Development of enucleated mouse oocytes reconstituted with embryonic nuclei

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Summary. The chromosomes of mouse oocytes at telophase of the first meiotic division were removed using micromanipulation and differential interference microscopy. The enucleated oocytes were used as recipients for nuclear transplantation, after culture for 4–6 h. The newly synthesized proteins of the enucleated oocytes showed the same pattern as those of secondary oocytes matured *in vivo*. When the enucleated oocytes received a nucleus from late 2- and 8-cell embryos, or a cell from the inner cell mass (ICM) of blastocysts, 23, 4 and 10%, respectively, of reconstituted embryos developed to blastocysts. After transfer to recipient females, live young were produced from the reconstituted eggs that received a nucleus from late 2-cell embryos.

Keywords: nuclear transplantation; mouse; oocytes; enucleation; live young

Introduction

Nuclear transplantation studies with mice and rats have shown that enucleated zygotes which received nuclei from either late 2-cell or more advanced embryos exhibited limited preimplantation development (McGrath & Solter, 1984; Robl *et al.*, 1986; Tsunoda *et al.*, 1987; Kono & Tsunoda, 1988). Transplantation of single nuclei from a 4-cell mouse embryo into one of the enucleated blastomeres of a 2-cell embryo resulted in the successful production of identical triplet mice (Kono *et al.*, 1991), but it is difficult to produce live young by such nuclear transplantation between highly asynchronous embryos. In amphibians, fertile adults have been produced from blastula nuclei (*Rana*: Briggs & King, 1952) and from intestine nuclei (*Xenopus*: Gurdon & Uehlinger, 1966) transferred into enucleated oocytes. These studies show that nuclei transferred into enucleated oocytes could be reprogrammed to support normal development (review in Briggs, 1979; DiBerardino, 1980). Based on this concept, offspring were successfully produced in sheep (Willadsen, 1986) and cattle (Prather *et al.*, 1987) from enucleated secondary oocytes which received nuclei from 8–16-cell embryos. At that time, however, the complete removal of metaphase II chromosomes from ovulated oocytes was unreliable as the chromosomes at this stage could not be readily recognized using phase-contrast or interference microscopy. The successful enucleation of the oocyte was associated with a significant reduction in ooplasmic volume.

Chromosomes at metaphase of the second meiotic division can be easily recognized when they are stained with Hoechst dye and viewed by fluorescence microscopy (Ebert *et al.*, 1985). Using this method, Tsunoda *et al.* (1988) reported a reliable procedure for the enucleation of secondary mouse oocytes in which the chromosomes, visible after Hoechst staining, were removed by standard micromanipulation techniques. However, ultraviolet irradiation is reported to damage the developmental ability of preimplantation mouse embryos, especially in combination with Hoechst staining (Eibs & Spielman, 1977; Ebert *et al.*, 1985).

In this paper, we report the removal of chromosomes at telophase of the first meiotic division without fluorescence staining, and the developmental potential of the reconstituted embryos. Using

the present method, we succeeded in producing live young after transfer to recipients of enucleated oocytes which had received a nucleus from late 2-cell embryos.

Materials and Methods

Collection of oocytes and embryos. F₁ hybrid (C57BL/6J × CBA) female mice were used as oocyte donors and female mice of strain CD-1 were used to produce 2- and 8-cell embryos. They were superovulated by injections of 5 iu pregnant mares' serum gonadotrophin (PMSG; Peamex, Sankyo Ltd, Tokyo, Japan) and 5 iu human chorionic gonadotrophin (hCG; Puberogen, Sankyo Ltd) given 48 h apart. Oocytes at telophase of the first meiotic division and freshly ovulated oocytes at metaphase II were released from ovarian follicles and oviducts at 9–11 and 12 h, respectively. Cumulus cells were removed by treatment with 300 iu hyaluronidase/ml in M2 medium (Quinn, 1982) and washed several times. After the injection of hCG, CD-1 females were paired with males of the same strain, and late 2- and 8-cell embryos were collected by flushing the oviducts with M2 medium 44–46 and 64–66 h after hCG injection, respectively. To obtain blastocysts for the preparation of inner cell mass (ICM) cells, spontaneously ovulating CD-1 females were mated with males of the same strain and the uterus was flushed at 3.5 days *post coitum*.

Hoechst staining. To examine the stage of meiotic maturation and position of chromosomes, oocytes were stained with Hoechst 33342 (Calbiochem-Behring Corp., San Diego, USA) in M2 medium (10 µg/ml) for 10 min at room temperature and washed with M2 medium several times. Oocytes were placed in a small drop of M2 medium on a micromanipulation glass slide covered with paraffin oil. The oocytes were secured by a holding pipette and observed using fluorescent microscopy.

Enucleation of oocytes. Oocytes were collected from ovarian follicles 10 h after hCG injection, and the telophase chromosomes were removed. The zonae pellucidae of the oocytes were slit with a glass needle along 10–20% of their circumference and placed in a small drop of M2 medium containing cytochalasin B (5 µg/ml) on a micromanipulation glass slide covered with paraffin oil. The oocytes were secured by a holding pipette opposite the slit in the zona. An enucleation pipette of 20 µm in diameter, with an unsharpened bevelled tip, was used to remove the meiotic chromosomes and associated spindle apparatus from the oocyte. The enucleation pipette was inserted into the perivitelline space through the slit and the tip was orientated to cover the first polar body. Following aspiration of the first polar body, telophase chromosomes with spindles were aspirated into the enucleation pipette with a small amount of cytoplasm (Fig. 1). If the chromosomes were not able to be identified in an enucleation pipette at the time of sucking, the oocytes were eliminated. In order to confirm that the enucleation method was reliable, the enucleated oocytes were stained with Hoechst 33342 and observed using fluorescent microscopy. None of the enucleated oocytes examined (50) had chromosomes. All procedures were performed using Nikon Diaphot microscopy and Narishige micromanipulators.

Fig. 1. Removal of anaphase chromosomes at the first meiotic division from a mouse oocyte by micromanipulation. Arrow shows the anaphase chromosomes with spindle when aspirated into an enucleation pipette (× 400).

Nuclear transplantation. Following enucleation, the oocytes were cultured in M16 medium for 4–6 h and were then subjected to nuclear transplantation. This gives a high activation rate in the reconstituted eggs (T. Kono, unpublished data). Nuclear transplantation was carried out as described by McGrath & Solter (1983) and Tsunoda *et al.* (1986). An ICM cell or a karyoplast from late 2- and 8-cell donor embryos was introduced with inactivated Sendai virus (HVJ) 2700 haemagglutinating activity units/ml into the perivitelline space of the enucleated and preincubated oocytes. The manipulated oocytes were cultured in M16 medium (Whittingham, 1971) in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37°C. Most oocytes fused with the nuclear karyoplasts within 15 min. At 15–30 min after fusion, the reconstituted eggs were activated with 7% ethanol for 7 min at room temperature (Cuthbertson, 1983).

Preparation of ICM cells. Single cells from the ICM were obtained from blastocysts by immunosurgery. Following removal of the zona pellucida with acid Tyrode solution, the blastocysts were incubated in M16 medium containing a rabbit anti-serum against mouse spleen (1:100 v/v) for 30 min in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37°C, and then treated with guinea-pig complement (1:20 v/v in M2) for 30 min at 37°C. The isolated ICM was incubated in M2 medium free from Ca²⁺ and Mg²⁺ and supplemented with 0.2% EDTA and 0.025% trypsin at 37°C for 15 min and pipetted into single cells.

Culture and transfer. After nuclear transplantation and activation, the reconstituted eggs were cultured in individual drops of M16 medium containing 100 µM EDTA, covered with paraffin oil, in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37°C. Blastocysts developed from reconstituted eggs were transferred to the right uterine horn of Day-3 (2.5 days *post coitum*) pseudopregnant recipients (CD-1 strain). Control blastocysts derived from CD-1 females mated with F₁ males were transferred to the left uterine horn of the same recipients.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Enucleated and nonenucleated oocytes were incubated for 2 h in M16 medium containing 200 µCi [³⁵S]-methionine (1000 Ci/mmol, NEG). Five to 10 oocytes were washed 5 times with protein-free M2 medium and placed in 10 µl double-strength SDS sample buffer (Laemmli, 1970) and stored at –80°C. Proteins were separated on 7.5–15% SDS polyacrylamide gradient gels as described by Flach *et al.* (1982). After electrophoresis, gels were processed as described by Bonner & Laskey (1974) and exposed to Fuji RX X-ray film for fluorography at –80°C.

Results

First meiotic division and removal of the chromosomes

Extrusion of the first polar body was observed in >80% of oocytes collected from ovarian follicles at 10 h after hCG injection and 90% were recognized to be at telophase of the first meiotic division (Table 1). The proportion of oocytes at this stage of the first meiotic division rapidly decreased with time after hCG injection. The majority of oocytes collected from oviducts at 12 h after hCG had progressed to metaphase II and only 10% of the oocytes had their chromosomes enclosed by the region destined to form the first polar body. In contrast, most oocytes collected at 9 h after hCG had not emitted the first polar body.

Table 1. Distribution of chromosome position in mouse oocytes

Time after injection with human chorionic gonadotrophin (h)	No. of oocytes examined	% of oocytes with chromosomes located in position*				
		1	2	3	4	5
9	82	100	0	0	0	0
10	102	90	7	3	0	0
11	101	62	23	9	6	0
12	100	10	41	20	17	12

*See Fig. 2.

These results demonstrated that the oocyte at 10 h after hCG injection was accessible to enucleation. The chromosomes with spindle were completely removed from 95% of oocytes: those in which chromosomes sucked into an enucleation pipette were readily identified using differential interference microscopy without the use of Hoechst staining (Fig. 1).

Fig. 2. Diagram of mouse oocyte showing 5 positions in relation to the first polar body (pb).

Development of the reconstituted oocytes

More than 90% of oocytes fused with a nuclear karyoplast from donor late 2- and 8-cell embryos or with an ICM cell, within 15 min of micromanipulation (Table 2). The proportion of oocytes that activated following exposure to ethanol and exhibited pronuclear-like swelling of the transferred nucleus was 72–90%. In most of the activated oocytes, emission of a polar body that contained chromosomes was observed within 2 h of the activation treatment, whether the nucleus was derived from a late 2-cell embryo, an 8-cell embryo or an ICM cell.

Table 2. Development of enucleated mouse oocytes reconstituted with embryonic nuclei *in vitro*

Nuclei of donor embryo	No. of oocytes fused/no. of oocytes manipulated	No. of eggs activated	No. developed to		
			2-cell	8-cell	Blastocyst
2-cell	102/110 (93)	88 (86)	79 (90)	30 (29)	20 (23)
8-cell	29/32 (91)	26 (90)	15 (58)	2 (8)	1 (4)
Inner cell mass	148/160 (93)	107 (72)	96 (90)	16 (15)	11 (10)

Values in parentheses are percentages.

After transfer to recipient females of 25 blastocysts derived from oocytes reconstituted with a nucleus from late 2-cell embryos, 3 live fetuses (8%) were recovered by Caesarean section on Day 19 of gestation (Table 3). However, development to term was not observed in mice that received blastocysts derived from oocytes reconstituted with 8-cell nuclei or ICM cells.

Table 3. Development of mouse blastocysts derived by transfer of nuclei to enucleated oocytes and after transfer to recipients

Nuclei of donor embryos	No. pregnant /no. of recipients	No. of embryos transferred	No. of live young (%)
2-cell	9/10	20	3 (15)
8-cell	1/1	1	0
Inner cell mass	6/6	11	0

Control blastocysts were transferred to the same recipients to ensure the pregnancy.

Protein profiles

The major proteins synthesized by freshly obtained oocytes and enucleated oocytes showed similar patterns (Fig. 3) and did not change significantly during maturation *in vivo* or *in vitro*,

Fig. 3. One-dimensional sodium dodecyl sulphate, polyacrylamide gel electrophoresis separation of [^{35}S]-methionine polypeptides synthesized during 2 h of labelling in control oocytes and enucleated oocytes. Control oocytes were labelled at 10 (1), 14 (2) or 16 (3) h after human chorionic gonadotrophin (hCG). Oocytes enucleated at 10 h after hCG were labelled after culturing for 0 (4), 2 (5), 4 (6), and 6 h (7). Arrow head indicates position of polypeptide, lower band of M_r 37×10^3 , whose synthesis is detectable in tracks (1), (2), (4) and (5) but not in tracks (3) and (7). The band 35×10^3 polypeptide that is a maternally encoded polypeptide (Howlett & Bolton, 1985) is clear.

through synthesis of the lower band of the 37×10^3 M_r complex (Fig. 3, arrow head) was reduced with time after ovulation (lanes 2, 3) or enucleation (lanes 6, 7).

Discussion

Offspring have been produced from nuclei of 8- to 16-cell sheep embryos fused with enucleated oocytes (Willadsen, 1986) and this method has been repeated in cattle (Prather *et al.*, 1987), rabbits (Stice & Robl, 1988) and sheep (Smith & Wilmut, 1989). In these studies, the enucleation of the oocyte was carried out at metaphase II. This method is not very reliable as it is often difficult, even by differential interference microscopy, to locate the position of the metaphase II chromosomes within the oocyte.

In the present study, to overcome the technical difficulties involved in the enucleation of secondary oocytes, we attempted to remove the chromosomes from oocytes at telophase of the first meiotic division by micromanipulation using differential interference microscopy. At this time, extrusion of the first polar body is not completed and the separated chromosomes at each pole are connected to each other by the meiotic spindle apparatus. The telophase chromosomes, spindle apparatus and extruding region of cytoplasm were, therefore, easily sucked into the enucleation pipette. The advantage of this method is that chromosomes can be reliably and quickly removed, with a small amount of cytoplasm, without any staining.

It has been reported that newly ovulated oocytes are difficult to activate with ethanol treatment, but that the activation rate increases following *in vitro* culture (Webb *et al.*, 1986). Although the oocytes at 10 h after hCG injection are also difficult to activate, most (93%) nonmanipulated oocytes can be activated after culturing for 4–6 h *in vitro* (data not shown). The activation rate of the reconstituted oocytes could also be significantly increased by culture of the enucleated oocytes before nuclear transplantation (data not shown). Thus the ability of the cytoplasm to mature and be activated is independent of the chromosomes, both *in vivo* and *in vitro*. Similar changes of cytoskeletal organization, activation rate and type of parthenogenesis were seen in mouse oocytes matured *in vivo* and *in vitro* (Webb *et al.*, 1986). The polypeptide profile of enucleated oocytes at anaphase of the first meiotic division and matured *in vitro* is not significantly different from that of oocytes matured *in vivo*. It is known that the protein synthesis required for the transition from metaphase I to metaphase II (Hashimoto & Kishimoto, 1988) is programmed by messenger RNAs in the oocyte cytoplasm (Globs & Stein, 1976; Crozet & Szollosi, 1980). Schultz *et al.* (1978) reported that nucleate and anucleate fragments of mouse oocytes produced following treatment of fully grown oocytes with cytochalasin B showed similar profiles of protein synthesis. These studies indicate that the complete transition from telophase of the first meiotic division to metaphase II can be accomplished without the presence of chromosomes.

The rate of development of the reconstituted oocytes *in vitro* and *in vivo* was investigated, to assess the suitability of the cytoplasm as recipient for nuclear transplantation. The oocytes that received a nucleus from a 2-cell embryo or ICM cell underwent ethanol-induced activation at a frequency equal to that observed in other studies in which oocytes were matured *in vivo*. Of the oocytes which received an ICM cell, 10% developed to blastocysts after culture for 4 days. Moreover, the oocytes that received a nucleus from late 2-cell embryos resulted in full-term development after transfer to recipients. This result clearly shows that the enucleated cytoplasm can be used as a recipient for nuclear transplantation.

In this study, most of the reconstituted oocytes emitted a polar body after activation treatment, in which the polar body certainly contained chromosomes derived from the donor nucleus. The polar body may be emitted as a result of mitosis of the donor nucleus. Czolowska *et al.* (1984) reported that interphase nuclei, in one case, fused with mouse oocytes underwent mitosis arranged on an extra spindle and emitted a polar body. Whether the reconstituted embryos with one polar body and pronucleus had a diploid set of chromosomes is unclear. However, at least some of them would have a normal diploid set of chromosomes after emission of the polar body, since live young have been produced from such embryos in the present study.

Enucleated mouse zygotes that reconstituted with a nucleus from 4- or 8-cell embryos or ICM cells were unable to develop *in vitro* (McGrath & Solter, 1984; Howlett *et al.*, 1987; Tsunoda *et al.*, 1987). Robl *et al.* (1986) reported that enucleated 2-cell embryos which received a nucleus from 4- and 8-cell embryos developed to blastocysts. This was confirmed by Tsunoda *et al.* (1987) and live young were produced from such reconstituted 2-cell embryos. Kono *et al.* (1991) reported that transplantation of a single nucleus from 2- or 4-cell embryos into one of the enucleated blastomeres of 2-cell embryo resulted in successful production of identical twin and triplet mice. However, the nuclei transplanted into enucleated 2-cell embryos were not fully transformed to normal 2-cell nuclei. In such embryos, therefore, the compaction occurred at the 4-cell stage (Tsunoda *et al.*, 1987) and the resulting blastocysts had significantly fewer cells (Robl *et al.*, 1986). Kono &

Tsunoda (1989) also reported that hatched blastocysts that were derived from single blastomeres from 8-cell mouse embryos fused into enucleated halves of 2-cell embryos lacked ICM completely. When ICM cells were transferred, the reconstituted 2-cell embryos did not develop at all (Tsunoda *et al.*, 1987).

The capacity for nuclear development after transfer to an enucleated oocyte differs among species. Transplantation of a nucleus from an 8–16-cell embryo to an enucleated oocyte resulted in production of offspring after transfer to a recipient in cattle (Prather *et al.*, 1987), in sheep (Willadsen, 1986; Smith & Wilmut, 1989) and in rabbits (Stice & Robl, 1988), but not in mice. In the present study, we succeeded in producing live young from enucleated oocytes that received nuclei from late 2-cell embryos. The stage of the donor embryo that is consistent with development of the reconstituted oocytes to term may coincide with the stage at which transcription of the embryonic genome begins; i.e. the middle of the 2-cell stage in mice (Johnson, 1981; Flach *et al.*, 1982) and the 8–16-cell stage in sheep, cattle and rabbits (Cotton *et al.*, 1980; Camous *et al.*, 1986; Crosby, 1988). Recently, however, Smith & Wilmut (1989) reported that ICM cells transferred to enucleated oocytes in sheep allowed development to term. In mice, it has been shown that nuclei from ICM (Tsunoda *et al.*, 1988; and the present results), primordial germ cells (Tsunoda *et al.*, 1989), and teratocarcinoma cells (Modlinski *et al.*, 1990) can undergo limited reprogramming after transplantation into enucleated oocytes.

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