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—Full Paper—

Spindle Formation and Microtubule Organization During First Division in Reconstructed Rat Embryos Produced by Somatic Cell Nuclear Transfer

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Abstract. The present study was conducted to demonstrate the spindle formation and behavior of chromosomes and microtubules during first division in reconstructed rat embryos produced by somatic cell nuclear transfer (SCNT) with cumulus cell nuclei. To demonstrate the effect of oocyte aging after ovulation on the cleavage of SCNT embryos, micromanipulation was carried out 11, 15 and 18 h after injection of hCG. SCNT oocytes were activated by incubation in culture medium supplemented with 5 μM ionomycin for 5 min followed by treatment with 2 mM 6-dimethylaminopurine (6-DMAP) in mR1ECM for 2–3 h. For immunocytochemical observation, the SCNT embryos were incubated with monoclonal anti-α-tubulin antibody and then fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Cleavage rates were significantly higher for oocytes collected after 15 and 18 h rather than for those collected 11 h after injection of hCG (56 and 53%, respectively *vs.* 28%; $P<0.05$). Premature chromosome condensation occurred before activation of the SCNT oocytes, but adequate spindle formation was only rarely observed. The distribution of microtubules in SCNT embryos after activation was different from those of fertilized and parthenogenic oocytes, i.e., a dense microtubule organization shaped like a ring was observed. Eighteen to 20 h post-activation, most SCNT embryos were in the 2-cell stage, but no nucleoli were clearly visible, which was quite different from the fertilized oocytes. In addition, first division with and without small cellular bodies containing DNA was observed in the rat SCNT embryos in some cases. The present study suggests that reorganization of transferred nuclei in rat SCNT embryos may be inadequate in terms of formation of the mitotic assembly and nucleolar reorganization.
Key words: Microtubule, Nuclear transfer, Rat, Somatic cell nuclear transfer (SCNT), Spindle formation

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Mammalian somatic cell cloning has progressed dramatically in recent years and now promises significant improvements in the generation of genetically modified animals for agricultural and biomedical purposes [1]. In addition, cloning provides us with unique experimental models for studying key mechanisms in mammalian development, such as genome reprogramming, genomic imprinting, DNA methylation, and telomere restoration, because it generates individual copies of donors by bypassing the normal reproduction process [2].

The rat is an important rodent model for studies in physiology, pathobiology, toxicology, neurobiology and a variety of other disciplines [3];

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this animal is of value because it is larger than the mouse and because a plethora of organ-specific physiologic and disease models have been developed for it over the last century [4]. The importance of the rat as a biological model has led to an intense effort to also establish it as a strong genetic model. However, for species such as the rat [5–7] and rhesus monkey [8], laboratories have failed to obtain offspring by SCNT; recently, fertile cloned rats have been obtained [9]. Previously, we showed that a combined treatment of electrical stimulation or ethanol and 6-DMAP induces parthenogenetic activation, and the reconstructed embryos produced by SCNT with cumulus cell nuclei using this method developed to the 2-cell stage *in vitro*, but not to term *in vivo* [10]. Normal development of cloned embryos depends on maintaining normal ploidy by coordinating the cell cycles of the recipient oocyte and donor nucleus [1]. For example, when mammalian oocytes arrested at metaphase II (MII) are used as recipients, normal ploidy is maintained if the donor nuclei are awaiting DNA replication (G0 or G1). Alternatively, advantage may be taken of the fact that ploidy is also changed if chromosomes are shed in a polar body. Although these general principles were established relatively quickly, much more information is needed concerning the mechanisms involved in different species [11]. In murine and bovine species, when a donor nucleus in the G0/G1 stage of the cell cycle is transferred to an enucleated oocyte, premature chromosome condensation (PCC) of the transferred nucleus is related to the success of cloning and probably reprogramming [12]. Therefore the timing of oocyte activation relative to SCNT must also be taken into consideration [13]. In rat SCNT embryos, however, there is little information available about the changes in donor nuclei after injection in the course of development to the first cleavage stage or indeed formation of the mitotic spindle assembly, especially considering thatspontaneous oocyte activation in the rat can be induced *in vitro* immediately after removal of the oviductal tube [14, 15], which may be seriously committed in course of production of SCNT embryos of this species.

In the present study, we demonstrated the effects of oocyte collection time after hCG injection on the pronuclear formation and cleavage of SCNT embryos and on the behavior of chromosomes and microtubules during first cleavage in an effort to examine the possibility of producing rat SCNT embryos with higher levels of developmental competence and normality.

Materials and Methods

The present study was approved by the Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research of the Graduate School of Agricultural Science, Tohoku University, Japan.

Animals, feed and housing

Wistar-Imamichi rats (WI; Institute for Animal Reproduction, Fukaya, Japan) were used throughout the experiments. Animals were kept in polycarbonate cages (25 × 40 × 20 cm) with wood shavings under controlled conditions with lights on at 0800 and off at 2000 h and were given food and tap water *ad libitum*.

Oocyte collection

Oocytes were collected as described previously [10]. Briefly, immature female rats were injected intraperitoneally with 10 IU of pregnant mare serum gonadotropin (PMSG, Serotropin; Teikoku zoki, Tokyo, Japan) followed by 10 IU of human chorionic gonadotropin (hCG, Puberogen; Sankyo, Tokyo, Japan) 48–52 h later. Oviducts were removed 11–18 h after administration of hCG, and cumulus-oocyte complexes (COCs) were collected by flushing the oviducts with culture medium (mR1ECM) [16] supplemented with 25 mM Hepes. COCs were treated with 0.1% (w/v) hyaluronidase (Sigma, St. Louis, MO, USA), and cumulus cells were collected as karyoplasts and stored until use. After being washed three times, oocytes were transferred to 100 μl of mR1ECM and used as recipient cytoplast.

Nuclear transfer

Micromanipulation was carried out using a micromanipulator (Olympus, Tokyo, Japan) with the piezo drive system as described previously [10]. In brief, after the oocytes were transferred to 100 μl of mR1ECM supplemented with 25 mM Hepes (Sigma) and 1 μg/ml of cytochalasin B (Sigma), they were enucleated by removing the MII spindle bodies with a small volume of ooplasm using a micropipette having an internal diameter of 20 μm

and injected with donor nuclei via a blunt-ended micropipette having an internal diameter of 6–9 μm. To demonstrate the effect of oocyte aging after ovulation on cleavage of the produced SCNT embryos, micromanipulation was carried out 11, 15 and 18 h after injection of hCG. The SCNT embryos were washed three times and cultured in highly humidified 5% CO_2 in air at 37 C until activation.

Activation

SCNT oocytes were activated by incubation in culture medium supplemented with 5 μM ionomycin (Sigma) for 5 min followed by treatment with 2 mM 6-DMAP (Sigma) in mR1ECM for 2–3 h. After activation, the SCNT embryos were transferred to 100 μl of culture medium and cultured.

Embryo transfer of SCNT embryos

After nuclear transfer using the oocytes collected 15 h after injection of hCG, the SCNT embryos developed to the pronuclear stage or the 2-cell stage were transferred into the oviducts of pseudopregnant WI females on Day 1 of pregnancy (the day a vaginal plug was detected). The recipient females were examined on Day 13 of pregnancy for assessment of fetal development.

Immunocytochemical examination of chromosomes and microtubules

After being washed three times in Dulbecco's phosphate buffered saline (PBS; Nissui, Tokyo, Japan) containing 0.1% polyvinyl alcohol (PBS-PVP; Sigma), the specimens were fixed in 2% paraformaldehyde (Wako, Osaka, Japan) in PBS-PVP supplemented with 0.2% Triton X-100 (Wako) for 40 min. *In vivo* fertilized embryos were used for comparison. After fixation, the specimens were washed in PBS-PVP twice for 15 min each, and stored in PBS-PVP containing 1% bovine serum albumin (BSA-PBS-PVP; Sigma) overnight. The following day, the specimens were treated for blocking with 10% fetal calf serum (Gemini Bio-Products, CA, USA) in BSA-PBS-PVP for 40 min, incubated in BSA-PBS-PVA containing a monoclonal anti-α-tubulin antibody (1:250 dilution; Sigma) at 4 C overnight, washed in BSA-PBS-PVP 3 times for 15 min each, and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:150 dilution; Organon Teknika, Durham, NC, USA) for 40 min at room temperature. After being washed in PBS-PVP 3 times for 15 min each, they were stained with propidium iodide (PI; Sigma) for 1 h and then mounted onto slide glasses. Immunocytochemical examination was carried out using a confocal microscope (MRC-1024, Bio-Rad; Hercules, CA, USA). The Laser Sharp Processing software (Bio-Rad) was used to analyze the confocal images [17].

Observation of spontaneous activation

To investigate the time course of spontaneous activation during *in vitro* culture, the oocytes were collected 15 h after injection of hCG and cultured for 24 h in mR1ECM. The specimens were fixed 0, 2, 5 and 24 h after culture and subjected to immunocytochemical examination as described above.

Statistical analysis

All data were analyzed with the χ^2 test using the StatView software (SAS Institute, NC, USA), and differences with $P<0.05$ were considered statistically significant.

Results

Effect of oocyte collection time after hCG injection on pronuclear formation and cleavage in SCNT embryos

The pronuclear formation and cleavage rates of the SCNT embryos are shown in Table 1. The rates of pronuclear formation and cleavage were

Table 1. Effect of oocyte collection time after hCG injection on pronuclear formation and cleavage in SCNT embryos

Time of collection after hCG injection (h)	No. of SCNT embryos cultured	No. (%) of SCNT embryos showing	
		pronuclear formation	cleavage
11	74	26 (35)[a]	21 (28)[a]
15	72	41 (57)[b]	40 (56)[b]
18	110	70 (64)[b]	58 (53)[b]

a–b: $P<0.05$.

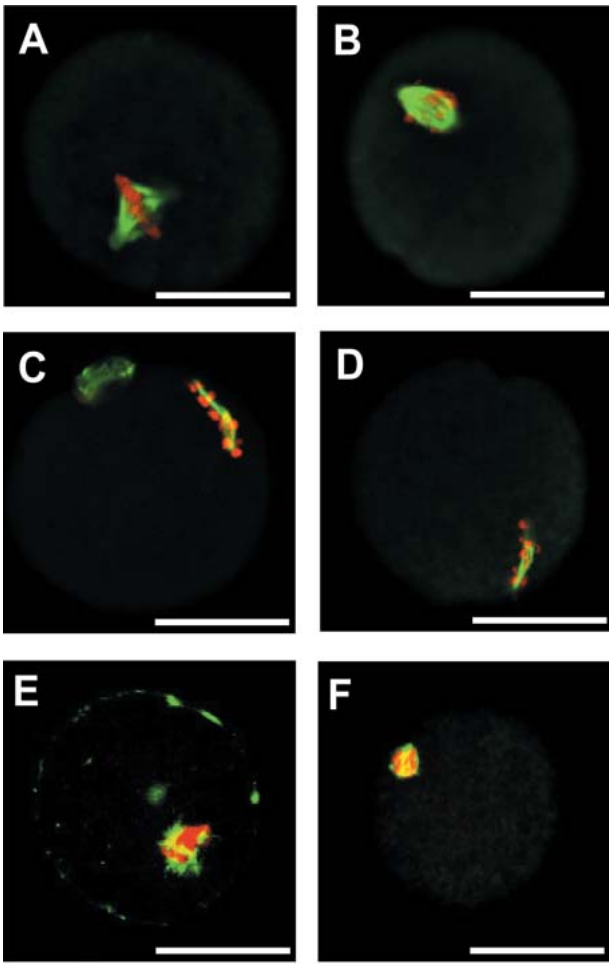

Fig. 1. The status of chromosomes (red) and microtubules (green) in SCNT oocytes just before activation. Proper spindle formation with PCC (A and B) was only observed in 20% (14/69) of SCNT oocytes. Aberrant spindle assembly with PCC (C and D) was observed in 38% (26/69) of SCNT oocytes, and the remaining 42% (29/69) of SCNT oocytes exhibited neither spindle formation nor PCC (E and F). Bars=50 μm.

significantly higher for oocytes collected 15 and 18 h rather than for those collected 11 h after injection of hCG (57 and 64%, respectively, *vs.* 35%; 56 and 53%, respectively, *vs.* 28%; $P<0.05$), which is thought to be just after ovulation. This result suggested that cytoplasmic maturation after ovulation is critical for support of formation of pronuclear-like structures and embryo cleavage after SCNT in rats. We selected 15 h after injection of hCG as the time of oocyte collection because the cytoplasmic maturation of oocytes at this point is considered to be the same as that at 18 h after hCG injection.

Transfer of SCNT embryos

A total of 571 SCNT embryos were transferred to the oviducts of 35 recipient females on day 1 of pseudopregnancy, which was the day after the recipient females were mated with vasectomized males. Examination of the uteri on Day 13 of pregnancy revealed 43 implantation sites in 21 recipients. None of these SCNT embryos developed into a fetus.

Behavior of chromosomes and microtubules during first cleavage in SCNT embryos

The oocytes collected 15 h after injection of hCG were reconstructed by SCNT and the resultant embryos were subjected to immunocytochemical analysis of the injected donor nuclei during first cleavage. Just before activation, that is, 2 h after the oocytes were collected, the status of the chromosomes in 20% (14/69) of the SCNT oocytes showed a transition to a prometaphase-like structure that seemed to be PCC (Fig. 1A and B). These SCNT oocytes exhibited an almost proper spindle formation in which microtubules formed

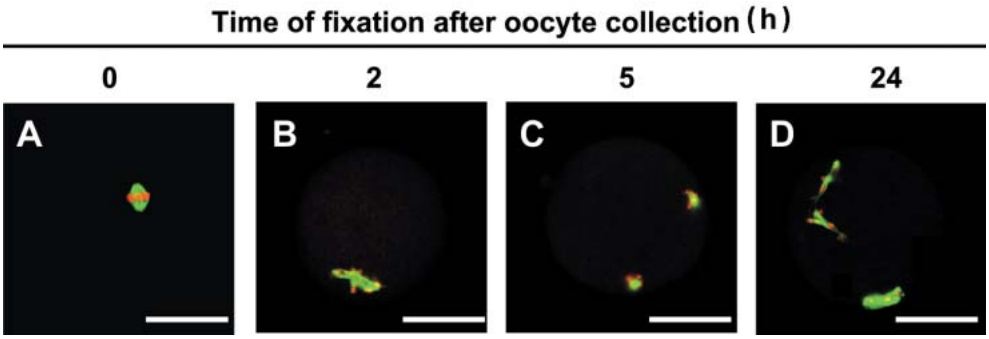

Fig. 2. Behavior of chromosomes (red) and microtubules (green) during spontaneous activation in oocytes cultured *in vitro* for 24 h. All ovulated oocytes immediately after collection (A) had chromosomes at the MII stage. Oocytes 2 (B) and 5 h after collection (C) showing spontaneous activation. Oocytes 24 h after collection (D) showing spontaneous activation but no formation of microtubules. Bars=50 μm.

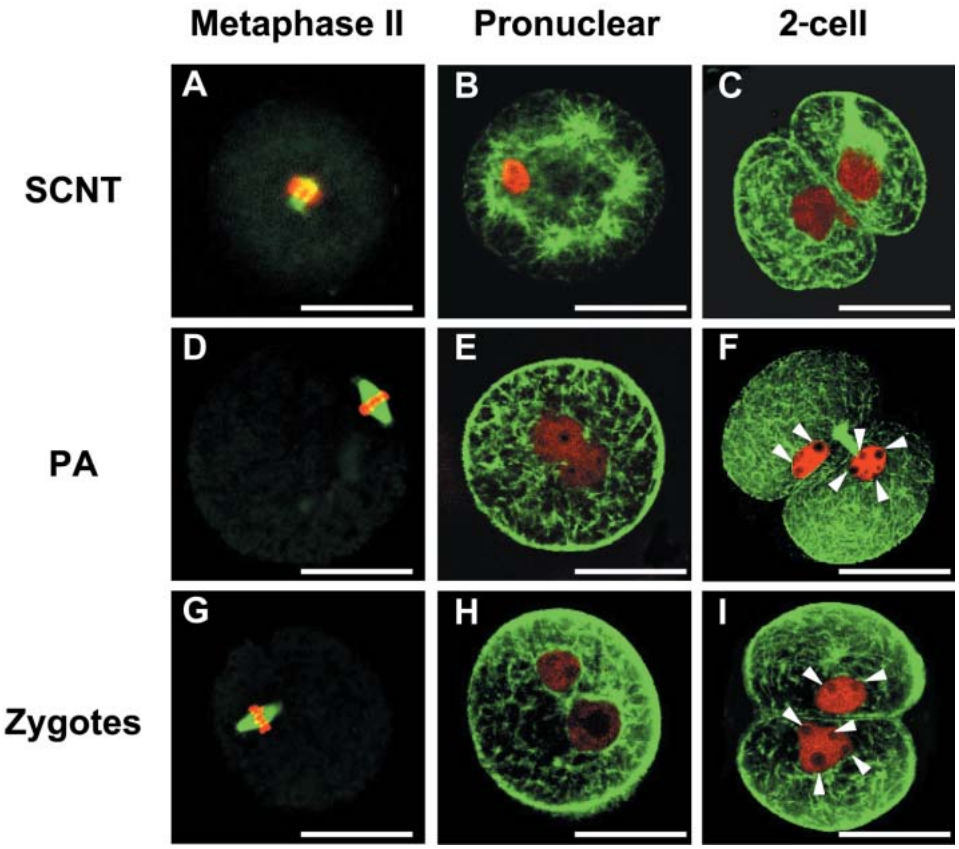

Fig. 3. Behavior of chromosomes (red) and microtubules (green) during first cleavage in SCNT embryos (SCNT), parthenogenic oocytes (PA) and oocytes fertilized *in vivo* (Zygotes). SCNT immediately before activation (A), ovulated oocytes (D and G); SCNT after activation (B), PA (E) and Zygotes (H) with pronuclei; SCNT (C), PA (F) and Zygotes (I) immediately after first cleavage. The arrowheads indicate nucleoli. Bars=50 μm.

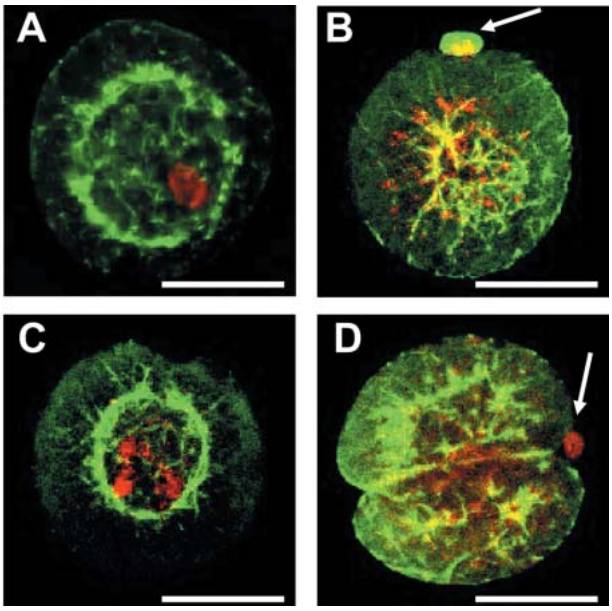

Fig. 4. SCNT embryos showing an abnormal appearance at the pronuclear stage (A) and first cleavage (B–D). Arrows show small cellular bodies containing DNA within the perivitelline space that were similar to polar bodies. Bars=50 μm.

bipolar spindles and organized arrays around the chromatin structure; however, this was not the same as seen in the meiotic spindle bodies of the ovulated oocytes (Fig. 3D and G) in which the chromosomes were aligned on a metaphase plate of distinct spindles. In contrast, 38% (26/69) of the SCNT oocytes had PCC without bipolar spindle bodies (Fig. 1C and D). The remaining 42% (29/69) of SCNT oocytes exhibited neither spindle formation nor PCC (Fig. 1E and F), although swollen nuclei were observed.

We also examined whether spontaneous activation during handling of oocytes *in vitro*

would affect formation and distribution of the microtubules in the oocytes. Immediately after their collection, all oocytes (31/31) were at the MII stage (Fig. 2A). During *in vitro* culture, the incidences of spontaneous activation, identified by the morphological changes in the MII spindle bodies, at 2, 5 and 24 h after the oocytes were collected were 84 (26/31), 81 (26/32) and 100% (26/26), respectively (Fig. 2B, C and D); the chromosomes were dividing and moving toward their respective poles 2 h after collection which corresponds to the point in time immediately before activation in the SCNT oocytes.

After activation, that is, 5h after the oocytes were collected, the distribution of microtubules in most of the SCNT embryos (21/32) that formed pronuclear-like structures (Fig. 3B) was different from that in the parthenogenetic oocytes and oocytes fertilized *in vivo* (Fig. 3E and H, respectively), and a dense microtubule organization that was shaped like a ring was observed (Fig. 3B). In the parthenogetic oocytes and oocytes fertilized *in vivo*, fibrous microtubules were distributed homogeneously in the ooplasm compared with the SCNT oocytes in which dense microtubules were localized heterogeneously in the middle layer of the ooplasm. This observation seems to be related to the observations made in our previous report in which dense microtubules were localized in the subcortical area of the cytoplasm and strong immunofluorescence was observed in the plasma membrane after enucleation [18]. At this point, metaphase-like spindles were still present or microtubules were barely observed in SCNT embryos that did not have pronuclear-like structures. These SCNT embryos did not show subsequent development. When the 1-cell embryos entered into first cleavage, 18 to 20 h post-activation, most of the SCNT embryos were at the 2-cell stage, but their nucleoli were scarcely visible (Fig. 3C), which was quite different from the conditions of the parthenogenetic (Fig. 3F, arrowheads) and fertilized oocytes (Fig. 3I, arrowheads). At this stage, the distribution of microtubules in the SCNT embryos resembled that in the parthenogenetic and fertilized oocytes, although fine microtubules were observed in the parthenogenetic and fertilized oocytes.

During *in vitro* culture for up to 24 h after their collection, however, none of the ovulated oocytes showed any cleavage or de novo synthesis of microtubules (Fig 2A, B, C and D), suggesting that spontaneous activation *in vitro* was not relevant to the malfunctions of the microtubules observed in the SCNT embryos, i.e., a dense microtubule organization that was shaped like a ring (Fig. 4A and C). In some of the SCNT embryos after first cleavage, small cellular bodies containing DNA were present within the perivitelline space that were similar to polar bodies (Fig. 4B and D, arrows). These pseudo-polar bodies were not observed until 18–20 h post-activation when the SCNT embryos were dividing. On the other hand, the second polar body was extruded just after fertilization in the fertilized oocytes; extrusion of a new body did not occur at the 2-cell stage. At this time, malfunctions were observed in some of the SCNT embryos (6/30). These malfunctions included dispersion of their chromosomes within the ooplasm (Fig. 4B, C and D) and disarrayed mitotic spindles with misaligned chromosomes as observed by DNA and microtubule imaging.

Discussion

The present study clearly showed that the timing of oocyte collection after an hCG injection affected spindle formation and cleavage of the produced rat SCNT embryos; the cleavage rate was higher when the oocytes were collected later compared with those collected just after ovulation. These findings are consistent with those in murine and bovine embryos. In both species, when a donor nucleus in the G0/G1 stage of the cell cycle is transferred to an enucleated oocyte, the PCC of the transferred nucleus is related to the success of cloning and probably reprogramming [12]. Donor nuclei in the G0/G1 stage show nuclear envelope breakdown (NEBD) and PCC when they are transferred into recipient cytoplasts with high levels of maturation/metaphase promoting factor (MPF) [19] before activation [20]. Methods that induce PCC in SCNT oocytes result in increased developmental potential, indicating that these processes may promote reprogramming of gene expression in the donor nucleus [12, 21–26]. In contrast, cytoplasts derived from activated oocytes or pronuclear zygotes lack active MPF and do not induce NEBD or PCC in transferred nuclei [25–27]. Previously, we found that most cumulus cells, which were used as donor cells in the present study, are at the G0/

G1 stage of the cell cycle [10]. In the case of the rat, spontaneous activation occurs immediately after oocytes are collected from the oviducts [14], and this spontaneous activation induces meiosis resumption and reduces the level of MPF activity [28]. In the present study, the dividing of chromosomes 2 h after collection (Fig. 2B) was caused by this spontaneous activation, and this explains why most of the SCNT oocytes with PCC did not have a proper spindle formation.

The first mitotic spindle of the SCNT embryos differed from zygotes during fertilization in terms of assembly and organization. Our results suggest incomplete formation of the metaphase spindle in rat SCNT oocytes (Fig. 1 and 3A). The rat SCNT oocytes seemed to have a small quantity of microtubules, and formation of the metaphase spindle was incomplete compared with mouse SCNT oocytes (data not shown). In mice, unfertilized oocytes contain cytoplasmic asters that act as microtubule organizers following fertilization [29]. Enucleation of MII oocytes caused no significant change in the number of cytoplasmic asters. The number of cytoplasmic asters, however, decreased after transfer of donor nuclei to these enucleated oocytes, probably because some of the asters participated in formation of the spindle that anchors the donor chromosomes. In our study, the rat MII oocytes contained no cytoplasmic asters that were detectable by staining with an anti-α-tubulin antibody. Therefore, rat SCNT oocytes may have less ability to reorganize microtubules and complete formation of the spindle leading to correct distribution of the chromosomes while maintaining normal ploidy. In primates, other factors may affect formation of the metaphase spindle in SCNT oocytes, as removal of the meiotic spindle depletes the ooplasm of NuMA and HSET, which are both vital for formation of mitotic spindle poles [8]. Proper mitotic spindles should be organized around somatic chromosomes if the meiotic spindle is left intact; for example, cloning in primates is challenged by understandable limits on oocyte availability and by cellular requirements during assembly of the first mitotic spindle that appear to be stricter than in the species that have been cloned successfully to date [8].

When somatic cells from various species are placed into enucleated oocytes and undergo reprogramming, one of the first morphological changes in the transferred nuclei is the rapid loss of nucleolar integrity. This is one of the most remarkable structural reorganizations in somatic nuclei during nuclear cloning. Proper disassembly and reassembly of nucleoli leads to normal development of cloned embryos because nucleoli have several roles in addition to ribosome synthesis, including cell cycle control and sequestration of telomerase [30]. However, the present study revealed that there was less formation of nucleoli in rat SCNT embryos after first cleavage. This is quite different from the situation in 2-cell embryos derived *in vivo*, suggesting that failure of nucleolar reorganization may be related to lack of fetal development.

In conclusion, the rat is widely used in biomedical research and is often the preferred rodent model in many areas of physiological and pathobiological research [3]. Although various genetic tools are available for the rat, methods to produce gene-disrupted knockout rats are greatly needed. Gene knockout has recently been achieved in rats [4], but the method utilizes techniques that are not readily available. Given the lack of ES-cell technology, which is available for mice, achievement of nuclear transfer in the rat is greatly desired [11]. The present study suggests that reorganization of transferred nuclei in rat SCNT embryos may be inadequate in terms of formation of the mitotic spindle and notably nucleolar reorganization. This problem may be overcome by preventing spontaneous activation of rat oocytes [11]; quite recently, oocytes collected in the presence of a protease inhibitor (MG 132) were stable for up to 3 h, and this allowed time for successful nuclear transfer resulting in fertile offspring [9].

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