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Reconstruction of mouse oocytes by germinal vesicle transfer: maturity of host oocyte cytoplasm determines meiosis

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We evaluated the maturational competence of mouse oocytes reconstructed by the transfer and electrofusion of germinal vesicles (GV) into anuclear cytoplasts of GV stage oocytes (both auto- and hetero-transfers), metaphase II stage oocytes or zygotes. Following in-vitro culture, the maturation rates of the reconstructed oocytes to metaphase II did not significantly differ between auto- and hetero-transfers (40/70 versus 95/144 respectively); these rates also did not differ from those of control oocytes (57/97) which were matured *in vitro* without micromanipulation and electrofusion. In contrast, when a GV was transferred into an enucleated metaphase II oocyte or a zygote, only a few reconstructed oocytes underwent germinal vesicle breakdown (5/30 and 2/21 respectively); moreover, none reached metaphase II stage. Cytogenetic and immunofluorescence analyses were conducted on hetero-GV oocytes that extruded a first polar body. Each oocyte showed two groups of chromosomes, one in the cytoplast and one in the polar body, as well as a bipolar spindle with twenty univalent chromosomes. Our findings suggest that oocytes reconstructed by GV transfer into a cytoplast of the same developmental stage mature normally *in vitro* through metaphase II. Such oocytes may be a useful research model to elucidate the cytoplasmic and nuclear mechanisms regulating meiosis and the relationships between meiotic errors and age-related changes in the oocyte.

Key words: cytogenetic analysis/GV transfer/oocyte maturation

Introduction

In mammals, the final stages of oocyte maturation are characterized by the first and second meiotic chromosomal divisions. The first meiotic division involves the breakdown of the nuclear envelope surrounding the germinal vesicle (GV) and the formation of a meiotic spindle that allows the even division of genetic material between the oocyte and its first polar body. Recent studies have shown that mouse and human oocytes acquire meiotic competence, i.e. the ability for GV breakdown (GVBD) and meiotic division, during pre-antral and antral follicle growth phases (Eppig and Schroeder, 1989; Cortvrindt *et al.*, 1996; Trounson *et al.*, 1998; Wu *et al.*, 1998; Zuccotti *et al.*, 1998).

In a preliminary study with human oocytes, we demonstrated that a GV can undergo GVBD and a normal first meiotic division even when removed and transferred by electrofusion into the enucleated cytoplast of another GV-stage oocyte (Zhang *et al.*, 1999). However, only small numbers of oocytes were available for this work and low success rates were associated with the GV micromanipulation and electrofusion steps. As a result, study of chromosome number and microtubule configuration in the reconstructed oocytes was very limited. A readily available animal model would help to improve our micromanipulation techniques and to evaluate GV maturation in different cytoplasmic environments. A mouse model has been described by others (Kono *et al.*, 1996; Takeuchi *et al.*, 1999).

The aims of this study are to establish a procedure for GV removal and transfer in mouse oocytes, to evaluate the meiotic behaviour of the GV following transfer and to assess chromosome number and meiotic spindle configuration during and following these final stages of nuclear maturation.

Materials and methods

Recovery of oocytes and zygotes

Female CB6F1 mice (6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) were injected i.p. with 5 IU pregnant mare serum gonadotrophin (PMSG; Sigma, St Louis, MO, USA). Immature, germinal vesicle stage oocytes (GV oocytes) were collected by puncturing the ovarian follicles at 48 h post-injection. A second group was injected i.p. with 5 IU human chorionic gonadotrophin (HCG; Sigma) at 48 h following PMSG and were killed 16–18 h later to collect mature oocytes (metaphase II stage). A final group of mice were mated immediately after HCG injection and then killed 20–22 h later to collect zygotes. Metaphase II oocytes and zygotes were harvested by opening the ampullae of excised Fallopian tubes. Cumulus cells were removed from all oocytes and zygotes by brief exposure to serum-free modified human tubal fluid (HTF) medium (Irvine Scientific, Irvine, CA, USA) containing 300 IU/ml hyaluronidase (Sigma).

Before and during GV transfer all the immature oocytes were incubated in modified HTF medium supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT, USA) and 50 μg/ml 3-isobutyl-1-methylxanthine (IBMX, Sigma). This inhibitor of cyclic nucleotide phosphodiesterase prevents GVBD. Control GV oocytes were incubated with IBMX but not were micromanipulated prior to in-vitro maturation.

Cytoplast preparation from enucleated GV oocytes, MII oocytes and zygotes

Oocytes and zygotes were exposed to modified HTF medium supplemented with 10% FCS and 7.5 μg/ml cytochalasin B (Sigma) for

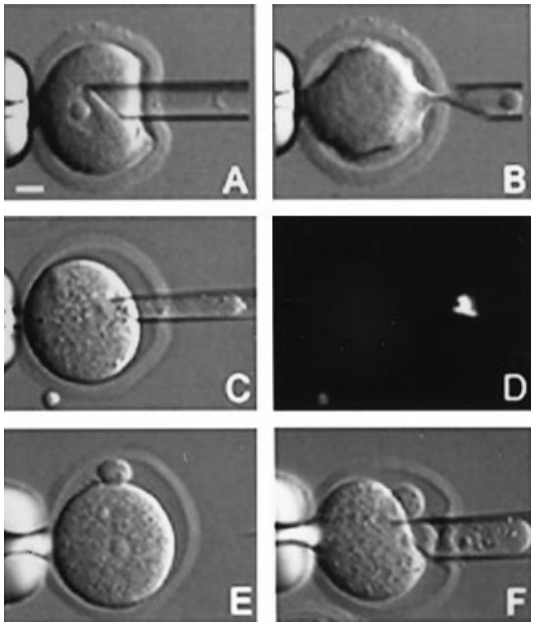

Figure 1. Preparation of enucleated cytoplasts from germinal vesicle (GV) stage, MII stage and zygotes: removal of GV from GV stage oocytes (**A** and **B**); removal of the meiotic apparatus from MII oocytes as illustrated by Hoechst 33342 staining (**C** and **D**); and removal of a pronucleus from a zygote (**E** and **F**). Bar = 20 μm.

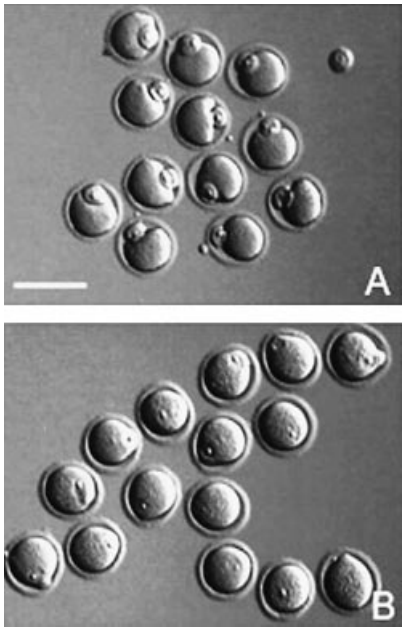

Figure 2. Reconstruction of oocytes by GV transfer: GV deposited into the subzonal space before electrofusion (**A**) and electrofusion of GV into an enucleated cytoplast (**B**). Bar = 100 μm.

30 min at room temperature prior to enucleation. Following lancing of the zona pellucida with a sharp-tipped pipette, GV and pronuclei were removed from immature oocytes and zygotes respectively, using a bevelled glass pipette (ID: 20–25 μm; Figure 1A, B, E and F). Removal of oocyte nuclei at metaphase II was achieved under UV light following staining with Hoechst 33342 (Tsunoda *et al.*, 1988; Figure 1C, D). These procedures were performed using micromanipulators attached to an inverted microscope equipped with a UV light source.

Preparation of karyoplasts (GV removal for transfer)

GV oocytes were incubated in microdrops of modified HTF with 10% FCS and 7.5 μg/ml cytochalasin B for 30 min at room temperature before GV aspiration as described above. Each GV was removed with a small, but consistent, amount of cytoplasm and appeared to be encapsulated by a membrane, then transferred individually into the subzonal space of an oocyte or zygote cytoplast (Figure 2A).

Germinal vesicle transfer and electrofusion

The following GV–cytoplast complexes were generated: GV–GV (both auto- and hetero-GV–GV in which the GV was removed and transferred back to the same or a different immature oocyte, respectively); GV–MII (GV transferred into an enucleated MII oocyte) and GV–PN (GV transferred into an enucleated zygote). These complexes were transferred into a drop of fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$ and 0.05 mM $MgSO_4$ in HTF) between the platinum electrodes of a fusion chamber. After alignment with an AC pulse of 6–8 V for 5–10 s, fusion of GV–cytoplast complexes was achieved with electrical pulses (1.8–2.5 kV/cm DC for 50 μs) delivered by a Model 2001 Electro Cell Manipulator (BTX, Inc., San Diego, CA, USA). A Model 500 Graphic Pulse Analyser (BTX) was used to monitor the amplitude of each pulse delivered to the chamber. Preliminary studies established that these are the minimal pulse parameters sufficient for GV transfer (Figure 2B).

After electrofusion the complexes were washed three times in modified HTF medium and then placed in HTF medium supplemented with 10% FCS for in-vitro maturation. The GV karyoplast was routinely incorporated into the cytoplast within 40 min. The oocytes were repeatedly monitored during a 24 h incubation for evidence of maturational changes.

Cytogenetic analysis

Oocytes extruding a first polar body within the 24 h incubation period were considered to be mature and were fixed for cytogenetic analysis as described (Tarkowski, 1966). Briefly, each oocyte was transferred into a 1% hypotonic trisodium citrate solution for 15 min before fixation with methanol:acetic acid (3:1) on a clean glass slide. The chromosome spreads were then air-dried and stained with 5 μg/ml Hoechst 33342 in phosphate buffer solution. The number and the structure of the chromosomes were determined under fluorescence microscopy.

Immunocytochemistry

Additional reconstructed oocytes with and without extruded *first* polar bodies were selected for immunocytochemistry of the meiotic spindle, which was performed using prior published methodology (Liu and Chen, 1994). After removing the zona pellucida by a brief treatment with 1% pronase (Sigma) in modified HTF, each oocyte was fixed in 3.7% formaldehyde (Sigma) in 0.1 mol/l PBS (pH 7.4) for 40 min, and then permeabilized in PBS buffer containing 0.25% Triton X-100 (Sigma) for 25 min at room temperature. Free aldehydes were reduced by a 20 min rinse in 0.1 mol/l PBS containing 150 mmol/l glycine (Sigma). Microtubules were localized with a mouse monoclonal antibody to β-tubulin (Sigma). The primary antibody was detected using a fluorescence labelled goat anti-mouse IgG (Sigma). Each antibody was applied overnight at 4°C; oocytes were rinsed with PBS containing 0.25% bovine serum albumin (BSA, Fraction V; Sigma) between antibody applications. Chromosomes were simultaneously detected with Hoechst 33342 (5 μg/ml in 0.1 mol/l PBS).

Table I. Survival and fusion following micromanipulation of oocytes and zygotes

Type of reconstructed oocyte/zygote	No. manipulated	Oocyte survival after nuclear removal *n* (%)	GV–recipient complexes fused *n* (%)
Auto–GV	88	81 (92)	70 (86)
Hetero–GV	196	185 (94)	144 (78)
GV–MII	45	43 (96)	30 (70)
GV–PN	30	27 (90)	21 (78)

GV = germinal vesicle; PN = pronuclei; MII = meiosis II.

Table II. Meiotic division of reconstructed oocytes/zygotes observed during incubation

Type of reconstructed oocyte/zygote	No. reconstructed oocyte/zygote	No. undergoing GVBD (%)	No. reaching MII (%)
Auto–GV	70	70 (100)[a]	32 (46)[a]
Hetero–GV	144	144 (100)[a]	95 (66)[a]
GV–MII	30	5 (17)[b]	0
GV–PN	21	2 (10)[b]	0
Control	97	95 (98)[a]	57 (59)[a]

GV = germinal vesicle; MII = metaphase II; PN = pronuclei; GVBD = germinal vesicle breakdown.
GV oocytes were pre-incubated in IMBX for 5 h before incubation for final maturation.
[a]Values are not significantly different from each other (χ^2 test).
[b]Values were significantly different from control ($P < 0.0001$; χ^2 test).

The stained oocytes were mounted whole on glass slides and examined using light phase and fluorescence microscopy.

Data analysis

Data were analysed using the χ^2 test with significance determined at $P < 0.05$.

Results

High survival rates were observed among the four groups of reconstituted GV oocytes/zygotes (Table I). Fusion was observed in >78% of the GV–oocyte and GV–zygote complexes. There were no significant differences between oocyte manipulation rates or fusion rates associated with the various reconstructions.

In the control oocytes GVBD routinely took place 2–3 h after their removal from IBMX-containing media, whereas GVBD occurred within 1–2 h in all the reconstructed auto-GV and hetero-GV oocytes (Table II). Following further incubation for 10–12 h, extrusion of the first polar body was noted in 46% of the auto-GV oocytes and 66% of the hetero-GV oocytes; these results did not differ significantly from those of the controls (59%; Table II). On the other hand, only 17% of the reconstituted GV-MII oocytes and 10% of the GV–PN zygotes underwent GVBD after 18–24 h incubation and none of these oocytes extruded a polar body (Table II).

To determine whether meiosis progressed normally following GV transfer into hetero-GV oocytes, 20 mature hetero-GV oocytes (with extrusion of the first polar body) were analysed following vital staining with Hoechst 33342. Two areas of fluorescence, one in the polar body and another in the cytoplast, were noted in each oocyte (Figure 3A). Fourteen oocytes were also stained with anti-β tubulin antibody; each showed a bipolar spindle with a metaphase chromosome alignment on the spindle equator (Figure 3).

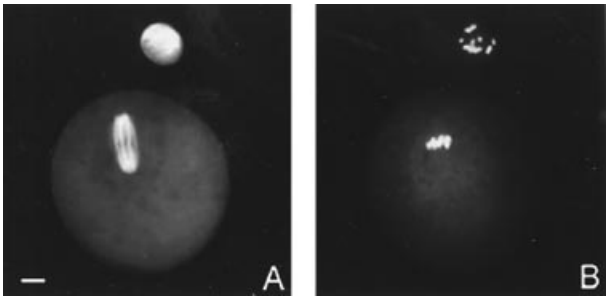

Figure 3. Matured reconstructed oocyte with polar body double stained with anti-B tubulin antibody and Hoechst 33342 to illustrate the bipolar spindle in the cytoplast (A) and groups of chromosomes aligned on the equatorial plate of the spindle and in the polar body (B). Bar = 10 μm.

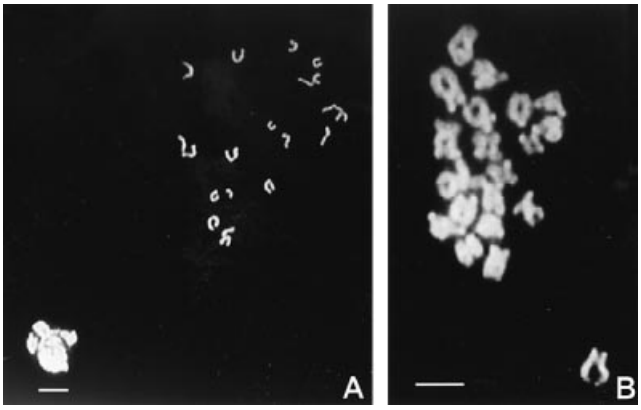

Figure 4. Chromosome spreads of mature and maturing reconstructed oocytes stained with Hoechst 33342: mature hetero-GV oocyte has 20 univalent chromosomes in upper right quadrant as well as dispersed polar body chromosomes in bottom left quadrant (A) and maturing hetero-GV oocyte without polar body with 20 bivalent chromosomes (B). Bar = 20 μm.

In another study, 20 of 34 mature hetero-GV oocytes were successfully fixed for cytogenetic study; all displayed univalent chromosomes (Figure 4A). Some oocytes which failed to extrude a polar body by 18 h following reconstruction were also analysed and displayed bivalent chromosomes (Figure 4B).

Discussion

The present study demonstrates that the GV of a mouse oocyte can undergo GVBD and form a structurally normal first meiotic metaphase following its removal and electrofusion into an enucleated cytoplast at the same developmental stage. This result confirms our previous finding with human pre-ovulatory oocytes (Zhang *et al.*, 1999). Moreover, our present results also suggest that the structural and functional integrity of the GVs and the recipient cytoplasts (enucleated GV oocytes) are

not compromised by the micromanipulation and electrofusion procedures. For example, the maturation rates of reconstructed auto-GV or hetero-GV oocytes do not differ from that of non-manipulated controls. In addition, the metaphase components of the matured hetero-GV oocytes show normal chromosomal structures and meiotic spindles.

We also tested if a GV can resume meiosis and mature when transferred into the cytoplasm of an anuclear zygote. It has been suggested that zygote cytoplast status may be similar to that of the GV oocyte (Campbell *et al.*, 1996). Zygotes have exited from metaphase arrest after fertilization and display a low level of maturation, or meiosis, promoting factor (MPF) activity. MPF is an important factor for meiosis resumption, including the induction of GVBD, chromosome condensation and final nuclear maturation (meiotic divisions). MPF is composed of p^{34cdc2} and cyclin B and the phosphorylation state of p^{34cdc2} in zygotes is similar to that reported in the ooplasm during the GV stage (Choi *et al.*, 1991). Moreover, cytoplasmic factor(s) in the zygote can decondense the MII oocyte chromosome and enclose it in a nuclear envelope, thereby enabling the cell to enter into a state of interphase and began new cell cycle (Balakier and Masui, 1986). Nonetheless, our results indicate that a GV transferred into an enucleated zygote cytoplast rarely undergoes GVBD and cannot extrude a polar body.

Finally, we tested if a GV resumes meiosis when transferred into the cytoplasm of an enucleated MII stage oocyte. Our results indicate that ~20% of such reconstructed oocytes undergo GVBD but none extrudes a first polar body. This result, also observed by others (Takeuchi *et al.*, 1999), is surprising considering that MPF activity is relatively high in oocytes at metaphase II (Hashimoto and Kishimoto, 1988). Moreover, during cloning studies (Wakayama *et al.*, 1998), it was noted that when a somatic cell nucleus (G0 or G1 cell cycle stage) is transferred into an enucleated MII oocyte, polar body extrusion normally occurs following activation. Mitosis and cell division only ensued when the reconstructed clone was suppressed with cytochalasin B treatment. There are two potential explanations for the different outcomes observed in this study and in that of Wakayama. The first is that chromosome number is an important determinant for the first stage of meiosis to progress. The oocyte GV (G2 stage) contains 4n chromosomes, whereas only 2n chromosome are present in somatic cells, especially those in cell cycle stage G0. The more likely explanation, however, involves the different procedures used to insert a cell nucleus into enucleated metaphase II eggs. Wakayama and his co-workers injected somatic cell nuclei directly, whereas we had to employ electrofusion to transfer a GV into an oocyte or zygote cytoplast due to the large size of the GV. Significantly, the minimal parameters for this electrofusion procedure are nonetheless sufficient to activate human oocytes (Zhang *et al.*, 1999). Thus, it is likely that the metaphase II cytoplast was activated during the transfer resulting in inappropriate cytoplasmic conditions for the progression of meiosis. Analysis of the effects of electrofusion on ooplasmic levels of MPF and other factors will be the topic of a future study.

In conjunction with in-vitro maturation, several advantages can be gained from the development of oocytes reconstructed by GV nucleus transfer. First, these reconstructed oocytes may be useful models to elucidate the cytoplasmic and nuclear interrelationships underlying the onset and progression of meiosis. Secondly, because we can exchange GVs between oocytes of different quality or age, GV transfer may be used to determine the role of the ooplasm in chromosome disjunction and whether its actions become compromised in oocytes which show genetic or developmental anomalies in maturation, e.g. the increased incidence of aneuploidy in oocytes of women of advanced maternal age (Eichenlaub-Ritter, 1996; Angell, 1997).

Further studies are necessary to determine whether normal offspring are produced using reconstructed oocytes. Even though reconstructed oocytes appear morphologically normal, their ability to fertilize and develop normally through early embryonic life must be established. Studies in mice have reported the birth of live offspring following the multiple nuclear transfers at the GV and MII stages (Kono *et al.*, 1996).

As we have stated previously (Zhang *et al.*, 1999), GV transfer could potentially become a new approach in treatment of human infertility due to advanced maternal age. Despite the fact that diagnostic, clinical and laboratory techniques have improved significantly since the introduction of IVF, donated oocytes remain the only solution for achieving pregnancy in women who are unable to produce normal quality oocytes. With the nuclear transfer technique it may be possible to construct normal quality oocytes for these women by transferring the GVs from their oocytes into cytoplasts generated from oocytes donated by young healthy women.

References

Angell, R. (1997) First-meiotic-division nondisjunction in human oocytes. *Am. J. Hum. Genet.*, **61**, 23–32.

Balakier, H. and Masui, Y. (1986) Interactions between metaphase and interphase factors in heterokaryons produced by fusion of mouse oocytes and zygotes. *Dev. Biol.*, **117**, 102–108.

Campbell, K.H.S., Loi, P., Otaegui, P. and Wilmut, I. (1996) Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.*, **1**, 40–46.

Choi, T., Aoki, F., Mori, M. *et al.* (1991) Activation of p34cdc2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development*, **113**, 789–795.

Cortvrindt, R., Smitz, J. and Van Steirteghem, A.C. (1996) In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepubertal mice in a simplified culture system. *Hum. Reprod.*, **11**, 2656–2666.

Eichenlaub-Ritter, U. (1996) Parental age-related aneuploidy in human germ cells and offspring: a story of past and present. *Environ. Mol. Mutagen.*, **28**, 211–236.

Eppig, J.J. and Schroeder, A.C. (1989) Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. *Biol. Reprod.*, **41**, 268–276.

Hashimoto, N. and Kishimoto, T. (1988) Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Dev. Biol.*, **126**, 242–252.

Kono, T., Obata, Y., Yoshizuma, T. *et al.* (1996) Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nature Genet.*, **13**, 91–94.

Liu, H. and Chen, D.Y. (1994) Dynamic changes of β-tubulin during the resumption of meiosis of mouse oocyte. *Dev. Reprod. Biol.* **3**, 17–22.

Takeuchi, T., Ergun, B., Huang, T.H. *et al.* (1999) A reliable technique of nuclear transplantation for immature mammalian oocytes. *Hum. Reprod.*, **14**, 1312–1317.

Tarkowski, A.K. (1966) An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics*, **5**, 394–400.

Trounson, A., Anderiesz, C., Jones, G.M. *et al.* (1998) Oocyte maturation. *Hum. Reprod.*, **13**, 52–62.

Tsunoda, Y., Shioda, Y., Onodera, M. *et al.* (1988) Differential sensitivity of mouse pronuclei and zygote cytoplasm to Hoechst staining and ultraviolet irradiation. *J. Reprod. Fertil.*, **82**, 173–178.

Wu, J., Zhang, L.Z. and Liu, P. (1998) A new source of human oocytes: preliminary report on the identification and maturation of human preantral follicles from follicular aspirates. *Hum. Reprod.*, **13**, 2561–2563.

Wakayama, T., Perry, A.C., Zuccotti, M. *et al.* (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, **394**, 369–374.

Zhang, J., Grifo, J., Blaszcyzk, A. *et al.* (1999) *In vitro* maturation of human preovulatory oocytes reconstructed by germinal vesicle transfer. *Fertil. Steril.*, **71**, 726–731.

Zuccotti, M., Rossi, P.G., Martinez, A. *et al.* (1998) Meiotic and developmental competence of mouse antral oocytes. *Biol. Reprod.*, **58**, 700–704.

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