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Monozygotic Twin Formation in Mouse Embryos in vitro

Abstract. *Monozygotic twins developed from cultured murine blastocysts at the ratio of approximately 1:100. The locus at which the denuded blastocysts attached to the culture dish was usually a random section of their mural trophoblasts, in which case single egg cylinders developed unilaterally. However, in those few blastocysts attaching with their antipolar mural trophoblasts, the inner cell mass became subdivided into two parts because of restrictions imposed on its growth by the apically situated polar trophoblasts and the plastic substrate. Each subdivision apparently incorporated totipotent cells, resulting in the bilateral formation of two egg cylinders sharing the same ectoplacental cone.*

The cause of monozygotic twinning in mammals, including humans, is not known (*1*). There is strong experimental evidence that in lower vertebrates monozygotic twinning can result from early-stage developmental retardation caused by such factors as lack of oxygen (*2*). In humans, the frequency of congenital malformations in monozygotic twins is nearly twice as high as that in single births, and there is no such increase in dizygotic twins (*3*, *4*). These results indicate that monozygotic twinning is related to some developmental malformation.

In the rat (*5*), rabbit (*6*), and mouse (*7*), it has been demonstrated that single blastomeres of cleaving embryos are totipotent and able to develop into individual animals. However, how and when the monozygotic twins are formed in utero is not known. In vitro, on the other hand, individually cultured mouse embryos are now able to develop far beyond the implantation state (*8*), and thus are amenable to the examination of twin phenomena. The development of monozygotic twins in vitro has been recorded by time-lapse cinematography (*9*).

Development of mouse embryos beyond the implantation stage requires specific macromolecules for growth and differentiation (*10*). Mouse embryos grown in vitro are indistinguishable from those grown in vivo (*11–14*), despite the fact that in vitro, blastocysts must attach to and develop on the rigid surface of the plastic culture dish instead of on the flexible uterine endometrium. Still, imposing the mechanical restriction of a culture dish on the developing mouse embryo may cause minor abnormalities. In the present study, we found that cultured blastocysts from random-bred CF 1 mice (6 to 8 weeks old; Charles River) form monozygotic twins at the ratio of nearly 1:100. The cause of these twinnings was traced to the very early development of the embryos at a stage after the attachment of single blastocysts. In this stage, the inner cell mass (ICM) was observed to subdivide and grow as two independent egg cylinders.

The female mice were injected with pregnant mare serum gonadotropin (5 IU; Organon) to stimulate growth of ovarian follicles; human chorionic gonadotropin (5 IU) was injected 48 hours later to stimulate ovulation. They were then placed with males (one pair per cage). The next morning, the females were checked for the presence of a copulatory plug (day 0 of pregnancy). On day 3 of pregnancy, they were killed by cervical dislocation.

All procedures in the preparation, cultivation, and observation of the mouse embryos were conducted at 37°C under either a horizontal or vertical flow hood. The uteri were removed from their mesometria and placed in 100-mm plastic culture dishes. Under a dissecting microscope, each uterine horn was flushed with 1 ml of CMRL 1066 culture medium plus 10 percent heat-inactivated fetal calf serum (FCS) (Grand Island Biological). Blastocysts were sucked into a capillary pipette and pooled in a 35-mm plastic culture dish containing 2 ml of the culture medium. The dish was constantly flushed with a mixture of 5 percent CO_2 and 95 percent air to maintain the *p*H of the medium at 7.4. Ten blastocysts were distributed by capillary pipette into separate culture dishes, each containing 2 ml of culture medium. The cultures were maintained at 38°C in humidified incubators containing 5 percent CO_2 and 95 percent air. The concentration of CO_2 was automatically regulated.

The culture medium was supplemented with 1 m*M* glutamine and 1 m*M* sodium pyruvate; no antibiotics were used at any stage. The concentration of FCS in the medium was increased as the embryos developed. Initially, all blastocysts were cultured in 2 ml of CMRL 1066 plus 10 percent heat-inactivated FCS (day 0 of culture). On day 2 of culture, when the blastocysts had attached to the dishes, the concentration of FCS

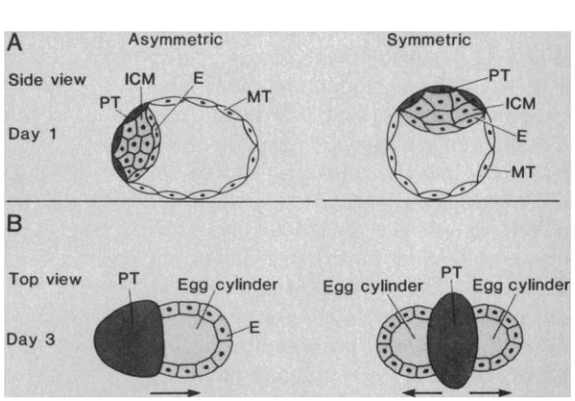

Fig. 1. Schematic drawing of blastocyst attachment (day 1 in culture) and subsequent elongation of the egg cylinder (day 3). (A) Side view of denuded blastocyst attached to the plastic substrate by mural trophoblasts (*MT*) (asymmetric attachment) and by antipolar trophoblasts (symmetric attachment). *E*, Primary endoderm; *ICM*, inner cell mass; *PT*, polar trophoblasts. (B) Top view of development and egg cylinder elongation in asymmetrically attached embryo (left) and symmetrically attached embryo (right). In the asymmetric attachment, while polar trophoblasts proliferate to the left to become the ectoplacental cone, the egg cylinder elongates toward the right. In the symmetric attachment, the polar trophoblasts are positioned above the ICM, and the egg cylinders grow bilaterally away from them.

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in the CMRL 1066 medium was increased to 20 percent. On day 3, the medium was changed to CMRL 1066 plus 20 percent heat-inactivated human placental cord serum (HCS). On days 4 and 5, the medium was renewed with CMRL 1066 plus 30 and 40 percent HCS, respectively.

After hatching, the blastocysts attached to the surfaces of the plastic culture dishes (Fig. 1A). The point of attachment was never the polar trophoblasts, which are apposed to the ICM, but usually a random section of the mural trophoblasts. Therefore, the orientation of the attached blastocysts was usually asymmetric with respect to the ICM. In a few cases, though, the point of attachment was symmetric with respect to the ICM; that is, the attaching cells were antipolar mural trophoblasts.

The blastocoel gradually diminished due to the radial migration of mural trophoblasts and enlargement of the ICM. At this stage, the ICM was covered with primary endoderm on the side of the blastocoel and by polar trophoblasts on the external side. In asymmetric attachment, in which the polar trophoblasts were positioned to one side of the embryo, the enlarged ICM was physically restricted from further downward expansion by the plastic substrate and the lack of space in the collapsed blastocoel. The egg cylinder became elongated sideward and then upward, protruding into the culture medium between the polar and mural trophoblasts (*13*).

The polar trophoblasts of the asymmetrically attached embryos continued to proliferate to form the ectoplacental cone in one direction, while the ICM, covered with primary endoderm, grew in the opposite direction to form the early egg cylinders (Fig. 1B). In contrast, the ICM of the more symmetrically attached blastocysts became separated into two parts due to restrictions placed on its growth between the apically attached polar trophoblasts and the plastic substrate (Fig. 2A). The polar trophoblasts, still attached to the spreading mural trophoblasts at several points, formed a cap that limited the upward growth of the ICM; the plastic substrate limited the downward expansion. At this stage, the polar trophoblasts tended to invaginate into the ICM to form extra embryonic ectoderm (*15*). The ICM was forced to migrate bilaterally away from the polar trophoblasts, presumably through breaks between the mural and polar trophoblasts. Thus monozygotic twins developed as a result of the physical incorporation of totipotent cells into each ICM subdivision.

With the lateral migration of each growing ICM, the ectoplacental cone became positioned basally with respect to the direction of growth. As the egg cylinder elongated, the embryo's direction of growth changed from horizontal to more upright (Fig. 2B), as did the asymmetrically attached embryos.

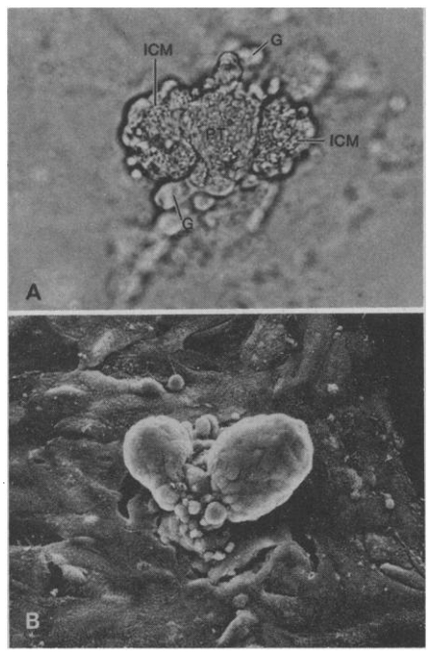

Fig. 2. (A) Light micrograph of a symmetrically attached blastocyst on day 2 of culture. The blastocoel has collapsed due to the outward migration of the mural trophoblasts. As a result, the polar trophoblasts sit on top of the ICM, which grows bilaterally from underneath them while the mural trophoblasts are transformed into giant cells (*G*). (B) Scanning electron micrograph of monozygotic mouse twinning after 3 days in culture.

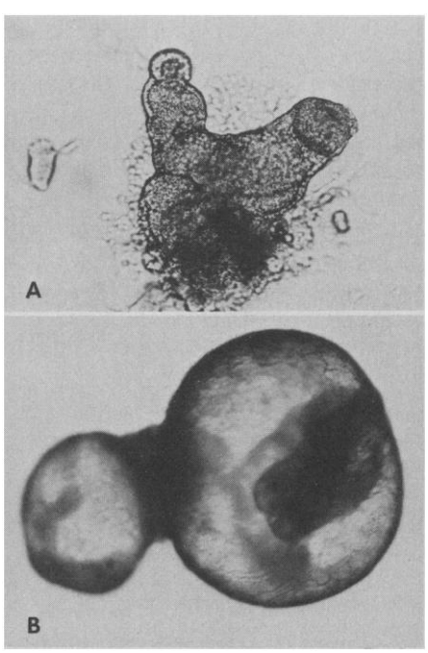

Fig. 3. Monozygotic twin mouse embryos in culture at (A) the egg cylinder stage (5 days in culture) and (B) the head-fold stage (7 days in culture).

Although one twin was usually larger than its counterpart and its growth stage slightly more advanced (Fig. 3B), this was not always the case if the attachment was truly symmetric (Fig. 3A). In most cases, both embryos developed to the early somite stage, including cardiac contraction.

Various kinds of conjoint monozygotic twins (Siamese twins) may be formed by different causes at each developmental stage. Monozygotic conjoined twins have been induced in fetuses on days 10 and 12 of gestation by giving the mothers single injections of vincristine on days 6, 7, and 8 of gestation (*16*).

The physical restriction placed on ICM growth by polar trophoblasts and the plastic substrate results in subdivision of the ICM into two parts. This subdivision, which results in the formation of two independent egg cylinders, is probably artificial monozygotic twinning—it usually does not occur in utero. It is the only kind of monozygotic twinning observed thus far in mouse embryos in vitro.

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