Zygote **11** (February), pp 53–59. © 2003 Cambridge University Press
DOI:10.1017/S0967199403001072 Printed in the United Kingdom

Development of mouse embryos derived from oocytes reconstructed by metaphase I spindle transfer

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Date submitted: 10.7.02. Date accepted: 10.9.02

Summary

Abnormal oocyte spindle is frequently associated with the infertility of aged women. Directly manipulating the metaphase I (MI) spindle may be a feasible method to overcome this kind of problem. Here, we report that the MI meiotic spindle can be removed from MI mouse oocytes and will autonomously divide into two daughter cells with the same size, morphology and an equal number of chromosomes after culture for 5 h in maturation medium. The division rate of the MI spindle reached 56% after 10–15 h of culture. After transferring the MI meiotic spindle into synchronous ooplasm by electrofusion, about 61% of the reconstructed oocytes continued to complete the first meiosis and extruded a normal first polar body. The matured reconstructed oocytes can also be fertilised. Approximately 50% of the 2-cell embryos developed to the morula stage after *in vitro* culture.

Keywords: IVF, MI spindle, Mouse, Nuclear transfer, Oocytes

Introduction

Oocyte maturation is a process of meiosis initiation and completion, including germinal vesicle breakdown (GVBD), chromosome condensation, spindle assembly and polar body extrusion. The formation of normal meiotic apparatus is critical to the progression of meiotic division. It has been shown that both microtubules and microfilaments are closely associated with the structure of the spindle and position of chromatin after GVBD and during the meiotic maturation of oocytes (Kim *et al*., 1996*a*; Rime *et al*., 1987; Messinger & Albertini, 1991). Oocytes with an abnormal spindle can not be fertilised for further embryo development. Abnormal spindle assembly and incorrect positioning of metaphase chromatin were frequently observed in *in vitro* matured oocytes (Kim *et al*., 1996*a*, *b*) or aged oocytes (Kim *et al*., 1996*b*; Battaglia *et al*., 1996), resulting in a high prevalence of aneuploidy and abnormal embryonic development. Nuclear transplantation by transferring the germinal vesicle of an oocyte from an aged woman to the recipient oocyte cytoplast from a young woman may not be helpful for treating the infertility associated with abnormal spindle function. Recently, transfer of the metaphase II (MII) spindle has been proved to be feasible to obtain live mice (Wang *et al*., 2001). However, the spindle and chromosomes first appear at the metaphase I (MI) stage; therefore, it is even more important and necessary to prove the feasibility of MI spindle transfer.

The purpose of the present study was to establish a model for separating the MI spindle from mouse oocytes, and to investigate the ability of meiotic resumption after MI spindle transfer as well as the development of embryos following fertilisation.

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Materials and methods

Preparation of immature oocytes

Donor oocytes at the GV stage were obtained from immature C57BL/6 mice (black) and Kunming strain mice (white) ovaries stimulated with 10 IU pregnant mare's serum gonadotropin (PMSG) for 48 h. The GV stage oocytes surrounded by several layers of cumulus cells were collected from large antral follicles by

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puncturing with a fine sharp needle and cultured in Tissue Cultured Medium 199 (TCM 199, Sigma product) supplemented with 10% fetal calf serum (FCS).

Separation and culture of MI spindle

The GV stage oocytes developed to the MI stage after culture for 5 h in M199 medium. Under an inverted phase-contrast microscope the MI spindle appeared transparent in the ooplasm in M_2 medium (Sigma product) containing 2% sucrose and 7.5 μg/ml cytocholasin B (CB) (Fig. 1*A*). Firstly, the zona pellucida of the oocyte was slit with a glass needle. Then, the MI meiotic apparatus with a little cytoplasm was aspirated into a blunt-tipped pipette with 20 μm inner diameter. Correct enucleation of the MI spindle was further proved by 5 μg/ml Hoechst 33342 (Sigma) staining. Some MI meiotic spindles were cultured in M199 medium for 10 h to observe their developmental behaviour, and the others were used for transfer with a Nicon micromanipulator (Fig. 1*B*, *C*).

MI spindle transfer and electrofusion

In the microdrops of micromanipulation medium, the MI spindle apparatus of C57BL/6 or Kunming mouse oocytes was aspirated into the enucleation pipette and inserted into the perivitelline space of another previously enucleated MI cytoplast of Kunming or C57BL/6 oocytes. The karyoplast was placed in contact with the cytoplast in order to prepare for electrofusion.

The MI–cytoplast complexes were transferred into a drop of M_2 medium between 0.25 mm diameter stainless steel electrodes of a fusion chamber. After manual alignment with a glass needle, fusion of reconstructed pairs was achieved with two DC electrical pulses (1.0 kV/cm DC for 50 μs) delivered by a Model 2001 Electro Cell Manipulator (BTX, San Diego, CA). The fused pairs were washed three times in M_2 medium and then cultured in M199 medium for 10 h to evaluate maturation as evidenced by extrusion of the first polar body (PBI) (see Fig. 3*A*, *B*).

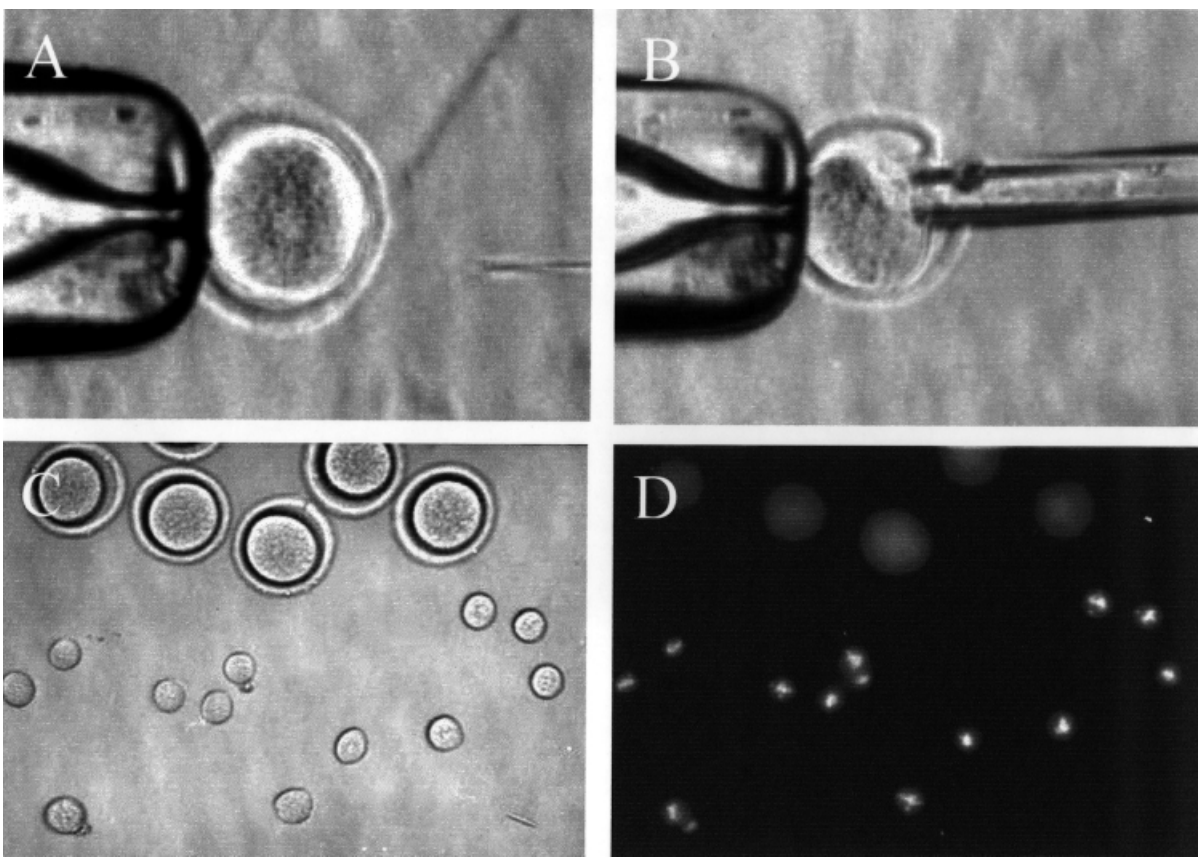


Figure 1 Micromanipulation of mouse MI stage oocyte. (*A*) Observation of the MI meiotic spindle, which is transparent in the ooplasm. (*B*) The removal of the MI spindle with the enucleation pipette. (*C*) The aspirated MI spindle and the enucleated MI cytoplast. (*D*) Fluorescent staining of chromosomes in the MI spindle with Hoechst 33342.

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In vitro fertilisation and embryo development

Caudal epididymides of C57BL/6 male mice were cut and torn in TYH medium to release sperm. After 1.5–2 h of capacitation, the reconstructed matured oocytes were added to the sperm drop for 4 h, then transferred into CZB medium to observe *in vitro* development. When the fertilised embryos had been at the 4-cell stage for 48 h, the CZB medium was supplemented with 1.1 mg/ml sucrose to support continuing development.

Cytogenetic analysis

Two daughter cells derived from MI meiotic apparatus transfer were fixed for chromosome analysis (Tarkowski, 1966). Briefly, two daughter cells were transferred into 1% hypotonic trisodium citrate solution for 15 min, and swollen cells were transferred onto a clean slide and fixative I (methanol:acetic acid, 3:1, v/v) added for 5 min. Then fixative II (methanol:acetic acid, 1:1, v/v) and fixative III (methanol:acetic acid, 1:3, v/v) were added for at least 5 min and the slide air-dried. Finally, fixed blastomeres were stained with Giemsa to score chromosomes.

Immunofluorescence staining

Two daughter cells from MI spindle transfer were selected for immunofluorescence staining and detection with confocal microscopy. Each pair of two daughter cells was fixed in 3.7% paraformaldehyde (Sigma) in 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4) for 40 min, and then permeabilised in PBS buffer containing 0.25% Triton X-100 (Sigma) for 40 min at room temperature. After rinsing clear in PBS buffer 4 times, the pairs were incubated with 1:50 fluorescein isothiocyanate (FITC)-labelled anti α-tubulin (Sigma) for 1 h. After being washed 3 times in PBS buffer, DNA was observed by exposure to 10 μg/ml propidium iodide (Sigma).

Results

Identification of the MI spindle and enucleation

When treated with 2% sucrose, the meiotic apparatus in MI oocytes appeared transparent, and could be distinguished from the surrounding cytoplasm under an inverted phase-contrast microscope (Fig. 1*A*). The enucleation accuracy was proved to be 100% by Hoechst 33342 fluorescent staining (Fig. 1*D*).

Table 1 Two daughter cells derived from the division of MI spindle karyoplast cultured in maturation medium

No. cultured	No. of replicates	No. divided (%)		
		5 h	10 h	15 h
121	6	48 (39.6)	–	–
1130	35	–	633 (56)	–
156	6	–	–	81 (51.9)

Behaviour of the MI spindle in maturation medium

The diameter of the MI meiotic apparatus with a little surrounding cytoplasm was about 25 μm. The karyoplast containing the MI spindle divided into two equal-sized daughter cells after culture in M199 medium for 5 h (Fig. 2*A*). The division rate increased to 56% at 10 h (Table 1). There was a midbody between the two cells, keeping a slight and weak connection between them. A metaphase spindle and a clutch of chromosomes were clearly shown to exist in each daughter cell by fluorescence staining (Fig. 2*B*). Each daughter cell displayed 20 normal univalent chromosomes (Fig. 2*C*).

In vitro maturation of reconstructed mouse oocytes after MI spindle transplantation

The results of *in vitro* maturation (IVM) are shown in Table 2. A total of 620 hetero-karyoplast-cytoplast complexes were reconstructed and used for electrofusion in 29 replicates. The fusion rate was more than 50%, without a significant difference between the C57BL/6–Kunming and Kunming–C57BL/6 groups. Following culture in maturation medium, about 60% of fused pairs extruded PB1.

Development of the constructed oocytes after *in vitro* fertilisation

A total of 67 matured reconstructed oocytes with PB1 were used for *in vitro* fertilisation (IVF). Among them, 28 were fertilised and developed to the 2-cell stage. The rate of development into morulae was about 50% in both groups, without a significant difference between the groups (Table 3, Fig. 3*C*).

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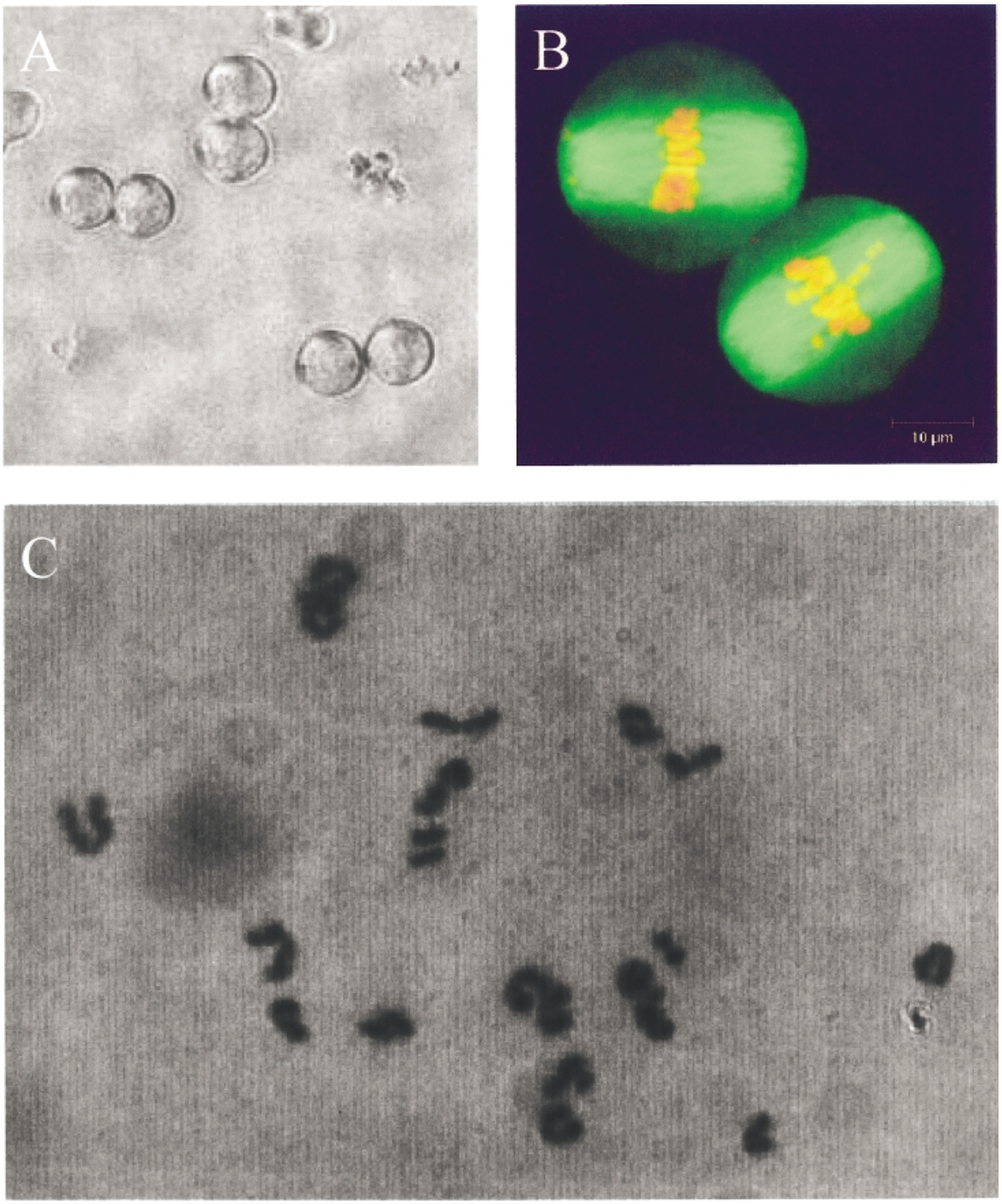


Figure 2 Division of the MI spindle into two daughter cells in maturation medium. (*A*) The two daughter cells have the same morphology and size under an inverted phase-contrast microscope. (*B*) A group of metaphase spindle and chromosomes in each daughter cell revealed by fluorescent staining. (*C*) A set of haploid chromosomes in each daughter cell.

Table 2 Maturation of mouse reconstructed oocytes after MI spindle transfer

Type of reconstructed oocyte (karyoplast–cytoplast)	No. of replicates	No. of reconstructed pairs	No. (%) fused	No. (%) reaching MII
C57BL/6–Kunming	29	620	353 (56.94)	215 (60.91)
Kunming–C57BL/6	29	548	316 (57.66)	165 (52.22)

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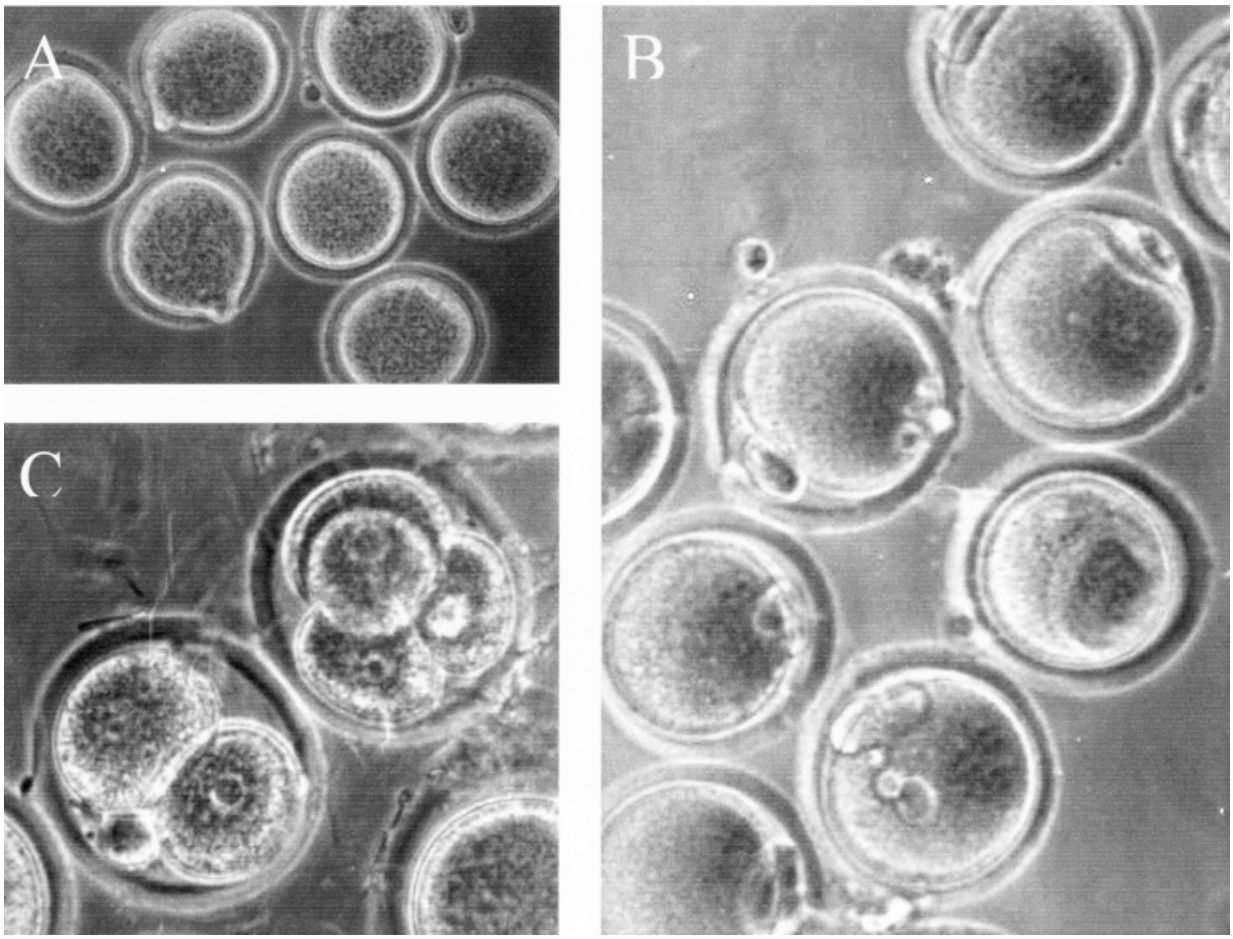


Figure 3 Electrofusion and maturation of reconstructed oocytes after MI spindle transfer and embryo development after *in vitro* fertilisation. (*A*) Electrofusion of oocytes reconstructed with a C57BL/6 MI spindle and an enucleated Kunming MI cytoplast. (*B*) The matured reconstructed oocytes extruded PB1 after fusion. (*C*) Two 4-cell embryos derived from the matured reconstructed oocytes after *in vitro* fertilisation.

Table 3 *In vitro* development of matured reconstructed oocytes after MI spindle transfer

Type of reconstructed oocyte (karyoplast–cytoplasts)	No. of replicates	No. emitting PB1	No. (%) of 2-cell	No. (%) of 4-cell	No. (%) of morulae
C57BL/6–Kunming	6	49	20 (40.81)	9 (45)	9 (45)
Kunming–C57BL/6	3	18	8 (44.44)	5 (62.50)	4 (50)

Discussion

The present study demonstrates that the MI spindle of mouse oocytes can be removed and divided into two daughter cells following culture in maturation medium. Each cell has a structurally normal MII meiotic spindle and haploid chromosomes. This division behaviour of the MI spindle may be related to the regulation of MAP kinase. When intact oocytes undergoing maturation were examined using a specific active-MAP kinase antibody, MAP kinase immunostaining was found at each pole of the first meiotic spindle (Verlhac *et al*., 1993). Previous studies have demonstrated that the level of MAP kinase was high in MI stage oocytes (Araki *et al*., 1996). MAP kinase was involved in the post-GVBD events during mouse, rat and goat oocyte maturation (Sobajima *et al*., 1993; Verlhac *et al*., 1994; Dedieu *et al*., 1996; Zernicka-Goetz *et al*., 1997). These events include the migration of the spindle, disassembly of microtubules, the separation of homologous chromosomes, and the formation and extrusion of the polar body. It is

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interesting that the two daughter cells with haploid chromosomes will keep a normal morphology for a period of time, unlike PBI, which quickly degenerates after extrusion.

Spindle dysfunctions frequently results in the disabled division of the spindle, a morphologically abnormal spindle and incorrect chromosome alignment as well as aneuploidy (Dailey *et al*., 1996). In mouse, with increasing age the meiotic spindle becomes shorter than normal (Eichenlaub-Ritter *et al*., 1998*a*, *b*) and shows chromosome misalignment (Eichenlaub-Ritter & Boll, 1989). Recent studies have demonstrated that spindle abnormalities are associated with the infertility of aged women (Munné *et al*., 1995; Battaglia *et al*., 1996; Angell, 1997). Possible methods to overcome these problems are to improve the cytoplasmic environment and chromosome functions. Recently, nuclear-cytoplasmic transfer using nuclear transplantation technique has obtained encouraging results in mammals. In order to improve ooplasmic deficiency, transfer or directly injection of ooplasm from donor oocytes into oocytes from patients with recurrent implantation failure has led to the birth of nearly 30 healthy babies worldwide (Cohen *et al*., 1997, 1998; Barritt *et al*., 2001). Additionally, germinal vesicle (GV) transfer has been established to achieve complete cytoplasm exchange between oocytes and resulted in the birth of rabbit (Li *et al*., 2001).

Operation on the spindle will probably become an important technique in chromosome engineering and for the treatment of infertility derived from dysfunction of the spindle or chromosomes. Recently, transfer of the MII spindle has been proved to be feasible and resulted in the birth of live mice (Wang *et al*., 2001). In this experiment we investigated the possibility of transferring the MI meiotic spindle at the first meiosis. About 60% of the reconstructed oocytes can complete the first meiosis, as characterised by the extrusion of PB1. The matured oocytes have the ability to form male and female pronuclei after fertilisation, and approximately 50% of the 2-cell embryos are able to develop to the morula stage after *in vitro* culture. Even though reconstructed oocytes have normal morphology and are able to be fertilised, and developed to the morula stage, further studies are necessary to determine whether normal offspring could be produced.

In conclusion, the karyoplast containing the MI meiotic spindle can autonomously divide into two daughter cells. Oocytes that have undergone MI spindle transfer can complete the following meiosis and be fertilised normally, and develop to the morula stage. This research provides a model that may be useful not only to understand the nuclear-cytoplasmic relationship, but also in infertility treatment in human assisted reproduction.

Acknowledgements

Special thank are extended to Ms Xiang-Fen Song and Shi-Wen Li for their technical assistance. The authors are also grateful to Drs Heng-Yu Fan, Min-Kang Wang, Ying Xu, Jin-Song Li, Zhi-Ming Han and Chun-Ming Bi as well as Ms Li Lian for their assistance and valuable discussions. This research was supported by the '973' Project (grant no. G1999055902), Knowledge Innovation Program of the Chinese Academy of Sciences (grant no. 39770098) and National Natural Science Foundation of China (grant no. 39970093 and 30225010).

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