Reichert's membrane and endoderm cells, have developed. × 160
Fig. C. Blastocyst cultured from eight-cell egg in Mulnard's medium and transferred to testis. A morphologically normal embryo, comparable to a 7½-day intra-uterine stage, has developed together with surrounding trophoblast. × 40
Figs. D, E. Two embryos developing in the testis following transfer of two blastocysts cultured from eight-cell eggs in Mulnard's medium. Figure E shows embryos at a later stage of differentiation. × 45
Fig. F. The single embryo which developed from an eight-cell oviducal egg transferred directly to the kidney. × 60
Fig. G. Embryo and trophoblast derived from a blastocyst cultured from a two-cell egg in Brinster's medium, and transferred to the kidney. × 60
represents a success rate of not less than 28%, and since five testes contained two distinctly separate growths (Plate 1, figs, D, E) the actual developmental rate is higher. Twenty-three out of 31 recipients of blastocysts cultured from four- to eight-cell stage eggs showed successful growths (74%) compared with 29 out of 63 recipients of blastocysts cultured from two-cell eggs (46%).

A total of 19 out of 57 growths contained differentiated embryos (33%), with a significantly higher proportion among blastocysts cultured from eight-cell stages (see Discussion).

Table 1. In vitro cultivation of oviducal mouse eggs to the blastocyst stage, and transfer to testis

<table>
<thead>
<tr>
<th>Medium</th>
<th>Stage at culture</th>
<th>Success of culture to blastocyst (%)</th>
<th>No. blastocysts transferred*</th>
<th>No. recipients</th>
<th>No. 'takes'</th>
<th>Tropheoblast only</th>
<th>Trophoblast and abortive e.e.m.</th>
<th>Trophoblast and embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brinster's</td>
<td>Morula†</td>
<td>57</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2-cell</td>
<td>75</td>
<td>76</td>
<td>39</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4- to 8-cell</td>
<td>84</td>
<td>47</td>
<td>47</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Mulnard's</td>
<td>2-cell</td>
<td>70</td>
<td>126</td>
<td>63</td>
<td>29</td>
<td>19</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4- to 8-cell</td>
<td>82</td>
<td>16</td>
<td>8</td>
<td>6‡</td>
<td>5</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8-cell</td>
<td>90</td>
<td>46</td>
<td>23</td>
<td>17§</td>
<td>9</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

Tissues developed

- Trophoblast only
- Trophoblast and abortive e.e.m.
- Trophoblast and embryo

*e.e.m.* extra-embryonic material.
* Blastocysts transferred either singly or in pairs.
† Cultured from two-cell stage in isolated ampulla.
‡ One testis contained two separate growths.
§ Four testes contained two separate growths.

Transfer to kidney series

Oviducal eggs at the two-cell and eight-cell stage were cultured to the blastocyst stage, and then transferred to the kidney. Transfers of two-cell, eight-cell, and uterine blastocyst stages were carried out as controls (Table 2). There were no significant differences between any of the groups in the percentage of successful 'takes', nor in the percentage of growths showing trophoblast only versus trophoblast with extra-embryonic membranes or undifferentiated embryonic tissue.

In the control transfers, two out of eight growths from uterine blastocysts contained differentiated embryos, as compared with one out of 21 from oviducal stages. The single recognizable embryo from an oviducal stage developed from an eight-cell egg (Plate 1, fig. F). When oviducal eggs were cultured to the blastocyst stage and then transferred, a total of nine out of 29 growths (31%) contained differentiated embryos (Plate 1, fig. G). This compares well with the proportion...
Development of cultured blastocysts

of embryos developing from uterine blastocysts, both in the present study and in earlier work (Kirby, 1962), and differs significantly from the fate of oviducal eggs transferred direct to the kidney \( (P < 0.01) \). Unlike in the testis series, the blastocysts from two-cell eggs differentiated into embryos as frequently as did those from eight-cell eggs.

Table 2. Transfer of tubal, uterine and cultured mouse eggs to the kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage at transfer</th>
<th>No. of transfers*</th>
<th>No. of 'takes'</th>
<th>Tropheblast only</th>
<th>Trophoblast + abortive</th>
<th>Tropheblast + embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oviducal controls</td>
<td>2-cell</td>
<td>12</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8-cell</td>
<td>22</td>
<td>13</td>
<td>3</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Uterine controls</td>
<td>Uterine blastocyst</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cultured in Brinster's medium</td>
<td>Blastocysts from 2-cell stage</td>
<td>19</td>
<td>14</td>
<td>2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Blastocysts from 8-cell stage</td>
<td>24</td>
<td>15</td>
<td>1</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

* One egg transferred to each recipient kidney.

e.e.m., extra-embryonic material

Discussion

Developmental capacity of oviducal eggs in ectopic sites

It would appear that oviducal mouse eggs are able to develop embryonic tissues ectopically, but that it is a very rare event. In the present investigation one oviducal eight-cell egg gave rise to an embryo in the kidney. Runner (1947) reported one instance of a similar development in the anterior chamber of the eye. Stevens (1967) has recently claimed that 29 out of 240 two-cell mouse eggs developed into disorganized embryonic tissues following transfer to the testis of strain 129 mice. In a similar investigation, but using randomly bred mice, none of the 71 two-cell eggs transferred to the testis had given rise to embryonic tissues when examined between 14 and 20 days later (Billington, unpublished results). Stevens's result may have been influenced by the use of strain 129 mice which have a very high incidence of spontaneous testicular teratomas. Furthermore, it is possible that the tissues were extra-embryonic membrane derivatives since it has been demonstrated that disorganized embryonic tissues can develop from ectopically transplanted rat yolk-sacs (Payne & Payne, 1961).

In other mammals the evidence is equivocal. Castle (1916) transected the utero-tubal junction of the pregnant guinea-pig and allowed the segmenting eggs to fall into the abdominal cavity. Development of embryos on the broad
ligament was reported. Nicholas (1934) repeated the experiment on 35 rats, and claimed that five foetuses were carried to term. Two of these foetuses had developed on the mesovarium and the other three on the intestinal mesentery. Nicholas was unable to obtain implantation of blastocysts experimentally transferred to the mesentery. More recent work by Jollie (1961) on the rat, and by Bland & Donovan (1965) on the guinea-pig, has failed to confirm these findings.

Nicholas (1933, 1942) also reported that two- to four-cell rat eggs gave rise to disorganized embryonic elements following transplantation to the kidney. Again this has not withstood recent investigation (Kirby, 1962), and Bland & Donovan (1965) have shown that neither oviducal nor uterine eggs of the guinea-pig develop into embryos in the testis or kidney.

In man, the numerous reports of ectopic pregnancy strongly suggest that the tubal human egg is totipotent. It is possible that some cases could occur by reflux of the uterine blastocyst back into the oviduct, but this is not widely accepted.

Embryonic cells may develop from both oviducal and uterine mouse eggs following explantation into intra-peritoneal diffusion chambers (Bryson, 1964). In vitro culture techniques show that oviducal mouse eggs can develop to the blastocyst stage, attach to the substrate, and give rise to migrating sheets of trophoblast and embryonic cells (Cole & Paul, 1965). In neither case, however, have well-differentiated embryonic structures been reported, nor is it even certain that the non-trophoblastic cellular outgrowths are truly embryonic, rather than extra-embryonic.

Developmental capacity of uterine blastocysts and influence of uterine environment

In the present investigation tubal mouse eggs cultured in vitro to the blastocyst stage formed well-differentiated embryos on subsequent transfer to ectopic sites (Table 3). The uterine environment is clearly not a necessary condition for totipotency of the mouse egg.

Cultured blastocysts also develop normally after transfer to receptive uteri, and give rise to normal foetuses or living young (McLaren & Biggers, 1958; Biggers, Moore & Whittingham, 1965), even after incubation from the 1-cell stage (Biggers, Gwatkin & Brinster, 1962). Cultured blastocysts can thus be both morphologically and developmentally normal in both uterine and ectopic sites.

On the other hand it is also clear that blastocysts which appear morphologically normal may vary widely in their developmental capacities. In the present study, the blastocysts cultured from either two-cell or eight-cell eggs in the kidney series, and those from eight-cell eggs in Mulnard's medium in the testis series, were at least as totipotent in their subsequent development as are uterine blastocysts. However, the blastocysts cultured from two-cell eggs in Mulnard’s medium gave fewer embryos on transfer, while those blastocysts cultured in
Brinster's medium in the testis series gave only a single embryo out of 90 transfers, and a much lower percentage of growths of any sort (Table 3).

The deleterious effect of Brinster's medium in the testis, but not in the kidney, series may have been due to the presence of phenol red in the medium, or to the lower concentration of albumin. (The two batches of Brinster's medium may have differed in other ways too, as they were made up in different laboratories.) Phenol red was present in similar concentration in Mulnard's medium, but other constituents of medium may have exerted a protective effect on the blastocysts. Use of a suboptimal culture medium may also explain the results of Whitten (1956), who cultured eight-cell eggs in vitro to obtain morphologically normal blastocysts, which were then transplanted beneath the kidney capsule and, according to a later publication (1958), gave rise only to trophoblastic and extra-embryonic tissues, not to differentiated embryos.

Table 3. The capacity of oviducal stages, and blastocysts exposed to varying conditions, to give rise to differentiated embryos in ectopic sites. (Data from Tables 1 and 2)

| Stage at transfer | Cultured from | Medium | Transfer site | No. transferred | Embryos (%)
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst</td>
<td>2-, 4-, 8-cell</td>
<td>Brinster's*</td>
<td>Testis</td>
<td>127</td>
<td>1</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>2-cell</td>
<td>Mulnard's</td>
<td>Testis</td>
<td>126</td>
<td>5</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>4-, 8-cell</td>
<td>Mulnard's</td>
<td>Testis</td>
<td>62</td>
<td>21</td>
</tr>
<tr>
<td>2-cell, 8-cell</td>
<td>—</td>
<td>—</td>
<td>Kidney</td>
<td>34</td>
<td>3</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>2-cell</td>
<td>Brinster's†</td>
<td>Kidney</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>8-cell</td>
<td>Brinster's†</td>
<td>Kidney</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>—</td>
<td>—</td>
<td>Kidney</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

* With phenol red. † With 3 mg/ml. albumin

The fate of tube-locked blastocysts is also relevant. Mouse eggs which are surgically prevented from entering the uterus develop into morphologically normal blastocysts in the oviduct. Kirby (1962) reported that some at least of such blastocysts were still able to produce normal embryos on transfer to the uterus, yet, of 20 developing successfully after transfer to the kidney, none gave rise to embryos. Unless the difference between the uterine and kidney transfers was an accident of sampling, this suggests either that a somewhat impaired blastocyst has a better chance of achieving normal development in the uterus than in the kidney, or that the in vitro culture media share some factor with the uterine environment which is lacking from the oviduct, and which constitutes the final requirement for totipotency of the mouse blastocyst. The last possibility seems unlikely, since Brinster's is a very simple, chemically defined medium, and certainly contains nothing comparable to the protein fraction 'blastokinin' extracted from the pregnant rabbit uterus and claimed to promote blastocyst development (Krishnan & Daniel, 1967).
The accumulated data now indicate that the pre-uterine stages of the mouse egg have the capacity for embryonic tissue development in an extra-uterine site, but that, for unknown reasons, this is only rarely realized. In addition, morphologically normal blastocysts which have not been subjected to the uterine environment may vary, according to the conditions to which they have been exposed, from those in which the capacity for complete embryonic differentiation is fully developed, to those in which it is gravely impaired.

**SUMMARY**

1. Two-, four- and eight-cell mouse eggs were cultured *in vitro* to the blastocyst stage.
2. Morphologically normal cultured blastocysts were transplanted to sites beneath the capsules of the testis and the kidney in recipient mice.
3. Five to nine days after transplantation the blastocysts developed into trophoblast, and, frequently, extra-embryonic material. In a number of cases morphologically normal embryos were formed.
4. With a single exception, embryos did not develop following transplantation of normal oviducal eggs.
5. Cultured mouse blastocysts have the capacity for normal embryonic development in extra-uterine sites; oviducal eggs also possess this potential, but it is very rarely realized. It is concluded that exposure to the uterine environment is not essential for totipotency of the mouse egg.

**RÉSUMÉ**

*Développement extra-utérin de blastocystes de souris cultivés in vitro à partir des premiers stades de la segmentation*

1. On a cultivé *in vitro* jusqu’au stade blastocyste des œufs de souris pris aux stades 2, 4 et 8 blastomères.
2. Des blastocystes cultivés, morphologiquement normaux, ont été transplantés sous les capsules testiculaire et rénale de souris réceptrices.
3. 5 à 9 jours après la transplantation, les blastocystes se sont développés en trophoblastes et, fréquemment, en matériel extra-embryonnaire. Dans nombre de cas, des embryons morphologiquement normaux se sont formés.
4. À l’exception d’un seul cas, il ne s’est pas formé d’embryons après transplantation d’œufs oviductaires normaux.
5. Les blastocystes de souris en culture ont la capacité de se développer normalement en dehors de l’utérus; les œufs oviductaires possèdent aussi ce pouvoir, mais il est très rarement réalisé. On conclut que l’exposition au milieu utérin n’est pas indispensable à la totipotence de l’œuf de souris.
We gratefully acknowledge financial support from the Medical Research Council (WDB & CFG) and the Ford Foundation (A.McL). We are indebted to Professor J. W. S. Pringle, F.R.S. for affording us facilities in his department (WDB & CFG), and to Dr Patricia Bowman for culturing the mouse eggs in the kidney series (A.McL.).

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