Positioning of inner cell mass determines the development of mouse blastocysts \textit{in vitro}

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SUMMARY

We have explanted mouse blastocysts into culture dishes and studied the positioning of the inner cell mass (ICM) at the time of attachment in order to establish what effect the location of the inner cell mass has on the subsequent development of embryos \textit{in vitro}. We show that blastocysts may attach to the substrate with any portion of the trophectoderm. The location of ICM within the attaching blastocysts is unpredictable. The directions of egg-cylinder development may be either upward or downward. The axis assumed by a developing egg cylinder \textit{in vitro} depends to a large extent on the initial positioning of the ICM at the time of attachment. Egg cylinders grown from blastocysts that have the ICM in the lower position (close to the plastic dish) reach advanced stages of development in greater numbers than egg cylinders grown from blastocyst with their ICM's in the upper lateral or upper position. Only upward-growing egg cylinders can develop further into somitic stage (which is equivalent to 8i- or 9-day \textit{in vivo} embryos). In contrast, downward growing egg cylinders become thwarted in their further development by the surrounding trophectoderm. If the downward growing egg cylinders are freed of trophectoderm and repositioned to face upwards they will develop in a manner similar to the originally upward growing egg cylinders. These findings indicate that the initial positioning of the ICM at the time of attachment is an important factor in governing the development of blastocysts to somite stage \textit{in vitro}.

INTRODUCTION

Many investigators have grown mouse blastocysts to the egg-cylinder stage \textit{in vitro} (Pienkowski, Solter & Koprowski, 1974; Sherman, 1975; McLaren & Hensleigh, 1975; Wiley & Pedersen, 1977), but only Hsu (1979, 1980) succeeded in growing preimplantation mouse embryos to somitic stages of development. This was accomplished by frequent changes of medium and the substitution of human cord serum (HCS) for foetal calf serum (FCS) during the later periods of culturing. Under these conditions, the \textit{in vitro} grown embryos resembled normal mouse embryos developing \textit{in utero} (Gonda & Hsu, 1980).

As outlined by Gonda and Hsu (1980), the blastocysts will hatch from the zona pellucida during the first day of culture and attach to the bottom of the

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culture dish 2 days after explantation. Upon attachment the trophoblast will spread laterally over the surface of the culture dish, the embryo proper will continue to develop and by day 3 in culture form an upward-projecting egg cylinder. The egg cylinder will further develop and finally by day 8 form a somitic embryo containing 5–12 somites. This advancement is achieved only under the conditions defined by Hsu (1979). The removal of macromolecular embryonic growth and differentiation factors from the FCS and HCS will retard and adversely affect the growth of embryos (Hsu, 1980). However, even under ideal conditions not all embryos will reach the somitic stages of development. The reasons for this differential development of mouse blastocysts explanted in vitro are not clear.

In this report we show that advanced development of embryos in vitro depends to a large extent on the type of initial attachment of the blastocyst to the culture dish as well as the positioning of the inner cell mass (ICM). Embryos attached to the culture dish with the polar (embryonic) trophectoderm develop in large numbers to the somitic stage. Blastocysts attached to the dish with the abembryonic mural trophectoderm will form egg cylinders projecting downwards and the full development of these egg-cylinders will be thwarted by the overlying trophectoderm. We also show that the downward growing egg cylinders have the same developmental potential as the upward growing egg cylinders and will develop into somitic embryos if freed of the overlying trophectoderm. Our data thus emphasize the importance of positional and mechanical factors in early mammalian development.

MATERIALS AND METHODS

Spontaneously ovulating Swiss Webster female mice (Perfection Breeders, Camden, NJ) were mated overnight and the day when the vaginal plug was found was designated as day one of pregnancy. Blastocysts were flushed from the pregnant uterine horns during the early afternoon hours of the fourth day with CMRL-1066 culture medium (Gibco Laboratories) containing 1 mM glutamine and 1 mM sodium pyruvate. The blastocysts were collected into uncoated plastic 35 mm Falcon culture dishes and incubated individually or ten per dish and cultured according to the protocol developed by Hsu (1979). This included maintenance of constant temperature (37 °C), and atmosphere (5 % CO₂ and 95 % air) and daily changes of the culture medium. During the first 4 days the culture medium was supplemented with 20 % foetal calf serum (FCS) (Gibco) and thereafter with heat-inactivated human placental cord serum (HCS). Embryos were inspected daily under the dissecting microscope and classified according to Witschi (1972). For histologic examinations, selected embryos were embedded in plastic (Sorvall Embedding Medium, Dupont), sectioned at 1–2 μm and stained with haematoxylin and eosin for light-microscopic examination.
RESULTS

(A) Embryonic development to the egg-cylinder stage in relation to the positioning of the ICM at the time of attachment

In order to determine whether the development of mouse embryos in vitro depends on the initial positioning of ICM, blastocysts were explanted individually, cultured for 4 days and observed continuously. The day of explantation was designated as day 0. On the basis of the initial ICM location, the blastocysts were classified into five groups (Fig. 1): (a) ICM in the down
position, (b) ICM in the lower lateral position, (c) ICM in the middle lateral position, (d) ICM in the upper lateral position and (e) ICM in the upper position. Blastocysts in position (a) attached to the dish with the trophectoderm overlying the ICM (polar or embryonic trophectoderm). Blastocysts in position (e) attached with the abembryonic mural trophectoderm whereas in positions (c) and (d), the attachment was with lateral mural trophectoderm. For blastocysts in position (b), the mechanism of attachment could not be exactly determined but was most likely mediated in part by embryonic and in part by lateral mural trophectoderm.

The axis of the developing egg cylinders was recorded for each blastocyst on day three and four. Embryos were classified into five groups according to the direction of their longitudinal axis (Fig. 1): (a) straight upward, (b) obliquely upward, (c) laterally, (d) obliquely downward, and (e) straight downward growing embryos. In the groups (a) and (b) the egg cylinder protruded through the abembryonic mural trophectoderm and one could see a continuous layer of trophoblast attached to the plastic dish around the embryo. In group (c) the egg cylinder protruded between the polar embryonic and lateral mural trophectoderm. In these cases the trophoblastic layer around the embryo was not continuous and a defect through which the egg cylinder presumably protruded could always be seen (Fig. 2).

We have explanted 127 blastocysts. On day one in culture, we were able to clearly localize the ICM in 100 hatched blastocysts. The remaining 13 unhatched blastocysts and the 14 blastocysts whose ICM we could not clearly see were discarded. Further observations were limited to the 100 blastocysts with exactly identifiable ICM.

From Table 1 it may be seen that the ICM was most often found (78/100) in the lateral position. Nineteen blastocysts were in the lower lateral position, 38 in the middle lateral position and 21 in the upper lateral position. Attach-
The first part of the table contains the schemes of five ICM positions, determining the type of blastocysts attachment, and the number of blastocysts that have attached to the dish in one of the possible positions: (a) ICM down, (b) ICM lower lateral, (c) ICM middle lateral, (d) ICM upper lateral, (e) ICM upper position.

The second part of the table indicates the number of blastocysts that have developed to egg cylinders on day 4. The egg cylinders were classified according to the direction of their axis: (a) straight upward, (b) obliquely upward, (c) laterally, (d) obliquely downward, (e) straight upward.

The third part of the table contains the cumulative data on the total number of upward (a, b, c) or downward (d, e) growing egg cylinders in each group of differently positioned blastocysts.

The fourth part of the table contains the data on upward growing egg cylinders that have reached stage 9–11 of Witschi or have undergone disorganization. The downward growing egg cylinders are not included because we could not always determine the stage of their development.

The data are expressed in absolute numbers and percentages for each group.
Blastocysts whose ICM was in the down position gave rise to only straight upward and obliquely upward growing egg cylinders (Table 1). Blastocysts with the ICM in the lower lateral position formed straight upward, obliquely upward and laterally growing embryos. Blastocysts with the ICM in the middle lateral position and upper lateral position formed obliquely downward growing egg cylinders in varying proportions (18% and 38% respectively) as may be seen in Table 1. The blastocysts attached with the abembryonic trophectoderm formed only downward growing egg cylinders. From 100 blastocysts we obtained 81 upward and 19 downward growing egg cylinders. Of the 81 upward growing egg cylinders, 47 have grown upwards penetrating through the abembryonic mural trophectoderm and of these nine were classified as straight upward and 38 as obliquely upward growing. The remaining 34 grew laterally and protruded through a slit between the polar embryonic and mural trophectoderm.

At the end of day 4, we have classified the upward growing 81 embryos in order to determine how many have reached Witschi's stage 9, 10 or 11. The downward growing embryos could not be adequately visualized. From Table 1, one may see that in total 15 embryos reached stage 9, 34 stage 10 and 30 stage 11, whereas 2 were obviously disorganized. It may be seen that all the 18 embryos grown from blastocysts attached with embryonic trophectoderm have reached stage 10 and 11, whereas the other groups contained significant numbers of less developed embryos. Furthermore, the egg cylinders obtained from groups (a) and (b) blastocysts showed less deviation from normality than the egg cylinders from groups (c) and (d) which were frequently defective or deformed.

(B) Development of somitic-stage embryos in vitro as related to the longitudinal axis at the egg-cylinder stage

In order to compare the developmental potential of upward and downward egg cylinders, we have explanted blastocysts and cultured them according to the protocol of Hsu (1979) for 8 days.

Two hundred ninety one blastocysts were flushed from pregnant uteri and explanted in vitro. The day of explantation was designated as day 0. By day 2 in culture 276 of all blastocysts had attached. By day 4, 222/276 (80%) of attached blastocysts formed an upward projecting egg cylinder and have reached Witschi's stage 9, 10, or 11. The remaining 54/276 (20%) of attached blastocysts were classified as downward growing and appeared as flat discs. This experiment gave results comparable with the data obtained in experiment A, and continued for an additional four days. By day 6, 118/276 (43%) of attached embryos or 53% of all upward growing embryos (118/222) had reached the primitive streak or presomitic neurula stage. By day 8, 59/276 (21%) of attached
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Fig. 3. Schematic presentation of polar embryonic (a) and abembryonic mural (b) attachment of the blastocyst. Trophoblast (Tb) surrounds the downward growing embryo from three sides but is present only on one side, adjacent to the ectoplacental cone (Epc) in the upward growing embryo. Trophoblastic giant cells (Tg) form only along the contact of the trophoblast and the surface of the dish. Distal, i.e. parietal endoderm (dEd) is formed only in downward growing egg cylinder. Note the positioning of the embryonic region (EmR) and the extraembryonic region (ExR). Only the upward growing egg cylinder develops into a somitic embryo. (Eb = embryo; Ys = yolk sac; Am = amnion; Al = allantois; Epc = ectoplacental cone.)

Embryos (or 27% of the upward growing embryos) were at the somite stage (corresponding to Witschi's stage 14-15) and showed a distinct rhythmic heartbeat. The remaining (73% of the 222 upward growing egg cylinders) developed into yolk-sac cysts and became disorganized.

The 54 embryos identified on day 4 as downward growing developed further in two directions. Twenty-one of these downward growing egg cylinders protruded laterally underneath the trophoblastic layer and continued developing to reach stage 11 and 12 of Witschi by day 5. All these egg cylinders appeared deformed. Further development of these embryos was abnormal and lead to formation of small yolk sacs devoid of embryo proper or containing non-descript or deformed embryonic structures. The remaining 33 of the downward growing embryos remained buried under the trophoblast and became disorganized. None of the downward growing embryos reached early somitic stage.

(C) Reorientation of downward growing embryos

In order to assess the developmental potential of downward growing embryos we have explanted 120 blastocysts. Twenty embryos found on day 4 to have a downward growing egg cylinder covered with trophectoderm were selected for further experimentation. The explants were gently lifted from the bottom of the plastic dish and turned around, whereupon the polar trophectoderm was placed on the surface of the plastic dish and the egg cylinder faced upward permitting direct contact with culture medium. Subsequent to this reorientation, these egg cylinders continued to develop to later stages with 10 reaching the somitic
Fig. 4. Schematic presentation of the development of the downward growing egg cylinders. (a) The egg cylinder protruding laterally under the trophoblast becomes an empty yolk sac (Ys). (b) The egg cylinder covered with trophoblast becomes disorganized. (c) The repositioned egg cylinder develops further into early somite stage embryo.

stages 14–15 of Witschi (Figs 3 & 4). The remaining 10 transformed into yolk-sac cysts.

(D) Histologic examination of embryos

Egg cylinder on day 4

Selected embryos from experiment C were embedded in plastic and histologically examined. Upward growing egg cylinders were composed of two layers corresponding to the epiblast and visceral endoderm. There was an ectoplacental cone interposed between the egg cylinder and trophoblast. No parietal endoderm was formed and there was no Reichert's membrane except for a small nubbin of basement membrane formed at the junction of the visceral endoderm and the placental cone (Fig. 5a). The downward growing egg cylinders resembled, in most respects, the upward growing egg cylinders except that the downward growing embryos were surrounded on three sides with trophoblastic cells and the upward growing embryos were not. Between the downward growing embryo proper and the trophoblast there was a well developed, although often discontinuous, Reichert's membrane lined by parietal endodermal cells (Fig. 5b). The downward growing embryos thus resembled to a greater extent the in vivo embryos than the upward growing embryos.
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**Fig. 5.** Comparison of an upward (a) and a downward (b) growing egg cylinder (stage 11) obtained after 4 days *in vitro* culture. The downward growing egg cylinder is surrounded on three sides by trophoblasts. (a) Only the trophoderm adheres to the bottom leaving the whole egg cylinder free floating above the surface of the dish. (b) Downward egg cylinders of the same stage. The whole egg cylinder is enclosed by multilayered trophoblasts (Tb) except the embryonic part which directly contacts with the bottom. Only the trophoblasts in contact with the plastic dish transforms into giant cells (Tg). In contrast to (a) the ectoplacental cone (Epc) is on the top. Distal endoderm (dEd) and Reichert’s membrane (Rm) are also seen (Ec = ectoderm; pEd = parietal endoderm).

**Somitic embryos**

Well differentiated somitic embryos that developed from upward growing cylinders were similar to the embryos that were obtained from downward growing egg cylinders manually reversed on day 4 of culture (Fig. 6). Developmentally most advanced embryos from both groups contained 5–12 somites, neural tube, heart and were surrounded with chorion, amnion and possessed a well developed allantoic stalk. The ten reversed embryos examined histologically on the 8th day of culture were somewhat less developed than the embryos obtained from upward growing egg cylinders, but the difference was not quantifiable.
In the present study, we have confirmed the reports of Hsu (1979, 1980) and shown that the sequential change of culture media with the addition of HCS on the fourth day in culture, allows mouse blastocysts to develop into somitic embryos. The rate at which the cultured embryos reach the egg-cylinder stage of development in our laboratory was comparable with the results of Hsu (1979). However, we were only able to grow 20\% of all blastocysts to stages 14–15, which is considerably lower than reported by Hsu (1979). The reasons for this are not clear, although the results could reflect some minor differences in technique or differences between embryos obtained by superovulation as in Hsu’s work, and the spontaneously ovulated eggs used in the present study. It is also possible that the randomly bred CF-1 mice used by Hsu (1979) yielded better results than the out-bred Swiss Webster mice of our colony.

In contrast to previous reports (Hsu, 1979, 1980; and Hsu & Gonda 1980; Pienkowski et al. 1974; Wiley & Pedersen, 1977) who claimed that the egg
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Cylinders almost invariably grow upward from the plastic dish to which the blastocysts have attached, we have shown that at least one fifth of all embryos grown in vitro do not develop in such a predictable way. Our data indicate that the egg cylinders grow upward or downward (Fig. 5) and that the direction of their growth depends primarily and to a large extent on the positioning of the ICM and the location of the blastocoel at the time of blastocyst attachment to the plastic dish. Like the normal egg cylinder developing in utero, the egg cylinder grown in vitro develops from the ICM and expands into the blastocoel. If the blastocyst attaches to the plastic dish with the polar (embryonic) trophectoderm, the blastocoel will be above the ICM and the egg cylinder will grow upward. On the other hand, if the blastocyst attaches to the plastic dish with the abembryonic mural trophectoderm, the blastocoel will be located underneath the ICM and the egg cylinder will grow downward. In both instances the direction of growth follows the path of least resistance, i.e. into the pre-existing cavity.

The upward growing egg cylinders invariably protrude through the trophectoderm. Hsu (1980) and Hsu & Gonda (1980) have convincingly shown that egg cylinder protrudes laterally between the polar embryonic and lateral mural trophectoderm. We have, however, noticed that the egg cylinders may protrude not only between the lateral mural and polar embryonic trophectoderm but also through the abembryonic mural trophectoderm. The latter mechanism appears actually to be more common (47%) than the type of protrusion described by Hsu (34%). Our data also show that the egg cylinders protruding through the abembryonic mural trophectoderm reach stage 10–11 at a higher rate than those protruding laterally between the polar embryonic and lateral mural trophectoderm. We believe that this difference is due to mechanical reasons and that the former type of protrusion damages and/or deforms less the egg cylinder than the protrusion on the lateral side.

We have observed the blastocysts at the time of attachment and noticed that approximately three fourths of all blastocysts attach to the plastic dish in such a manner that the ICM is located in the lateral portion of the spherule. The attachment with the abembryonic mural trophectoderm is rare. Although our data suggest a preferential positioning of ICM at the time of attachment more experiments would be needed to prove or disprove this statement. Our data are at variance with the results of Hsu & Gonda (1980) who asserted that mouse blastocysts never attach to the plastic dish with the polar (embryonic) trophectoderm. It remains to be determined whether the structural and functional differences between the mural and polar trophectoderm (Copp, 1978 & 1979) may affect the attachment of the blastocyst to the plastic, and whether the inner cell mass migrates during the attachment assuming a position relatively closer to the dish surface (Gardner, Papaioannou & Barton, 1973; Wilson & Jenkinson, 1974).

When viewed histologically, the downward growing embryos were more
representative of in vivo embryos than the upward growing embryos. Presumably, the downward growing egg cylinders are initially better protected from adverse environmental influences than the freely exposed upward growing embryos. Only later on does the overlying trophoblast impede the development of downward growing embryos. Upon reversal, the downward growing egg cylinders exhibited the same developmental potential as the upward growing egg cylinders and reached the somitic stages at comparable or higher rates than the upward growing egg cylinders.

Our data thus show that the direction of egg-cylinder outgrowth depends on the positioning of the inner cell mass at the time of attachment. It is not clear why the downward growing embryos do not develop beyond the egg-cylinder stage, but several explanations are possible: (1) that the trophectoderm prevents the access of nutrients in the medium to the embryo and disposal of metabolic wastes away from the embryo proper into the medium (Wilson & Jenkinson, 1974); (2) since the Reichert’s membrane forms around the egg cylinder, it is possible that this membrane prevents the diffusion of nutrients into the embryo or expansion of the embryo as suggested by New (1978) for in vitro-grown postimplantation rat embryos; (3) it is furthermore possible that the trophoblast cells damage the enclosed embryos by releasing histolytic enzymes into the embryonic sac; (4) it is also possible that the mechanical factors exerted by the trophoblast from above and the plastic dish from below do not allow expansion of the developing embryo. The existing data do not favour any of the above mentioned explanations.

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