Aust. J. Biol. Sci. 1982, **35**, 187–93

Development of Preimplantation Mouse Embryos *in vivo* and *in vitro*

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Abstract

The culture conditions for the development *in vitro* of (C57BL/6 × CBA) F_2 hybrid two-cell embryos to the blastocyst stage have been optimized. Commercially available pre-sterile disposable plastic culture dishes supported more reliable development than re-usable washed glass tubes. The presence of an oil layer reduced the variability in development. An average of 85% of blastocysts developed from hybrid two-cell embryos cultured in drops of Whitten's medium under oil in plastic culture dishes in an atmosphere of 5% O_2 : 5% CO_2 : 90% N_2. The time taken for the total cell number to double in embryos developing *in vivo* was 10 h, and in cultured embryos 17 h. Embryos cultured *in vitro* from the two-cell stage to blastocyst stage were retarded by 18–24 h in comparison with those remaining *in vivo*. Day-4 blastocysts *in vivo* contained 25–70 cells (mean 50) with 7–28 (mean 16) of these in the inner cell mass. Cultured blastocysts contained 19–73 cells (mean 44) with 8–34 (mean 19) of these in the inner cell mass. In the uterine environment, inner-cell-mass blastomeres divided at a faster rate than trophectoderm blastomeres and it is suggested that a long cell cycle is associated with terminal differentiation. Although cultured blastocysts and inner cell masses contained the same number of cells as blastocysts and inner cell masses *in vivo*, the rate of cell division in cultured inner cell masses was markedly reduced.

Extra keyword: Cleavage.

Introduction

The reliability of investigations on cultured mouse embryos rests on the assumption that their development *in vitro* and *in vivo* are comparable. However, cultured embryos from random-bred mice have a slower rate of cell division and metabolic activity (Bowman and McLaren 1970*a*; Quinn and Wales 1973), are less viable when transferred to pseudopregnant foster mothers (Bowman and McLaren 1970*b*) and are generally less well differentiated (McReynolds and Hadek 1972) than embryos developing entirely *in vivo*. These results indicate that the culture conditions used were suboptimal. More recently, it has been reported (Snow 1975; Harlow and Quinn 1979*a*) that conditions *in vitro* have been improved to the extent that, upon transfer to recipients, cultured blastocysts produced the same number of young as uterine blastocysts, indicating they were not less viable. Greater success in pre implantation development has also been reported when F_1 hybrid mice are used rather than inbred or random-bred mice (Whitten 1971; Whittingham 1975; Quinn and Harlow 1978).

The initial investigations described here were designed to optimize conditions for the culture of (C57BL/6 × CBA) F_1 hybrid two-cell embryos to the blastocyst stage. Comparisons were made between the two major types of culture vessels used—culture dishes in which small drops of medium were placed under oil (Brinster 1963) and test tubes containing 1 ml of medium (Whitten 1971; Quinn and Harlow 1978).

Studies using serial sections of blastocysts that had developed *in vivo* have shown that inner-cell-mass blastomeres divide more rapidly than trophectoderm blastomeres (Barlow *et al.* 1972) and that the rate of cell division in the inner cell mass (ICM) is compatible with its rate of increase of cell number (Copp 1978). Furthermore, the ICM appears to be more adversely affected by culture than the trophectoderm as foetuses developing from cultured blastocysts were smaller than those developing from freshly collected blastocysts although there was no difference in placental weight (Harlow and Quinn 1979*a*). Therefore, blastomere numbers in the trophectoderm and ICM of blastocysts developing *in vivo* and *in vitro* were also studied.

Materials and Methods

Embryos

Embryos from superovulated 3–5-week-old virgin mice of the F_1 hybrid cross (C57BL/6 × CBA, or reciprocal) were either freshly collected at various times after administration of human chorionic gonadotrophin (HCG) or collected at the two-cell stage (42–44 h after administration of HCG) and cultured as previously described (Harlow and Quinn 1979*b*).

Embryo Culture

The culture medium used was that of Whitten (WM_1) prepared as indicated by Hoppe and Pitts (1973). Preliminary studies showed that development would proceed in medium prepared with water distilled one to three times and stored for up to 4 days at 4°C. The humidified atmosphere surrounding the cultured embryos consisted of 5% CO_2:5% O_2:90% N_2.

Plastic tissue culture dishes (No. 301V; Sterilin, Middlesex, U.K.) or microtest plates (No. 3034; Falcon, Oxnard, California, U.S.A.) were used, each containing 10–20-μl drops of culture medium under paraffin oil (Harlow and Quinn 1979*b*). Glass culture tubes, 12 by 75 mm, (Kimax; c/o A. E. Stansen, Mt Waverley, Vic.) stoppered with autoclavable white rubber stoppers (No. S43; West Company, Phoenixville, Pennsylvania, U.S.A.) contained 1 ml of medium and were prepared as described by Quinn and Harlow (1978). These tubes were pre-treated by soaking overnight in 1 M HCl, followed by washing as described by Quinn and Harlow (1978).

The percentages of two-cell embryos forming blastocysts after 70–74 h of culture were recorded. The results were accumulated over a 3-year period. All replicates contained 10–20 embryos.

Cleavage Rate

Embryos from replicates that had been cultured in the optimal conditions (i.e. in plastic dishes under oil) and produced greater than 90% blastocysts, were collected at the required time and incubated in 0·5 μg ml^{-1} colchicine (Calbiochem, Carlingford, N.S.W.) for 3 h. The number of blastomeres in some embryos was counted using the air-drying technique of Tarkowski (1966). ICMs were isolated from the remaining embryos by immunosurgery using a 1:50 dilution of rabbit anti-mouse spleen serum followed by a 1:10 dilution of guinea-pig complement (Commonwealth Serum Laboratories, Parkville, Vic.) (Harlow and Quinn 1979*b*). The number of ICM cells was counted. The times taken for the total cell number to double *in vivo* and *in vitro* were calculated by regression analysis after the data were transformed to base-2 logarithms.

The percentage of embryos containing at least one blastomere in metaphase or anaphase was recorded. The mitotic duration (t, in hours) at each stage of development was calculated using the formula:

$$t = M \times T/\ln 2,$$

where M is the mitotic index (the percentage of cells in metaphase or anaphase per embryo of the whole embryo sample) and T is the doubling time (estimated from the regression line) (Smith and Dendy 1962).

Differences in cell numbers and mitotic indices were analysed by Student's t-test.

Table 1. Number of blastomeres per embryo during development *in vivo* or *in vitro*

Time after administration of HCG (h)	No. of mice	*In vivo* development			*In vitro* development[A]		
		Total No. of embryos	No. of blastomeres per embryo $\bar{x}\pm$s.e.	Range	Total No. of embryos	No. of blastomeres per embryo $\bar{x}\pm$s.e.	Range
30	3	96	1·3±0·1	1–2			
42	3	101	2·0±0·0	2			
48	4	140	2·0±0·0	2			
54	3	51	3·2±0·1	2–4			
66	3	29	6·8±0·2	5–10	29	5·3±0·3	3–8
72	3	14	11·9±0·7	8–16			
90	6	27	39·5±2·2	25–60	30	21·7±1·4	10–32
96	5	38	41·5±1·8	27–70			
114					50	44·0±1·8	19–73

[A]Two-cell embryos collected for *in vitro* development at 42 h after administration of HCG.

Results

In the preliminary studies to evaluate the effects of the purity of water and storage of media, 85 ± 2% (67 replicates; range 55–100) of two-cell embryos formed blastocysts when cultured in plastic dishes under oil. However, when two-cell

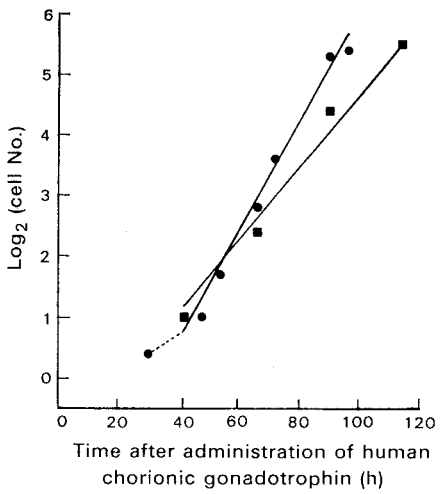

Fig. 1. Relationship between time after administration of human chorionic gonadotrophin and cell number in pre-implantation embryos developing *in vivo* (●) or *in vitro* (■). Points represent mean number of cells at each time of development (from Table 2). Regression lines are plotted from the equations $y = 0{\cdot}9x - 3{\cdot}00$ (*in vivo*) and $y = 0{\cdot}06x - 1{\cdot}33$ (*in vitro*).

embryos were cultured in glass tubes without oil, there was a large variability in development to blastocysts (51 ± 6%; 33 replicates; range 0–100). This variability was partially reduced by the presence of an oil layer (70 ± 9%; five replicates; range 35–90) but not by the use of plastic tubes (48 ± 15%; eight replicates; range 0–100).

The numbers of cells per embryo at different times of development are given in Table 1. 34% of embryos had undergone the first cleavage by 30 h after administration of HCG and 100% had cleaved by 42 h. By 66 h, all cells had undergone two cleavage divisions and by 72 h, all had undergone three divisions. The regression lines describing the increase in cell number from 42 to 96 h are given in Fig. 1. Both regressions were highly significant ($P<0{\cdot}001$) and had different slopes from each

Table 2. Proportion of embryos with at least one cell in metaphase or anaphase (P), their mitotic index (M) and mitotic duration (t) during development *in vivo* or *in vitro*

Time after administration of HCG (h)	*In vivo* development				*In vitro* development			
	n	P (%)	M ($\bar{x}\pm$s.e.)	t (h)	n	P (%)	M ($\bar{x}\pm$s.e.)	t (h)
66	11	36	24±11	3·8	29	42	14±4	3·4
72	14	64	12±3	1·9				
90	17	88	11± 3	1·7	30	80	17±3	4·2
96	38	89	9± 1	1·4				
114					50	92	9±1	2·2

other ($t_{539}=2{\cdot}9$, $P<0{\cdot}01$). From the regression lines it was estimated that the time taken for the total cell number to double in embryos developing *in vivo* was 10 h and in cultured embryos 17 h. The number of cells in cultured blastocysts at 114 h after administration of HCG did not differ significantly from the number of cells in blastocysts freshly collected at 90 h ($t_{75}=1{\cdot}35$, $P>0{\cdot}1$) or 96 h ($t_{86}=1{\cdot}14$, $P>0{\cdot}2$). Therefore, cultured blastocysts were 18–24 h behind their *in vivo* counterparts.

Table 3. Number of cells and mitotic index (M) of inner cell masses and trophectoderm from blastocysts developing *in vivo* and *in vitro*

Stage of development	Inner cell mass				Trophectoderm	
	No.	Cell No.[A]	% with at least 1 mitosis	M	Cell No.	M
In vivo						
90 h after administration of HCG	4	16±2 (9–20)	100	20±4	24	4
96 h after administration of HCG	18	17±1 (7–28)	72	16±3	25	4
In vitro						
114 h after administration of HCG	48	19±1 (8–34)	48	6±1	25	12

[A]Mean±s.e. (range).

Some aspects of the cell cycle of embryos developing *in vivo* or *in vitro* are given in Table 2. As development proceeded, the embryos were more likely to have at least one cell in metaphase or anaphase, and the mitotic index (M) of the whole embryo

sample decreased. The mitotic duration (t) decreased with development both *in vivo* and *in vitro*, and was greater in cultured embryos than in embryos developing *in vivo*.

The number of cells and the mitotic indices (M) of ICMs from blastocysts developing *in vivo* or *in vitro* are given in Table 3. There was no difference in the number of cells in ICMs from cultured blastocysts and blastocysts collected at 90 h ($t_{51} = 0{\cdot}99$, $P>0{\cdot}2$) or 96 h after administration of HCG ($t_{64} = 1{\cdot}20$, $P>0{\cdot}2$). Only half of the cultured ICMs showed evidence of any cells in metaphase or anaphase. The mitotic index of cultured ICMs was significantly lower than that of ICMs from blastocysts *in vivo* ($t_{68} = 4{\cdot}37$, $P<0{\cdot}001$). Using this data and those in Tables 1 and 2, the number of cells and mitotic index in the trophectoderm were estimated and are also given in Table 3. These estimates indicated that the mitotic index of trophectoderm cells *in vivo* was low while *in vitro* it was high.

Discussion

The large variation in development found when embryos were cultured in glass tubes probably arises from the washing and re-use of the tubes. The prevention of evaporation by oil may play a role in reducing variability of development in tubes. The optimum culture conditions, which were subsequently used routinely, included medium prepared with triple-distilled water and stored for up to 4 days, placed in disposable plastic culture dishes covered by oil.

The time of first cleavage in this study agrees well with previous reports for ova from superovulated random-bred mice (28–36 h after administration of HCG; Barlow *et al.* 1972) and naturally ovulated ova from inbred mice and their hybrids (23 h after the midpoint of the dark cycle; Whitten and Dagg 1961). The doubling time of 10 h for the hybrid embryos *in vivo*, in this study, is the same as that for random-bred mice (Bowman and McLaren 1970*a*; Allen and McLaren 1971; Barlow *et al.* 1972). The doubling time of 17 h for cultured embryos is less than Bowman and McLaren (1970*a*) found and suggests that culture conditions in the present study are more favourable.

Blastocysts cultured from the two-cell stage in the optimum culture conditions are not more than 18–24 h behind day-4 blastocysts *in vivo*. Furthermore, the number of cells in day-4 embryos from hybrid mice (range 25–70) is comparable to reported ranges of cell numbers in day-4 embryos from random-bred mice (28–57, Bowman and McLaren 1970*a*; 29–57, Allen and McLaren 1971; 31–55, Barlow *et al.* 1972; 22–45, Smith and McLaren 1977; 29–83, Handyside 1978; 25–45, Kiessling and Weitlauf 1979). This indicates there is no fundamental difference in cleavage rates of embryos from inbred, random-bred or hybrid mice.

As the time taken for the total cell number to double remained constant and the mitotic index decreased with development from two-cell embryos to blastocysts, a decrease in the mitotic duration was observed. This could indicate two processes. Firstly, a decrease in the mitotic duration may be accompanied by an increase in the non-mitotic period of the cell cycle, which would allow more time for embryonic genome expression as differentiation occurs. A similar process was observed in the formation of mesoderm in a 7-day-old mouse embryo in which the total cell cycle increased but duration of mitosis remained the same (Solter *et al.* 1971). Secondly, a decrease in mitotic duration may reflect an overall decrease in cell-cycle length as described by McLaren (1972) and Graham (1973).

However, the above discussion has assumed that the doubling time for the whole embryo is the same as that for each cell within the embryo. This is not so, because an increase in asynchrony requires cells to develop different cell cycles and it has been shown that the labelling index of inside cells is consistently higher than that of outside cells (Barlow *et al.* 1972). As more cells in the ICM are dividing than in the trophectoderm in blastocysts developing *in vivo* (Table 3), it appears that as cells become enclosed, their cell-cycle length decreases such that in day-4 blastocysts, approximately one-fifth of the cells in the ICMs are dividing. Kiessling and Weitlauf (1979) also indicated that the cell-division time decreased during the formation of the ICM and was related to an increase in DNA polymerase activity. Tissue derived from the ICM maintains this rapid rate of division during the period of growth immediately after implantation (Snow 1976, 1977). Those cells which remain on the outside do not show a decrease in the length of their cell cycle and have few cells undergoing division. Therefore, a lengthy cell cycle may be associated with terminal differentiation of trophectoderm blastomeres. Mural trophectoderm, which forms primary giant cells, ceases dividing altogether while the polar trophectoderm continues dividing but at a lower level than ICM cells (Copp 1978).

The rates of cell division in the ICM and trophectoderm of cultured blastocysts and blastocysts developing *in vivo* are markedly different, although the total cell numbers in these blastocysts (disregarding their 24-h age difference) are similar. The rate of cell division in trophectoderm cells of cultured blastocysts has not decreased below that in day-3 embryos and indicates two possible processes. Firstly, they may be retarded with respect to the expression of one of their characteristic properties, namely slow cell division. Culture beyond the 40- to 50-cell stage would be needed to determine if the rate of division of trophectoderm blastomeres begins to decrease in a similar manner to that *in vivo* as described by Copp (1978, 1979). During culture, the initial formation of the ICM may not be retarded, resulting in the number of cells in ICMs from cultured blastocysts and blastocysts *in vivo* appearing the same. By the 40- to 50-cell blastocyst stage, the culture conditions may have begun to preferentially inhibit further cell division in the ICM as revealed by the low mitotic index. Secondly, the trophectoderm cells may have ceased division in a state of suspended anaphase or metaphase, resulting in the high mitotic index observed *in vitro*.

Acknowledgments

We are grateful to Mrs C. Lloyd for skilled technical assistance. This work was supported by a grant from the Australian Research Grants Committee. One of us (G.M.H.) was supported by a Commonwealth of Australia Postgraduate Scholarship.

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Manuscript received 10 July 1981, accepted 17 February 1982