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Production of mouse by inter-strain inner cell mass replacement

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Summary

The developmental ability of reconstructed blastocysts from C57BL/6 strain mouse inner cell masses (ICMs) and Kunming strain mouse trophoblasts was assessed. The procedure of ICM replacement was as follows: C57BL/6 ICMs were separated from the blastocysts using immunosurgery. A slit was made in the zona pellucida of a Kunming blastocyst to allow its ICM to extrude. The C57BL/6 ICM was injected into the Kunming blastocoele, and the extruded Kunming ICM was cut off. The reconstructed blastocysts were able to re-expand (77%) and hatch (27.3%) *in vitro*. A total of 64 reconstructed blastocysts and 124 Kunming blastocysts were co-transferred into the uteri of 11 pseudopregnant Kunming mice, and an ICM replacement offspring was born. The results indicate that reconstructed embryos obtained by inter-strain ICM replacement have the ability to develop to term. This technique may provide a method to solve the pregnancy failure in interspecific cloning.

Keywords: Inner cell mass replacement, Mouse, Reconstructed blastocyst, Trophoblast

Introduction

Blastocyst is composed of trophoblast and inner cell mass (ICM). The trophoblast interacts with the maternal uterus at the implantation stage and gives rise to the placenta, whereas the ICM develops into the tissues of the fetus. Blastocyst injection provides a tool for analysing cellular differentiation and development. Mice produced by injecting embryonic stem (ES) cells into heat-treated or tetraploid blastocysts had normal fertility, and their germ cells were of ES cell origin (Amano *et al*., 2001). This technique can also be used as a means of obtaining chimeric animals (Rossant *et al*., 1983; Butler *et al*., 1987; Polzin *et al*., 1987; Nagashima *et al*., 2004). *M. caroli* mouse blastocysts transferred to *M. musculus* mouse uterus failed to survive to term, but injection of *M. caroli* mouse ICM cells into *M. musculus* mouse blastocysts resulted in viable interspecific chimeras, suggesting that trophoblast layer may mediate the survival or death of the foreign embryonic cells in the uterus (Rossant *et al*., 1983).

Interspecific pregnancy is a useful model for the study of maternal–fetal interactions and assists in the preservation of endangered species. Interactions between the trophoblast and the uterus play an important role in determining the development of embryos. Isolation of ICMs and blastocyst injection can be used to permit interspecific embryo transfer by allowing development of a fetus in the uterus of a different species. A viable *M. caroli* mouse was produced by blastocyst reconstruction using trophoblast of *M. musculus* and ICM of *M. caroli*, showing that protection of the *M. caroli* fetus in the *M. musculus* uterus may be provided by *M. musculus* trophoblast (Rossant *et al*., 1983). Reconstructed blastocysts from Kunming trophoblasts and Kunming ICMs were transferred into Kunming recipients, and two intra-strain ICM replacement mice were born in our laboratory (Bi *et al*., 2003). In this experiment, blastocysts were reconstructed using C57BL/6 ICMs and Kunming trophoblasts, and the developmental ability of the inter-strain ICM replacement blastocysts was evaluated.

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Materials and methods

Collection of blastocysts

Kunming (white) and C57BL/6 (black) female mice (8–12 weeks old) were superovulated with 10 IU pregnant mare serum gonadotropin (PMSG, Tianjin Experimental Animal Center) followed by 10 IU human chorionic gonadotropin (hCG, Institute of Zoology, CAS, Beijing) 48 h later. Then the superovulated mice were mated with male mice (12–15 weeks old) of the same strain. The females were checked the next morning for the presence of a vaginal plug to confirm mating. On the fourth day post-coitus they were killed by cervical dislocation, and embryos were flushed from the uteri with M2 medium. The blastocysts were cultured in CZB medium at 37 °C with 5% CO_2 in air.

Preparation of antiserum and complement

Rabbit anti-mouse serum was produced in a Japanese Big Eared White rabbit (4 months old), which was injected with 4×10^8 mouse spleen cells through the ear vein four times at weekly intervals. The rabbit was bled 10 days after the last injection. Serum was heated at 56 °C for 30 min to inactivate rabbit complement. Guinea pig serum was used as the source of complement.

Isolation of ICMs

ICMs were isolated from blastocysts by immunosurgery. Briefly, C57BL/6 blastocysts were treated with 0.5% pronase (Calbiochem) for 5 min to remove the zona pellucida (ZP). The ZP-free blastocysts were cultured in rabbit anti-mouse serum diluted at 1:16 in CZB medium for 20 min. Then the blastocysts were washed three times and incubated in guinea pig serum diluted at 1:16 in CZB medium for 40 min. The trophoblast cells were swollen and lysed (Fig. 1A) after immunosurgery, and were dissociated from the ICMs by gentle pipetting. Isolated ICMs were washed and cultured in CZB medium.

Blastocyst injection

A Kunming blastocyst was positioned with the ICM at 12 o'clock, and a slit was made on the ZP just over the ICM by a glass needle (Fig. 1B). The blastocysts were then cultured in CZB medium at 37 °C for 3–4 h, and those with extruded ICMs were selected for injection. The C57BL/6 ICM isolated by immunosurgery was cut into two parts by a glass needle, aspirated into an injection pipette with an outer diameter of 20–25 μm and a beveled tip of 40°, and then gently injected into the blastocoel of Kunming blastocyst (Fig. 1C, D). The extruded Kunming ICMs were cut off after the injection of C57BL/6 ICMs (Fig. 1E, F). The reconstructed blastocysts were cultured in CZB medium at 37 °C for 3–4 h.

Embryo transfer

The foster mothers for embryo transfer were Kunming females mated with vasectomized Kunming males. Reconstructed blastocysts were surgically transferred into the uterus of pseudopregnant recipients at 2.5–3.5 days post-coitus. Because the number of reconstructed blastocysts was limited, Kunming blastocysts and reconstructed blastocysts were co-transferred into recipients. Each recipient was transferred about 6 reconstructed embryos and 10 Kunming blastocysts.

Results

Isolation of ICMs by immunosurgery

C57BL/6 blastocysts treated with antiserum and complement showed lysed trophoblasts and intact ICMs. The ICMs were obtained after the trophoblasts were removed by pipetting. The isolation rate of ICM was 100%.

Re-expansion and hatching of reconstructed blastocysts *in vitro*

A total of 239 reconstructed blastocysts were cultured at 37 °C for 3–4 h, and 184 (184/239; 77%) of the embryos re-expanded *in vitro*. Twenty-two re-expanded reconstructed blastocysts were cultured for 24 h, and 6 (6/22; 27.3%) developed to the hatched blastocyst stage (Fig. 1G).

Transfer of reconstructed blastocysts

A total of 162 re-expanded reconstructed blastocysts were transferred into 25 pseudopregnant Kunming mice. Of these, 98 blastocysts were transferred into 14 fosters, but no offspring was born. The remaining 64 reconstructed blastocysts and 124 Kunming blastocysts were co-transferred into 11 recipients. A recipient receiving 7 reconstructed blastocysts and 11 Kunming blastocysts delivered five mice, among which there was one pup with black eyes and a black coat (derived from reconstructed blastocysts) and four with red eyes and white coats (from Kunming blastocysts) (Fig. 1H). The rate of reconstructed blastocysts developing to term was 1.6% (1/64) in the co-transfer group.

Discussion

Preincubation with antiserum followed by exposure to complement can selectively kill trophoblast cells. Antiserum is prevented from gaining access to ICM

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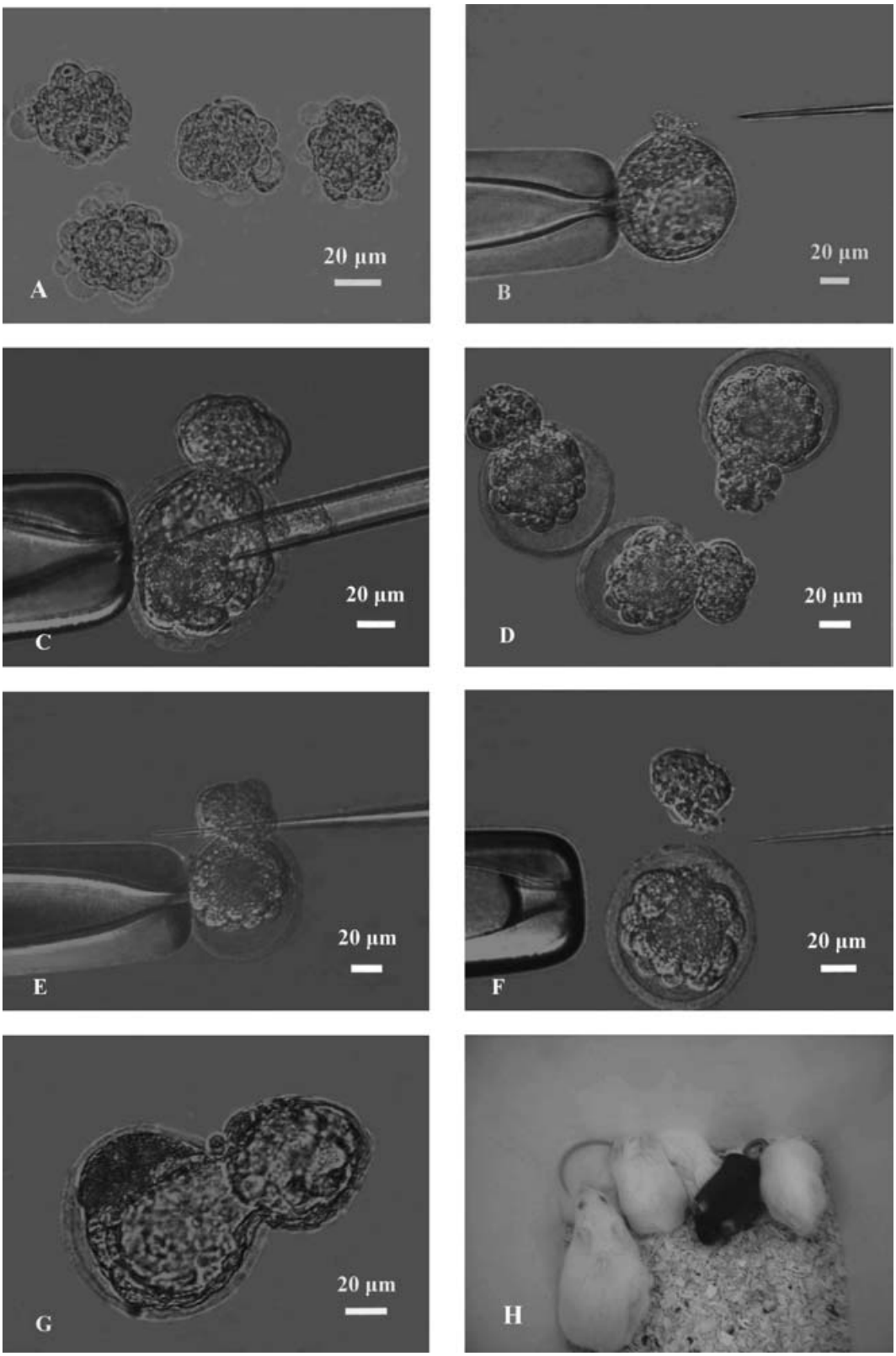

Figure 1 Inter-strain mouse inner cell mass replacement. (*A*) C57BL/6 ICMs isolated by immunosurgery. (*B*) A slit was made in the zona pellucida over the Kunming ICM by a glass needle. (*C*), (*D*) The C57BL/6 ICM was injected into the Kunming blastocoele. (*E*), (*F*) The Kunming ICM was cut off by a glass needle after blastocyst injection. (*G*) A hatched reconstructed blastocyst after 24 h of culture *in vitro*. (*H*) The ICM replacement (black) and Kunming (white, small) offspring, together with the foster mother (white, large).

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by the tight junctions between the surrounding trophectoderm cells, and only trophoblast is lysed by the addition of complement, leaving the ICM intact. In this experiment, C57BL/6 ICMs were separated from blastocysts using this technique, and the isolation rate was 100%, indicating that immunosurgery is an efficient method to obtain ICMs from blastocysts. In this experiment, a C57BL/6 ICM was cut into two parts so that it could be easily aspirated into the injection pipette. The two ICM parts injected into the blastocoel could aggregate together.

The blastocoele collapsed after Kunming ICMs were removed from the blastocysts. In the experiment conducted by Bi *et al*., the extruded Kunming ICMs were cut off before injection of C57BL/6 ICMs. The Kunming trophoblasts without ICMs needed to be cultured for 5–6 h so that they could expand before being used for injection (Bi *et al*., 2003). In this experiment, the extruded Kunming ICMs were cut after injection of C57BL/6 ICM. Thus the time interval between cutting of Kunming ICM and injection of C57BL/6 ICM was reduced.

Blastocyst formation is essential for implantation and establishment of pregnancy. It is mediated by a trans-trophectoderm ion gradient(s) established by Na^+/K^+-ATPase, which drives the movement of water through aquaporins to form the fluid-filled blastocoele (Watson & Barcroft, 2001). Blastocyst hatching occurs as a result of protease-mediated lysis and blastocoel tension, and the activity of actin filaments may have a crucial role in this process (Cheon *et al*., 1999). In this experiment, the trophoblast collapsed after the injection pipette was withdrawn from the blastocoele, but the reconstructed blastocysts retained the ability to re-expand and hatch *in vitro*.

The presence of a sufficient number of trophoblast and ICM cells in the blastocysts is a prerequisite for successful implantation and embryogenesis. Reduction in ICM and trophoblast cells may affect implantation and development (Chan *et al*., 2000; Modlinski *et al*., 2002). Blastocysts with relatively large ICMs implant at a higher rate than those with smaller ICMs (Richter *et al*., 2001). Failure to form a fetus may be due to the absence of egg cylinder development, which is correlated with the reduced number of the ICM cells (Wang *et al*., 1990). Blastocysts reconstructed from Kunming trophoblasts and C57BL/6 ICMs have a lower cell number than blastocysts of either strain, but the morphology and cytoskeletal structure of the reconstructed embryos do not differ from those of normal blastocysts (Bi *et al*., 2003). Although the rates of re-expansion and hatching of the reconstructed blastocysts were relatively high, the ability of reconstructed blastocysts to develop to term was low in this study. Cutting of C57BL/6 ICM and its aspiration into the injection pipette may damage the ICM, and injection of the C57BL/6 ICM and separation of Kunming ICM from the injected blastocysts may damage the Kunming trophoblast, which would impair implantation and development of the reconstructed embryos *in vivo*.

After transfer of micromanipulated embryos, recognition and maintenance of pregnancy play key roles in the production of live offspring. Glycoproteins on the cell surface of the embryo are involved in intercellular recognition and adhesion between the embryo and endometrial epithelium (Kitamura *et al*., 2003). Parthenogenetic embryos appear to be as effective as fertilized embryos in producing endocrine signals to maintain pregnancy. After co-transfer of three fertilized embryos with 55–60 parthenogenetic embryos, two healthy piglets were delivered at term (King *et al*., 2002). No offspring was produced after transfer of inter-strain ICM replacement blastocysts into pseudopregnant mice (Bi *et al*., 2003). In this experiment, the inter-strain reconstructed blastocysts and Kunming blastocysts were co-transferred into pseudopregnant recipients, and a live ICM replacement mouse was born, suggesting that co-transfer of fertilized embryos may be beneficial to implantation and development of the reconstructed blastocysts.

Trophoblast plays an important role in embryonic implantation and formation of placenta. The inferior developmental potential of trophectoderm cells underlies the low developmental rate of cloned embryos and high rate of postnatal death of cloned fetuses (Amano *et al*., 2002). The high rate of pregnancy failure may result from abnormal development of placenta, which is correlated with the trophoblast development of cloned embryos (Tanaka *et al*., 2001; Hashizume *et al*., 2002). Although interspecific cloning technique has been successfully applied to save some endangered species (Lanza *et al*., 2000; Loi *et al*., 2001), whether cloned embryos can normally implant and develop to term in the uterus of a different species remains a key issue in interspecific cloning (Chen *et al*., 2002). ICM replacement may provide a method to solve the problem in interspecific cloning. If the ICM of interspecific cloned embryos were injected into trophoblast vesicle from normally fertilized blastocyst, and the reconstructed blastocysts were then transferred into the uterus of the same species as the trophoblast, the implantation and development to term of interspecific cloned embryos may be possible.

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