1. ABSTRACT

Previous reports have indicated that failure in cloning monkey is attributed to the removal of nuclear mitotic apparatus (NuMA) during enucleation and subsequent abnormal organization of mitotic apparatus. This study investigated the transformation and assembly of tubulin and NuMA protein during the first cell cycle of cloned monkey embryos reconstructed by using enucleated rabbit oocytes as recipients. After the oocyte fused with a fibroblast, extensive microtubule organization was observed around the introduced nucleus in most reconstructed embryos, suggesting the introduction of a somatic cell centrosome. A high proportion of fibroblast nuclei transferred into non-activated oocytes underwent premature chromosome condensation (PCC), transient spindle organization and chromosomes separation, followed by the formation of two pronucleus-like structures. In contrast, fibroblast nuclei in pre-activated cytoplast rarely underwent PCC, but formed a swollen pronucleus-like structure. Normal spindles were observed in about one third of the cloned embryos reconstructed by both methods. After transferring monkey fibroblasts into NuMA-removed enucleated rabbit oocytes, NuMA was localized in pseudo-pronuclei and gradually moved to mitotic spindle poles at the first mitotic spindle poles. NuMA antibody microinjection resulted in spindle disorganization and chromosome misalignment, but did not significantly affect early cleavage. Our findings indicate that: 1. NuMA in donor monkey fibroblast may contribute to form a normal spindle in enucleated rabbit oocyte; 2. when non-activated cytoplasts and pre-activated cytoplasts are used as recipients, the donor nuclei undergo different morphological changes, but yield similar early embryo development; 3. although abnormal spindle organization and chromosome alignment may cause low efficiency of animal cloning, these abnormalities do not significantly affect early cleavage.
2. INTRODUCTION

Somatic cell cloning has been performed successfully in several mammalian species, including sheep, cattle, mice, goat, pig, cat, rabbit, mule, horse and rat (1-10). Up to now, non-human primate live birth has not been obtained from somatic cell nuclear transfer (SCNT), and only two offspring were obtained from embryonic cell nuclear transfer (11). It is possible that the biological and technical factors that affect the efficiency of cloning differ from species to species. Understanding the cellular and molecular changes in SCNT is crucial to cloning success. It is generally accepted that the coordinated behavior of microtubules and donor chromosomes in nuclear transferred oocytes is a prerequisite for the subsequent normal development of cloned embryos (7, 12-13). In mice, bovine and rabbit, although misaligned metaphase plates were found, normal spindle could be formed after somatic donor cell introduction into the enucleated oocyte (3,7,14,15).

Primates are reported to be different from other animals. Disarrayed abnormal mitotic spindles with misaligned chromosomes were formed in all SCNT embryos, and no pregnancies were obtained from SCNT embryos. It was suggested that NuMA and HSET removal during enucleation (meiotic spindle removal) might cause primate SCNT anomalies (12). However, donor cell NuMA was recently reported by us to contribute to mitotic spindle formation in nuclear transfer embryos (16).

Unlike mouse and domestic animal SCNT, primate oocyte availability, quality and price all limit non-human primate NT research. However, rabbit oocytes have been proved to be an ideal model for many types of studies due to their numerous advantages, such as high competency to support the development of heterogeneous cell after nuclear transfer, and easiness to handle and harvest(17-21). Thus in our research the rabbit oocytes were chosen as the recipients to construct monkey SCNT embryos, which would be helpful to clarify the early development mechanism of cloned monkey embryos.

Successful monkey production of SCNT embryo requires proper oocyte activation and introduction of a nucleus into oocyte. However, there are no reports about the influence of the activation time on the behavior of microtubules and development in the reconstructed monkey embryos.

In this study, we investigated the configuration of chromatin and microtubule organization following transfer of monkey somatic cell nuclei into pre-activated or non-activated rabbit oocytes, and then observed the NuMA distribution and function in the first cell cycle of reconstructed monkey–rabbit cloned embryos.

3. MATERIALS AND METHODS

3.1. Animals

Animal care and handling were in accordance with the policy on the Care and Use of Animals of the Ethical Committee, Institute of Zoology, Chinese Academy of Sciences. Female Japan Big Eared white rabbits (purchased from Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were housed in stainless steel cages, and were fed with regular rabbit fodder and water ad libitum.

3.2. Preparation of donor cells

Fibroblast cells were collected from an ear-skin biopsy of a 4-year-old female macaque (Macaca mulatta teleniensis). Primary cell culture was performed with the same method as described previously (22). Fibroblasts at passages 4–10 were used as donors.

3.3. Oocyte collection and enucleation

Female rabbits were superovulated by administering PMSG and hCG (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 100 IU PMSG and with 100 IU hCG 96 hours after the PMSG injection. Rabbits were killed 14h after the hCG treatment. Matured metaphase II stage (MII) rabbit oocytes were flushed from the oviducts with 199 medium (Gibco BRL, NY) and were treated shortly thereafter with 300IU/ml of hyaluronidase (Sigma Chemical Co, St. Louis, MO) in M199 medium. Cumulus cells were stripped from the oocytes by repeated gentle pipetting. The cumulus-free eggs were transferred to M199 medium containing 7.5 µg/ml cytochalasin B (Sigma) and 10% FBS for 10 min, and then manipulated under an inverted microscope (Nikon’s ECLIPSE TE300, Nikon Corporation, Japan). Removal of the meiotic spindle and chromosomes was accomplished in the following way. The “squish” enucleation method was used for metaphase-II spindle aspiration. A cumulus-free oocyte was held with a holding micropipette (170 µm outer diameter, 20 µm inner diameter) and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm containing the metaphase II spindle were squeezed and extruded with a 20-µm pipette gently. After enucleation, the karyoplast was stained with 1µg/ml Hoechst 33342 (Sigma) and exposed to the ultraviolet light to confirm the removal of chromosomes.

3.4. Reconstruction of monkey–rabbit cloned embryos

Single donor cells were introduced into the perivitelline space of enucleated oocytes. The coupllets were pre-equilibrated in fusion medium consisting of 0.25M sorbitol, 0.5mM Hapes, 0.1mM Ca(CH_3COO)_2, 0.5mM Mg(CH_3COO)_2 and 1 mg/ml bovine serum albumin, and then placed between the electrodes of a fusion chamber in fusion medium. Two direct current pulses (1.4 kV/cm, 80 µs each, 1 s apart) were applied with an ECM2001 Electrocell Manipulator (BTX inc. San Diego).

3.5 Activation and embryo culture

Coupllets were checked for fusion under an inverted microscope and fused coupllets were activated by double DC pulses of 1.2 kV/cm for 20µs, and then cultured in G_1 medium (Vitrolife, Mo’indalsva’gen, Goteborg, Sweden). The 8-cell NT embryos were transferred into G_2 medium (Vitrolife) for subsequent development. Oocytes
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were either pre-activated with electric pulse 2 hours prior to membrane fusion or activated at 2 hours after fusion.

3.6. Immunofluorescent confocal microscopy

After removing the zona pellucida in acidic M2 medium (pH 2.5), oocytes were fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Oocytes and reconstructed embryos were permeabilized with 1% Triton X-100 for 1 hour at 37°C, followed by blocking in 1% BSA for 1 h and incubation for 1 hour at 37°C or overnight at 4°C with mouse anti-human NuMA antibody (Oncogene, EMD Biosciences, San Diego USA) diluted 1:50 in blocking solution. After three washes in PBS containing 0.1% Tween-20 and 0.01% Triton X-100 (washing solution) for 5 min each, the oocytes were labeled with FITC-conjugated goat anti-mouse IgG diluted 1:100 for 1 hour at 37°C. After extensive washing, DNA of samples was counterstained with propidium iodide (PI). Finally, samples were mounted between a cover slip and glass slide supported by four columns of a mixture of petroleum jelly and paraffin (9:1). The slides were sealed with nail polish. As for microtubule staining, samples were treated exactly the same as those described above except that the first antibody was replaced by monoclonal anti-alpha-tubulin-FITC antibody (Sigma).

Digested and dispersed monkey fibroblast were placed into Petri dishes containing a cover glass slide, then cultured in DMEM-F12 containing 10% FBS. When reached 60–80% confluence, the cells were fixed. The following steps were the same as the treatment of reconstructed embryos.

Each experiment was repeated at least three times, and at least 10 randomly selected oocytes were examined each time. Slides were scanned using Leica TCS4D microscope (Leica Laser Technik, GmbH, Heidelberg, Germany) with an argon/krypton laser at 488 and 563 nm and two-channel scanning for detection of fluorescein isothiocyanate and PI, respectively.

3.7. Antibody microinjection

In order to observe the role of NuMA in spindle assembly and the first cell division, NuMA antibody was microinjected into monkey-rabbit interspecies reconstructed embryos 6 hours post-fusion (hpf). Mouse IgG was used as a control. Each experiment was performed at least three times. A total of 20-30 reconstituted embryos were microinjected per group per experiment. To minimize the damage to reconstituted embryos, microinjection needle with a diameter of about 1μm was used. The injection amount of antibody or control IgG was roughly 8 pl/oocyte, and the operation of each group was completed within 20 mins.

3.8. Experimental design

This study is composed of five experimental groups. In experiment I, the microtubule organization and chromatin dynamics in the first cell cycle of reconstructed embryo was detected. In experiment II, the effect of non-activation and pre-activation rabbit ooplasm on monkey embryo development was investigated. In experiment III, NuMA distribution in monkey fibroblasts was stained. In experiment IV, NuMA distribution in monkey-rabbit interspecies SCNT embryos was observed; and in experiment V, the effect of NuMA antibody injection on monkey-rabbit early embryos was studied. The distribution patterns of the microtubules, NuMA, and chromosomes were observed at 0.5, 2, 4, 6, 8, 10, 12-13, and 15 hpf.

3.9. Statistical analysis

The data were pooled from at least five replicates and analyzed by Chi-square test. If P < 0.05, difference was considered as statistically significant.

4. RESULTS

4.1. microtubule and chromatin dynamics in monkey-rabbit interspecies SCNT embryos

The nuclear and microtubular dynamics in eggs reconstructed by non-activated and pre-activated oocytes were studied. Compared with the MII rabbit oocytes (Figure 1A), no microtubules could be detected in the enucleated oocytes (Figure 1B). After these oocytes were activated and fixed at different time points, no microtubules were found in all cytoplasts examined. The image of the digested monkey fibroblast was displayed in Figure 1C.

Immediately after fusion of non-activated oocyte and fibroblast, a microtubule meshwork was seen around the transferred nucleus in most oocytes (Figure 1D). Within 1-4 hours, most (78.0%, 46/59) fibroblast nuclei underwent PCC and spindle-like structures were formed (Figure 1E), and then divided into two masses of chromosomes (Figure 1F). The others (22.0%, 13/59) did not show PCC but exhibited microtubule assembly. At 5-9 hpf, two pronucleus-like structures formed and they were surrounded by abounding microtubules in most of the eggs (Figure 1G). After the nuclear membrane of the two pronucleus-like structures had broken down (Figure 1H), a normal mitotic spindle formed by 10-13 hpf (Figure 1I), and the egg entered anaphase (figure. 1J) quickly. Most monkey-rabbit reconstructed embryos (about 80%) underwent the first cleavage by 14 hpf.

When nuclear transfer was conducted by using pre-activated oocytes as recipients, microtubules were also organized around the introduced cell nucleus (Figure 1K, L), but only 14.7% (5/34) of the fibroblast nuclei underwent PCC, and most eggs formed a swollen pronucleus-like structure enveloped in microtubules (Figure 1M). The two-nuclei formation rate (8.2%, 4/49) was significantly lower than that in non-activation group (table 1). Normal mitotic spindle and anaphase entry could also be observed (Figure 1N, O).

The majority of reconstructed embryos underwent various abnormal changes, including formation of mitotic spindles but with improper capture of condensed chromosomes (Figure 1P); formation of two mitotic spindles (Figure 1Q); absence of chromosome condensation (Figure 1R), absence of microtubule assembly (Figure 1S),

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and abnormal chromosome separation during anaphase (Figure 1T). Only about one third embryos had normal chromosome and microtubule organization in both pre-activation and non-activation groups (28.6%, 32.0% respectively, table 2).

4.2. impact of pre-activation and post-activation on early development of the SCNT embryos

The blastocyst development rate of SCNT embryo was similar in pre-activation and post-activation groups. The blastocyst rates were 9.1% and 8.5% in post-activation group and pre-activation group respectively (table 3). These results suggest that introduction of a monkey fibroblast into a pre- or non-activation enucleated oocyte during somatic cell nuclear transfer has similar effect on subsequent development of reconstituted eggs.

4.3. distribution of NuMA in monkey fibroblasts

In monkey fibroblasts, NuMA was detected in the nucleus during interphase (Figure 2A). By prometaphase, NuMA had translocated to the future spindle poles (Figure 2B) and located in the spindle poles during metaphase and anaphase (Figure 2C, D), but by telophase and during cytokinesis, the intensity of NuMA staining was observed on chromosomes (Figure 2E) and re-forming daughter cell nuclei (Figure 2F). As donor cells in SCNT, fibroblast cells were often treated with contact inhibition (Figure 2G) and serum starvation (0.5 % FBS) (Figure 2H) for 3-5 days. In these situations, NuMA could also be detected in the nuclei of most cells.

4.4. distribution of NuMA in monkey-rabbit interspecies SCNT embryos

Before enucleation, NuMA was located in the MII spindle poles (Figure 3A), and after enucleation,
Mictrotubules and NuMA in cloned embryos

Table 1. Chromatin and microtubule configuration in the reconstructed embryos 5 to 9 hr after fusion

<table>
<thead>
<tr>
<th>Recipients</th>
<th>No. of oocytes fused(r)</th>
<th>No. of reconstructed embryos in metaphase or anaphase</th>
<th>Chromatin and microtubule configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-activation</td>
<td>49(5)</td>
<td>45(91.8)(^a)</td>
<td>4(8.2)(^a)</td>
</tr>
<tr>
<td>Post-activation</td>
<td>60(5)</td>
<td>13(21.7)(^b)</td>
<td>41(68.3)(^b)</td>
</tr>
</tbody>
</table>

r, Replication; \(^a\), \(^b\). Values with different superscripts within each column are significantly different (at least P<0.05). NMNC, normal microtubule and chromosome assembly; AMNC, abnormal microtubule assembly and normal chromosome change; ACNM, abnormal chromosome change and normal microtubule assembly; AMAC, abnormal microtubule and chromosomes assembly.

Table 2. Chromatin and microtubule configuration in the reconstructed embryos 10 to 13 hr after fusion

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No. of oocytes fused(r)</th>
<th>No. of reconstructed embryos in metaphase or anaphase</th>
<th>Chromatin and microtubule configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-activation</td>
<td>34(5)</td>
<td>21</td>
<td>6(28.6)(^a)</td>
</tr>
<tr>
<td>Post-activation</td>
<td>36(5)</td>
<td>25</td>
<td>8(32.0)(^b)</td>
</tr>
</tbody>
</table>

r, Replication; \(^a\), \(^b\). Values with different superscripts within each column are significantly different (at least P<0.05). NMNC, normal microtubule and chromosome assembly; AMNC, abnormal microtubule assembly and normal chromosome change; ACNM, abnormal chromosome change and normal microtubule assembly; AMAC, abnormal microtubule and chromosomes assembly.

Table 3. In vitro development of monkey-rabbit embryos following nuclear transfer

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No. of recipients (r)</th>
<th>No.of fused (%)</th>
<th>Two-cell (%)</th>
<th>8-cell (%)</th>
<th>blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-activation</td>
<td>201(10)</td>
<td>118(58.7)(^a)</td>
<td>90(76.3)(^a)</td>
<td>21(17.8)(^a)</td>
<td>10(8.5)(^a)</td>
</tr>
<tr>
<td>Post-activation</td>
<td>252(13)</td>
<td>165(65.5)(^a)</td>
<td>133(80.6)(^a)</td>
<td>46(27.9)(^a)</td>
<td>15(9.1)(^a)</td>
</tr>
</tbody>
</table>

r, Replication; \(^a\), \(^b\). Values with different superscripts within each column are significantly different (at least P<0.05).

NuMA at spindle poles was removed together with the spindle (Figure 3B). After nuclear transfer, NuMA, which was initially located in the nucleus of donor cells (figure 3C, D), dispersed among the chromosomes (Figure 3E) after the donor cell nucleus had undergone PCC. NuMA was translocated into pseudo-pronuclei (Figure 3F) and gradually moved to mitotic spindle poles after pseudo-pronuclear membrane broke down (Figure 3G, H, I). NuMA was relocalized in the interphase nuclei when embryos cleaved into 2 cells (Figure 3J).

4.5. effect of NuMA antibody microinjection on the development of monkey-rabbit interspecies reconstituted embryos

After the NuMA antibody or mouse IgG (as control) was injected, monkey–rabbit SCNT embryos were fixed 12-13 hrpf and scored for developmental progression by immunofluorescent microscopy. Spindles were observed in 23 of 50 embryos in the control group and 18 of the 50 embryos in NuMA antibody microinjection group. What is worth to mention is that 78.3%(18/23) of the spindles were normal in the control group, while all 18 spindles were abnormal and over half (10/18) had misaligned chromosomes in the NuMA microinjected embryos (Figure 4A, B, C, D). Unexpectedly, cell division of SCNT embryos was not markedly affected by NuMA antibody injection. The first cell division rates were compared and no difference was found between the NuMA microinjected group (71.8%, 51/71) and the control (70.4%, 28/37).

5. DISCUSSION

It is crucial to understand the cellular and molecular changes during the first cell cycle in designing strategies for successful SCNT. To study the microtubule dynamics, enucleated rabbit oocytes were fixed every hour after activation till 13 hours of culture in vitro. In these oocytes, few microtubules could be detected. However, following transfer of the monkey fibroblast into the ooplasm, extensive microtubule assembly was observed adjacent to the monkey fibroblast nucleus and a normal spindle was organized in one third of the reconstructed embryos, which suggests that the rabbit egg cytoplasm appears to tolerate exogenous centrosome transfer as in bovine oocytes (23), and to support the monkey somatic cell morphological reconstruction.

We compared the nuclear and microtubular dynamics in embryos reconstructed by using non-activated and pre-activated oocytes as recipients. Most fibroblast nuclei transferred into non-activated oocytes underwent PCC, transient spindle formation, chromosome segregation, and finally the formation of two-pronucleus-like structures. In contrast, fibroblast nuclei in pre-activated oocytes rarely underwent PCC, but formed one swollen pronuclear-like structure. Previous studies showed that non-activated oocyte cytoplasm was beneficial for remodeling of the donor nucleus following nuclear transfer and newborns were reported to come from this kind of recipients (24-26). However, several other researches demonstrated that activating oocytes prior to membrane fusion prevented the PCC process but increased the development of cloned embryos (27-29). In this study, there was no difference in the rate of normal organization of chromosomes and microtubules and in early embryo development between the pre- and non-activation groups, which is in agreement with Kurosaka’s study (30).
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**Figure 2.** Immunofluorescent localization of NuMA (nuclear mitotic apparatus) in monkey fibroblasts during mitosis (A–H), NuMA appears in green and DNA appears in red. Yellow reflects the overlapping of NuMA and DNA images. A) interphase, B) prometaphase, C) metaphase, D) anaphase, E) telophase, F) cytokinesis, G) contact inhibition for five days H) serum starvation (0.5 % FBS) for five days. Bar=10 μm.

**Figure 3.** Distribution of NuMA in monkey-rabbit interspecies SCNT embryos. DNA and NuMA were stained red and green, respectively. A) rabbit MII oocyte; B) enucleated rabbit oocyte; C) monkey fibroblast as donor; D) 0.5 h after electrofusion, NuMA was located in the donor nucleus; E) After 3.5 hours of fusion, transient spindle was formed and NuMA was dispersed between the chromosomes and translocated to the two poles of the spindle; F) NuMA was located in the pseudo-pronucleus; G) The membrane of one pseudo-pronucleus broke down, and NuMA moved to the future spindle poles; H) The envelope of both pseudo-pronuclei broke down; I) metaphase of the first division; J) NuMA was located in 2-cell stage interphase nuclei. Bar=20 μm.

NuMA is an intranuclear matrix protein (31) that is present in the nucleus during interphase (32). During mitosis, NuMA is localized to spindle poles and essential for the terminal phases of chromosome separation and/or nuclear reassembly (33). Impairment of NuMA function causes abnormal spindle formation (34). Taimen and his co-workers (35) found that some fibroblasts were absent of NuMA and that a gradual degradation of NuMA took place when the cells passed into a resting stage and the amount of NuMA-negative cells correlated with the confluence of culture. Our results showed that NuMA was normally present in the nucleus and mitotic spindle poles in monkey fibroblasts. Furthermore, we also found that NuMA distributed in most of the nuclei when the cells were in

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contact inhibition or after serum starvation, which made them rest in G₀ or G₁ phase.

Although no NuMA could be detected in the enucleated oocytes as reported previously (12), we found that NuMA from the monkey donor cell could contribute to the formation of a normal spindle in rabbit oocyte. In the reconstructed embryos, NuMA concentrated between the disarrayed chromosomes 3.5 hpf and was translocated to transient spindle poles. NuMA translocated into pseudonuclei and then to the spindle poles at first mitotic metaphase. These results indicate that donor cell NuMA participates in transient spindle assembly and the first mitotic spindle assembly in embryos reconstituted by monkey fibroblasts and enucleated rabbit oocytes.

Microinjection of anti-NuMA antibody into one of two blastomeres of 2-cell-stage mouse embryos inhibited normal cell division(36). While Yang and Snyder (37) showed that anaphase cells injected with NuMA antibodies completed cell division and that these cells had little or no NuMA in their nuclei. Our data demonstrated that early mitotic cell division of reconstructed embryos is not markedly affected by NuMA antibody microinjection, but the spindle assembly and chromosome arrangement were abnormal in NuMA antibody microinjected embryos. So NuMA could participate in the formation and stabilization of the spindle possibly by bundling microtubules and keeping the force balance of microtubules. A similar result was also obtained in pig–mouse interspecies NT embryos (16).

It was found in this experiment that, when monkey somatic cell was introduced into the rabbit ooplasm, no or few microtubules and NuMA were detected in some of the oocytes, suggesting that not only the presence of somatic cells but also the quality of oocyte recipients, as well as the proper interaction between them are required for the normal spindle assembly. We additionally found that about two thirds reconstructed embryos had abnormalities in chromosome alignment, microtubule assembly or NuMA organization. Further studies are needed to clarify which factors contribute to these abnormalities.

In this study we find that neither oocyte chromosome depletion nor the meiotic spindle (including NuMA) removal contributes to the low efficiency of normal spindle assembly. We speculate that the occurrence of abnormalities may be due in part to technical problems, such as the operation of removing the cumulus cells, the NT manipulation at room temperature, the enucleation, the amount of removed cytoplasm, and oocytes quality especially. Our data support the conventional conclusion that incomplete nuclear re-modeling is likely to be the main reason for the lack of live births in primates, as reported by Ng et al (38). The major distinction between development via sperm and egg fertilization versus SCNT is a profound perturbation of gene expression in the latter. The deviations arise from various sources, including influence of the culture medium and factors in the nucleoplasm and cytoplasm. When all going well, morphogens interact to effectively reprogram the new nucleus, which is vital to resetting the developmental program (39). Much more work is required to understand the molecular and cellular events that occur after the introduction of a somatic cell into an enucleated oocyte.

In summary, normal spindle organization and NuMA function are observed at least in part of the cloning embryos reconstructed by transferring monkey somatic cells into enucleated rabbit oocytes. The failure of non-human primate cloning by nuclear transfer may be caused mainly by epigenetic abnormalities.

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**Key Words:** Non-Human Primate, Somatic Cell Nuclear Transfer, First Cell Cycle, Nuclear Mitotic Apparatus Protein, Microtubule, Activation

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