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Asynchronous Cytoplast and Karyoplast Transplantation Reveals That the Cytoplasm Determines the Developmental Fate of the Nucleus in Mouse Oocytes

YONG CHENG, HENG-YU FAN, DUAN-CHENG WEN, CHAO TONG, ZI-YU ZHU, LEI LEI, QING-YUAN SUN, AND DA-YUAN CHEN*

State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

ABSTRACT The relationship between nucleus and cytoplasm can be well revealed by nuclear transplantation. Here, we have investigated the behavior changes of the reconstructed oocytes after transferring the karyoplasts from mouse GV, MI, and MII oocytes into the cytoplasts at the different developmental stages. When the GV cytoplast was used as recipient and MI or MII karyoplast was used as donor (MI-GV pair and MII-GV pair), the reconstructed pairs extruded a polar body after electrofusion and culture. Both the cytoplasm and the polar body had a metaphase spindle in the MI-GV pair, while only a clutch of condensed chromatin was observed in the cytoplasm and polar body of the MII-GV pair. When the MI cytoplast was used as recipient and GV or MII karyoplast was used as donor (GV-MI pair and MII-MI pair), the reconstructed pairs also extruded a polar body. Each had one spindle and a group of metaphase chromosomes in the cytoplasm and polar body, respectively. When the MII cytoplast was used as recipient and GV or MI karyoplast was used as donor (GV-MII pair and MI-MII pair), the reconstructed pairs were activated, became parthenogenetic embryos and even developed to hatching blastocysts after electrofusion. The result from immunoblotting showed that MAP kinase activity was high in the MI and MII cytoplasts, while not detected in GV cytoplast. The results demonstrate that the cytoplasmic environment determines the behavior of asynchronous donors. *Mol. Reprod. Dev. 65: 278–282, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: nuclear–cytoplasmic interaction; nuclear transplantation; oocyte; parthenogenesis; MAP kinase

INTRODUCTION

Oocyte maturation includes the procedure of nuclear maturation and cytoplasmic maturation (Eppig et al., 1994). The former means germinal vesicle breakdown (GVBD), chromatin condensation and the first polar body emission (Albertini, 1992). The latter refers to the expression and organization of cytoplasmic factors required for fertilization (Eppig et al., 1994; Antczak and Van Blerkom, 1997; Cheung et al., 2000). The interactions between nucleus and cytoplasm in oocytes have become one of the fundamental questions in the field of reproductive biology. Recent achievements in the nuclear–cytoplasmic relationship have been practically applied in the production of cloned animals and the treatment of infertility by assisted reproductive technologies.

The development of nucleus is influenced by the surrounding cytoplasmic environment, which has been identified in the nuclear transplantation experiments. It has been shown that GV karyoplasts with a very thin rim of cytoplasm seldom underwent GVBD (Takeuchi et al., 1999). The amount of perinuclear cytoplasm present in an isolated karyoplast has a positive effect on nuclear maturation (Fulka et al., 1998). The cytoplasm transfer will be helpful to improve the developmental capacity of incompetent oocytes. In amphibian and monkeys, immature oocytes resume meiosis after injected into a small amount of cytoplasm from maturing oocytes (Wasserman and Masui, 1975; Flood et al., 1990). In order to overcome the ooplasmic deficiency, Cohen et al. (1997, 1998) transferred ooplasm from healthy eggs at MII stage into a synchronous patient egg, normal fertilization was achieved and birth of one baby was resulted. Recently, a model of GV transfer between synchronous immature oocytes has been established in mouse (Takeuchi et al., 1999; Liu et al., 1999), human (Zhang et al., 1999), and rabbit (Li et al., 2001a). This method enables us to transfer nearly complete cytoplasm at the early stage, placing the donors into a better host cytoplasmic environment.

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*Correspondence to: Da-Yuan Chen, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 19 Zhongguancun Rd, Haidian, Beijing 100080, China.
E-mail: chendy@panda.ioz.ac.cn

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In the present experiment, we have designed a series of experiments of nuclear transplantation between asynchronous cytoplasts and karyoplasts to prove a hypothesis that cytoplasm plays a critical role in determining nucleus behavior. The behavior changes of the nucleus from mouse GV, MI, and MII oocytes in the asynchronous cytoplasm are investigated, and the possible effect of cytoplasmic factors on nuclear behavior is discussed.

MATERIALS AND METHODS

Preparation of Mouse GV, MI, and MII Stage Oocytes

Female Kunming mice (3–4 weeks old) were injected, i.p., with 10 IU pregnant mare's serum gonadotropin (PMSG). Immature GV stage oocytes were collected by puncturing the ovarian follicles at 48 hr post-injection. In another group, Kunming mice (6–8 weeks old) were injected, i.p., with 10 IU human chorionic gonadotropin (hCG) at 48 hr following PMSG administration and killed 16–18 hr after hCG injection to collect mature MII stage oocytes from the ampullae of Fallopian tubes. Cumulus cells were removed with 300 IU/ml hyaluronidase (Sigma).

The oocytes at the metaphase of the first meiosis (MI stage) were obtained by culturing GV stage oocytes for 5 hr in M199 medium (Sigma) containing 10% fetal bovine serum (FBS, Sigma).

Preparation of Karyoplasts and Cytoplasts From GV, MI, and MII Stage Oocytes

The enucleation of GV stage oocytes was performed according to the method as described by Takeuchi et al. (1999). Briefly, GV stage oocytes were cultured in M199 supplemented with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 2 hr to develop a perivitelline space. Then, the zona pellucida was penetrated by pressing a glass microneedle tangentially into the perivitelline space against the holding pipette. The enucleation was performed after the oocytes were exposed to M_2 medium containing 20 μg/ml cytochalasin B (CB; Sigma) for 15 min. The GV karyoplast was removed by a micropipette with 20 μm inner diameter (Fig. 1A,B).

The enucleation of MI or MII stage oocytes was carried out by a method similar with that of MII stage oocyte enucleation (Wang et al., 2001). The MI or MII spindle appeared transparent in the ooplasm in the M_2 medium (Sigma) containing 2% sucrose and 7.5 μg/ml CB. Following lancing of the zona pellucida with a microneedle, MI or MII karyoplast was removed with an enucleation pipette (Fig. 1C,D,E, and F).

Transfer and Electrofusion of Karyoplast of GV, MI, or MII Oocyte Into the Asynchronous Cytoplast

Six groups of karyoplast–cytoplast complexes were generated:

A. MI-GV (the karyoplast containing MI spindle was transferred into an enucleated GV oocyte);

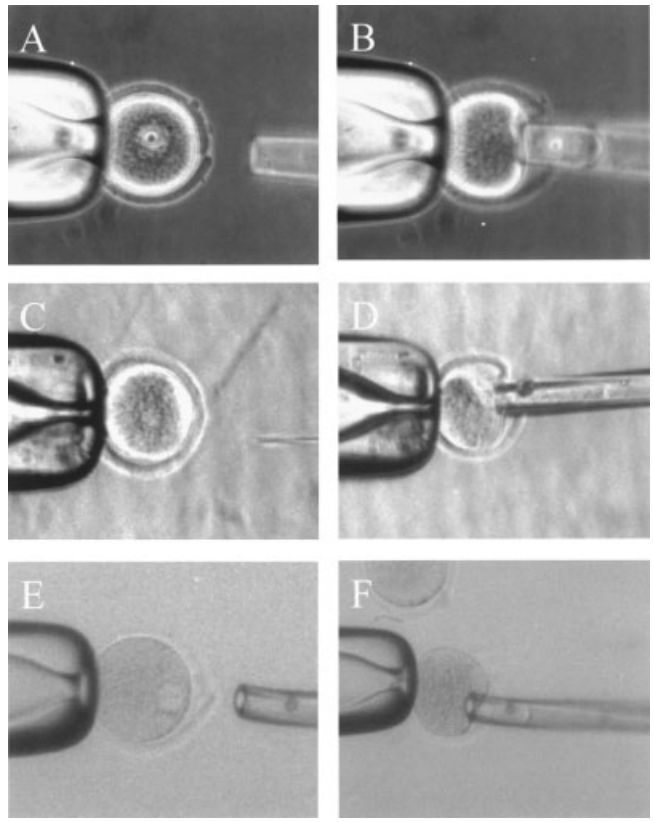

Fig. 1. Preparation of karyoplasts and cytoplasts from GV, MI, and MII stage oocytes. **A**, **B**: Removal of GV karyoplast from a GV stage oocyte; (**C**, **D**) Removal of MI meiotic apparatus from a MI stage oocytes; (**E**, **F**) Removal of MII meiotic apparatus from a MII stage oocyte.

B. MII-GV (the karyoplast containing MII spindle was transferred into an enucleated GV oocyte);
C. GV-MI (the karyoplast containing GV was transferred into an enucleated MI oocyte);
D. MII-MI (the karyoplast containing MII spindle was transferred into an enucleated MI oocyte);
E. GV-MII (the karyoplast containing GV was transferred into an enucleated MII oocyte);
F. MI-MII (the karyoplast containing MI spindle was transferred into an enucleated MII oocyte).

These complexes were transferred into a drop of M_2 fusion medium between 1 mm apart electrodes of a fusion chamber. Fusion of karyoplast–cytoplast complexes was achieved with two electrical pulses (1–1.4 kV/cm DC for 50 μsec) delivered by a Model 2001 Electro Cell Manipulator (BTX, Inc., San Diego, CA).

After electrofusion, the complexes of A, B, C, and D groups were washed three times in M_2 medium and then placed in M199 with 10% FCS for maturation culture for 24 hr, and finally observed with an inverted phase-contrast microscope. The fused complexes of E and F groups were firstly placed in M199 with 10% FCS and 5 μg/ml CB for 4 hr to inhibit the PB emitting, then cultured in CZB medium to observe the following development.

Immunofluorescence

Reconstructed oocytes (A, B, C, and D) with extruded polar body (PB) were selected for immunofluorescent detection of microtubules by confocal laser microscopy. Each oocyte was fixed in 3.7% paraformaldehyde (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 40 min at room temperature. After rinsed in PBS buffer for three times, the oocytes were incubated with 1:50 FITC-labbled anti-α tubulin (Sigma) for 1 hr. After being washed three times in PBS buffer, DNA was stained by exposure the oocytes to 10 μg/ml propidium iodide (Sigma). Samples were observed with Leica TCS-4D confocal laser microscopy (Leica, Heidelberg, Germany).

Electrophoresis and Immunoblotting

The enucleated cytoplasts from GV, MI, and MII oocytes were collected in the SDS sample buffer and heated to 100°C for 4 min, and frozen at −20°C until use. The proteins were separated by SDS–PAGE with a 4% stacking gel and a 10% separating gel at 90 V, 0.5 hr and 120 V, 2 hr, respectively and transferred onto nitrocellulose membrane for 2 hr, at 200 mA and 4°C. After washing in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) twice and blocking in 5% skimmed milk in TBST (TBS containing 0.1% Tween-20) overnight at 4°C, the membrane was incubated in TBST containing 1:1000 polyclonal anti-active MAP kinase antibody (Santa Cruze Biotechnology, Santa Cruz, CA) at 37°C for 2 hr. After three washes of 15 min each in TBST, the membrane was incubated for 1 hr at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig G (Jackson Immunoresearch Laboratories, Inc., PA) diluted 1:1000 in TBST. The membrane was washed twice in TBST and then processed using the ECL detection system (Amersham). For all of the treatments, 40 eggs were loaded into each lane.

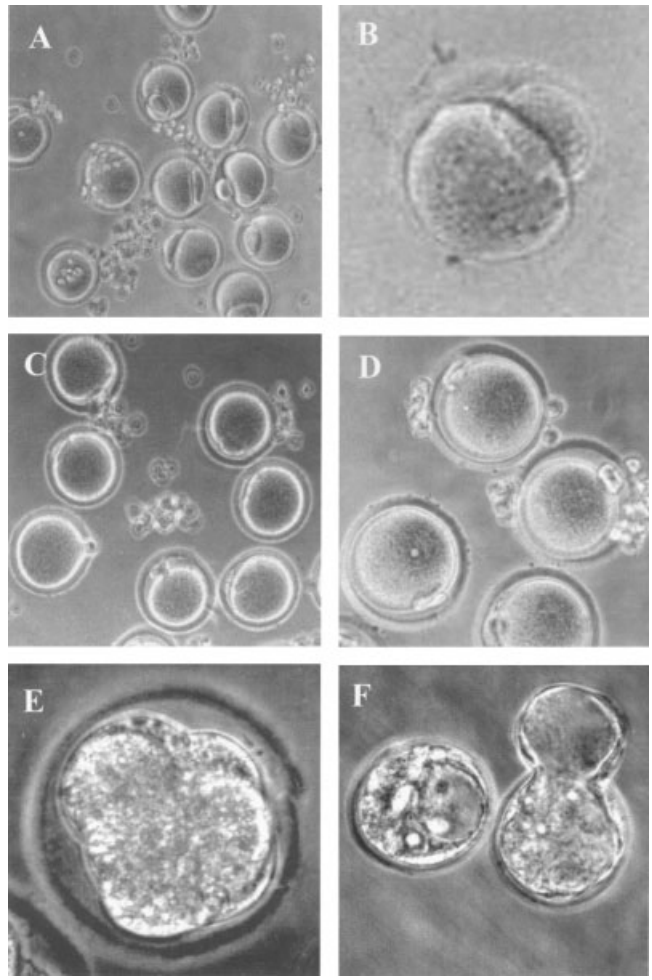

Fig. 2. In vitro development of oocytes reconstructed by transferring the karyoplast of GV, MI, or MII oocyte into asynchronous cytoplast. **A**: A big polar body extruded from an MI-GV pair; (**B**) A polar body extruded from an MII-GV pair; (**C**) A small polar body extruded from a GV-MI pair; (**D**) A polar body extruded from an MII-MI pair; (**E**) A morula derived from a GV-MII pair; (**F**) A hatching blastocyst developed from an MI-MII pair.

RESULTS

In Vitro Maturation of Oocytes Reconstructed by Using GV Cytoplast as Recipient and MI or MII Spindle as Donor

About 2/3 of the MI-GV reconstructed oocytes and 1/3 of the MII-GV reconstructed oocytes extruded a PB after culture for 24 hr (Table 1; Fig. 2A,B). The size of PB in MI-GV oocyte was bigger. Immunofluorescent staining revealed that both the cytoplasm and the PB had one spindle in MI-GV pair. The chromosomes were clearly aligned in the center plate of spindle of the second meiotic division (Fig. 3A). MII-GV pair showed condensed chromosomes in the cytoplasm and PB without tubulin staining (Fig. 3B).

TABLE 1. In Vitro Development of Mouse Oocytes Reconstructed by Transferring the Karyoplasts of GV, MI, and MII Oocytes Into Asynchronous Cytoplasts

No. of karyoplast-cytoplast	Replicates	No. (%) fused	No. (%) emitted PB1	No. (%) embryo			
				Pronuclei	2-Cell	Morula	Blastocyst
A: MI-GV 63	3	38 (60.31)	26 (68.42)				
B: MII-GV 60	3	28 (46.66)	10 (35.71)				
C: GV-MI 64	3	49 (76.56)	24 (48.97)				
D: MII-MI 102	5	39 (38.23)	24 (61.53)				
E: GV-MII 68	3	51 (75)		25 (49.01)	21 (41.17)	5 (9.8)	—
F: MI-MII 91	5	63 (69.23)		39 (61.90)	29 (46.03)	21 (33.33)	19 (30.15)

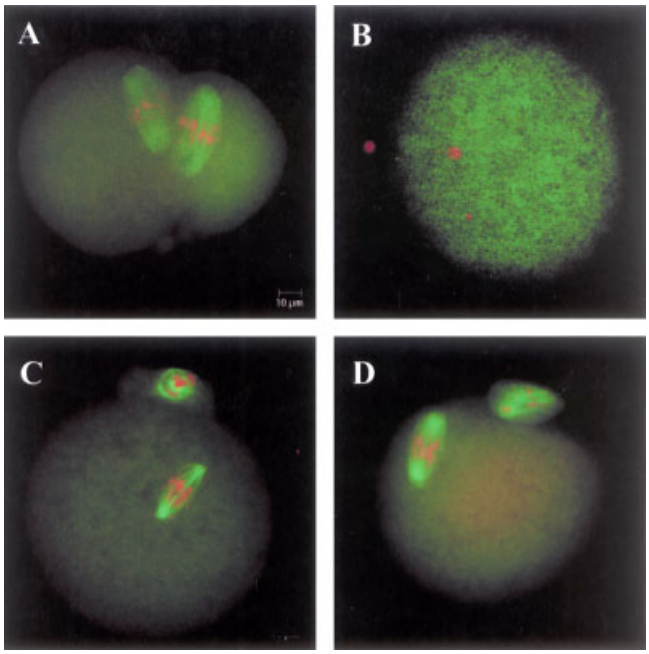

Fig. 3. Immunofluorescent staining of reconstructed oocytes with anti-α tubulin antibody (green) and propidium iodide (red) to illustrate the spindle and chromosomes. An individual metaphase spindle and chromosomes existed in the ooplasm and PB of (**A**) MI-GV pair; (**C**) GV-MI pair; (**D**) MII-MI pair. Condensed chromatin was observed in (**B**) MII-GV pair.

In Vitro Maturation of Oocytes Reconstructed by Using MI Cytoplast as Recipient and GV or MII Spindle as Donor

The reconstructed complexes of GV-MI (48.97%) and MII-MI (61.53%) pairs also extruded a PB after fusion and culture in vitro (Table 1; Fig. 2C,D). The PB of the GV-MI pair was smaller than that of MI-GV pair. Both of GV-MI pair and MII-MI pair had one metaphase spindle and one PB as revealed by immunofluorescent detection (Fig. 3C,D).

In Vitro Development of Oocytes Reconstructed by Using MII Cytoplast as Recipient and GV or MI Spindle as Donor

The reconstructed complexes of GV-MII (49.01%) and MI-MII (61.90%) pairs did not extrude a PB, but started early embryo development (Table 1; Fig. 2E,F). The parthenogenetic embryos even developed to hatching blastocyst (Fig. 2F).

MAP Kinase Activity in the Cytoplasts of GV, MI, and MII Oocytes

The cytoplasts of enucleated GV, MI, and MII oocytes were probed with anti-active MAP kinase antibody to show MAP kinase activity. The results of immunoblotting showed that the active MAP kinase was detected in the MI and MII cytoplasts, but not in GV cytoplasts (Fig. 4).

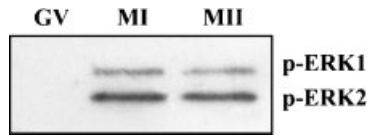

Fig. 4. Immunoblotting of active MAP kinase in the GV, MI, and MII cytoplasts. Both active anti-ERK_1 and ERK_2 were showed to exist in the MI and MII cytoplasts while not in GV cytoplasts.

DISCUSSION

In the present study, the interaction between nucleus and cytoplasm has been investigated by various combinations of mouse oocyte cytoplasts and karyoplasts. The GV cytoplast and MI cytoplast enable asynchronous karyoplasts to extrude a PB. The explanation to this may be related with the cytoplasmic factors: maturation promoting factor (MPF) and MAP kinase. MPF is an important factor for meiosis resumption, inducing nuclear envelope breakdown (GVBD), chromosome condensation, and the maintenance of the meiotic arrest (Masui and Markert, 1971; Muggleton-Harris et al., 1982). In addition to MPF, MAP kinase is also known to play a role in the regulation of meiosis in oocytes. MAP kinase is involved in the events following GVBD, such as the migration of spindle, disassembly of microtubule, and the extrusion of PB (Sobajima et al., 1993; Verlhac et al., 1994). Our result of immunoblotting shows that there is no MAP kinase activity in the cytoplast of GV oocyte. However, when GV cytoplast was cultured in vitro, MAP kinase activity reached a high level (Sugiura et al., 2001). Additionally, it has been known that MAP kinase activity in the GV and MI cytoplasts is not reduced after electric stimuli (our unpublished data), just opposite with the decline of MAP kinase activity in electrically stimulated MII oocytes. Therefore, MPF and MAP kinase activity may contribute to induce asynchronous karyoplast to extrude a PB.

The organization of spindle is also regulated by the activity of important cytoplasmic factors such as MPF and MAP kinase. MPF activity appears shortly before GVBD, maintains a high level during MI, decreases prior to extrusion of the first PB, to rise again throughout the MII stage (Fulka et al., 1992, 1996; Campbell et al., 1996). Different from MPF, MAP kinase activity remains high between MI and MII stage (Araki et al., 1996). Therefore, MAP kinase may be involved in the resumption procedure of the active MPF after the first meiosis, which makes the secondary oocytes normally enter into the metaphase of the second meiotic division (Araki et al., 1996). Our immunofluorescent results show that there is one metaphase spindle arrested in the cytoplasm and PB of MI-GV, GV-MI, and MII-MI pairs, which may be related with the high activity of MPF and MAP kinase.

MII cytoplasm has been found to be able to induce the donors of GV and MI karyoplasts to form parthenogenetic embryos. Previous studies showed that mouse GV karyoplasts seldom underwent GVBD in the enucleated mouse (Liu et al., 1999) and rabbit (Li et al., 2001b) MII

cytoplasts during a 24-hr culture. We observed here the continuous development of the reconstructed oocytes after transferring the karyoplasts from GV and MI oocytes into the MII cytoplasts. Both the GV-MII pair and MI-MII pair had the ability to become parthenogenetic embryos and developed up to hatching blastocysts following continuously cultured for 96 hr. This may be related to the activity of MPF and MAP kinase in MII oocytes. The high level of MPF in MII oocytes will be reduced after activation by electric current, allowing MII oocytes to undergo parthenogenetic division (Whitaker, 1996). During cloning studies (Wakayama et al., 1998), reconstructed oocytes after activation can also undergo cleavage division when a somatic cell nucleus (G0 or G1 cell cycle stage) is transferred into an enucleated MII oocyte and exposed to MPF. On the other hand, the present result from immunoblotting shows that the enucleated MII cytoplast has the high MAP kinase activity, which is consistent with the previous detection in intact MII oocytes (Araki et al., 1996). The inactivation (dephosphorylation) of MAP kinase is involved in regulation of pronuclear envelope assembly/disassembly after mouse egg fertilization (Moos et al., 1995, 1996). It was also found that MAP kinase was still in a phosphorylated form at 4 hr postinsemination, then dephosphorylated at 8 hr, when most eggs formed pronuclei (Sun et al., 1999). Therefore, the decline of MPF and MAP kinase activity may contribute to the parthenogenesis derived from transferring mouse GV and MI karyoplasts into the MII cytoplasts.

In summary, the present study using a new approach, shows that mouse GV and MI cytoplasts have the ability to induce the asynchronous karyoplast donors to extrude a PB. MII cytoplast can induce the GV and MI karyoplasts to undergo the parthenogenesis. The data suggest that the cytoplasm controls the nucleus behavior in mouse oocytes.

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