Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 306 (2005) 35 – 46

Experimental Cell Research

www.elsevier.com/locate/yexcr

Function of donor cell centrosome in intraspecies and interspecies nuclear transfer embryos

Zhi-Sheng Zhong[a], Gang Zhang[a,b], Xiao-Qian Meng[a,b], Yan-Ling Zhang[a], Da-Yuan Chen[a], Heide Schatten[c], Qing-Yuan Sun[a,*]

[a]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China*
[b]*Key Laboratory of Animal Resistance, College of Life Science, Shandong Normal University, Jinan, 250014, China*
[c]*Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri-Columbia, MO 65211, USA*

Received 1 October 2004, revised version received 20 January 2005
Available online 17 March 2005

Abstract

Centrosomes, the main microtubule-organizing centers (MTOCs) in most animal cells, are important for many cellular activities such as assembly of the mitotic spindle, establishment of cell polarity, and cell movement. In nuclear transfer (NT), MTOCs that are located at the poles of the meiotic spindle are removed from the recipient oocyte, while the centrosome of the donor cell is introduced. We used mouse MII oocytes as recipients, mouse fibroblasts, rat fibroblasts, or pig granulosa cells as donor cells to construct intraspecies and interspecies nuclear transfer embryos in order to observe centrosome dynamics and functions. Three antibodies against centrin, γ-tubulin, and NuMA, respectively, were used to stain the centrosome. Centrin was not detected either at the poles of transient spindles or at the poles of first mitotic spindles. γ-tubulin translocated into the two poles of the transient spindles, while no accumulated γ-tubulin aggregates were detected in the area adjacent to the two pseudo-pronuclei. At first mitotic metaphase, γ-tubulin was translocated to the spindle poles. The distribution of γ-tubulin was similar in mouse intraspecies and rat–mouse interspecies embryos. The NuMA antibody that we used can recognize porcine but not murine NuMA protein, so it was used to trace the NuMA protein of donor cell in reconstructed embryos. In the pig–mouse interspecies reconstructed embryos, NuMA concentrated between the disarrayed chromosomes soon after activation and translocated to the transient spindle poles. NuMA then immigrated into pseudo-pronuclei. After pseudo-pronuclear envelope breakdown, NuMA was located between the chromosomes and then translocated to the spindle poles of first mitotic metaphase. γ-tubulin antibody microinjection resulted in spindle disorganization and retardation of the first cell division. NuMA antibody microinjection also resulted in spindle disorganization. Our findings indicate that (1) the donor cell centrosome, defined as pericentriolar material surrounding a pair of centrioles, is degraded in the 1-cell reconstituted embryos after activation; (2) components of donor cell centrosomes contribute to the formation of the transient spindle and normal functional mitotic spindle, although the contribution of centrosomal material stored in the recipient ooplasm is not excluded; and (3) components of donor cell centrosomes involved in spindle assembly may not be species-specific.

Keywords: Centrosome; Nuclear transfer; Early development; Embryo; Donor cell nucleus

Introduction

Centrosomes, the major microtubule-organizing centers (MTOCs) of animal cells, play a pivotal role in microtubule nucleation and in establishing spindle bipolarity that is required for fundamental cellular processes such as cell division, embryogenesis, and subsequent development [1,2]. Centrosomes are composed of a pair of perpendicularly oriented centrioles and numerous surrounding proteins called pericentriolar material (PCM) [3]. Typically coordinated with the replication of DNA, centrosomes also replicate during the S phase of the cell cycle. Precise reproduction of centrosomes and precise assembly of centrosome proteins to form the bipolar mitotic spindle

* Corresponding author. Fax: +86 10 62565689.
E-mail address: sunqy1@yahoo.com (Q.-Y. Sun).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.02.009

are vital for normal mitosis. Prior to the onset of mitosis, the centrosome has to duplicate exactly once during the cell cycle to form the mitosis-competent bipolar centrosome. Centrosome separation occurs before centrosomes move to the opposite sides of the cell, where each forms one pole of the bipolar mitotic spindle. In mature mammalian oocytes, centrioles do not exist but functional MTOCs are present at spindle poles that do not contain centrioles. In most animal species, the sperm introduces its proximal centriole into the oocyte during fertilization and recruits components from the oocyte to restore a fully functional centrosome [4,5]. However, a sperm centriole is not introduced into the egg during mouse fertilization, and no centriole exists in early embryos from fertilization to implantation. Acentriolar mouse zygotes accomplish early embryonic cleavages using MTOCs until late pre-implantation development [6,7].

Centrins are members of a highly conserved subgroup of the EF-hand superfamily of Ca^{2+}-binding proteins. Commonly associated with centrioles, centrin is an intrinsic component of centrosomes and it has an essential role in the duplication of the centrosomes [8]. γ-tubulin, a key component of centrosomal material, directly participates in microtubule nucleation and assembly. The recruitment of γ-tubulin from the ooplasm by the sperm centriole after fertilization is an essential step to rebuild full functional centrosome [9,10]. NuMA is a nuclear-matrix protein, which plays an important role in somatic cells or early embryonic mitosis. NuMA is located as a crescent around spindle poles during mitosis and meiosis and translocates into the nucleus at interphase [11,12].

During somatic cell nuclear transfer (SCNT), the donor cell's centrosome is introduced into the anuclear recipient oocyte. Centrosomal material in the recipient ooplasm and the centrosome of the donor cell coexist in the reconstituted embryo. Whether and how the donor cell centrosomes assemble mitotic spindles remains to be elucidated. Up to now, there are still no persuasive experiments describing the destiny of the donor cell centrosomes in the intraspecies or interspecies SCNT reconstituted embryos.

In this experiment, we used mouse MII oocytes as the recipients, mouse fibroblast, rat fibroblast, or porcine granulosa cells as donor cells to reconstruct intraspecies/interspecies SCNT embryos. We used three antibodies against centrin, γ-tubulin and NuMA, respectively, to label the centrosome and analyze the donor cell's centrosome behavior in spindle assembly.

Materials and methods

The somatic cell culture

Mouse fibroblasts, rat fibroblasts, HeLa cells, or porcine granulosa cells were used for examining the antibodies' centrosome-tagging ability. Mouse fibroblasts, rat fibroblasts, or porcine granulosa cells were also used as nuclear transfer donor cells.

For collection of primary porcine granulosa cells, ovaries were collected at a local slaughterhouse and placed into saline containing 75 μg/ml penicillin, 50 μg/ml streptomycin sulfate. Ovaries were carried back to the laboratory within 1.5 h. Oocyte–cumulus complexes (OCCs) were aspirated from antral follicles (2–6 mm in diameter) with an 18-gauge syringe and settled for 5 min. Small masses of cumulus cells were picked up from the deposit, rinsed 2 or 3 times, and then cultured in DMEM-F12 containing 10% FBS.

Collection of mouse oocytes

Eight-week-old female Kunming mice were intraperitoneally injected with 10 IU pregnant mares' serum gonadotropin (PMSG) and sacrificed 48 h later. Ovaries were removed and placed in culture medium. Antral follicles were punctured with a sterile needle. Germinal vesicle (GV) stage oocytes were collected and rinsed. GV stage oocytes were fixed for immunofluorescence microscopy, or cultured in M16 medium for further maturation. GVBD stage oocytes were collected at 3–4 h of culture. After maturing in vitro for 8–9 h, MI stage oocytes were collected. After maturing in vitro for 12 h, mature (MII) oocytes were collected.

Eight-week-old female Kunming mice were superovulated with intraperitoneal injections of 10 IU PMSG and 10 IU human chorionic gonadotropin (hCG) at 48 h intervals. They were sacrificed at 15 h after hCG injection. Oocyte–cumulus cell complexes were collected from the Fallopian tubes and then digested with 300 IU/ml hyaluronidase to remove the cumulus cells. After rinsing 35 times, MII oocytes were used for immunofluorescence observation or nuclear transfer.

Donor cell preparation

Fibroblasts at 3–7 passages or porcine granulosa cells at 2–3 passages were used as nuclear transfer donor cells. When fibroblasts reached 80%–90% confluence, the concentration of FBS in the medium was decreased from 10% to 0.5% for serum-starvation. Donor cells were synchronized to G_0 phase of the cell cycle by 3–5 days culture under serum-starvation.

Mouse intraspecies/interspecies nuclear transfer

Mouse intraspecies/interspecies nuclear transfer manipulation was based on the protocol developed in our laboratory [13]. This somatic cell nuclear transfer manipulation was a combination of the Roslin technique and the Honolulu technique [14,15]; after culture under serum-starvation culture, a 3 h post-electrofusion interval and strontium

activation were adopted. The nuclear transfer manipulation solution is CZB medium supplemented with 3% sucrose, 10% FCS, and 5 μg/ml cytochalasin B [16]. The CZB culture medium free of glucose was used for early embryo development [15,17].

The region of the MII spindle became a clearly transparent area under high osmotic pressure caused by 3% sucrose, and it was easy to distinguish the spindle region from other parts of the cytoplasm. The larger perivitelline space caused by high osmotic pressure was also beneficial for slitting the zona pellucida. Oocytes were placed in a drop of manipulation solution for 3–5 min and enucleated with a 10-μm pipette by aspirating the metaphase II spindle with a minimum of cytoplasm. The zona pellucidae of oocytes were slit with a glass needle along 1/4–1/5 of their circumference near the position of the spindle or the swelling. The metaphase II chromosomes of recipients were removed with an enucleation pipette. A donor cell was placed into the perivitelline space of an anuclear oocyte. To ensure the efficiency of removal of MII chromosomes, some oocytes were stained with 5 μg/ml Hoechst 33342 and checked under UV light [18]. These donor-recipient pairs were rinsed in CZB medium 3 times then placed in the CZB medium for another 30 min for equilibrium.

Membrane fusion between enucleated oocytes and donor cells was performed at room temperature (RT). The donor-recipient pairs were washed 3 times in electrofusion solution (0.3 M mannitol, 0.1 mM $MgSo_4$, 0.1 mg/ml polyvinyl alcohol, and 3 mg/ml bovine serum albumin) [19]. Donor-recipient pairs were manually aligned. The fusion of donor-recipient pairs was induced by a DC pulse of 1.8 kv/cm for 10 μs delivered by ECM2001 (BTX, San Diego, CA). After the electric stimuli, the reconstructed pairs were rinsed 3 times in CZB and then incubated in 5% CO_2, 95% air at 37°C. After 30 min in culture, fusion of donor-recipient pairs was assessed using a dissecting microscope. The fused pairs were cultured in CZB medium with 5 μg/ml CB for 3 h and then placed in activation solution (CZB culture medium free of Ca^{2+}, supplemented with 10 mM $SrCl_2$, 5 μg/ml CB) for 6 h for chemical activation. After activation, reconstituted embryos were washed 3 times in CZB then cultured in CZB culture medium free of glucose [15,19]. Reconstituted embryos were fixed at different developmental stages.

Antibody microinjection

In order to observe the role of γ-tubulin in spindle assembly, we microinjected γ-tubulin antibody into mouse intraspecies reconstituted embryos [20]. Reconstituted embryos microinjected with rabbit IgG were designed as control. γ-tubulin antibody microinjection was performed about 12 h after activation. Reconstituted embryos were fixed for immunofluorescence observation 20 h after activation or scored for the rate of progression to the 2-cell stage. By using porcine granulosa cells as donor cells and mouse MII oocytes as recipients, we reconstituted pig–mouse interspecies reconstituted embryos. NuMA antibody was microinjected into pig–mouse interspecies reconstituted eggs 10 h after activation. Microinjection of mouse IgG was used as control. Each experiment was performed 3 times. A total of 20–30 reconstituted embryos were microinjected per group per experiment. To minimize the harm to reconstituted embryos, microinjection needle diameter was smaller than 1 μm. The amount of antibodies or control IgG microinjected was roughly 8 pl/oocyte, and the time of operation in each group was completed within 30 min.

Laser confocal microscopy

Porcine granulosa cells were placed in a Petri dish containing a coverslip then cultured in DMEM-F12 containing 10% FBS. Digested and dispersed mouse fibroblasts, rat fibroblasts, and HeLa cells were placed into Petri dishes, each containing a coverslip. When the cells reached 60–80% confluence, they were fixed for immunofluorescence microscopy.

Zona pellucidae of oocytes from GV stage to MII stage were removed with acidified CZB (pH 2.5). Because acidified CZB causes severe morphological distortion on nuclear transfer reconstituted embryos, zona pellucidae of reconstituted embryos were kept intact until embryos were ready for mounting on glass slides. Zona pellucidae of the reconstituted embryos were removed by repeated pipetting using a pipet with a diameter that was slightly smaller than that of the reconstituted embryos. Except for specimens stained for centrin, the samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at RT. Specimens to be stained for centrin were fixed in 2% paraformaldehyde in PBS (pH 7.4) for 30 min at RT, followed by fixation in −20°C cold methyl alcohol for 3–5 min. All specimens were permeabilized in permeabilization solution (0.5% Triton X-100, 20 mM Hepes, pH 7.4, 3 mM MgCl2, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3 in PBS) for 45 min at RT. Except for specimens tagged with α-tubulin, the other was blocked in 1% BSA for 1 h at RT. Specimens were incubated with different concentrations of antibodies (1:100 α-tubulin, Sigma, Cat # T6793; 1:400 γ-tubulin, Sigma, Cat # T5192, 1:200 centrin, Sigma, Cat # C-7736; 1:100 NuMA, Oncogene, Cat # NA08) for 1 h at RT or overnight at 4°C. Specimens tagged with α-tubulin were rinsed 3 times and stained with 10 μg/ml propidium iodide. Specimens tagged with γ-tubulin or centrin antibody were rinsed 3 times and incubated with 1:100 FITC-conjugated goat anti-rabbit IgG for 1 h, while specimens tagged with NuMA antibody were rinsed and incubated with 1:100 FITC-conjugated goat anti-mouse IgG for 1 h. The specimens were rinsed 3 times followed by staining with 10 μg/ml propidium iodide. Finally, all specimens were mounted on glass slides with DABCO

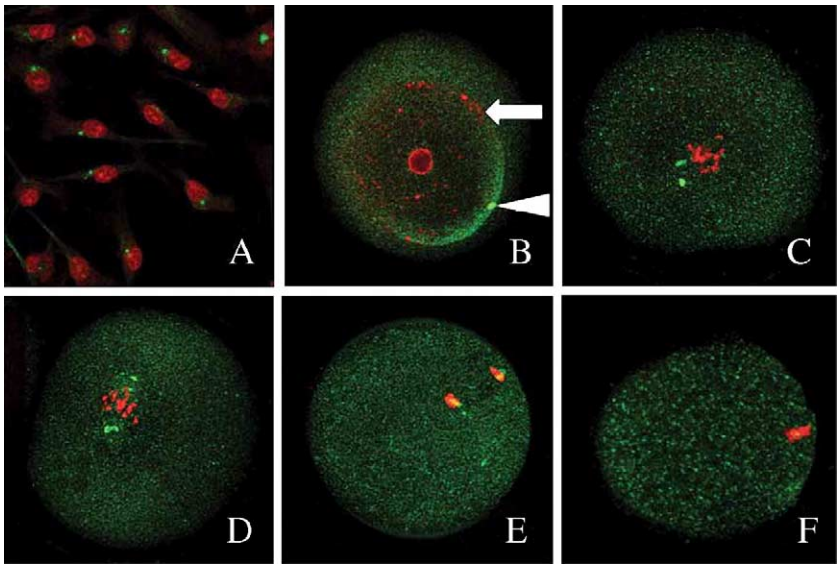

Fig. 1. Localization of centrin in mouse fibroblast and different stage oocytes as revealed by confocal microscopy. DNA, red; centrin, green. Centrin dots were localized near interphase nuclei of mouse fibroblasts (A). One or two centrin bright dots were localized near the GV (arrow pointed to the GV and arrowhead pointed to the centrin dot) (B). After GVBD, two centrin dots separated (C). Two groups of dots distributed to the MI (D) or telophase I (E) spindle poles; centrin disappeared from the MII oocyte spindle poles (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1,4-diazobicyclo-[2,2,2]-octane) and scanned using a TCS-4D laser confocal microscope (Leica Microsystems).

Statistical analysis

Data were analyzed by Chi-square test. If $P < 0.05$, difference was considered as statistically significant.

Results

Tagging of centrosomes in mouse fibroblasts and oocytes by centrin

Centrin dots were located near the nuclei of mouse interphase fibroblasts. One or two centrosomes were found

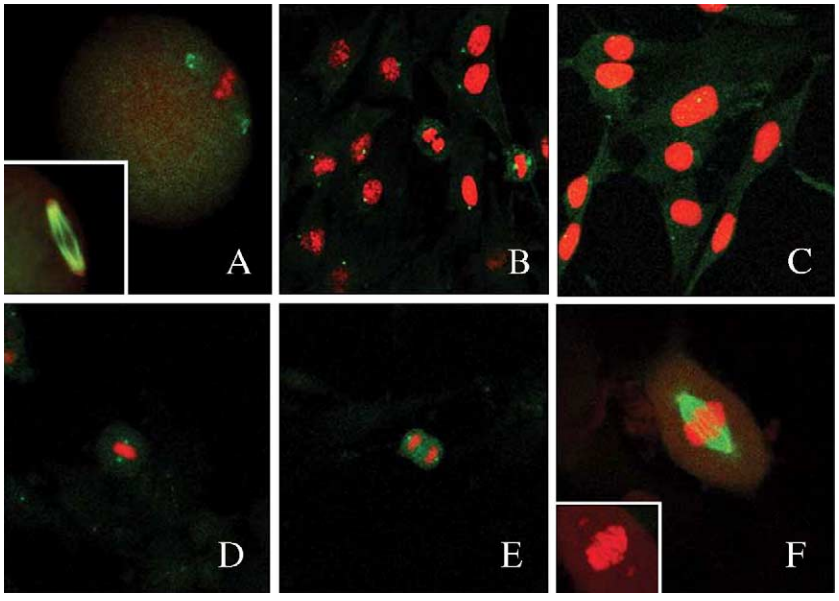

Fig. 2. Localization of γ-tubulin in mouse MII oocyte, mouse fibroblasts, and rat fibroblasts. Except when specifically noted, DNA, red; γ-tubulin, green. (A) γ-tubulin localized at the poles of mouse oocyte MII spindle. The inserted diagram shows double staining of α-tubulin (green) and γ-tubulin (red). (B and C) γ-tubulin dots localized near the rim of the mouse (B) and rat (C) interphase fibroblast nuclei. (D and E) γ-tubulin localized at the rat fibroblast metaphase (D) or anaphase (E) spindle poles. (F) Double staining of metaphase spindle microtubules and γ-tubulin. In inserted diagram, two lateral red dots representing γ-tubulin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

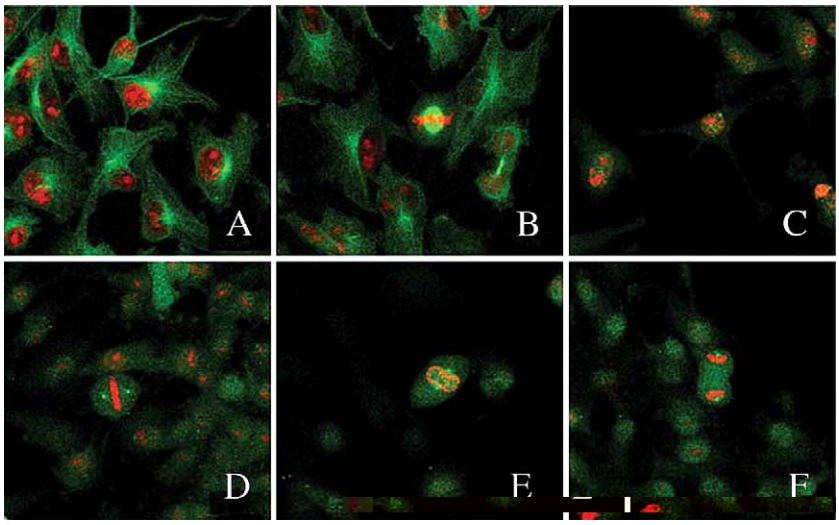

Fig. 3. Localization of γ-tubulin and microtubule assembly in porcine granulosa cells. Except when specifically noted, DNA, red; γ-tubulin, green. (A, B) Microtubule assembly stained with α-tubulin (green) in porcine interphase (A) and mitotic phase (B) granulosa cells. (C, D, E, and F) γ-tubulin dots located near the porcine granulosa cells' interphase nuclei (C), metaphase (D), anaphase (E), and telophase (F) spindle poles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to be tightly connected with the nucleus of the GV stage oocytes. After GVBD, the two centrosomes detached and translocated to the opposite sides of the oocyte, where each formed a pole of the meiotic spindle. Centrosomes gradually degraded during subsequent development and disappeared at the MII stage (Fig. 1).

The distribution of γ-tubulin in mouse fibroblasts, rat fibroblasts, porcine granulosa cells, and mouse oocytes

γ-tubulin was located at spindle poles of mouse MII oocyte. Double-staining with α-tubulin and γ-tubulin revealed that microtubules were displayed between γ-tubulin dots and metaphase chromosomes (Fig. 2). γ-tubulin dots were located near the interphase nuclei of mouse fibroblasts and rat fibroblasts, or in spindle poles at mitotic metaphase (Fig. 2). The distribution of γ-tubulin in porcine granulosa cells was similar to that of mouse fibroblasts and rat fibroblasts (Fig. 3).

The distribution of NuMA in somatic cells and oocytes

The NuMA antibody we used was produced to human NuMA epitope in mouse. The product manual notes that this

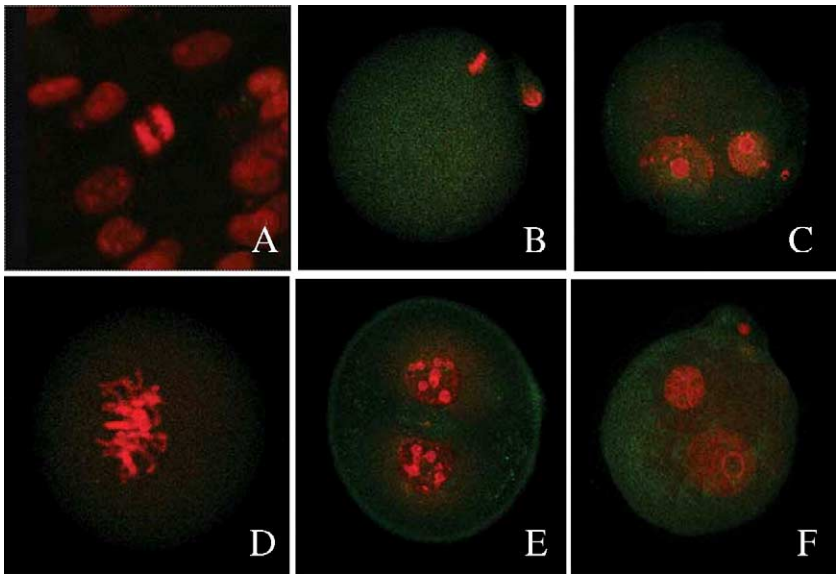

Fig. 4. NuMA antibody negatively stained NuMA epitope of mouse fibroblasts, MII oocytes, zygotes, or parthenogentically activated oocytes. DNA, red; NuMA, green. (A) Fibroblasts. (B) MII oocyte. (C) Pronucleus-stage zygote. (D) First mitotic-phase zygote. (E) 2-cell stage embryo. (F) Parthenogenetically activated pronucleus-stage egg. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antibody has no immune-cross reactivity to rat or mouse cells. Laser confocal microscopy showed that this NuMA antibody could not tag NuMA protein of mouse fibroblasts, parthenogentically activated oocytes, or fertilized eggs, confirming the antibody's lack of immune-cross reactivity to mouse cells (Fig. 4). However, this antibody had immune-cross reactivity to human or pig cells. NuMA was located in the nuclei of HeLa cells and porcine granulosa cells. During mitosis, from metaphase to anaphase, NuMA was localized in the spindle poles and then disappeared later at telophase (Fig. 5).

The distribution of centrin in mouse intraspecies SCNT reconstituted embryos and normal fertilized embryos

The enucleation of the MII spindle was proven to be 100% accurate after checking with Hoechst 33342 staining. Mouse intraspecies embryos were reconstituted by using fibroblasts as donor cells. Centrin was initially located near the nuclei of donor cells after electrofusion and then spread to the area surrounding the nucleus. Centrin did not noticeably concentrate in the poles of the transient spindle. Centrin was localized to the pseudo-pronuclei. Centrin also did not noticeably concentrate in the poles of first mitotic spindle but migrated to interphase nuclei of 2-cell stage reconstituted embryos. Similar to the reconstituted embryos, centrin showed no obvious concentration in mitotic spindle poles of normal fertilized embryos. Centrin was also localized in the pronuclei and 2-cell stage embryo interphase nuclei after fertilization (Fig. 6).

The distribution of γ-tubulin and spindle assembly in mouse intraspecies and rat–mouse interspecies SCNT reconstituted embryos

Mouse intraspecies SCNT embryos were reconstructed by using mouse fibroblasts as donor cells and rat–mouse interspecies SCNT embryos were reconstructed by using rat fibroblasts as donor cells. Recipients of both intraspecies and interspecies SCNT reconstituted embryos were mouse MII oocytes. γ-tubulin, which was localized to meiotic spindle poles, was removed during enucleation (Fig. 7). γ-tubulin was initially localized near the donor cell nucleus after electrofusion and then dispersed between chromosomes after the donor cell nucleus had undergone premature chromosome condensation. At 1–2 h of activation, γ-tubulin was concentrated in transient spindle poles. γ-tubulin dots were not detected outside of the pseudo-pronuclear membrane. When reconstituted embryos entered mitosis, γ-tubulin was distributed in spindle poles. Similar to that in pseudo-pronuclear stage embryos, γ-tubulin dots were not detected near the outside of interphase nuclear membranes in 2-cell stage reconstituted embryos (Fig. 8). Although γ-tubulin, which originally resided in the ooplasm (not in the MII spindle poles), possibly could be recruited and participate in the spindle assembly of reconstituted embryos, γ-tubulin that came from the donor cell obviously participated in the spindle assembly of reconstituted embryos. Except for the formation of the transient spindle, the microtubule assembly and spindle assembly in the reconstituted embryos were similar to that in fertilized eggs and parthenogentically activated eggs (Fig. 9).

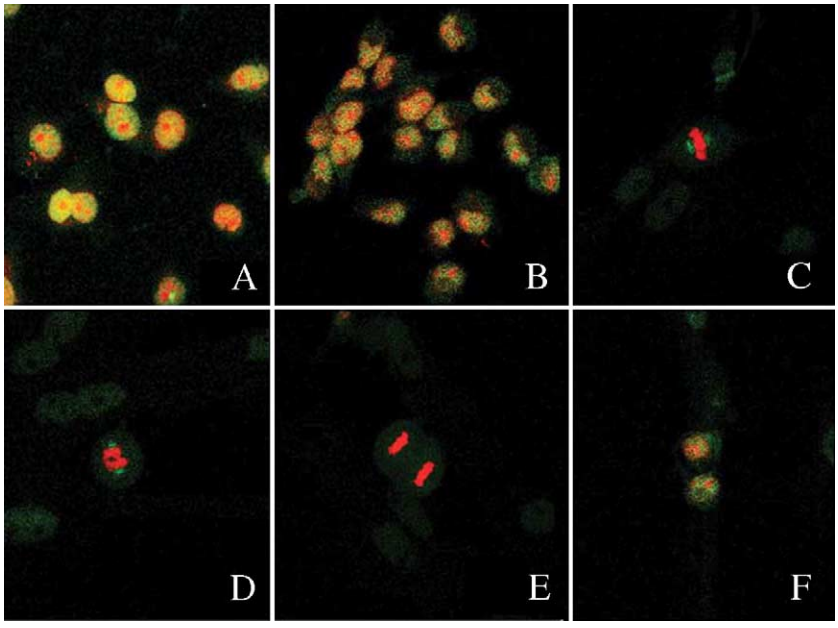

Fig. 5. Subcellular localization of NuMA in HeLa cells and porcine granulosa cells. DNA and NuMA were stained red and green, respectively. NuMA was located in HeLa cell (A) and porcine granulosa cell (B) interphase nuclei. NuMA was located in the two poles of porcine granulosa cell metaphase (C) and anaphase (D) spindles. NuMA disappeared from porcine granulosa cell telophase spindle poles (E). NuMA returned to two daughter cells nuclei (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

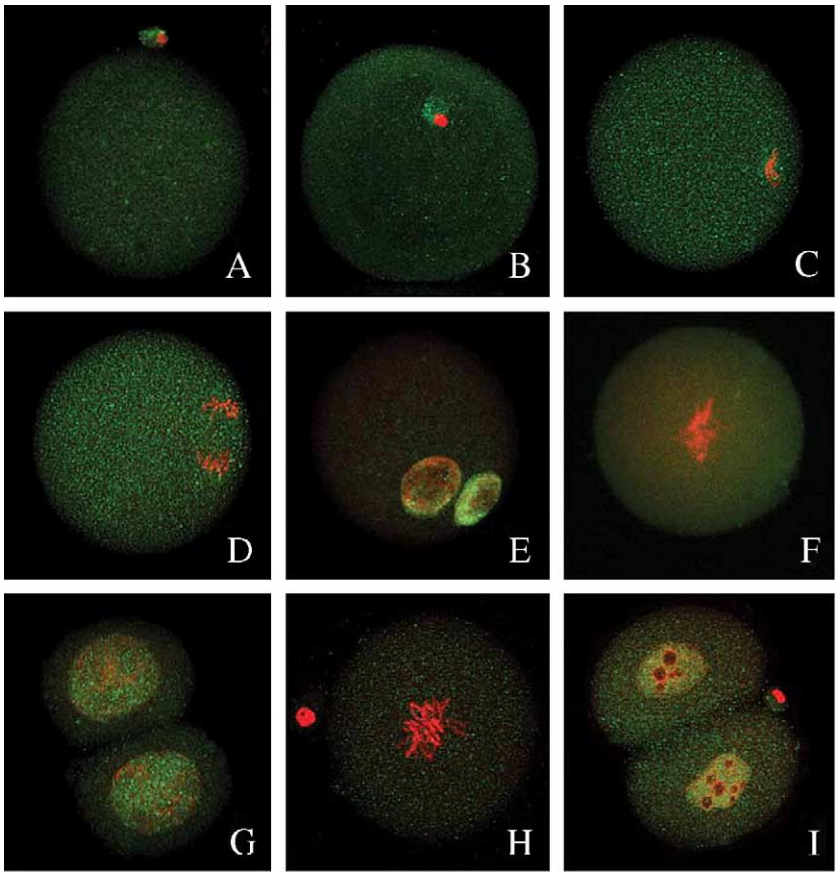

Fig. 6. Distribution of centrin in intraspecies reconstituted embryos and fertilized eggs. DNA and centrin were stained red and green, respectively. (A) Before electrofusion, centrin was located near the donor nucleus. (B) One hour after electrofusion, centrin was still located near the donor nucleus. (C) Centrin spread to the area surrounding the donor nucleus 15 min after activation. (D) One hour after activation, a transient spindle formed. No obvious centrin concentration at the transient spindle poles was observed. (E) Centrin was located in two pseudo-pronuclei 6 h after activation. (F) In the first mitotic metaphase of reconstituted embryos, centrin showed no obvious concentration at the spindle poles. (G) Centrin was located in 2-cell stage reconstituted embryo interphase nuclei. (H) In the first mitotic metaphase of fertilized eggs, centrin showed no obvious concentration at the spindle poles. (I) Centrin was located in 2-cell interphase nuclei after fertilization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The distribution of NuMA in pig–mouse interspecies SCNT reconstituted embryos

SCNT embryos were reconstituted by using porcine granulosa cells as donors. Similar to the distribution of γ-tubulin in reconstituted embryos, NuMA, which was initially located within the nucleus of donor cells after electrofusion, dispersed between the chromosomes after the donor cell nucleus had undergone premature chromosome condensation. NuMA was translocated into pseudo-pronuclei and moved to mitotic spindle poles after the pseudo-pronuclear membrane broke down and was relocalized in the interphase nuclei when embryos cleaved into 2 cells (Fig. 10).

Interspecies SCNT embryos were also reconstituted by using non-enucleated mouse oocytes. These pig–mouse interspecies embryos had both murine and porcine chromosomes after electrofusion. One hour after activation, both the set of donor cell chromosomes and the set of MII oocyte chromosomes split into two groups of chromosomes, and there were two pairs of chromosome groups. NuMA dispersed between one or two pairs of chromosome set. One or two transient spindles could be observed in these reconstituted embryos 1–2 h after activation. Accordingly, two or four pseudo-pronuclei could be found in these reconstituted embryos. After the pseudo-pronuclei broke down, NuMA translocated to the poles of mitotic spindle. After embryo cleavage, NuMA returned to the interphase nuclei (Fig. 11).

The effect of γ-tubulin antibody microinjection on spindle assembly and development of mouse intraspecies reconstituted embryos

Mouse intraspecies SCNT embryos were reconstituted by using mouse fibroblasts as donor cells and mouse MII oocytes as recipients. The antibody injected and sham injected control reconstituted embryos were scored for developmental progression to 2-cell stages or processed for immunofluorescence microscopy. The 2-cell rate of reconstituted embryos was decreased from 70.7% (29/41) in

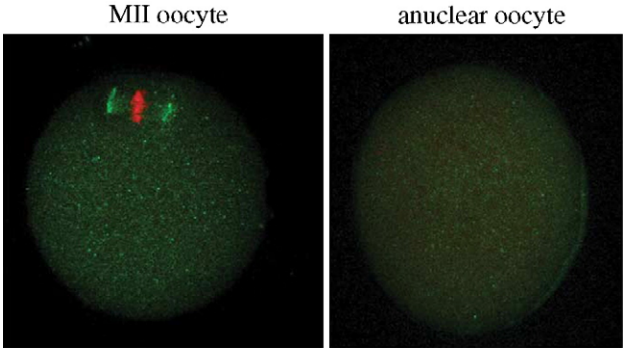

Fig. 7. Distribution of γ-tubulin in mouse MII oocytes before and after enucleation. DNA and γ-tubulin were stained red and green, respectively. Left: before enucleation, γ-tubulin was located in the MII spindle poles. Right: after enucleation, spindle pole γ-tubulin was removed together with the spindle during the enucleation process. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the control group to 29.7% (11/37) in the microinjected group ($P < 0.05$). Immunofluorescence microscopy showed that the percentage of normal spindle formation was decreased from 33.3% (9/27) in the control group to 9.1% (2/22) in the microinjected group ($P < 0.05$). Abnormal spindle could exhibit asymmetrical bipolar spindle or small spindle (Figs. 12B, C).

The effect of NuMA antibody microinjection on spindle assembly in pig–mouse interspecies reconstituted embryos

Pig–mouse SCNT interspecies embryos were reconstituted by using porcine granulosa cells as donor cells and mouse MII oocytes as recipients. The NuMA antibody injected and mouse IgG injected (as control) reconstituted embryos were fixed 16 h after activation and scored for developmental progression by immunofluorescent microscopy observation. At this time, most reconstructed embryos from both control (24/42) and microinjected (32/37) groups were at interphase and 1–4 pronucleus-like structures were observed in the ooplasm. Spindles were observed in 15 of 42 embryos in the control group and 4 of the 37 embryos in NuMA antibody microinjected group. What is worth to note is that over half (8/15) of the spindles were normal in the sham control group, while all 4 spindles observed in the NuMA microinjected embryos were abnormal. The abnormal spindles could be blunt spindle or tri-polar spindle (Figs. 12E, F). The percentage of 2-cell embryos was decreased from 7.1% (3/42) in the control group to 2.7% (1/37) in the microinjected group.

Discussion

Few studies have been reported on the fate of donor cell centrosomes in SCNT reconstituted embryos. In the present study, three antibodies against centrin, γ-tubulin, and NuMA, respectively, were used to tag centrosomes in SCNT embryos. Centrin dots were located near the nuclei of mouse interphase fibroblasts. Bright centrin dots were also found near the GV of oocytes and then deteriorated gradually during the subsequent maturation process. The results showed on one hand that centrin antibody could be used as a centrosome core marker and on the other hand that centrosomes containing two centrioles, gradually degenerated during oocyte maturation, which is in agreement with previous reports [6,7]. During fertilization, centrosomes or centrioles, typically introduced by sperm, recruit some pericentrosomal material from the ooplasm and initiates

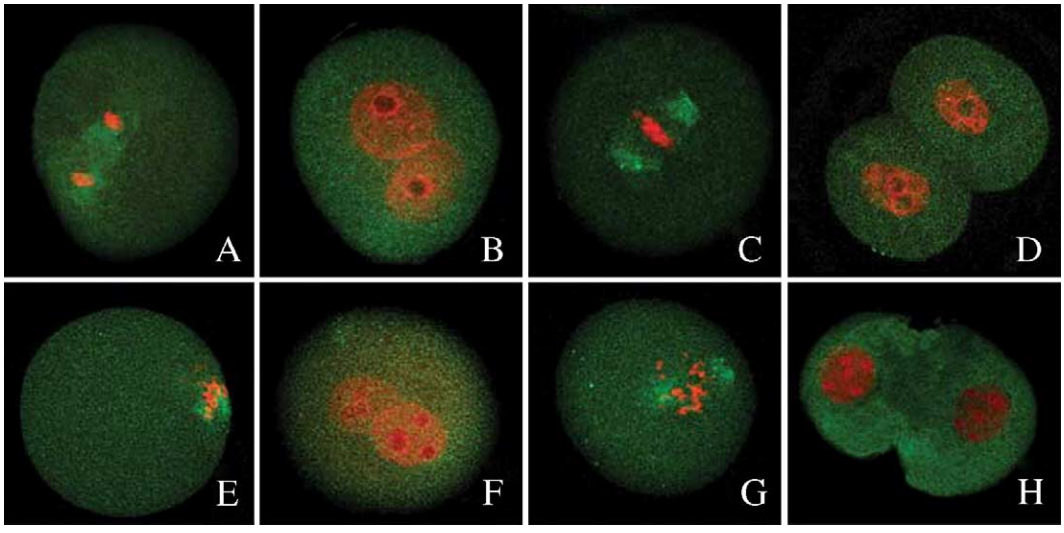

Fig. 8. Distribution of γ-tubulin in SCNT embryos reconstituted by using mouse fibroblasts or rat fibroblasts as donor cells. DNA and γ-tubulin were stained red and green, respectively. The distribution of γ-tubulin in the intraspecies reconstituted embryo (A–D) was similar to the interspecies reconstituted embryo (E–H). γ-tubulin was mainly concentrated in transient spindle poles (A and E). At pseudo-pronucleus stage, γ-tubulin showed no obvious concentration (B and F). γ-tubulin concentrated in two poles of the mitotic spindle (C and G). γ-tubulin showed no obvious concentration in 2-cell stage embryos (D and H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

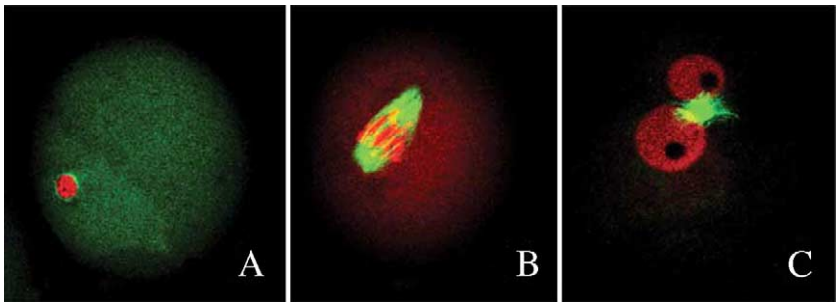

Fig. 9. Microtubule assembly in embryos reconstructed by using mouse fibroblasts as donor cells and mouse MII oocytes as recipients. DNA and α-tubulin were stained red and green, respectively. (A) Microtubules assembled into a ring around the donor nucleus 2.5 h after electrofusion. (B) Transient spindles formed 2 h after activation. (C) Two pseudo-pronuclei formed and enlarged 6 h after activation and a microtubule aster was assembled between them. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the restoration of fully functional centrosomes [21]. However, in the case of the mouse, no centrioles exist in fertilized eggs or embryos from fertilization through blastocyst stages. Centrioles degrade at mouse meiosis I, but centrosomal components participate in oocyte meiosis spindle assembly [22]. The mouse round spermatid centrosome contains a pair of centrioles. The distal centriole degenerates during the testicular stage of spermiogenesis, while the proximal centriole is lost during the epididymal stage [23]. In mouse intraspecies reconstituted eggs, centrin was observed near the donor cell nucleus but was detected neither in transient spindle poles nor in the spindle poles of the first mitosis. Although centrin signal was detected in pseudo-pronuclei and interphase nuclei, centrin dots were not detected outside the interphase nuclei. Because centrin is considered a reliable marker for centrioles, our results suggest that donor cell centrioles disappear or deteriorate in mouse SCNT embryos. We further observed the distribution of γ-tubulin, the permanently centrosome-associated microtubule nucleating protein, in mouse intraspecies and rat–mouse interspecies SCNT embryos. The distribution of γ-tubulin was similar in mouse intraspecies and rat–mouse interspecies SCNT embryos. Although γ-tubulin was located in the first mitotic spindle poles, γ-tubulin dots were not detected outside the pseudo-pronuclear and interphase nuclear membrane. The distribution of γ-tubulin in pseudo-pronuclear stage SCNT embryos was different from that in pronucleus-stage fertilized eggs. In the latter, several γ-tubulin dots were located at the region of ooplasm near the pronuclei [24,25]. Taken together, these results indicate that centrosomes introduced by donor cells disappear or deteriorate in mouse SCNT embryos and de novo assembly of centrosomes containing a pair of centrioles does not occur during the first mitotic cell cycle.

Previous reports showed that γ-tubulin participated in spindle assembly of reconstituted cow embryos; γ-tubulin was located in spindle poles, and one or two γ-tubulin dots were detected in association with interphase nuclei and were considered to represent reconstituted centrosomes [26]. In our experiments, the distribution of γ-tubulin was similar in

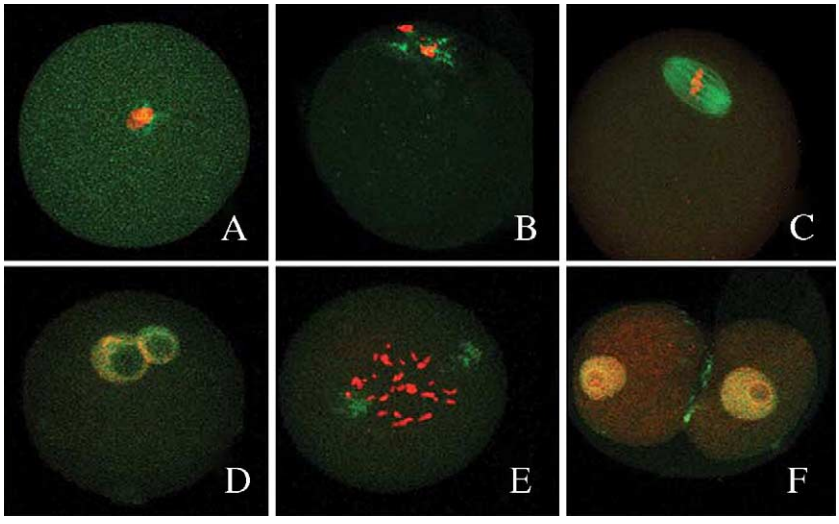

Fig. 10. Distribution of NuMA in interspecies SCNT embryos reconstituted by using porcine granulosa cells as donor cells. Except when specifically noted, DNA and NuMA were stained red and green, respectively. (A) After electrofusion, NuMA was located near the donor nucleus. (B) After 30 min of activation, NuMA dispersed between the chromosomes. (C) Transient spindles formed, which were stained with α-tubulin (green). (D) NuMA was observed in pseudo-pronuclei. (E) NuMA concentrated in two poles of the mitotic spindle. (F) NuMA translocated to 2-cell stage interphase nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

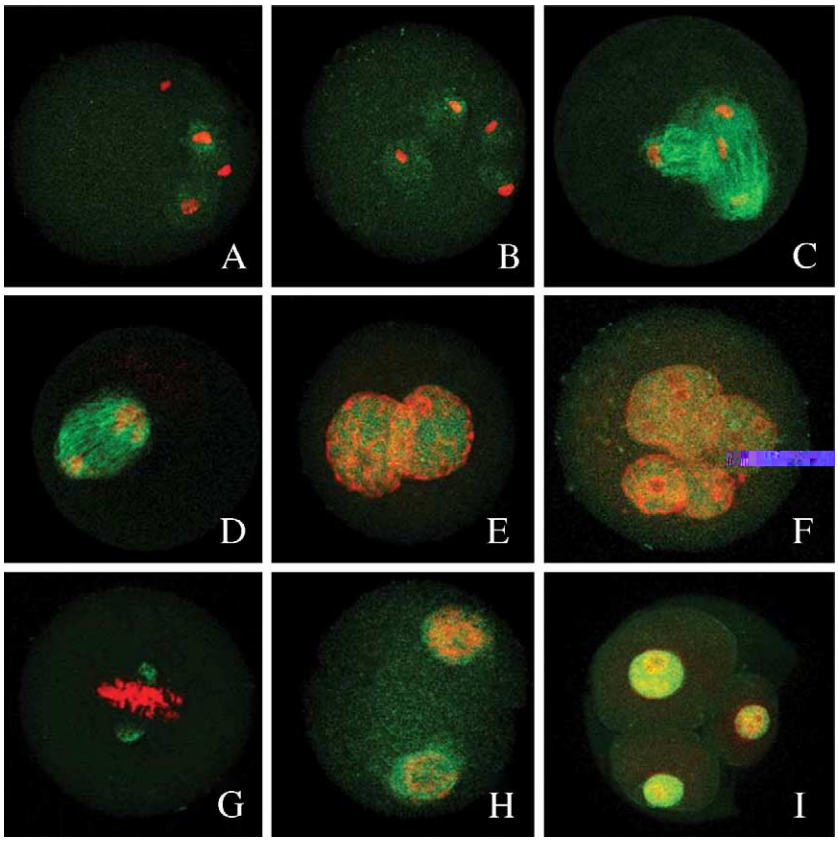

Fig. 11. Distribution of NuMA in interspecies embryos reconstituted by using pig granulosa cells and non-enucleated mouse oocytes. DNA and NuMA were stained red and green, respectively. One hour after activation, NuMA concentrated near one of two groups of separated chromosomes (A) or both groups of separated chromosomes (B). One hour after activation, one (C) or two (D) transient spindles formed. Green, α-tubulin. NuMA was located in two (E) or four (F) pseudo-pronuclei of reconstituted embryos 20 h after activation. NuMA was located in the first mitotic spindle poles (G). NuMA translocated to interphase nuclei of reconstituted embryos (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

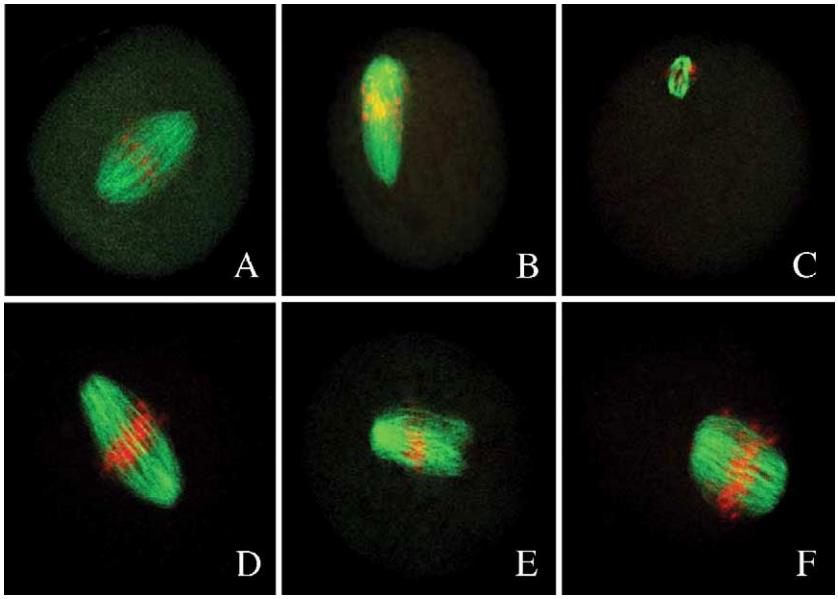

Fig. 12. Spindle assembly was disturbed after γ-tubulin or NuMA antibody microinjection into reconstituted embryos. DNA and α-tubulin were stained red and green, respectively. (A and D) Control group's normal first mitotic spindle. (B and C) γ-tubulin antibody microinjection group's abnormal first mitotic spindles. (E and F) NuMA antibody microinjection group's abnormal first mitotic spindles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mouse intraspecies and rat–mouse interspecies SCNT embryos. γ-tubulin dots were observed near the donor nucleus after electrofusion and then translocated to poles of the transient spindle during activation of reconstituted embryos. γ-tubulin translocated to the poles of the first mitotic spindle and participated in the assembly of the spindle. γ-tubulin antibody microinjection resulted in spindle disorganization and retardation of cell division. Abnormal spindles included asymmetrical bipolar spindles or small spindles accompanying with misalignment of chromosomes. Previous researches had reported that cells primed by microinjection of antibody failed to restore microtubules arrays after depolymerization induced by nocodazole or cold [20]. Depletion of γ-tubulin by RNAi increased the percentage of monopolar or asymmetrical bipolar spindle [27]. Our findings imply that γ-tubulin is essential for spindle assembly in reconstituted embryos. Donor cell centrosomes, defined as pericentriolar material containing a pair of centrioles, disassembled or deteriorated during the activation of mouse SCNT embryos. In contrast to somatic cells, no γ-tubulin staining dots were observed outside pseudo-pronuclei in reconstituted eggs. It is possible that γ-tubulin cannot assemble into an aggregated structure without a centriole as an adhering scaffold. Mouse MII oocytes possibly contain some factors that may cause centrioles disassembly or degradation. The mechanisms of this phenomenon remain to be elucidated. It also remains to be determined when centrioles are restored and reappear in reconstituted mouse embryos.

NuMA is a transiently associated centrosome protein that carries out important functions during mitosis. It is localized to spindle poles during mitosis and to the nucleus at interphase in somatic cells. Impairment of NuMA function causes abnormal spindle formation [28]. NuMA is concentrated at the broad meiotic spindle poles and cytoplasmic cytasters of mouse M II oocytes and then is translocalized to the male and female pronuclei after fertilization. Microinjection of anti-NuMA antibody into one of two blastomeres of 2-cell-stage mouse embryos inhibited normal cell division [11]. The removal of some rhesus oocyte proteins including NuMA during the process of nuclear transfer has been thought to be related to the high rate of abnormal first mitotic spindles [29].

In order to further prove the hypothesis that centrosomal material of donor cells participates in the spindle assembly in reconstituted embryos, NuMA distribution was observed in reconstituted embryos. The NuMA antibody used in this experiment did not recognize NuMA protein of mouse oocytes but recognized NuMA protein of porcine granulosa cells at different stages, so this antibody can be used for tracking the fate of NuMA protein that comes from the donor cell (porcine somatic cell), without interference from NuMA protein that comes from recipient mouse oocyte. In the pig–mouse interspecies reconstructed embryos, NuMA concentrated between the disarrayed chromosomes 15 min after activation and was translocated to transient spindle poles 1 h after activation. When pseudo-pronuclei formed, NuMA translocated into pseudo-pronuclei. After pseudo-pronuclear envelope breakdown, NuMA was located between the chromosomes and then translocated to spindle poles at first mitotic metaphase. The results indicate that NuMA participates in the formation of the transient spindle and the first mitotic spindle; NuMA also participates in transient spindle assembly and the first mitotic spindle assembly in embryos that were reconstituted by fusion of porcine granulosa cells and non-enucleated mouse oocytes. Since NuMA was considered to contribute to the formation and maintenance of focused microtubule arrays [28], NuMA microinjection was carried out. In our experiment, all spindles observed in NuMA antibody microinjected embryos were disorganized, showing blunt spindle and multipolar spindle (Figs. 12E, F). Disturbance of chromosome alignment was also evident (Fig. 12E). NuMA participates in the formation and stabilization of the spindle possibly by mechanisms that bundle microtubules and keep the force balance of microtubules. Taken together, these observations suggest that NuMA from donor cells contributes to the mitotic spindle function in reconstituted embryos and pericentriolar material might not be species-specific in nucleating microtubules and assembling mitotic spindles. Although the centrosomal material located in MII spindle poles were removed during enucleation, we cannot exclude the involvement of centrosomal material stored in the ooplasm, such as cytoplasmic γ-tubulin, in assembling mitotic spindles.

In conclusion, our findings indicate that (1) the donor cell centrosome containing a pair of centrioles is degraded in the SCNT reconstituted mouse embryos; (2) the components of donor cell centrosomes contribute to the formation and function of mitotic spindles of reconstituted embryos; and (3) components of donor cell centrosomes that participate in the regulation of spindle formation may not be species-specific.

Acknowledgments

This study was supported by grants from National Natural Science Foundation of China (30371037, 30225010, 30430530), NIH grant (R03-HD43829-02) and CAS Knowledge Innovation Program (KSCX2-SW-303).

References

[1] A. Kalt, M. Schliwa, Molecular components of the centrosome, Trends Cell Biol. 3 (1993) 118–128.
[2] D.R. Kellogg, M. Moritz, B.M. Alberts, The centrosome and cellular organization, Annu. Rev. Biochem. 63 (1994) 639–674.
[3] W. Zimmerman, C.A. Sparks, S.J. Doxsey, Amorphous no longer: the centrosome comes into focus, Curr. Opin. Cell Biol. 11 (1999) 122–128.
[4] M.M. Krioutchkova, G.E. Onishchenko, Structural and functional characteristics of the centrosome in gametogenesis and early embryogenesis of animals, Int. Rev. Cytol. 185 (1999) 107–156.
[5] G. Schatten, The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization, Dev. Biol. 165 (1994) 299–335.

[6] G. Schatten, C. Simerly, H. Schatten, Maternal inheritance of centrosomes in mammals? Studies on parthenogenesis and polyspermy in mice, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 6785–6789.
[7] H. Schatten, G. Schatten, Motility and centrosomal organization during sea urchin and mouse fertilization, Cell Motil. Cytoskeleton 6 (1986) 163–175.
[8] J.L. Salisbury, Centrin, centrosomes, and mitotic spindle poles, Curr. Opin. Cell Biol. 7 (1995) 39–45.
[9] R. Jeng, T. Stearns, Gamma-tubulin complexes: size does matter, Trends Cell Biol. 9 (1999) 339–342.
[10] A.M. Tassin, M. Bornens, Centrosome structure and microtubule nucleation in animal cells, Biol. Cell 91 (1999) 343–354.
[11] C.J. Tang, H.M. Hu, T.K. Tang, NuMA expression and function in mouse oocytes and early embryos, J. Biomed. Sci. 11 (2004) 370–376.
[12] C.H. Yang, M. Snyder, The nuclear-mitotic apparatus protein is important in the establishment and maintenance of the bipolar mitotic spindle apparatus, Mol. Biol. Cell 3 (1992) 1259–1267.
[13] M.K. Wang, D.Y. Chen, J.L. Liu, G.P. Li, Q.Y. Sun, J.L. Lui, In vitro fertilisation of mouse oocytes reconstructed by transfer of metaphase II chromosomes results in live births, Zygote 9 (2001) 9–14.
[14] I. Wilmut, A.E. Schnieke, J. McWhir, A.J. Kind, K.H. Campbell, Viable offspring derived from fetal and adult mammalian cells, Nature 385 (1997) 810–813.
[15] T. Wakayama, A.C. Perry, M. Zuccotti, K.R. Johnson, R. Yanagimachi, Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei, Nature 394 (1998) 369–374.
[16] M.K. Wang, J.L. Liu, G.P. Li, L. Lian, D.Y. Chen, Sucrose pretreatment for enucleation: an efficient and non-damage method for removing the spindle of the mouse MII oocyte, Mol. Reprod. Dev. 58 (2001) 432–436.
[17] C.L. Chatot, C.A. Ziomek, B.D. Bavister, J.L. Lewis, I. Torres, An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro, J. Reprod. Fertil. 86 (1989) 679–688.
[18] Y. Tsunoda, Y. Shioda, M. Onodera, K. Nakamura, T. Uchida, Differential sensitivity of mouse pronuclei and zygote cytoplasm to hoechst staining and ultraviolet irradiation, J. Reprod. Fertil. 82 (1988) 173–178.
[19] A. Ogura, K. Inoue, K. Takano, T. Wakayama, R. Yanagimachi, Birth of mice after nuclear transfer by electrofusion using tail tip cells, Mol. Reprod. Dev. 57 (2000) 55–59.
[20] H.C. Joshi, M.J. Palacios, L. McNamara, D.W. Cleveland, Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation, Nature 356 (1992) 80–83.
[21] C. Simerly, S.S. Zoran, C. Payne, T. Dominko, P. Sutovsky, C.S. Navara, J.L. Salisbury, G. Schatten, Biparental inheritance of gamma-tubulin during human fertilization: molecular reconstitution of functional zygotic centrosomes in inseminated human oocytes and in cell-free extracts nucleated by human sperm, Mol. Biol. Cell 10 (1999) 2955–2969.
[22] A. Can, O. Semiz, O. Cinar, Centrosome and microtubule dynamics during early stages of meiosis in mouse oocytes, Mol. Hum. Reprod. 9 (2003) 749–756.
[23] G. Manandhar, P. Sutovsky, H.C. Joshi, T. Stearns, G. Schatten, Centrosome reduction during mouse spermiogenesis, Dev. Biol. 203 (1998) 424–434.
[24] M.J. Palacios, H.C. Joshi, C. Simerly, G. Schatten, Gamma-tubulin reorganization during mouse fertilization and early development, J. Cell Sci. 104 (1993) 383–389.
[25] X.Q. Meng, H.Y. Fan, Z.S. Zhong, G. Zhang, Y.L. Li, D.Y. Chen, Q.Y. Sun, Localization of gamma-tubulin in mouse eggs during meiotic maturation, fertilization, and early embryonic development, J. Reprod. Dev. 50 (2004) 97–105.
[26] M.R. Shin, S.W. Park, H. Shim, N.H. Kim, Nuclear and microtubule reorganization in nuclear-transferred bovine embryos, Mol. Reprod. Dev. 62 (2002) 74–82.
[27] B. Raynaud-Messina, L. Moisand, A. Cirinesi, A. Mazzolini, M. Wright, Elongation of centriolar microtubule triplets contributes to the formation of the mitotic spindle in gamma-tubulin-depleted cells, J. Cell Sci. 117 (2004) 5497–5507.
[28] A.A. Levesque, L. Howard, M.B. Gordon, D.A. Compton, A functional relationship between NuMA and kid is involved in both spindle organization and chromosome alignment in vertebrate cells, Mol. Biol. Cell 14 (2003) 3541–3552.
[29] C. Simerly, T. Dominko, C. Navara, C. Payne, S. Capuano, G. Gosman, K.Y. Chong, D. Takahashi, C. Chace, D. Compton, L. Hewitson, G. Schatten, Molecular correlates of primate nuclear transfer failures, Science 300 (2003) 297.