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Somatic nucleus remodelling in immature and mature *Rassir* oocyte cytoplasm

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Summary

Successful production of cloned animals derived from somatic cells has been achieved in sheep, cattle, goats, mice, pigs, rabbits, etc. But the efficiency of nuclear transfer is very low in all species. The present study was conducted to examine somatic nucleus remodelling and developmental ability *in vitro* of rabbit embryos by transferring somatic cells into enucleated germinal vesicle (GV), metaphase I (MI) or metaphase II (MII) oocytes. Microtubules were organized around condensed chromosomes after the nucleus had been transferred into any of the three types of cytoplasm. A bipolar spindle was formed in enucleated MII cytoplasm. Most of the nuclei failed to form a normal spindle within GV and MI cytoplasm. Some chromosomes scattered throughout the cytoplasm and some formed a monopolar spindle. Pseudopronucleus formation was observed in all three types of cytoplasm. Reconstructed embryos with MI and MII cytoplasm could develop to blastcysts. Nuclei in GV cytoplasm could develop only to the 4-cell stage. These results suggest that (1) GV material is important for nucleus remodelling after nuclear transfer, and (2) oocyte cytoplasm has the capacity to dedifferentiate somatic cells during oocyte maturation.

Keywords: Cytoplasm, Nuclear transfer, Rabbit, Somatic

Introduction

Somatic cell nuclear transfer has been developed and used successfully to produce cloned sheep, cattle, mice, goats, pigs, rabbits, etc. (Miyoshi *et al*., 2003). However, the efficiency of nuclear transfer in all species is very low. In all somatic cloned embryos developed to term, metaphase II (MII) cytoplasm was used as recipient. Whether germinal vesicle (GV) and metaphase I (MI) cytoplasm can support the development of the somatic nucleus is not known.

There are many factors affecting the efficiency of nuclear transfer. The environment of the oocyte cytoplasm is one critical factor besides the donor cells. Recent studies have shown that somatic nuclei can be dedifferentiated in MII cytoplasm, but the materials causing somatic nucleus dedifferention are not known. Does the cytoplasm of GV oocytes or MI oocytes have dedifferentiation capability like MII cytoplasm? The present study was conducted to examine nucleus remodelling and embryo developmental ability after transferring somatic cells into enucleated GV, MI or MII oocytes.

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Materials and methods

Animals

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethics committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Female Japanese Big-Eared white rabbits were housed in stainless steel cages, and were fed regular rabbit fodder and water *ad libitum*.

Cell lines

Fibroblast cells were obtained from ear skin tissue of a mature female Japanese Big-Eared white rabbits. Cell culture and assessment procedures have been described previously (Chen *et al*., 2002; Yang *et al*., 2003). Skin ear tissue was taken, cut into pieces and digested with 0.25% trypsin for 30 min at 37 °C. The digested cells and tissues were cultured in DMEM/F12 (1:1) supplemented with 20% fetal bovine serum (FBS) under 5% CO_2 in air in an incubator. Cells were passed for use when 70–80% of them had converged. After reaching 90–100% confluence, the cells were disaggregated with 0.25% (w/v) trypsin and then resuspended in DMEM/F12 (1:1). Cells that had passed 3–9 generations were used as donors.

Animal superovulation and oocyte collection

Rabbits were superovulated by PMSG and hCG. Female rabbits were injected intraperitoneally with 120–150 IU PMSG and killed 72 h after injection. GV-stage oocytes were recovered by aspiration of follicles > 3 mm in diameter, using an 18 gauge needle and a 1 ml syringe. The other group was injected with 80–100 IU hCG (Institute of Zoology, Academia Sinica) at 72 h following PMSG priming and killed 13–15 h later to collect MII-stage oocytes. Cumulus cells of all oocytes were removed by exposure to M_2 medium containing 500 IU/ml hyaluronidase. One group of GV-stage oocytes were cultured in M199 medium supplemented with 10% fetal calf serum (FCS) and 50 μg/ml 3-isobutyl-1-methylxanthine (IBMX) for 2 h to prevent spontaneous germinal vesicle breakdown and to develop a perivitelline space. GV-stage oocytes were cultured in 50 μl M_2 medium droplets with 10% FCS, 50 μg/ml IBMX for 10 min before GV aspiration. The zona pellucida was penetrated by pressing a glass microneedle tangentially into the perivitelline space against the holding pipette, and the GV surrounded by a small amount of cytoplasm (GV karyoplast) was removed indirectly (Meng *et al*., 1996; Li *et al*., 2001) by increasing the pressure inside the holding pipette to expel a GV karyoplast through the slit made in the zona (Fig. 1).

In order to remove the MI karyoplast a slit was made in the zona just above the GV (after IBMX incubation as mentioned above), then the oocyte was cultured in M199 supplemented with 10% FCS for 5 h (Yu *et al*., 2002) and the karyoplast removed by increasing the pressure inside the holding pipette to expel the karyoplast through the slit. Enucleation of MII oocytes was performed by removing the metaphase II spindle with a small amount of ooplasm and the first polar body (PB).

Microinjection

The microinjection process was as described previously (Li *et al*., 2002). The pipette was introduced through the same slit in the zona pellucida made during enucleation, and the cell was wedged between the zona and the cytoplast membrane for subsequent fusion.

Activation and embryo culture

The reconstructed oocytes were equilibrated in M199 plus 10% FCS medium for 10 min and then transferred into a drop of fusion medium (0.27 M mannitol, 0.1 mM $CaCl_2$, 0.05 mm $MgSO_4$). Electrofusion was stimulated with two electrical pulses (150 V/mm DC for 20 μs) delivered by a Kefa Electro Cell manipulator (Academia Sinica). Fusion was examined 30 min later. Then the reconstructed oocytes were activated with 2 pulses (150 V/mm DC for 80 μs) and cultured in M199 with 10% FCS and 5 μg/ml cytochalasin B for 15 h (GV reconstituted), 8 h (MI reconstituted) or 6 h (MII reconstituted), and transferred to M199 with 10% FCS.

Immunocytochemical staining of reconstructed oocytes

Samples were taken at 1 h intervals after fusion, and immunocytochemical staining performed as described previously (Zhu *et al*., 2003). Reconstructed oocytes were fixed with 3.7% (w/v) paraformaldehyde in PBS for 40 min at room temperature. Fixed oocytes were stored in PBS containing 0.3% (w/v) bovine serum albumin (BSA) for up to 1 week at 4 °C. Fixed oocytes were permeabilized by transferring them into PBS

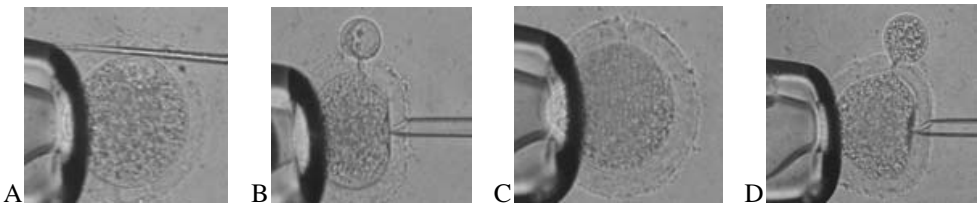

Figure 1 Enucleation of oocytes at the GV and MI stages. (*A*) Zona of a GV oocyte is cut by a sharp needle. (*B*) The GV is expelled. (*C*) A slit is formed the GV in an MI oocyte. (*D*) The MI spindle is removed from the slit.

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Table 1 Development of embryos reconstructed by transferring fibroblast cell nuclei into GV, MI or MII cytoplasm

Enucleation stage	No. of oocytes surviving enucleation	No. of oocytes fused (%)[a]	Development after culture (%)[b]			
			2-cell	4-cell	Morula	Blastocyst
GV	57	48 (84.0)[c]	12 (25.0)[c]	7 (14.5)[c]		
MI	69	51 (73.9)[cd]	31 (60.7)[d]	16 (31.4)[d]	7 (13.7)[d]	3 (5.9)[d]
MII	73	50 (68.5)[cde]	35 (70.0)[de]	28 (56.0)[e]	19 (38.0)[e]	11 (22.0)[e]

[a] Percentage of oocytes surviving enucleation.
[b] Percentage of oocytes fused.
[cde] Values with different superscripts differ significantly ($p < 0.05$).

containing 0.2% (w/v) Triton X-100 and 0.1% Tween-20 and then incubated for 40 min at room temperature. Then the oocytes were transferred into blocking solution (PBS containing 1% BSA) for 40 min at room temperature. The microtubules were localized with a mouse monoclonal antibody against beta-tubulin (Sigma), which was diluted in the blocking solution (1:200 [w/v]) before use. Oocytes were incubated for 40 min at room temperature, followed by three washes of 5 min each. Oocytes were incubated with fluorescein isothiocyanate-labelled goat-anti-mouse by three washes of 5 min each. Chromatin was stained with 10 μg/ml propidium iodide (Sigma). Finally oocytes were mounted on slides with antifluorescence-fade medium (DABCO). The samples were examined with a laser-scanning confocal microscope (Leica TCS-4D, Bensheim, Germany). Images were processed with Photoshop 6.0 software (Adobe Systems, San Jose, CA).

Statistical analysis

The results were evaluated using a chi-square test. A p value of < 0.05 was considered significant.

Results

Microtubule patterns and nucleus changes after nuclear transfer

Nuclear swelling occurred after the somatic cell was fused into any of the three types of cytoplasm. A good proportion (31/49) of the somatic nuclei showed little change in the GV cytoplasm. Microtubule patterns and nuclear changes after transferring fibroblast cells into cytoplasm enucleated at the GV stage are shown in Fig. 2. Chromosomes were distributed throughout the cytoplasm 4 h after fusion in MI cytoplasm and usually formed a monopolar spindle. A spindle began to organize 2 h after fusion in MII cytoplasm. A well-organized spindle formed and the chromosomes aligned on the metaphase plate 4 h after fusion (29/37). All three types of cytoplasm were able to induce pseudopronucleus formation after the oocyte was activated.

After fusion of fibroblast cell nuclei into MI cytoplasm (after fixation with 3.7% (w/v) paraformaldehyde), 12 of 59 contained two pseudopronuclei and 30 of 59 formed one pseudopronucleus at 12 h. In MII cytoplasm, 30 of 50 formed two pseudopronuclei, and 13 of 50 formed one pseudopronucleus at 8 h; the others showed little change and remained at the spindle stage.

Development of fibroblast cells in GV, MI and MII cytoplasm

The percentage of cleavage of the reconstructed embryos was not different between MI and MII cytoplasm, but the development rate of blastocysts between the two types of oocytes differed significantly ($p < 0.01$) (Table 1). GV cytoplasm could support somatic nuclear development only to the 4-cell embryo stage.

Discussion

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division. It is generally accepted that maturation promoting factor (MPF) plays an important role in the G2/M transition. MPF is an important factor for meiosis resumption, inducing germinal vesicle breakdown (GVBD), chromosome condensation, and the maintenance of meiotic arrest (Taieb *et al*., 1977; Muggleton-Harris *et al*., 1982). In addition to MPF, mitogen-activated protein kinase (MAPK) also plays a role in the regulation of meiosis in oocytes. MAPK is involved in the events following GVBD, such as the migration of the spindle, disassembly of the microtubules, and extrusion of the polar body (PB) (Sobajima *et al*., 1993; Verlhac *et al*., 1994; Yu *et al*., 2002).

Studies have shown that nuclear components stored in the GV facilitate remodelling of somatic nuclei in *Xenopus* oocytes, and the candidate molecules include nucleoplasmin and N1/N2, both of which can mediate the transfer of core histones to DNA and the assembly of nucleosomes (Laskey *et al*., 1978; Kleinschmidt *et al*., 1986). Nucleoplasmin is important in removing sperm protamines and decondensing the sperm chromatin to

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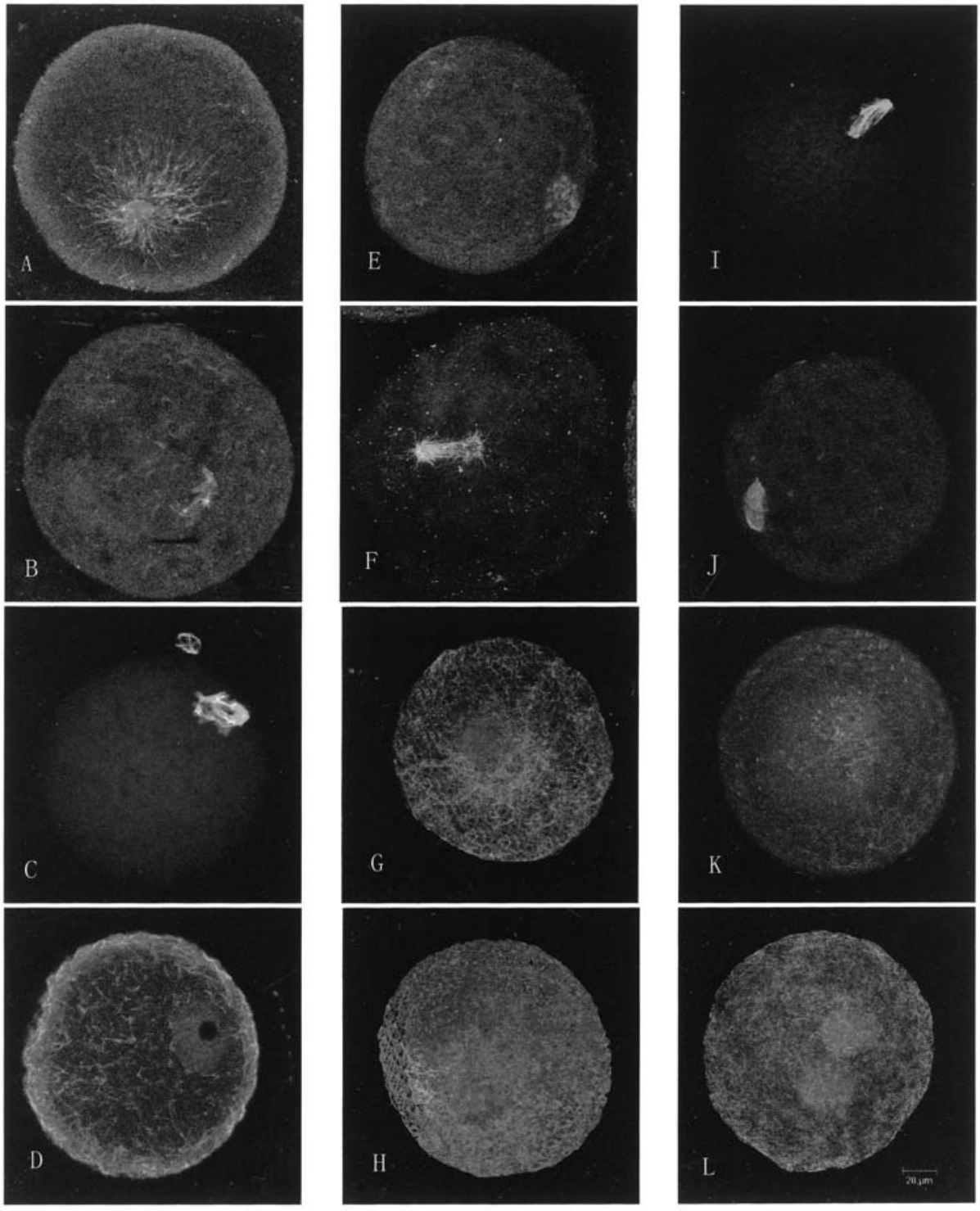

Figure 2 Laser-scanning confocal microscopic images of microtubules and chromatin in reconstructed oocytes. Green, microtubule; red, chromatin. (*A*)–(*D*) Enucleated GV oocytes fused with fibroblast cell nuclei. (*A*) Microtubules associated with swelling nuclei. (*B*) Chromosome distribution throughout the cytoplasm 13 h after fusion. (*C*) Well-organized with disorganized chromosomes on the pole of the spindle. (*D*) One pseudopronucleus was formed 25 h after fusion. (*E*)–(*H*) Enucleated MI oocytes fused with fibroblast cell nuclei. (*E*) Chromosomes were distributed throughout the cytoplasm 4 h after fusion. (*F*) A monopolar spindle was formed. (*I*)–(*K*) Enucleated MII oocytes fused with fibroblast cell nuclei. (*I*) Two hours after nucleus transfer. (*J*) A well-organized spindle and chromosomes aligned on the metaphase plate 4 h after fusion. (*K*)–(*L*) Pseudopronuclei were formed 8 h after fusion.

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allow the assembly of the paternal pronucleus (Hiyoshi *et al*., 1991; Leno *et al*., 1996). The poor development of the embryos reconstructed using GV cytoplasm suggests that those materials in the GV which are necessary for fertilization may also be important for mammalian somatic cell remodelling. Which molecules released from the GV regulated the reprogramming of somatic nuclei needs further investigation.

Porcine MI oocytes have a potential to develop into blastocysts after nuclear transfer of somatic cells. When embryos reconstructed with confluent cells and MI oocytes were cultured, 1.5% developed to blastocysts, a significantly lower proportion than when embryos are produced by transferring confluent cells into MII oocytes. (Miyoshi *et al*., 2001). The blastocyst formation rate of reconstructed embryos produced by transferring confluent cells into MI oocytes was significantly lower than that of embryos reconstructed with confluent cells and MII oocytes, although there were no significant differences in percentages of embryos cleaved to the 2-cell stage. These results suggest that the reprogramming of donor nuclei may be started but is not completed in some embryos reconstructed with MI oocytes. The factors required for reprogramming of donor nuclei may be present near the MI chromosomes and removed with the MI plate during enucleation, or the unknown factors required for reprogramming of nuclei are present in MII oocytes but not present or active in GV and MI oocytes.

Our results show that rabbit GV oocyte cytoplasm could support the development of somatic nuclei only to the 4-cell stage. MI oocytes have the potential to develop into blastocysts after nuclear transfer; however, the reprogramming ability was different compared with MII oocytes. When Gao *et al.* (2002) enucleated mouse oocytes at the GV or pro-metaphase I stage, cultured the cytoplasm to the MII stage and then injected embryonic stem (ES) nuclei into both types of cytoplasm, they found that chromosomes distributed throughout the mature GV cytoplasm. When activated no pseudopronucleus was formed. In pro-MI and MII cytoplasm an ES-cell derived bipolar spindle formed.

The development of reconstructed embryos derived from MI cytoplasm was much lower than that of embryos derived from MII cytoplasm. MPF activity is maximal at both MI and MII during oocyte maturation. In theory, MI oocytes may be more suitable as cytoplast recipients because their use ensures the donor nucleus is exposed to high levels of MPF activity for a long time (Miyoshi *et al*., 2003).

Wells *et al*. (1999) demonstrated that exposure of the somatic nucleus to enucleated MII oocytes for 4–6 h before activation resulted in an increased proportion of fused embryos developing into blastocysts. Similarly nuclei introduced either by electric fusion in cattle (Cibelli *et al*., 1998; Wells *et al*., 1998) or direct nuclear injection in pigs (Onishi *et al*., 2000) were subjected to a 2–6 h exposure to MII cytoplast before activation.

However, Miyoshi *et al*. (2001) and our results have shown that the rate of blastocyst formation in embryos reconstructed with MI oocytes is significantly lower than that of embryos reconstituted with MII oocytes. These results suggest that MII oocytes rather than MI oocytes are more appropriate recipients for production of differentiated cell-derived cloned embryos in mammals and that MPF is required but not sufficient for maximum developmental ability of reconstructed embryos.

Our results show that the development of rabbit MI reconstituted oocytes is much higher than that of porcine MI oocytes. One reason may be species differences and another that we developed a new method for enucleating the MI plate without using ultraviolet, which is harmful to cells.

Nuclear reprogramming is characterized by functional modification of the transferred nucleus to direct normal embryo development. We demonstrated that the developmental potential of reconstructed MII oocytes is much higher than that of reconstructed GV or MI oocytes. The molecular mechanism for reprogramming of the donor nuclei following somatic cell nuclear transfer has not been fully elucidated. The present study suggests that the remodelling of introduced somatic nuclei including PCC, the development of two pronuclear-like structures and the apposition of nuclei with microtubule asters may help the reprogramming of the transferred nuclei and increase the developmental potential of the reconstructed oocytes as Shin *et al*. (2002) reported.

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