

Comparison of Microtubule Distributions between Somatic Cell Nuclear Transfer and Parthenogenetic Porcine Embryos

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ABSTRACT

The aim of this study was to examine the microtubule distributions of somatic cell nuclear transfer (SCNT) and parthenogenetic porcine embryos. Porcine SCNT embryos were produced by fusion of serum-starved fetal fibroblast cells with enucleated oocytes. Reconstituted and mature oocytes were activated by electric pulses combined with 6-dimethylaminopurine treatment. SCNT and parthenogenetic embryos were cultured *in vitro* for 6 days. Microtubule assembly of embryos was examined by confocal microscopy 1 hr and 20 hr after fusion or activation, respectively. The proportions of embryos developed to the blastocyst stage were 25.7% and 30.4% in SCNT and parthenogenetic embryos, respectively. The frequency of embryos showing β -tubulins was 81.8% in parthenogenetic embryos, whereas 31.3% in SCNT embryos 1 hr after activation or fusion. The frequency of the embryos underwent normal mitotic phase was low in SCNT embryos (40.6%) compared to that of parthenogenetic ones (59.7%) 20 hr after fusion or activation ($p < 0.05$). The rate of SCNT embryos with an abnormal mitosis pattern is about twice compared to that of parthenogenetic ones. The spindle assembly and its distribution of SCNT embryos in the first mitotic phase were not different from those of parthenogenetic ones. The result shows that although microtubule distribution of porcine SCNT embryos shortly after fusion is different from parthenogenetic embryos, and the frequency of abnormal mitosis 20 hr after fusion or activation is slightly increased in SCNT embryos, microtubule distributions at the first mitotic phase are similar in both SCNT and parthenogenetic embryos.

(Key words : Somatic cell nuclear transfer, Microtubule distribution, Nuclear progression, Confocal microscopy, Porcine embryos)

INTRODUCTION

The nuclear reprogramming by which differentiated somatic cells are restored back to totipotent state is for the development of nuclear transfer (NT) embryos (Collas and Robl, 1991; Niemann and Reichelt, 1993). It means that the NT embryos develop to blastocysts with same cell numbers and timing as fertilized embryos and to term. This might be achieved through a nuclear remodeling, epigenetic modification, normal microtubule organization, and so on.

Typically, microtubules are organized in a radial fashion due to their anchorage at a centrally located microtubule organizing center (MTOC), centrosome, in almost animal cells. In somatic cells, centrosome is consisted of two centrioles and surrounded by pericentriolar materials (PCM) that is responsible for microtubule

nucleation (Kirshner and Mitchison, 1986). During the S phase, the centrosome is duplicated and the duplicated centrosomes are separated to serve as two mitotic spindle poles.

The construction of a bipolar spindle is essential for the accurate segregation of chromosomes in mitosis. Microtubules are necessary for the formation of a spindle and chromosome movement during mitosis. Microtubule configuration following NT using blastomere nuclei has been demonstrated in rabbits and cattle (Collas *et al.*, 1992; Pinto-Correia *et al.*, 1993; Nabara *et al.*, 1994). Despite of the importance of integrated events of nuclear and microtubule remodeling during somatic cell NT (SCNT), little information relevant to this subject is available. This study was conducted to examine the configuration of microtubules and nuclear progression of parthenogenetic and SCNT porcine embryos by immunofluorescent confocal microscopy.

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MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Cumulus-oocyte complexes (COCs) were aspirated from the antral follicles of the porcine ovaries collected from a slaughterhouse. Approximately 50~100 COCs were cultured in a 500- μ l drop of NCSU-23 medium (Petters and Wells, 1993) supplemented with 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 10 IU/ml equine chorionic gonadotropin (eCG; Intervet International B.V., Boxmeer, Holland), 10 IU/ml human chorionic gonadotropin (hCG; Intervet International B.V.), 10 ng/ml epidermal growth factor (EGF; Sigma), 10% (v/v) porcine follicular fluid, and 50 μ g/ml gentamicin (Sigma) covered with paraffin oil and incubated at 39°C in 5% CO₂ in air for 22 hr. COCs were then cultured in the same medium without hormones for 20 hr, and cumulus cells were removed by vortexing the COCs in PBS (Gibco-BRL, Grand Island, NY, USA) containing 0.1% PVA and 0.1% hyaluronidase (Sigma).

Preparation of Porcine Fetal Fibroblasts

A day 50 porcine female fetus was obtained from a pregnant gilt. The head and inner organs were removed, and the remaining tissues were chopped into small pieces. The tissues were enzymatically digested with 0.05% trypsin-EDTA in PBS for 30 min at 37°C with occasional stirring. The digested tissues were allowed to settle and the supernatant containing disaggregated cells was centrifuged. The cell pellet was resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 50 μ g/ml gentamicin (Sigma) and 15% fetal bovine serum (FBS, Gibco-BRL). Cells were subsequently passaged 3~5 times before being frozen and stored in liquid nitrogen. Before NT, cells were cultured for 2~3 days, and serum starved by replacing the culture medium with DMEM supplemented with 0.5% FBS for 3~5 days (Wilmut *et al.*, 1997).

Micromanipulation

Micromanipulation was carried out in HEPES-buffered TCM-199 (Gibco-BRL) supplemented with 3 mg/ml BSA and 5 μ g/ml cytochalasin B (Sigma). Matured oocytes were enucleated by aspiration the first polar body and metaphase II (MII) plate in a small amount of surrounding cytoplasm (about 20% of total volume). Enucleation was confirmed by staining the oocytes with 1 μ g/ml Hoechst 33342 (Sigma) for 15~20 min at 39°C. Fetal fibroblast cells were trypsinized and held in TCM-199 containing 3 mg/ml BSA before and during NT. A single cell was transferred into the perivitelline space of an enucleated oocyte.

Electrofusion and Activation

Reconstituted oocytes were placed between 0.2 mm diameter wire electrodes (1 mm apart) in a fusion chamber overlaid with 0.3 M mannitol (Sigma) solution containing 0.1 mM MgSO₄ (Sigma), 0.5 mM CaCl₂ (Sigma), and 3 mg/ml BSA. A single DC pulse of 1.5 kV/cm was applied for 30 μ sec using a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA, USA). SCNT embryos were further activated 1 h after the fusion treatment by exposure to two DC pulses of 1.0 kV/cm for 50 μ sec, followed by treatment with 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) for 4 h before *in vitro* culture (Kwon *et al.*, 2007).

Parthenogenesis and *In Vitro* Culture

Parthenogenetic embryos (parthenotes) were produced by exposing oocytes to the same electrical pulses and chemicals as those used for the activation of SCNT embryos. After activation of SCNT embryos and parthenogenesis, the embryos were cultured in 50 μ l droplets of PZM-3 (Yoshioka *et al.* 2002) supplemented with 3 mg/ml BSA overlaid with paraffin oil for 6 days at 39°C under an atmosphere of 5% CO₂ in air.

Immunofluorescent Staining and Confocal Microscopy

At 1 hr or 20 hr after fusion or parthenogenetic activation, nuclear progression and microtubule distribution of embryos were examined by confocal microscopy. Microtubules and DNA were detected by indirect immunocytochemical technique. SCNT and parthenogenetic embryos were fixed with 3.7% (w/v) paraformaldehyde in PBS for 1 hr at room temperature. Fixed embryos were stored in PBS containing 0.3% (w/v) BSA and 0.02% sodium azide for up to 1 wk at 4°C. Fixed embryos were permeabilized by incubation with PBS containing 0.1% (w/v) Triton X-100 and 3 mg/ml BSA, and 0.02% sodium azide for 40 min at 39°C. After washing twice with PBS containing 0.01% Triton X-100 and 0.3% BSA, oocytes were incubated in blocking solution of PBS containing 150 mM glycine, 0.3 mg/ml BSA, and 0.02% sodium azide for 30 min at 39°C. To evaluate β -tubulin and DNA, multiple fluorescence labeling using double-analysis was performed. The microtubules were immunolabeled with a mouse monoclonal antibody against β -tubulin (Sigma). Primary antibody was detected using Alaxa-488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Antibodies were diluted in the blocking solution [1:200 (v/v)] before use. Embryos were incubated with primary and secondary antibody for each 40 min at 39°C, followed by three washes for 5 min each. DNA was stained with 10 μ g/ml propidium iodide (PI, Sigma) for 20 min at 39°C. Finally, Embryos were mounted on glass slides in Vecta-Shield anti-fade (Vector Laboratories, Burlingame, CA, USA) under coverslip. The samples were examined

with a laser-scanning confocal microscope (Zeiss LSM 510, Jena, Germany).

Statistical Analysis

At least five replicate trials for each treatment were conducted. Data were analyzed by Duncan's multiple range test using General Models procedure in Statistical Analysis System (SAS Institute, Inc., Cary, NC).

RESULTS

In Vitro Development of Embryos

The proportions of SCNT embryos cleaved (76.2%, 80/105) and developed to the blastocyst (25.7%, 27/105) were not different from those of parthenogenetic embryos (81.4% and 30.4%, respectively, Table 1). Mean cell numbers in blastocysts were also similar in both NT (24.8±2.2) and parthenogenetic embryos (26.4±3.1).

Microtubule Distribution Shortly after Fusion or Activation

The microtubule distributions of SCNT and parthenogenetic embryos 1 hr after fusion or activation were examined. As shown in Table 2, the frequency of embryos showing a β -tubulin was 31.3% (15/48) in SCNT embryos (Fig. 1A), and 68.7% of SCNT embryos did not show the microtubules in their cytoplasm (Fig. 1B). Whereas, 81.8% (45/55) of parthenogenetic embryos showed the microtubules (Fig. 1C).

Table 2. Microtubule organization of SCNT and parthenogenetic embryos 1 hr after fusion or activation

Treatment	No. of oocytes	Microtubule distribution (%)	
		β -tubulin	Non-tubulin
Parthenotes	55	45(81.8) ^a	10(18.2) ^a
NTs	48	15(31.3) ^b	33(68.7) ^b

* Parthenotes, parthenogenetic embryos; NTs, nuclear transfer embryos.

^{a,b} Values with different superscripts in the same column are significantly different ($p < 0.05$).

Microtubule Distribution in the First Mitotic Phase

The nuclear progression and microtubule distribution in the first mitotic phase of SCNT and parthenogenetic embryos were examined 20 hr after fusion or activation. As shown in Table 3, the frequency of embryos did not induce the mitosis and showing an interphase nucleus (Fig. 2A, B) was observed 33.3% (23/69) and 26.9% (18/67) in SCNT and parthenogenetic embryos, respectively. The frequency of the embryos showing a normal mitotic phase (Fig. 2C and Fig. 3) in SCNT embryos (40.6%, 28/69) was significantly lower ($p < 0.05$) than that of parthenogenetic embryos (59.7%; 40/67). The proportion of embryos in abnormal mitosis (Fig. 2D) was not significantly different between SCNT (26.1%) and parthenogenetic (13.4%) embryos, but the rate of SCNT embryos with an abnormal mitosis pattern is about twice compared to that of parthenogenetic ones.

Table 1. *In vitro* development of SCNT and parthenogenetic embryos

Treatment*	No. of oocytes	No. (%) of embryos			Cell no. in blastocysts (Mean±SEM)
		Cleaved	Fragment	Blastocyst	
Parthenotes	102	83(81.4)	15(14.7)	31(30.4)	26.4±3.1
NTs	105	80(76.2)	16(15.2)	27(25.7)	24.8±2.2

* Parthenotes, parthenogenetic embryos; NTs, nuclear transfer embryos.

Table 3. Nuclear progression of SCNT and parthenogenetic embryos 20 hr after fusion or activation*

Treatment	No. of NTs	Nuclear progression				Abnormal mitosis**
		1 N	Normal mitosis		Total	
			M-phase	2 N		
Parthenotes	67	18(26.9)	25(37.3) ^a	15(22.4) ^a	40(59.7) ^a	9(13.4)
NTs	69	23(33.3)	15(21.7) ^b	13(18.8) ^a	28(40.6) ^b	18(26.1)

* Parthenotes, parthenogenetic embryos; NTs, nuclear transfer embryos; M, mitotic; PN, pronucleus.

** Chromatin condensation and NEBD were occurred but microtubules were not detected.

^{a,b} Values with different superscripts in the same column are significantly different ($p < 0.05$).

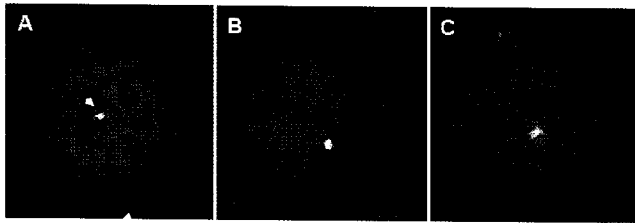


Fig. 1. Microtubule distribution in SCNT and parthenogenetic embryos 1 hr after fusion or activation. Nucleus (PI; red) and β -tubulin (green) of parthenogenetic or SCNT embryos were visualized by laser scanning confocal microscope. (A) SCNT embryos showing microtubule (arrow head) or (B) non-tubule. (C) Parthenogenetic embryos showing microtubules

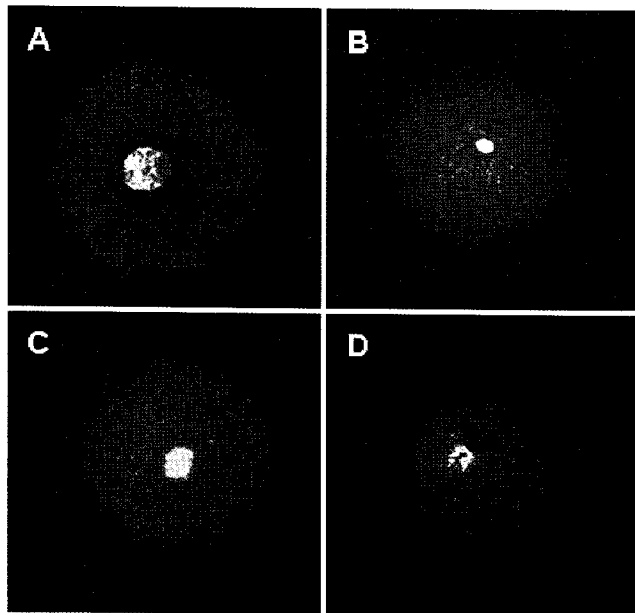


Fig. 2. Microtubule distribution in SCNT embryos 20 hr after fusion or activation. Nucleus (PI; red) and β -tubulin (green) of SCNT embryos were visualized by laser scanning confocal microscope. Large (A) and small (B) interphase nuclei were organized but microtubules were not assembled. C) Microtubules were associated with condensed chromosome. D) Chromatin condensation and NEBD were occurred but microtubules were not detected. This is classified as an abnormal pattern.

Microtubule distributions in the first mitosis were very similar in both SCNT and parthenogenetic embryos (Fig. 3).

DISCUSSION

In previous studies, bovine parthenogenetic embryos unexpectedly formed functional bipolar spindles at mitotic phase which permit embryo development to the blastocyst stage (Navara *et al.*, 1994). During the first interphase stage, microtubules were not detected but

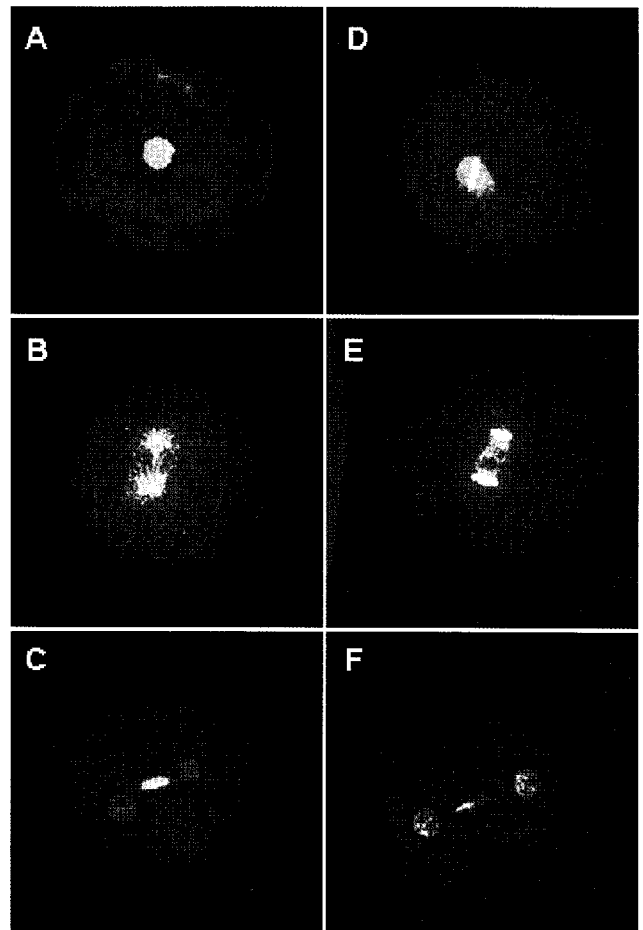


Fig. 3. Microtubule distribution in SCNT and parthenogenetic embryos 20 hr after fusion or activation. Nucleus (PI; red) and β -tubulin (green) of SCNT (A~C) and parthenogenetic (D~F) embryos were visualized by laser scanning confocal microscope. A, D) Chromosomes became aligned at the equator of spindle by microtubules barrel-shaped in metaphase. B, E) As the aligned chromosomes separated, the spindle elongated and assembled astral microtubules at anaphase or telophase. C, F) Finally, chromosomes were distributed into two daughter cells by cytokinesis and spindle microtubules were disappeared followed formation of pronucleus.

reorganized and associated with the chromosome condensation and NEBD in pro-metaphase. In metaphase, anastral bipolar microtubules are organized, and each chromosome is surrounded by astral microtubules in anaphase. After then microtubules were gradually disappeared and not observed in two pronucleus stage. It was suggested that the porcine oocyte are able to form a functional MTOC instead of contribution by sperm (Navara *et al.*, 1994; Kim *et al.*, 1996; Thomson *et al.*, 1998; Campbell, 1999).

Normal microtubule organization is important for the reprogramming of SCNT embryos (Zhong *et al.*, 2005). Microtubule configuration following embryonic cell NT in rabbit (Collas *et al.*, 1992) and cattle (Navara *et al.*, 1994), and SCNT in mouse (Nguyen *et al.*, 2004) and

rat (Tomioka *et al.*, 2007) has been demonstrated. In mouse cumulus cell NT, most of SCNT embryos organized monopolar spindle at 10 min after NT, then, monopolar spindle were transformed into bipolar spindles during 30-60 min after NT (Nguyen *et al.*, 2004). In rat SCNT, ring-shaped microtubule was organized after activation (Tomioka *et al.*, 2007). In this study, spot-liked microtubule was observed in only 31% of SCNT embryos, and microtubule was not seen in the majority of SCNT embryos 1 hr after fusion. The microtubules were reorganized and associated with the condensed chromosome after NEBD in pro-metaphase as shown in parthenogenetic embryos. When the donor cells were transferred into the porcine oocyte cytoplasm, the γ -tubulins of donor cells were rapidly disappeared (Zhong *et al.*, 2007), which difference between mouse, rat and pig might be due to the nuclear remodeling, observation time of microtubules or species specificity (Nguyen *et al.*, 2004; Tomioka *et al.*, 2007; Zhong *et al.*, 2007).

Our results showed that although microtubule distribution of porcine SCNT embryos shortly after fusion was different from parthenogenetic ones, microtubule distribution at the first mitotic phase was similar in both SCNT and parthenogenetic embryos on the whole (see Fig. 3). However, the proportion of SCNT embryos showing abnormal mitosis 20 hr after fusion was twice as much as parthenogenetic embryos. These may reflect an incomplete reprogramming of SCNT embryos (Shin *et al.*, 2002; Dai *et al.*, 2006).

In conclusion, our result shows that although microtubule distribution of porcine SCNT embryos shortly after fusion is different from parthenogenetic embryos, and the frequency of abnormal mitosis 20 hr after fusion or activation is slightly increased in SCNT embryos, microtubule distributions at the first mitotic phase are similar in both SCNT and parthenogenetic embryos. Further investigation about relationship between nuclear reprogramming and microtubule organization is needed.

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