Haploid mouse embryos obtained by microsurgical removal of one pronucleus

By JACEK A. MODLIŃSKI

From the Department of Embryology,
Zoological Institute, University of Warsaw

SUMMARY

A pronucleus can be microsurgically removed from the fertilized mouse egg. Out of 145 haploid eggs obtained by this method and transplanted into the oviduct of pseudopregnant recipients, 36 multicellular embryos were recovered on the 4th or 5th day. On the 4th day all embryos were morulae composed of 8–50 cells, with the majority containing 8–16 cells. After an additional 24 h in vivo or in vitro the cell number increased considerably, sometimes up to as many as 80. Out of 36 multicellular embryos only one developed into a blastocyst while the others remained at the morula stage. Karyological investigations confirmed that the embryos were haploid and revealed that all were gynogenetic. Possible reasons for the absence of the androgenones and for the scarcity of blastocysts are discussed.

INTRODUCTION

Haploid embryos of mammals represent very interesting material from the point of view of embryology as well as genetics. The question of how far the haploid genome can promote development remains open. Haploid embryos might prove useful in two ways. First, they could provide a source of haploid cell lines suitable for genetic analysis of recessive mutations which in haploid cells are not masked by the dominant alleles. Secondly, by doubling the haploid chromosome set during cleavage one should be able to produce fully homozygous diploid embryos and examine the effect of this condition on embryogenesis.

Spontaneous haploid embryos seldom occur in mammals. In the mouse, Beatty & Fischberg (1951) and Beatty (1957) found seven 3½-day-old haploid embryos out of 1000 examined. Haploid mouse embryos are frequently obtained in studies on experimental parthenogenesis (Braden & Austin, 1954; Tarkowski, Witkowska & Nowicka, 1970; Graham, 1970, 1971, 1972; Kaufman, 1973a, b; Witkowska, 1973; Graham & Deussen, 1974; Kaufman & Gardner, 1974; Kaufman & Surani, 1974). Graham (1970) on the 5th day after activation obtained blastocysts which after being transplanted into testes gave rise to ‘growth’. Cytophotometric investigation revealed that one of them consisted

1 Author's address: Department of Embryology, Zoological Institute, University of Warsaw, 00–92/71 Warszawa, Krakowskie Przedmieście 26/28, Poland.
of haploid cells. Witkowska (1973) from among 5-day parthenogenetic blastocysts produced by electric stimulation of eggs in the oviduct described 60% as haploid and Kaufman & Gardner (1974) provided evidence that haploid blastocysts can evoke a decidual reaction in the uterus.

Haploid mouse eggs were also obtained in experiments aimed at producing either gynogenesis, by irradiation of spermatozoa with X-rays (Edwards, 1957a) or ultraviolet rays (Edwards, 1957b) or by the action of chemical compounds (Edwards, 1958a), or androgenesis, by treatment of ova with colcemid (Niemierko, personal communication) or colchicine (Edwards, 1958b; McGaughey & Chang, 1969) or by using delayed insemination (Marston & Chang, 1964).

So far no attempts have been undertaken to obtain haploid embryos in mammals by microsurgical removal of one of the two pronuclei from fertilized eggs. Such an experiment was performed with success on sea-urchin eggs (Hiramoto, 1962). This method was applied in the present work to obtain haploid mouse embryos.

**MATERIAL AND METHODS**

The inbred strains A, CBA/T6T6,129/terSv and randomly bred Swiss albino mice were used. One of the parents originated always from the CBA/T6T6 strain, so that on the basis of the presence or absence of the marker chromosome in the embryo’s karyotype, it could be verified which of the two pronuclei had been removed. The ova were obtained from spontaneous ovulation. The mice were killed between 10 a.m. and 1 p.m.

Enucleation was performed by means of a mechanical micromanipulator (Carl Zeiss, Jena) connected with an NfpK microscope (Carl Zeiss, Jena). The eggs to be treated were placed in a drop of Whitten medium or Ringer solution in a chamber filled with liquid paraffin. Two instruments were introduced into the chamber: a wide mouth-controlled pipette for immobilizing the egg and a micropipette for removing the pronucleus. The latter was connected by a Teflon tube with a syringe the piston of which was pushed by a screw with a fine pitch of thread. The whole system, with the exception of the very tip of the micropipette, which contained medium and air, was filled with liquid paraffin. Enucleation was performed on eggs in which the pronuclei were already visible but had not yet shifted to the centre and occupied their characteristic positions, namely the female pronucleus near the second polar body, the male pronucleus usually on the opposite side near the cell surface. Eggs at this stage were convenient for operation for two reasons: first it was usually possible to establish which pronucleus (male or female) had been removed and, secondly, removal of a small nucleus reduced the possibility of damaging the cell membrane.

Micropipettes used for enucleation were drawn on a pipette-puller and then with the use of a microforge and microburner the end of the pipette was bent
twice, so that the tip of the pipette (3-4 mm long) would move in the chamber in a horizontal plane.

Three series of experiments were carried out: In series I thick-ended pipettes (inner diameter of the tip 4-5 μm) were used and the pronucleus was removed in toto by aspiration to the end of the pipette or by sucking (Fig. 9). In series II and III very fine pipettes were used (inner diameter of the tip not exceeding 1 μm); the nuclear membrane was aspirated to the tip of the pipette and drawn out, while most of the karyoplasm and the nucleoli remained in the ovum. In series I Ringer solution was used and the manipulations were carried out at room temperature. In further experiments the ova were kept in Whitten's medium (1971) and the operation was performed either at room temperature (series II) or 37 °C (series III).

The operated eggs were transplanted into one oviduct of recipients (Tar-kowski, 1959) on the first day of pseudopregnancy (day of vaginal plug = first day). On the 4th or 5th day the recipients were injected with 0·02 ml of 0·02% solution of colchicine per 1 g body weight and killed 4–5 h later. From the recovered embryos chromosome preparations were made using the air-drying method of Tarkowski (1966). Embryos obtained on the 4th day from other recipients were cultured in vitro for 24–30 h, and chromosome preparations were made after 2–3 h exposure to colchicine or colcemid (1 μg/ml) of culture medium.

RESULTS

The results of the experiments are summarized in Table 1 and in Fig. 1.

In series I the wide pipettes (4–5 μm in diameter) caused severe damage to the cell membrane and most ova degenerated immediately after treatment. From among the 405 ova treated in this way only 90 (22·2%) survived and were transplanted. After 3½ or 4½ days of development in vivo, 15 morulae were recovered; they consisted of 8 to about 50 cells. All 12 embryos which contained metaphase plates were exclusively haploid. On the basis of the presence or absence of the T6 marker chromosome it was found that the chromosome set in all the embryos was derived from the female pronucleus.

Apart from the morulae, 13 two-cell eggs were recovered from these recipients; they were either transplanted eggs which did not develop further or activated eggs of the recipients.

For the elimination of one chromosome set, the removal of the nuclear membrane of the pronucleus proved to be sufficient. Twelve eggs enucleated in this way were placed in Whitten's medium with colcemid and after 24 h air-dried chromosome preparations were made. All seven eggs in which chromosome condensation had already occurred proved to be haploid. In view of this observation all the subsequent experiments were performed in this way (series II and III). The effectiveness of the operation increased above 50%. The course
Table 1

<table>
<thead>
<tr>
<th>Series</th>
<th>No. of operated ova</th>
<th>No. of ova which survived enucleation and were transplanted</th>
<th>Duration and site of development</th>
<th>No. and stage of recovered embryos</th>
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<tr>
<td>I</td>
<td>340</td>
<td>67 (20 %)</td>
<td>in vivo 3½ days</td>
<td>11 × 2-cell, 9 morulae</td>
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<td>2 × 2-cell, 6 morulae</td>
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<tr>
<td>II</td>
<td>65</td>
<td>23 (28 %)</td>
<td>in vivo 4½ days</td>
<td>1 × 4-cell, 8 morulae</td>
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</tr>
<tr>
<td>III</td>
<td>51</td>
<td>22 (45 %)</td>
<td>in vivo 3½ days + 24 h in culture</td>
<td>11 morulae, 1 blastocyst</td>
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Fig. 1. Number of cells in 3½-day-old (□) and 4½-day-old (□) haploid embryos.

of enucleation by removal of the nuclear membrane is shown in Figs. 2–9 and the appearance of the egg after operation in Fig. 11.

After removal of the nuclear membrane the nucleoli and at least some of the karyoplasm remain in the cytoplasm. Immediately after the operation the nucleoli are distinct (Figs. 12–13) but they rapidly decrease in size (Figs. 14–15) and after 15 min are no longer visible either in living eggs or in eggs fixed and stained with haematoxylin. Reformation of the nucleus after removal of the nuclear membrane was never observed. The rate of disappearance of the
Figs. 2–9. The course of enucleation of the female pronucleus by removal of the nuclear membrane. The nuclear membrane is aspirated to the end of the pipette and slowly withdrawn. The nucleolus remains in the cytoplasm (8) but rapidly disappears. During the operation the egg is immobilized by aspiration to a broad pipette with very narrow lumen. × 90.
Haploid mouse embryos 903

nucleoli compares well with Sorensen's (1973) observations, that during matura-
tion of the mouse oocyte the nucleolus disappears 11 min after the first sign of
nuclear membrane disintegration.

Of 105 ova treated in this way, 55 (52 %) survived and were transplanted. After 72 h in recipients 21 (38 %) 3½-day-old embryos were recovered (Fig. 16). As in exp. I the embryos displayed great variation in cell number. After 24 h of culture the number of cells usually increased and in some cases reached 60–80 but only one morula became a blastocyst (Fig. 18). All embryos in which metaphase plates were found proved to be gynogenetic haploids, i.e. the chromosome set was always derived from the female pronucleus (Fig. 17).

More eggs with a female pronucleus than with a male pronucleus were trans-
planted: removal of the female pronucleus was more injurious to the ovum than
removal of the male one, so that only a small number of ova with male pro-
nuclei survived. It may be that the cell membrane in the region of the female
pronucleus is more delicate and sensitive to injury than in other parts of the
ovum, because of the abstriction at this site of the second polar body.

In experiments in which the female pronucleus was removed, only 10 out of
38 operated ova survived. After 3½ days of development in vivo, six embryos
were found consisting of 4–7 cells. Unfortunately none of these embryos
possessed metaphase plates, so their androgenetic character could not be
confirmed. The results of these experiments are not included in Table 1.

Control experiments involved inserting the micropipette into the ovum
without enucleation. Twenty-six ova operated in this way were transplanted to
six pseudopregnant females and on the 4th day 16 blastocysts, 4 morulae, 1 four-
cell and 5 uncleaved eggs were found (in addition to 16 fragmented and de-
genereated eggs and empty zonae, derived presumable from the recipients). Thus
it seems that puncture itself is not harmful to zygotes.

Figures 10–18

Fig. 10. Enucleation by removal of the whole pronucleus. The pronucleus is seen
attached to the tip of a micropipette. × 225.

Fig. 11. Two living eggs several hours after removal of the male pronucleus. × 225.

Figs. 12–13. An egg 3 min after enucleation of male pronucleus photographed at
two focal planes. Fig. 12 shows a female pronucleus and 2PB. Fig. 13 shows a
nucleolus (arrow) left in the cytoplasm. Permanent preparation, haematoxylin. × 320.

Figs. 14–15. An egg 10 min after enucleation. The female pronucleus and 2PB are
visible on Fig. 14 and a small disappearing nucleolus of the male pronucleus (arrow)
on Fig. 15. Permanent preparation, haematoxylin. × 320.

Fig. 16. Morulae, 3½ days old, developed after removal of the male pronucleus.
× 225.

Fig. 17. A haploid metaphase plate from a 3½-day-old morula derived from a
CBA/T6T6 male parent. × 900.

Fig. 18. A 4½-day haploid blastocyst. × 225.
The results obtained in the present study show that fertilized mouse ova from which one pronucleus is surgically removed can develop to the morula stage and sporadically to the blastocyst stage. Their development is, however, slower than that of normal diploid embryos since on the 4th day the majority of the embryos are morulae composed of relatively small numbers of cells (8–16). Only a few of the embryos were composed of greater numbers of cells (up to 50). Although during the subsequent 24–30 h the number of cells increases considerably, sometimes up to as many as 80, the overwhelming majority of embryos remain in the morula stage (35 out of 36). The fact that haploid morulae obtained as a result of enucleation do not generally transform into blastocysts seems to contradict observations of Witkowska (1973) according to which 60% of parthenogenetic blastocysts recovered on the fifth day were haploid. From these observations Witkowska drew the conclusion that transformation of morulae into blastocysts does not require diploidy and can occur on the basis of the haploid genome.

To reconcile this discrepancy the following tentative explanation is proposed. First, haploid cells may not be capable of differentiating into trophoblast nor of secreting blastocoelic fluid. Secondly, for the transformation of morula into blastocyst it is not necessary that all cells are diploid, a few may suffice. Thirdly, parthenogenetic blastocysts which – like the only one obtained in the present experiment – were identified as haploid, were actually mosaic embryos with a small number of diploid cells. Their mosaicism was not discovered because the number of metaphase plates examined was too small. Haplo-diploid mosaicism was observed in 20% of parthenogenetic embryos activated with an electric current (Tarkowski et al. 1970; Witkowska, 1973). In embryos developed from eggs in which one pronucleus was removed diploid cells were not detected, even on the 5th day of development. This seems to indicate that after removal of one pronucleus, duplication to the diploid level seldom or never occurs (at least up to 5th day). It is not clear why gynogenetic embryos should differ in this respect from haploid parthenogenetic ones. However, one cannot exclude the possibility that the arrest of gynogenetic haploids at the morula stage is due simply to damaging the eggs during the operation.

All embryos suitable for karyological analysis possessed the chromosome set derived from the female pronucleus. It should be remembered that eggs with a male pronucleus constituted only a small percentage of the transplanted ova because the removal of the female pronucleus led most often to the destruction of the egg. It may be, therefore, that the absence of androgenetic embryos was due merely to the inadequate number of experiments. Alternatively, the absence of an X-chromosome might impair the viability of the 50% of androgenones which carried a Y-chromosome and no X, as Morris (1968) suggested that an X-chromosome was probably necessary for cleavage to occur. If this were so, X-bearing androgenones should not differ in developmental potential from gynogenetic eggs.
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REFERENCES


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