Zygote **23** (February), pp. 76–82. © Cambridge University Press 2013
doi:10.1017/S0967199413000634 First Published Online 18 December 2013

Abnormal dynamic changes in β-tubulin in somatic nuclear transfer cloned mouse embryos

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Date submitted: 01.02.2013. Date accepted: 28.03.2013

Summary

The efficiency of somatic cell nuclear transfer (SCNT) cloning remains low, thus limiting the applications of this technique. In this study, we used immunochemistry and confocal microscopy to detect the microtubule component, β-tubulin, in SCNT, parthenogenetic (PA), and intracytoplasmic sperm injection (ICSI) embryos before the first mitotic division. β-Tubulin is the component subunit of microtubule, which plays critical roles in regulating localization of cellular organelles, and the growth, maturation and fertilization of oocytes. Our results demonstrated similar changes of spindle patterns in PA and ICSI embryos. The second meiotic division resumed 1 h post-treatment, and the cytoplasmic asters (CAs) disappeared. After about 4–6 h of treatment, pronuclei formed with the midbodies connecting each other. Meanwhile, the CAs reappeared and a microtubule network developed in the cytoplasm. However, SCNT embryos showed abnormal multipolar spindles, and the pseudopronuclei that contained many nucleoli existed after 6 h of $SrCl_2$ activation. Enucleated oocytes alone did not form spindle-like structures when they were artificially activated for 6 h, indicating that somatic cell chromosomes might be necessary for spindle formation in SCNT embryos. These results demonstrated abnormal changes of β-tubulin in mouse SCNT embryos, compared with PA and ICSI embryos.

Keywords: Enucleated oocytes, ICSI, Mouse, SCNT, β-Tubulin

Introduction

SCNT has been performed successfully in many mammals; however, its efficiency in terms of delivering live, cloned offspring remains low. Inadequate reprogramming of the somatic cells is believed to be the key problem responsible for this low efficiency. Reprogramming by a somatic nucleus introduced into an enucleated oocyte involves morphologic remodelling of the donor nucleus and its accompanying cytoskeleton, including critical roles in premature chromosomal condensation, mitotic spindle formation, pseudopronuclei formation and migration (Van Thuan *et al.*, 2006). SCNT is a multistep process that requires the removal of oocyte chromosomes and spindles, introduction of a somatic nucleus, and activation of the reconstructed oocyte. Enucleation is thought to be potentially harmful when important cytoskeletal proteins such as nuclear mitotic apparatus (NuMA) and γ-tubulin are removed along with the meiotic spindle (Simerly *et al.*, 2003). Donor cell chromosomes and cytoskeletal proteins need to change coincidently with the cytoskeleton of the enucleated oocytes to ensure that the first cell cycle of the reconstructed embryos can take place. Unlike those in other mammals, mouse oocytes include two microtubule-containing structures: the meiotic spindle, which is responsible for chromosome separation, and the cytoplasmic asters (CAs), which are crucial for pronuclei apposition (Branzini *et al.*, 2007). Cytoplasmic asters act as microtubule-organizing centres (MTOCs) in

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murine oocytes (Schatten *et al.*, 1985). However, in other mammals, MTOCs are mainly derived from the centrosome inherited from the fertilizing sperm.

Nuclear transfer cloned rat embryos had fewer microtubules and incomplete spindle formation, possibly associated with the low level of CA detection in MII oocytes (Tomioka *et al.*, 2007). This situation suggests that CAs may act as a source of microtubules for mitotic spindle formation in SCNT cloned rodent embryos. Van Thuan *et al.* (2006) indicated that, even though the enucleation process reduced the levels of γ-tubulin and NuMA, initial spindle morphogenesis was not disturbed because of regulation by the donor cell centrosome. After removal of the meiotic spindle, the oocytes retained their microtubule structure and CAs (Miki *et al.*, 2004). However, the ability of the remained microtubule proteins to form the spindle without donor chromosome interaction remains unknown.

It has been demonstrated that removal of the genetic material and meiotic spindles from oocytes may disrupt the arrangement of the cytoskeletal proteins in reconstructed oocytes and induce abnormal spindle formation, chromosome separation, and cleavage in SCNT embryos (Tomioka *et al.*, 2007; Yoo *et al.*, 2007). In the present study, we compared the dynamic changes that occur in microtubules and chromosomes in mouse parthenogenetic (PA), intracytoplasmic sperm injection (ICSI), and SCNT embryos. We also investigated the changes of β-tubulin in enucleated oocytes and during activation.

Materials and methods

Animals and reagents

ICR male and female mice (8–10 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. They were kept under controlled temperature (24 ± 2°C) and light conditions (14 h light/10 h dark regime). Food and water were available *ad libitum*. Animals were handled according to the Code of Practice of Harbin Medicine University Ethics Committees. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. CZB-HEPES, CZBG and KSOM media for oocyte collection, micromanipulation, and embryo culture were prepared in accordance with Nagy *et al.* (2003).

Oocyte collection

Female ICR mice were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Hangzhou, China), followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Hangzhou, China). Metaphase II oocytes were collected from oviduct ampullae 14 h after hCG injection. Cumulus cells were removed by treatment with 0.1% hyaluronidase for 3–5 min at room temperature. Oocytes were washed three times in CZB-HEPES and then incubated in CZBG droplets under mineral oil at 37°C in an atmosphere of 5% CO_2 in air, until use.

ICSI

ICSI was performed as described previously, with modifications (Yoshida & Perry, 2007). Males of the same strain (≥3 months old) were sacrificed and spermatozoa were collected from the caudae epididymides by dicing with fine scissors in CZB-HEPES. Spermatozoa and tissue fragments were incubated in CZB-HEPES at 37°C in an atmosphere of 5% CO_2 in air for 10 min, and supernatant with actively motile spermatozoa was then transferred to a new 0.5 ml tube. Spermatozoa suspensions were mixed with 10% PVP solution to a final PVP concentration of 3%. The heads of the spermatozoa were separated using a piezo impact drive unit (PMM-150 FU, Prime Tech, Japan). An MII oocyte was held gently using the holding pipette (outer diameter, 80–100 μm; inner diameter, 10–15 μm), and a sperm head was injected into the oocyte using the piezo unit and micromanipulators (Eppendorf, Germany) using a blunt-ended, mercury-containing pipette (inner diameter, 6–7 μm) at room temperature. After injection, the oocytes were maintained in CZB-HEPES at room temperature for 10 min. They were then transferred to KSOM droplets under oil and cultured at 37°C in an atmosphere of 5% CO_2 in air. Oocytes were extracted and fixed at different time points for tubulin detection.

Enucleation

MII oocytes were transferred in CZB-HEPES droplets supplemented with 5 μg/ml cytochalasin B (CB) to avoid disintegration, and observed under Hoffman optics. The position of the spindle was adjusted by the enucleation pipette to around 3 o'clock. A piezo pulse was used to help the enucleation pipette to penetrate the zona pellucida. The MII spindle was removed with a minimal volume of cytoplasm using the pipette. In the case of SCNT, the first polar body of the oocyte was also removed.

SCNT

Electrofusion was employed to maintain the microtubules in donor fibroblast cells. Fibroblast cells for SCNT were derived from E13.5 fetal mice, and were cultured in Dulbecco's modified Eagle's

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Table 1 Developmental potential of PA, ICSI, and SCNT preimplantation embryos *in vitro*

	Oocytes	1-cell (%)	2-cell (%)	Blastocyst (%)
PA	208	196 (94.2)	184 (93.9)[a]	151 (82.1)[a]
ICSI	135	109 (80.7)	107 (98.2)[a]	98 (89.9)[a]
SCNT	136	122 (89.7)	82 (67.2)[b]	23 (18.9)[b]

[a,b]Values with different superscripts in the same column indicate significant differences ($P < 0.05$).
PA: parthenogenetic activation. ICSI: intracytoplasmic sperm injection. SCNT: somatic cell nuclear transfer. 1-cell refers to oocytes surviving after 6 h activation or microinjection and electrofusion.

medium (DMEM) with 0.5% serum for 48 h before micromanipulation, to synchronize the cell cycle to the G0/G1 stage. A fibroblast cell was inserted into the space between the zona pellucida and the oolemma of the enucleated oocyte. Electrofusion was induced by a DC pulse of 1.8 kV/cm for 10 μs, using an ECM2001 (BTX Instruments, Genetronics, San Diego, CA, USA) in 275 mM mannitol supplemented with 0.1 mM $MgSO_4 \cdot 7H_2O$, 0.05 mM $CaCl_2 \cdot 2H_2O$, and 1 mg/ml bovine serum albumin (BSA). Reconstructed oocytes were then washed three times in CZB-HEPES and cultured in KSOM. Fusion results were checked 30 min later using a stereomicroscope, and successful fusion was defined by the absence of the donor cell in the perivitelline space. Reconstructed oocytes were then transferred to fresh KSOM droplets (up to 30 oocytes in a 30 μl droplet), and unfused pairs were subjected to a second electrofusion.

Artificial activation

MII oocytes, enucleated oocytes, and reconstructed oocytes were incubated separately in Ca^{2+}-free CZB that contained 10 mmol/l $SrCl_2$ and 5 μg/ml CB for 6 h. For PA activation and SCNT oocytes, the presence of pseudonuclei was taken as evidence of activation. Oocytes were then washed and cultured in KSOM medium at 37°C in an atmosphere of 5% CO_2 in air. Oocytes were extracted and fixed at different time points for tubulin detection.

Immunofluorescence staining

The immunofluorescence staining procedure has been described previously (Zhu *et al.*, 2003). Briefly, the zona pellucida was removed in acidic CZB-HEPES (pH 2.5), and oocytes or preimplantation embryos were then fixed for 40 min in 3.7% paraformaldehyde at room temperature, and incubated in 0.5% Triton-X100 at 37°C for 50 min. They were blocked in Dulbecco's phosphate-buffered saline (DPBS) with 10% fetal bovine serum and 1% BSA at 37°C for 50 min, followed by incubation with mouse monoclonal anti-β-tubulin antibody (Sigma; 1:120 dilutions) for 1 h at 37°C, or overnight at 4°C. The second antibody used was fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin (Ig)G (Sigma; 1:100 dilution), which was coincubated with samples at 37°C for 1 h. The nuclei were stained with 10 μg/ml propidium iodide. Finally, the samples were mounted on a slide with diazobicyclo-octane and observed under a laser-scanning confocal microscope (Olympus+ FV 300, Japan). Experiments were repeated three times for each group, and at least 10 samples in each group were detected at one time.

Data analysis

Data were analyzed using χ^2 tests. Significant differences were determined at P-values < 0.05.

Results

Preimplantation development

As shown in Table 1, the developmental potential of SCNT preimplantation embryos was significantly lower than that of ICSI or PA embryos ($P < 0.05$). The rate of blastocyst development in SCNT was 18.9%, compared with 89.9% in ICSI and 82.1% in PA embryos, respectively.

Dynamic changes of β-tubulin in PA embryos

Matured mouse oocytes showed a clear β-tubulin-positive structure and the dipolar meiotic spindle, which was located close to the cortex and parallel to the oolemma. Chromosomes were arrayed on the equator of the spindle, and some CAs were distributed in the cytoplasm (Fig. 1*A*). After 1 h exposure to the activation medium, MII oocytes entered into anaphase II, and the CAs disappeared. Sister chromatids started to separate and move along the microtubules towards the spindle poles (Fig. 1*B*). At 2 h of activation, sister chromatids arrived at the poles of the spindle and the oocytes entered into telophase II. Confocal images showed that the spindle was still parallel to the oolemma (Fig. 1*C*). Pronuclei started to form at 3–4 h of activation, and they were connected by internuclear microtubules (remnants of the spindle). CB, which was

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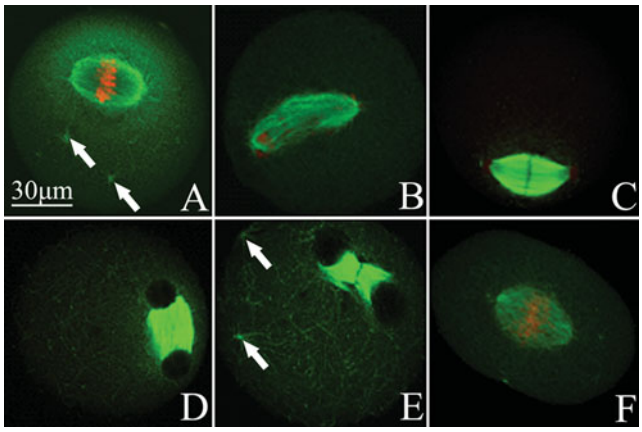

Figure 1 Dynamic changes in β-tubulin expression in PA embryos. MII oocytes showed barrel-like spindles with chromosomes at the equatorial plate. Cytoplasmic asters (CAs, arrows) were distributed in the cytoplasm (*A*). At 1 h after exposure to artificial activation medium, chromosomes started to be pulled toward the opposite poles of the spindle (*B*). At 2 h, the spindle was parallel to the oolemma, and the chromosomes were located at the two spindle poles (*C*). At 3–4 h, the two female pronuclei formed (*D*). At 4–6 h, the two pronuclei were close to each other (*E*), and CAs (arrows) appeared. At 20 h after activation, the mitosis spindle formed (*F*). Green indicates β-tubulin, red indicates chromatids.

applied in the medium, inhibited the extrusion of the second polar body (Pb_2), and two maternal pronuclei were present in the cytoplasm (Fig. 1*D*). During 5–6 h of PA activation, CAs reappeared and connected to each other to form a microtubule network (Fig. 1*E*). The first mitosis started at 20 h post-activation, and the mitotic spindle formed. Meanwhile, the microtubule network disappeared and only a few asters remained in the cytoplasm (Fig. 1*F*).

β-Tubulin in ICSI embryos

The injected sperm activated the MII oocytes to resume the second meiosis. At 1 h after microinjection, the head of the sperm showed a condensation structure inside the cytoplasm of the MII oocytes (Fig. 2*A*, arrowhead), and the chromatids were moving towards the poles of the spindle. At 2 h after injection, the head of sperm decondensed (Fig. 2*B*, arrowhead), and the chromatids were located at the poles of the spindle. The pronuclei were not formed at about 3–4 h after sperm injection (Fig. 2*C*), and did not present until 5–6 h after injection. Meanwhile, the Pb_2 was extruded but still connected to the female pronucleus by microtubules (Fig. 2*D*). At 8 h after injection, the two pronuclei had started to move towards each other (Fig. 2*E*).

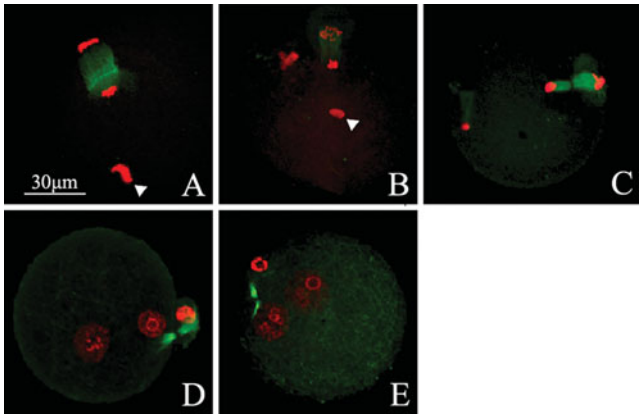

Figure 2 Changes in β-tubulin expression in ICSI embryos. At 1 h after sperm injection, oocytes resumed the second meiosis and a condensed sperm head (arrowhead) was seen inside the cytoplasm (*A*). At 2 h after injection, the chromatids had migrated to the spindle poles (*B*) and the head of the sperm had decondensed (arrowhead). At 3–4 h after injection, male and female pronuclei had formed (*C*), and the second polar body was connected to the female pronucleus (*D*). The two pronuclei started to move towards each other at 8 h after sperm injection (*E*).

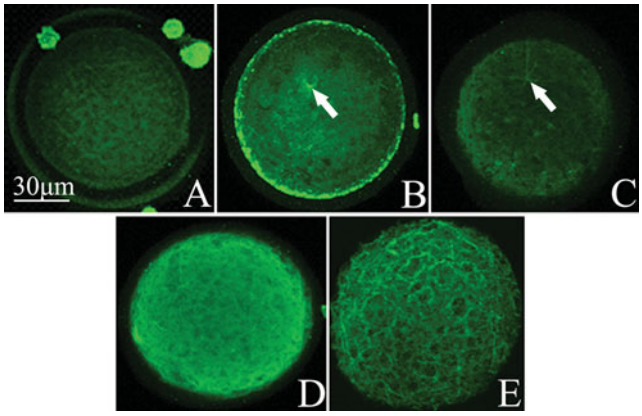

Figure 3 Spindle formation was disturbed in enucleated oocytes. Immediately after enucleation, no β-tubulin staining was detected in the cytoplasm and few cumulus cells were attached to the zona pellucida (*A*). After 1–2 h exposure to activation medium, few cytoplasmic asters (CAs, arrow) appeared and β-tubulin expression was restricted to the cortex of the oocyte (*B*). At 3–4 h of activation, the β-tubulin ring had disappeared, but CAs remained (arrow) (*C*). At 5–6 h of activation (*D*), and 20 h (*E*) after activation, no spindle-like structures were seen in the oocyte.

β-Tubulin in enucleated oocytes

Immediately after enucleation, β-tubulin was barely detectable in the cytoplasm of the MII oocytes (Fig. 3*A*). However, restricted β-tubulin expression appeared in the cortex in enucleated oocytes activated in Ca^{2+}-free CZB that contained 10 mmol/l $SrCl_2$ and 5 μg/ml CB for 1–2 h, and CAs also appeared (Fig. 3*B*).

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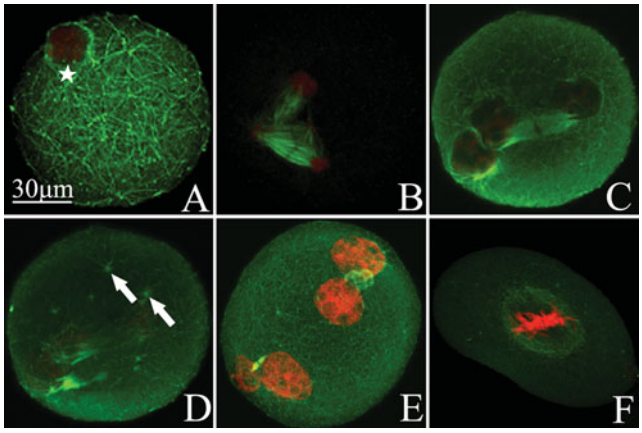

Figure 4 Multipolar spindle leads to formation of multiple pseudopronuclei in reconstructed oocytes. At 30 min after electrofusion, fibroblast cells were fused to the enucleated oocytes. β-Tubulin lattice work (asterisk) and cytoplasmic asters (CAs) were seen in the cytoplasm (*A*). Multipolar spindles formed after 1–2 h in activation medium (*B*), leading to the formation of 3–4 pseudopronuclei at 3–4 h of activation (*C*), and the reappearance of CAs (arrows, *D*). Four pseudopronuclei formed with clear nucleoli inside (*E*). Reconstructed oocytes underwent the first mitosis at 20 h after activation (*F*). Green indicates β-tubulin, red indicates chromatids.

The number of CAs was increased at 3–4 h, but cortical β-tubulin was decreased (Fig. 3*C*). Enucleated oocytes exhibited β-tubulin-positive lattice work, with no spindle-like structures at 6 h (Fig. 3*D*) and 20 h after activation (Fig. 3*E*).

Changes of β-tubulin in SCNT embryos

The β-tubulin-positive microtubule network and somatic nucleus (Fig. 4*A*, asterisk) were clearly observed in reconstructed oocytes immediately after electrofusion (Fig. 4*A*). One hour later, the reconstructed oocytes resumed the second meiosis, and the meiotic spindle reappeared with the chromatids arranged in a disordered fashion (Fig. 4*B*). The chromatids were then pulled towards opposing poles of the spindle. However, multipolar spindles were frequently detected in SCNT embryos (Fig. 4*B*,*C*). The spindle disassembled in the SCNT embryos at around 4 h of $SrCl_2$ activation, and pseudopronuclei were present in SCNT embryos, linked by midbodies (Fig. 4*C*). Meanwhile, the CAs were detectable again (Fig. 4*D*). At 4–6 h activation of SCNT embryos, pseudopronuclei formed with distinct multiple nucleoli in each and sometimes more than two pseudopronuclei exist in reconstructed embryos (Fig. 4*E*). However, SCNT embryos formed one mitotic spindle about 20 h after activation (Fig. 4*F*).

Discussion

The results of this study showed that the developmental potential of SCNT embryos was significantly lower than that of PA or ICSI embryos. This result is in accordance with the data from Rybouchkin *et al.* (2002). Unlike the situation in most mammals, mouse MII oocytes have two microtubule-containing structures, a spindle and CAs. In the present study we detected dynamic changes in the microtubules in PA, ICSI, enucleated cytoplasm, and SCNT embryos. The changes in both microtubule-containing structures were similar in PA and ICSI embryos. $SrCl_2$ has been widely used as an effective PA agent to evoke oscillations in cytosolic Ca^{2+}, which is similar to those evoked by sperm. Mouse MII oocytes resumed the second meiosis about 1–2 h after activation by Sr^{2+} or sperm. Previously abundant CAs disappeared, and the maternal chromatids were arranged on the metaphase plate and pulled toward the poles of the spindle. Pronuclei formed after 4–6 h of treatment, and were in similar sizes in PA embryos, while the male pronucleus was slightly larger in ICSI embryos. CB was used during PA activation to inhibit the extrusion of Pb_2 and ensure diploid chromosomes in PA embryos.

In mouse MII oocytes, CAs, one form of the MTOCs (Schatten *et al.*, 1985), degenerated shortly after the oocytes resumed the second meiosis and reappeared after pronuclei formation. They might anticipate mitotic spindle formation, as they were less abundant when the first mitotic spindle developed in PA or ICSI embryos.

Enucleation has been reported to have no significant effect on the number of CAs, nor on their absence and reappearance, during the SCNT procedure (Miki *et al.*, 2004). However, it was unknown if dramatic changes of microtubule-containing structures occurred in enucleated oocytes during artificial activation. The results of the current study indicated that the microtubules were restricted to the cortex, with few CAs in the cytoplasm, when enucleated oocytes were activated for 1–2 h. Despite exposure to activation medium for 6 or 20 h, no spindle-like structures appeared in the enucleated oocytes. Interactions between microtubule asters have previously been found to account for the formation of bipolar or multipolar spindles in chromatin-lacking oocyte fragments (Brunet *et al.*, 1998). However, in our study, spindle formation was not observed until 1 h after the somatic cells were introduced into the enucleated oocytes. This situation could be because both microtubule and microfilament assemblies are required for the dispersion of the meiotic chromosomes, pronucleus formation, and movement (Kim *et al.*, 1997). The addition of CB, an inhibitor of microfilament polymerization, to the

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enucleating medium to prevent oocyte lysis might impair the function of the microfilaments, which in turn obstructed the formation of the meiotic spindle in the enucleated oocytes.

Chromosomes have also been shown to be important for promoting microtubule polymerization and stabilizing the forming spindle poles (Woods *et al.*, 1999). Our results demonstrated that activated enucleated oocytes without chromosomes could not form spindle-like structures.

Mammalian SCNT has been achieved in many species, although the development of SCNT embryos to term remains inefficient. A significantly high proportion of abnormal chromosome or microtubule distribution was observed in the SCNT bovine embryos (Kwon *et al.*, 2010). Non-human primate SCNT studies have demonstrated that some critical molecules required for mitotic spindle formation, such as NuMA, were lost during enucleation, which could have caused the abnormal development (Simerly *et al.*, 2003). Unlike in primate SCNT, however, enucleation of mouse oocytes did not significantly decrease NuMA expression, but did decrease the levels of γ-tubulin, another MOTC candidate (Van Thuan *et al.*, 2006). Liu *et al.* (2006) indicated that NuMA was derived from the somatic cells in cloned porcine embryos, while Dai *et al.* (2006) showed that approximately half of the total bovine SCNT embryos exhibited centrosomal abnormalities, leading to failure of spindle morphogenesis. This outcome raises the question of which molecule is more important for spindle formation in mouse SCNT embryos, given that they may contain both centrosomes and NuMA derived from the donor somatic cells, and cytoplasmic asters or γ-tubulins from the enucleated oocytes. Further studies using specific markers for these molecules, or interspecies SCNT, are needed to clarify the relative importance of these molecules.

In this study, we constructed mouse SCNT embryos by electrofusion, ensuring the number of microtubules in the donor cells. The meiotic spindle formed 1 h post-activation, followed by a multipolar spindle. At 4–6 h of activation, pseudopronuclei appeared with many nucleoli in them. Nucleolar proteins have been shown to be activated at late 4-cell stage in mouse SCNT embryos, which is later than the time in PA embryos (Svarcova *et al.*, 2009). The multiple nucleoli in the pseudopronuclei of mouse SCNT embryos might represent this delayed nucleologenesis.

In conclusion, the dynamic changes in β-tubulin in SCNT embryos before the first mitotic division differ from those observed in PA or ICSI embryos. Multipolar spindles and many nucleoli in pseudopronuclei are frequently formed in SCNT embryos. Enucleated oocytes are unable to form spindle-like structures until chromosomes are introduced into the cytoplasm.

Acknowledgements

This work was supported by the Project of Abroad Researcher Foundation of Heilongjiang (Grant No. 1151hz031), the National Natural Science Foundation of China (No. 30900413), the Weihan Yu foundation of Harbin Medical University and China Postdoctoral Science Foundation (No. 201104421)

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