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Cytoskeletal and Nuclear Organization in Mouse Embryos Derived From Nuclear Transfer and ICSI: A Comparison of Agamogony and Syngamy Before and During the First Cell Cycle

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ABSTRACT In this study, somatic cell nuclear transfer (SCNT) and intracytoplasmic sperm injection (ICSI) are used as models of agamogony and syngamy, respectively. In order to elucidate the reasons of low efficiency of somatic cell cloning, cytoskeletal and nuclear organization in cloned mouse embryos was monitored before and during the first cell cycle, and compared with the pattern of ICSI zygote. A metaphase-like spindle with alignment of condensed donor chromosomes was assembled within 3 hr after NT, followed by formation of pronuclear-like structures at 3–6 hr after activation, indicating that somatic nuclear remodeling depends on microtubular network organization. The percentage of two (pseudo-) pronuclei in cloned embryos derived from delayed activation was greater than that in immediate activation group (68.5% vs. 30.8%, $P < 0.01$), but similar to that of ICSI group (68.5% vs. 65.5%, $P > 0.05$). The 2-cell rate in NT embryos was significantly lower than that in zygotes produced by ICSI (64.8% vs. 82.5%, $P < 0.01$). Further studies testified that the cloned embryos reached the metaphase of the first mitosis 10 hr after activation, whereas this occurred at 18 hr in the ICSI zygotes. Comparision of the pattern of microfilament assembly in early NT embryos with that in syngamic zygotes suggested that abnormal microfilamental pattern in cloned embryos may threaten subsequent embryonic development. In conclusion, agamogony, in contrast to syngamy, displays some unique features in respect of cytoskeletal organization, the most remarkable of which is that the first cell cycle is initiated ahead distinctly, which probably leads to incomplete organization of the first mitotic spindle, and contributes to low efficiency of cloning. *Mol. Reprod. Dev. 74: 655–663, 2007.* © 2006 Wiley-Liss, Inc.

Key Words: nuclear transfer; ICSI; microtubule; microfilament; oocyte; mouse

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is used to elucidate fundamental biological issues such as reversibility of cell differentiation and interactions between the cytoplasm and nucleus (Park et al., 2004). Although somatic cell cloning has been performed successfully in several mammalian species, to date, the overall efficiency in all species is only 1%–5% (Gao et al., 2003). A majority of cloned embryos arrest development before implantation, and most other remaining embryos arrest before or during fetal development. Many cloned animals show abnormal symptoms at and after birth such as increased body weight, pulmonary hypertension, placental overgrowth, and respiratory problems (Wakayama and Yanagimachi, 1999; Lanza et al., 2000; Tamashiro et al., 2000). The reasons for the low success rate of cloning and abnormal symptoms of offspring are complicated and have not been elucidated. Evidently, the donor genome reprogramming is absolutely crucial in nuclear transfer (Hiiragi and Solter, 2005). Studies by other and us indicate that incomplete epigenetic reprogramming, for instance, severe DNA methylation aberrations is the major cause of the developmental failure of cloned embryos (Dean et al., 2001; Ohgane et al., 2001; Chen et al., 2005, 2006). Asynchronous cell cycles of donor nucleus and recipient oocyte lead to chromosome segregation errors during the first mitotic divisions after NT, which may contribute significantly to the low efficiency (Miyoshi et al., 2003; Shi et al., 2004). Although cloned offspring have been obtained in more mammalian species and the developmental rates of cloned embryos have been increased in a certain degree,

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it is difficult to improve reprogramming of donor nuclei by technical approaches (Miyoshi et al., 2003).

The donor genome reprogramming is accompanied with assembly of the first mitotic spindle, which is important for embryonic development. Nuclear and microtubules dynamics following SCNT have been described in cattle (Shin et al., 2002), rabbit (Chesne et al., 2002), pig (Yin et al., 2003), mouse (Gao et al., 2002; Miki et al., 2004), and nonhuman primate (Ng et al., 2004; Simerly et al., 2004), but the integrated and systematically analysis between microtubules and nucleus have not been investigated before and during the first cell cycle, which has a major impact on subsequent embryo development. Additionally, little is reported about interactions between microfilaments and nucleus following SCNT. So far, it is unclear whether cytoskeleton system is disturbed by NT procedure and ties in the low efficiency. A previous study indicates that the low efficiency is not due to inhibition of cytokinesis, which resulted from the inhibitor of microfilament polymerization, commonly cytochalasin B (CB), used during enucleation and activation (Wakayama and Yanagimachi, 2001). Recently, some researchers proposed that mechanical operation may disturb mitotic spindle assembly, which leads to chromosome segregation errors during the first cleavage (Shi et al., 2004).

Although intracytoplasmic sperm injection (ICSI) belongs to syngamic category, like natural fertilization or in vitro fertilization (IVF), it is quite different from normal fertilization in the cytoskeletal events, for example, an injected sperm does not undergo typical oocyte interactions, which may result in abnormal remodeling of the male pronucleus (Hewitson et al., 2002). Unlike ICSI, somatic cell cloning, conceptually pertained to agamogenetic technology, may be very different from normal fertilization in interactions between cytoskeleton and nuclei, which contributes apparently to the low efficiency after SCNT. The present study analyzed the dynamic changes of microtubules, microfilaments, and nuclei before two-cell stage of mouse embryos constructed by SCNT and ICSI, both of which require micromanipulation. The purpose of the study was to compare interactions between cytoskeleton and nuclei before and during the first cell cycle of agamogony and syngamy, as well as to reveal the influence of cytoskeletal reorganization on nuclear reprogramming and cloning efficiency.

MATERIALS AND METHODS

Superovulation and Collection of Mouse Oocytes

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethical committee of Institute of Zoology, Chinese Academy of Sciences.

Female Kunming White mice (age, 6–8 weeks) were injected with 7.5 IU pregnant mare serum gonadotropin (PMSG, Tianjin Animal Hormone Factory, Tianjin, China), followed by 7.5 IU human chorionic gonadotropin (hCG, Ningbo Animal Hormone Factory, Ningbo, Zhejiang, China). The females were killed 14–15 hr post-hCG administration, and the cumulus-oocyte complexes (COCs) were released from the ampullae of oviducts into pre-equilibrated HEPES-buffered-CZB medium (HCZB). The cumulus cells were removed by brief exposure to HCZB containing 300 IU/ml of hyaluronidase (Sigma), and then the cumulus-free, MII-stage oocytes were used for the following experiments after being washed at least three times with HCZB.

Enucleation of Oocytes and Nuclear Transfer

Nuclear transfer procedure was performed by using a piezo-driven micromanipulator according to a reported method (Wakayama and Yanagimachi, 2001), with slight modifications. In brief, an aliquot of the fresh cumulus cells was transferred to a droplet of HCZB, containing 10% (w/v) polyvinylpyrrolidone (PVP-360; Sigma, St. Louis, MO). A group of oocytes (usually 15–20) was transferred to a droplet of HCZB containing 5μg/ml cytochalasin B (CB). Oocyte zona pellucida was "drilled" by applying several piezo pulses to the tip of an enucleation pipette (~9 μm inner diameter). The metaphase II chromosome–spindle complex was pulled gently away from the oocyte with a small amount of accompanying ooplasm. All enucleated oocytes in one group were transferred into CB-free CZB and restore themselves for up to 1 hr at 37°C, then used for further manipulation. A cumulus cell was gently aspirated in and out of the injection pipette (~7 μm inner diameter), and sometimes combined with a few piezo pulses until the cumulus cell membrane was broken, followed by injecting the donor nucleus into a separate enucleated oocyte. At 2–3 hr or less than 0.5 hr of donor nucleus injection, activation was carried out by placing the oocytes in Ca^{2+}-free CZB containing both 10 mM $SrCl_2$ and 5 μg/ml CB for 6 hr. Then they were transferred to Sr^{2+}-free, CB-free CZB medium to continue incubation in a humidified atmosphere of 5% CO_2 at 37°C.

Preparation of Spermatozoa and ICSI Procedure

Spermatozoa were collected from caudae epididymides of male Kunming mice (age, 8–12 weeks). Spermatozoa were squeezed into a 500 μl drop of HCZB under mineral oil, and allowed to disperse at 37°C for 30 min. The sperm samples from at least three mice were pooled into a 10 ml centrifuge tube, and 5 ml HCZB was added for washing by centrifuging at 600*g* for 5 min at 4°C. Injection of sperm heads into mouse oocytes was performed as reported by Kimura and Yanagimachi (1995) using a piezo-driven injection pipette (~6 μm inner diameter). A single sperm was drawn tail first, and a few piezo-pulses were applied in the neck region to separate the head from the flagella in HCZB containing 10% PVP. A single sperm head was injected immediately into the oocyte in HCZB, then the zygotes were cultured in fresh CZB medium in 5% CO_2 in air at 37°C.

Immunofluorescent Staining and Confocal Microscopy

Immunostaining of microtubules and microfilaments were carried out as described previously with minor modifications (Zhu et al., 2003). In brief, reconstituted eggs or embryos were fixed with 4% (w/v) paraformaldehyde in PBS for 40 min at room temperature. Fixed samples were stored in PBS containing 0.3% (w/v) BSA for up to 1 week at 4°C. The samples were permeabilized in PBS containing 0.1% (w/v) Triton X-100 and 0.3% BSA for 30–40 min at 37°C. After washing twice with PBS containing 0.01% Triton X-100, they were incubated in block solution (PBS containing 150 mM glycine and 0.3% BSA) for 30 min at 37°C. The samples were used either for microtubule analysis or microfilament localization. Some samples were incubated with FITC-labeled mouse monoclonal antibody against α-tubulin (Sigma) diluted in the blocking solution (1:100) for 40 min at 37°C or overnight at 4°C. Analogously, additional samples were incubated with FITC-Phalloidin (1:50, Sigma) to tag microfilaments. Following three washes of 5 min each, chromatin was stained with 10 μg/ml of propidium iodide (Sigma). Finally, the samples were mounted on slides with antifluorescence-fade medium (DABCO). They were then examined with a laser-scanning confocal microscope (Zeiss LSM 510 Meta, Jena, Germany). Images shown in results are representatives of at least 50 samples from more than 3 replicates.

RESULTS

Early Development of Mouse Embryos Derived From SCNT and ICSI

The results shown in Tables 1 and 2 are similar to previous reports about the early development of embryos derived from SCNT and ICSI in mouse (Kimura and Yanagimachi, 1995; Wakayama and Yanagimachi, 2001), indicating the reliability of samples used for cytoskeletal and nuclear pattern analysis. Eggs reconstructed by SCNT formed pseudo-pronuclei at 3–6 hr following activation, and the percentage of two pseudo-pronuclei was greater in delayed activation (2–3 hr after NT) group than that in immediate activation (<0.5 hr after NT) group (68.5% vs. 30.8%, $P < 0.01$, Table 1). But similar two-cell rates were observed, regardless of the interval between activation and NT (64.8% and 57.5%, respectively, $P > 0.05$, Table 1). When oocytes were injected with a single spermatozoon, 65.5% of oocytes injected successfully formed two pronuclei (PN) and a second polar body (Pb2) at 3–7 hr after injection, and 82.5% of oocytes injected successfully developed to two-cell stage at 24–26 hr after injection (Table 2). Integrating the data from Tables 1 and 2, the percent of two pseudo-pronuclei eggs activated at 2–3 hr after NT was similar to percent of Pb2 + 2PN zygotes produced by ICSI with single sperm (68.5% vs. 65.5%, respectively, $P > 0.05$), but two-cell rate in NT group was significantly lower than that in ICSI group (64.8% vs. 82.5%, respectively, $P < 0.01$, Tables 1 and 2).

Comparison of Microtubule and Nuclear Patterns After SCNT and ICSI Before and During the First Cell Cycle

Immunofluorescent staining showed that microtubules only distributed at metaphase spindle and no cytoplasmic aster was visible in almost all MII-stage oocytes from Kunming mouse (Fig. 1A). Soon after introducing a cumulus cell into enucleated oocyte, donor cell became swellen and was surrounded by numerous microtubules (Fig. 1B). A symmetrical, transient bipolar spindle was organized, and donor chromatin condensed in nonactivated cytoplasm, followed by alignment of condensed chromosomes on the metaphase-like spindle within 3 hr after NT (Fig. 1C,D). When the oocytes were treated with $SrCl_2$ at 2–3 hr after NT, donor chromosomes in majority of reconstructed oocytes segregated to form two pronuclear-like structures at 3–6 hr following activation (Fig. 1E, F; Table 1). At 10 hr after activation, most of the cloned embryos developed to prometaphase or metaphase of the first mitotic division, as initially evidenced by re-condensing of chromosomes. During the early prometaphase, the condensed chromosomes were surrounded by dense microtubule arrays, indicating the mitotic spindle was assembling (Fig. 1G). After that, the embryos entered M-phase, and a big metaphase spindle with a regular configuration was clearly observed in the center of normal one-cell cloned embryo (Fig. 1H). Completion of the first cleavage mostly appeared at 20–24 hr after activation (Fig. 1I). In some cases, absence of cytoplasmic asters or functionary imperfectness of spindle after NT resulted in formation

TABLE 1. Development of Mouse Embryos Reconstructed by SCNT Before the First Cleavage

Interval between NT and activation	Number eggs reconstituted/ operated	Number of cloned zygotes with (%)*				Number of cloned embryos to (%)**		
		2PN	1PN	0PN	Deformed extensively	2-cell	1-cell	Fragmented
2–3 hr	162/207	111 (68.5)[a]	33 (20.4)[a]	10 (6.2)	8 (4.9)	105 (64.8)[a]	43 (26.5)	14 (8.6)
<0.5 hr	120/155	37 (30.8)[b]	64 (53.3)[b]	11 (9.2)	8 (6.7)	69 (57.5)[a]	35 (29.2)	16 (13.3)

*After activation (6–7 hr) treatment with $SrCl_2$; PN, pseudo-pronucleus.
**After activation (20–24 hr).
[a,b]Values with different superscripts within each column are significantly different ($P < 0.01$). The data were analyzed using the χ^2 test.

TABLE 2. Development of Mouse Embryos Derived From ICSI Before the First Cleavage

Number of sperm heads injected	Number of eggs reconstituted/ operated	Number of zygotes with (%)*				Number of embryos to (%)**		
		1Pb + 2PN	1Pb + 1PN	0Pb + 0PN	Abnormal	2-cell	1-cell	Fragmented
1	177/198	116 (65.5)[a]	34 (19.2)[a]	19 (10.7)	8 (4.5)	146 (82.5)[a]	25 (14.1)	6 (3.4)
0	75/83	2 (2.7)[b]	14 (18.7)[a]	54 (72.0)	5 (6.7)	11 (14.7)[b]	55 (73.3)	9 (12.0)

*Seven hours after ICSI; 0Pb + 0PN: oocytes unfertilized or inactivated; abnormal: oocytes contained 0Pb + 1PN, deformed and so on. Pb, the second polar body; PN, pronucleus.
**After sperm injection (24–26 hr).
[a,b]Values with different superscripts within each column are significantly different ($P < 0.01$).

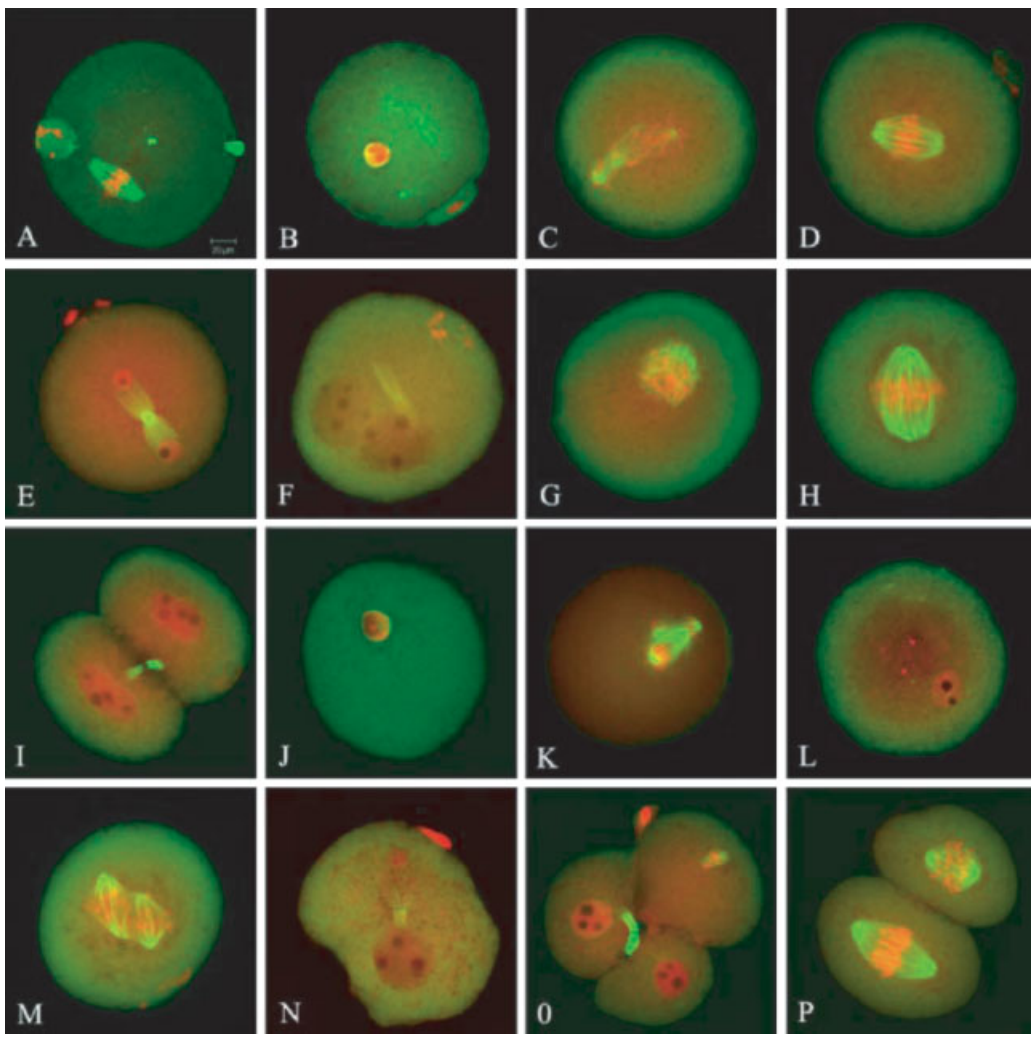

Fig. 1. Microtubule and nuclear patterns in mouse embryos reconstructed by SCNT. Green, microtubules; red, chromatin. **A**: Recipient MII oocyte. **B**: Within 0.5 hr after NT, microtubules reassemble. **C**, **D**: Within 3 hr, donor chromatin condense, and then condensed chromosomes align on the metaphase-like transient spindle in nonactivated cytoplasm. **E**, **F**: Majority of reconstructed oocytes form two pseudo-pronuclei at 3–6 hr following activation, performed 2–3 hr after NT. **G**, **H**: Most of cloned embryo enter the prometaphase or metaphase of the first mitosis at 10 hr after activation, and a big metaphase spindle appears in the center of one-cell embryo. **I**: The first cleavage mostly completes at 20–24 hr after activation. **J**–**L**: Formation of one pseudo-pronucleus, which is more common in immediately activated oocyte. **M**–**O**: The mitotic spindles of a few reconstructed embryos assemble abnormally, resulting in abnormal division during the first mitosis. **P**: A normal two-cell cloned embryo, in which the two blastomeres enter prophase and metaphase of mitosis, respectively.

of one pseudo-pronucleus, which was more common in immediately activated reconstructed oocytes (Fig. 1J–L; Table 1). The mitotic spindles of a few reconstructed embryos assembled abnormally, and resulted in abnormal division during the first mitosis (Fig. 1M–O). A normal two-cell cloned embryo was shown in the end of Figure 1P.

One hour after intracytoplasmic injection with a single sperm, microtubules assembled obviously around the swelled sperm head (Fig. 2B). Two hours after ICSI, microtubules surrounding decondensing sperm nucleus formed spindle-like structure, and maternal chromosomes separated toward the two spindle poles and entered anaphase, reminiscent of resuming of meiosis. At the moment, asynchronous behavior of paternal and maternal chromatin was observed (Fig. 2C,D). Majority of the fertilized oocytes developed to telophase of meiosis and extruded the second polar body (Pb2) 3 hr after ICSI (Fig. 2E). At 3–9 hr, female and male pronuclei (fPN, mPN) were observed in most zygotes; only fPN was seen in a few eggs (Fig. 2F,G; Table 2). Within 18 hr, fPN and mPN apposed in the center of each zygote, and microtubules fill the entire cytoplasm (Fig. 2H). At 18–22 hr after ICSI, zygotes entered the first mitotic cell cycle which was characterized by microtubules reorganizing to form mitotic spindle, and biparental chromosomes recondensed and merged at metaphase. Subsequently, zygotes developed to two-cell embryos at 22–26 hr (Fig. 2I–L; Table 2).

Comparison of Microfilament and Nuclear Patterns After SCNT and ICSI Before and During the First Cell Cycle

The pattern of microfilament assembly of cloned embryos was different from that of embryos derived from ICSI. Although cytochalasin B was applied to disrupt microfilaments in favor of enucleation and introduction of donor nucleus, when PCC of the donor nucleus occurred after NT, the assembly of microfilaments intensively increased in the cortex of the oocyte, especially adjacent to the donor chromosomes (Fig. 3B–D). After ICSI, enriched microfilaments distributed mainly in the cortical surface adjacent to maternal chromosomes, without distribution in vicinity of sperm nucleus (Fig. 4A–C).

During development of pseudo-pronuclei in cloned embryos, cortical microfilaments disassembled, irrespective of number of pronuclei (Fig. 3E,F), while they uniformly distributed in the cytoplasm of syngamic ICSI zygotes during pronuclear formation and apposition (Fig. 4D,E). Following initiating mitosis, microfilaments reassembled and uniformly localized in the cortex of

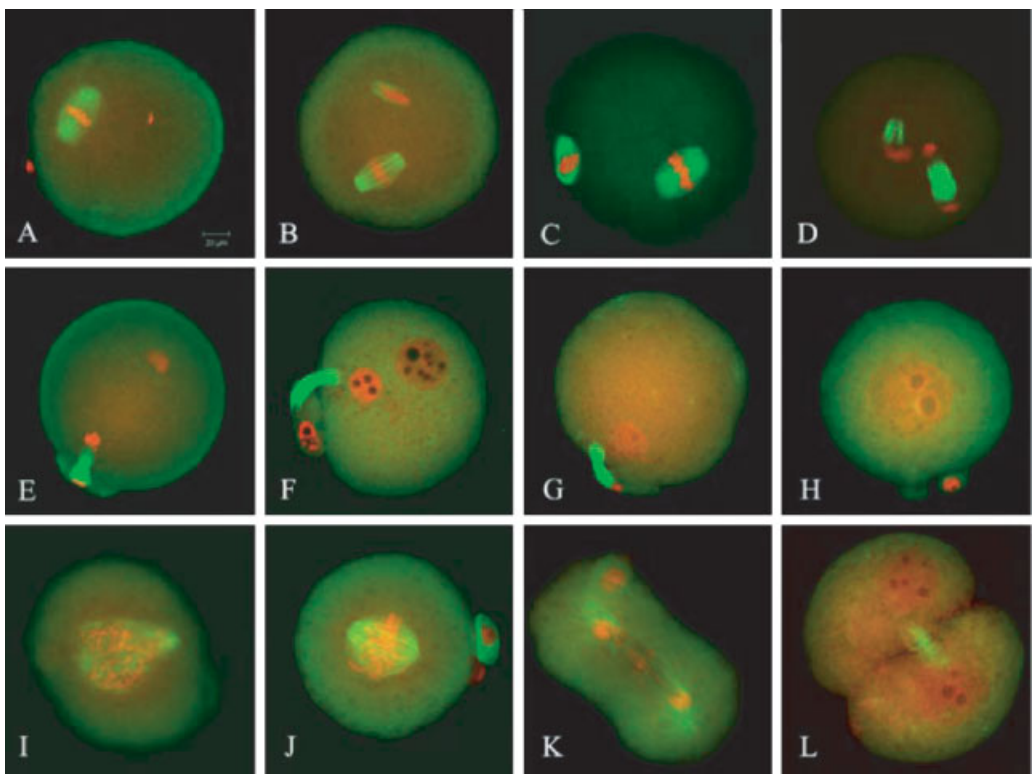

Fig. 2. Microtubule and nuclear patterns in mouse embryos derived from ICSI. Green, microtubules; red, chromatin. **A**: A fertilized oocyte displays a meiotic apparatus and a sperm head just injected. **B**: One hour after ICSI, microtubules assemble in an area close to the swelled sperm head. **C**, **D**: At 2 hr, microtubules surrounding sperm nucleus form a spindle-like structure, and maternal chromosomes enter anaphase II. **E**: At 3 hr, majority of oocytes develop to telophase II. **F**: At 3–9 hr, female and male pronuclei (fPN, mPN) are observed in most zygotes. **G**: Only fPN is seen in a few zygotes. **H**: Within 18 hr, fPN and mPN appose in the center of each zygote. **I**, **J**: At 18–22 hr, zygotes enter the first mitotic cell cycle. **K**, **L**: Two-cell embryos forms at 22–26 hr following ICSI.

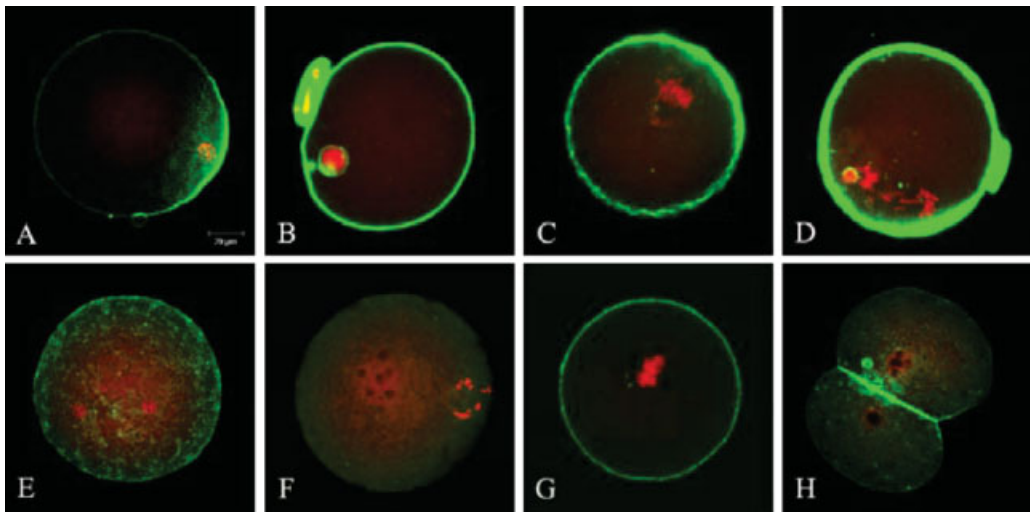

Fig. 3. Microfilament dynamics in mouse embryos derived from SCNT. Green, microfilaments; red, chromatin. **A**: Microfilaments are mainly located at oocyte surface adjacent to cortical nucleus before enucleation. **B**: After NT, microfilaments uniformly distribute in cortical surface. **C**, **D**: Following PCC, microfilaments enrich in cortical surface adjacent to donor chromosomes before activation treatment. **E**, **F**: Microfilaments disassemble during pseudo-pronuclei phase 3–6 hr following activation, irrespective of number of pronuclei. **G**: Microfilaments reassemble in cortical surface in cloned embryos at mitotic metaphase 10 hr after activation. **H**: Microfilaments only distribute in cleavage furrow of two-cell cloned embryos.

cloned embryos, but they maintained uniform distribution in entire cytoplasm of the syngamic zygotes (Fig. 3G; Fig. 4F,G). Finally, microfilaments were localized only in the cleavage furrow of two-cell embryos, derived from either NT or ICSI (Fig. 3H; Fig. 4H)

DISCUSSION

Despite the feasibility of cloning animals from adult somatic cells, cloning remains an extremely inefficient process, which is the major obstacle to widespread use of this technology. Incomplete nuclear reprogramming following the transfer of donor nuclei into recipient oocytes has been implicated as a primary reason for the low efficiency of the cloning procedure (Miyoshi et al., 2003; Hiiragi and Solter, 2005).

The success of SCNT depends on several parameters that impact on the ability of the cytoplast to reprogram the nucleus of the donor cell, or to reverse the epigenetic changes that occur during development (Wilmut et al., 2002). The morphological remodeling of introduced somatic nuclei including PCC, the development of two pronuclear-like structures and the apposition of nuclei

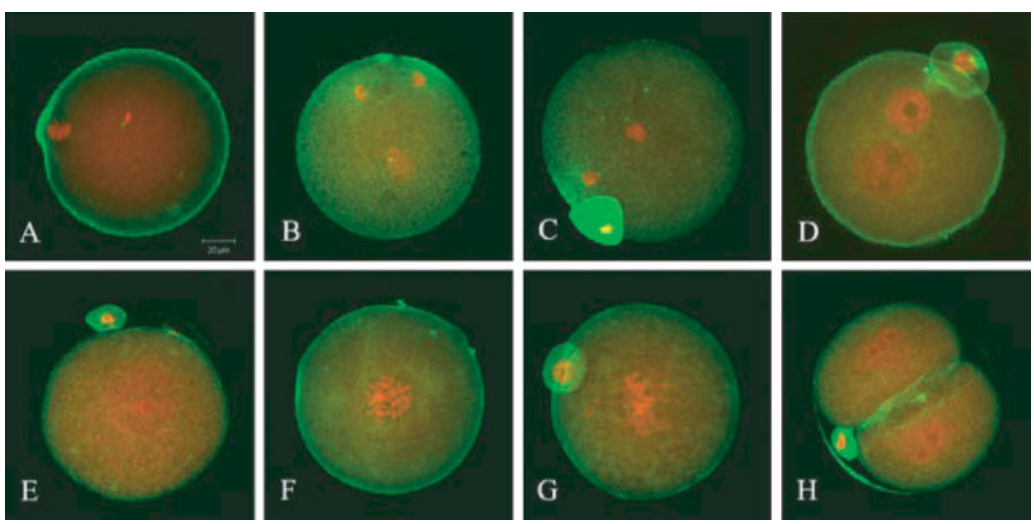

Fig. 4. Microfilament dynamics in mouse embryos derived from ICSI. Green, microfilaments; red, chromatin. **A**–**C**: After ICSI, microfilaments just enrich in cortical surface adjacent to maternal chromosomes or female pronucleus, without distribution in vicinity of sperm nucleus or male pronucleus. **D**, **E**: At the period between later pronuclear phase (6 hr) and pronuclear apposition phase (18 hr), microfilaments uniformly distribute in ooplasm, instead of cortex. **F**, **G**: Following entering the first mitosis, microfilaments maintain the uniform distribution in entire ooplasm. **H**: Microfilaments distribute in cleavage furrow of two-cell embryos.

with microtubule asters may help the reprogramming of donor nuclei and increase the developmental potential of the reconstructed embryos (Shin et al., 2002). In this study, we systematically analyzed the interactions of cytoskeleton with nuclei before and during the first cell cycle of SCNT and ICSI mouse embryos.

Before forming pseudo-pronuclei in NT-reconstructed mouse embryos, the pattern of microtubule assembly is similar to that of prometaphase of meiosis I. It has been reported that there were two discrete populations of centrosomes in the mouse oocyte (Meaainger and Albertini, 1991). One population that is consistently associated with chromatin may be removed at the enucleation procedure, whereas a second population of centrosomes exist in the ooplasm. Ooplasm centrosomal materials organize the microtubular asters that may play a crucial role during the first cell cycle of murine NT embryos (Miki et al., 2004), or organize a microtubular network which is responsible for separation of donor chromosomes and apposition of pseudo-pronuclei following NT. The microtubule asters were also observed in association with decondensed chromatin following SCNT in those species which belong to paternal centrosome inheritance, such as bovine (Shin et al., 2002), rabbit (Chesne et al., 2002), pig (Yin et al., 2003), and nonhuman primates (Simerly et al., 2004). This phenomenon equally indicates introduction of a somatic cell centrosome during SCNT, which probably plays a role in nuclear remodeling and subsequent development (Shin et al., 2002). In the absence of the donor cell centrosomes, assembly of the bipolar spindle of reconstructed mouse oocyte seems to occur through the self-organization of microtubules around donor chromatin (Van Thuan et al., 2006). Our study also demonstrates that somatic nuclear remodeling depends on microtubular network organized by maternal centrosomes in cloned mouse embryos.

Somatic nuclei exposed to nonactivated ooplasm undergo nuclear envelope breakdown (NEBD) and subsequent premature chromosome condensation (PCC) due to the high level of maturation/metaphase promoting factor (MPF) activity, within 2 hr after NT. This change for the configuration of chromatin has been thought the characteristic of chromatin remodeling, which is a crucial beginning of reprogramming of differentiated nucleus. Therefore, delayed activated oocytes mostly form two pseudo-pronuclei due to adequate remodeling. In contrast, reconstructed oocytes activated immediately within 0.5 hr after NT commonly fail to undergo NEBD and form only one pseudo-pronucleus (Wakayama and Yanagimachi, 2001). Although similar two-cell rates of cloned embryos activated at different times after NT were obtained in the present study, a prolonged interval between nuclear injection and oocyte activation is apparently beneficial for chromatin remodeling, and also for both pre- and post- implantation development (Wakayama et al., 1998). It may be resulted from the following reasons: one is that MII-stage ooplasm contains "reprogramming factors," which activity disappears after parthenogenetic activation (Tani et al., 2003), and the other is that NEBD may play an important role in nuclear reprogramming by allowing direct interactions of chromosomes with cytoplasmic environment (Kim et al., 2002).

Although our results cannot display the difference of pseudo-pronuclear/pronuclear morphology between cloned and ICSI derived one-cell stage embryos, it has been reported that pseudo-pronuclear structure in cloned embryos presented evident abnormality by the identification of several markers, for example, nuclear mitotic apparatus (NuMA) protein, comparing with the embryos derived from ICSI or IVF and parthenogenesis (Pedro et al., 2003; Zhong et al., 2005; Liu et al., 2006; Yan et al., 2006). And it is presumed that deficiencies in somatic nuclear reprogramming might emanate at least partly from failure to remodel the somatic nucleus morphologically into a functional embryonic nucleus (Pedro et al., 2003).

To our surprise, the present study shows that the mouse zygotes derived from ICSI enter the first mitotic metaphase 18 hr after injection, whereas this timing occurred in the cloned embryos is 10 hr after activation. It seems to be a common phenomenon in mammal that one-cell embryos from NT enter the first cell cycle obviously earlier than zygotes from ICSI. In the early one-cell stage mouse embryo, DNA transcription is silent, which correlates with remodeling of the differentiated nucleus into a totipotent nucleus. Under the pattern of syngamy, the two highly differentiated cells, sperm and oocyte, should eliminate their program of gene expression inhibition after fertilization to make totipotent zygote. The mouse zygotic genome starts its intrinsic transcription at the late one-cell stage after a transcriptionally silent period regardless of zygotic derivation from fertilization or cloning (Aoki et al., 1997; Kim et al., 2002). Our results indicate that differentiated nuclear remodeling following NT is deficient in the transcriptionally silent period, that is, the early one-cell stage of agamogony, unlike ICSI (syngamy), which can obtain the totipotent zygotes, as the same as natural fertilization. The length of the first DNA replication is positively correlated with further successful embryo development. Comizzoli et al. (2000) demonstrated that the paternal component directly regulates the time of onset of DNA replication for both pronuclei during the G_1-phase. So we presume that cloned mouse embryos without incorporation of sperm exhibit incomplete donor nuclear remodeling, with initiating the first DNA replication ahead distinctly and curtailing the transcriptionally silent period, which probably leads to incomplete organization of the first mitotic spindle, and contributes significantly to the low efficiency of cloning.

Our study describes the dynamic images of microfilaments assembly of the early agamogenetic embryo from cloning and their difference from the syngamic zygotes derived from ICSI. Cytochalasin B is used to microoperation to disrupt microfilaments in favor of enucleation and introduction of donor nucleus, but

during the early nuclear remodeling until beginning the activation procedure, removal of CB leads to remarkable increase of microfilaments in the oocyte cortex, especially adjacent to the donor chromosomes. We hypothesize that the compensatory assembly of microfilaments may be useful for moving the donor chromosomes to the proper position of the oocyte to form two pseudo-pronuclei, implying that delayed activation is beneficial to early nuclear remodeling by increasing assembly of microfilaments. During the formation and development of (pseudo-) pronuclei, addition of CB to activated medium leads to disassembly of microfilaments in NT reconstructed oocytes, whereas microfilaments uniformly distribute in cytoplasm of the syngamic zygotes. Following initiating mitosis, microfilaments reassemble and uniformly localize in the cortex of cloned embryos, but they maintain uniform distribution in entire cytoplasm of the syngamic zygotes, indicating that employment of the microfilament disrupting agent CB probably contributes to abnormal microfilamental pattern in early cloned embryos. The results suggest that employment of CB in manipulated medium in common NT procedure threatens potentially subsequent development of cloned embryos. Although there is a report that the low efficiency of cloning is not due to inhibition of cytokinesis resulted from CB (Wakayama and Yanagimachi, 2001), it has been testified recently that microfilament disruption before enucleation is required for germinal vesicle stage but not for MII stage human oocytes (Tesarik et al., 2003). Further study is needed to determine whether the efficiency of cloning can be improved by omitting recipient MII oocytes exposure to CB at the enucleation step.

Due to the complexity and comprehensiveness of factors affecting nuclear reprogramming and efficiency of cloning, the new standpoint has been suggested that the only way to improve nuclear reprogramming is to modify the chromatin structure of somatic cells before nuclear transfer, to provide the oocyte with a chromosomal structure that is more compatible with the natural reprogramming machinery of the oocyte (Loi et al., 2003). Recently, there was a report that condensed chromosomes, which were obtained by permeabilization of a somatic nucleus in a mitotic cell extract prior to transplantation, were directly transferred into enucleated bovine oocytes (Sullivan et al., 2004). This procedure may facilitate subsequent chromatin remodeling in recipient oocyte and full-term development.

In conclusion, we demonstrate that owing to the mouse oocyte cytoplasm reprograms a somatic nucleus rather than a sperm nucleus, agamogony, in contrast to syngamy, displays some unique features in respect of cytoskeletal reorganization. Especially, we find the phenomenon that one-cell embryos from NT enter the first cell cycle obviously earlier than the zygotes from ICSI. These distinctions between agamogony and syngamy facilitate to elucidate the reasons of low efficiency of cloning.

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