Microtubular spindle dynamics and chromosome complements from somatic cell nuclei haploidization in mature mouse oocytes and developmental potential of the derived embryos

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BACKGROUND: The aim of this study was to investigate haploidization of somatic cell nuclei in non-enucleated mature oocytes regarding spindle formation, chromosomes and developmental potential. METHODS: Mouse cumulus cells were injected into metaphase II oocytes. Some injected oocytes were examined for morphological changes of chromosomes and the spindle immediately, and at 30 min, 1 h or 2 h after the injections. The remaining oocytes were activated by Sr2+ after various incubation periods and observed for formation of a second polar body and pseudo-polar body. Cytogenetic analysis was performed for some of the resulting zygotes. The progress to blastocysts in vitro and the possibility of conception in vivo were assessed. RESULTS: Immediately after injection, the cumulus cell nucleus was still in interphase without spindle formation. The occurrence of premature chromosome condensation (PCC) and spindle formation increased as the incubation time increased. The percentages of activated oocytes increased with the incubation time after nuclear transfer, but the difference was not significant between 1 (58%) and 2 h (62%). The incidence of chromosomal aberrations was high for the derived embryos. Development in vitro was poor, and no procreation of pups occurred after transfer of the 324 embryos. CONCLUSIONS: The PCC and spindle formation induced by cumulus cell nuclei in mature oocytes was time dependent, as was the chance for successful activation. The chromosomal abnormalities from segregation errors presented one obvious cause, apart from the potential epigenetic defects, of developmental failure of the semi-cloned embryos.

Key words: chromosomes/haploidization/microtubular spindle/nuclear transfer

Introduction

The possibility of creating offspring using haploidization of somatic cell nuclei in mature oocytes (reproductive semi-cloning) has remained elusive (Lacham-Kaplan et al., 2001; Tesarik et al., 2001; Tateno et al., 2003b). Tesarik et al. (2001) proposed that the cytoplasm of metaphase II (MII) oocytes was able to drive G0/G1 somatic cell nuclei to premature M phase without previous S phase. They claimed that after activation, segregation of the two sets of parental homologous chromosomes and production of a pseudo-second polar body could occur in the absence of recombination. They performed fluorescence in situ hybridization (FISH) for two pseudo-second polar bodies extruded after injection of cumulus cells to mature human oocytes. The results revealed normal haploid chromosome complements for 13, 18, 21, X and Y.

Using a confocal microscope, Lacham-Kaplan et al. (2001) analysed seven mouse oocytes activated with cumulus cells and showed separation of cumulus cell chromosomes into two sets. One of these sets extruded into a pseudo-second polar body. They transferred 20 semi-cloned embryos to foster females; however, none of them developed to live pups. The process of haploidization, accuracy of chromosomal segregation and developmental potential of the resulting embryos needs further investigation.

Microtubules are crucial for the events following activation of oocytes, i.e. completion of meiosis and second polar body formation (Schatten et al., 1985). Defects in meiotic spindle formation may be one of the reasons for failure of normal chromosomal division and termination of subsequent development. In this study, we used a mouse model to evaluate changes of the microtubular spindle and chromosomes of somatic cell nuclei after injection into non-enucleated mature oocytes. The relationship between the timing of activation and activation outcome was investigated. Cytogenetic analysis of the resulting embryos was performed. We explored the
development of semi-cloned embryos in vitro and their pregnancy potential in vivo.

Materials and methods

Preparation of oocytes
Each female C57BL/6 × DBA/2 F₁ hybrid mouse (8–12 weeks old) was injected i.p. with 5 IU of pregnant mare serum gonadotrophin (Sigma, Saint Louis, MO). After 48 h, 5 IU of HCG (Sigma) was administered to induce ovulation. After 14 h, cumulus–oocyte complexes were collected in HEPES-buffered Chatot–Ziomek–Bavister (CZB) medium (Chatot et al., 1989). Cumulus cells were dispersed by medium containing 80 IU/ml hyaluronidase (Sigma). Mature oocytes with a first polar body were picked up for experiments. They were cultured with CZB medium in an atmosphere of 5% CO₂ in air at 37°C.

Preparation of cumulus cells
Cumulus cells were washed in CZB medium using centrifugation at 300 g for 5 min, and the pellet was kept and suspended in 0.1 ml of medium. A 1 μl aliquot of cell suspension was mixed with 3 μl of 12% polyvinylpyrrolidone (PVP; mol. wt 360 000, Sigma) in HEPES-buffered CZB medium. Cumulus cells were dispersed by medium containing 80 IU/ml hyaluronidase (Sigma). Mature oocytes with a first polar body were picked up for experiments. They were cultured with CZB medium in an atmosphere of 5% CO₂ in air at 37°C.

Injection of cumulus cells into oocytes
Microinjection was performed with the aid of two hydraulic micromanipulators (Narishige, Tokyo, Japan) and microsyringes (IM-6; Narishige) mounted on an inverted microscope (Olympus, Tokyo, Japan) equipped with Hoffman modulation optics. The needle holder for injection was attached to a piezo electric actuator that was driven by a controller (Prime Tech Ltd, Ibaraki, Japan). The procedures of microinjection are demonstrated in Figure 1A–D. Because mouse oocytes were vulnerable to injection at 37°C, we performed this procedure at room temperature (Wakayama et al., 1998). This condition might cause depolymerization of the spindle and segregation of chromosomes. To reduce these effects, we usually injected five oocytes at a time, which took ~5 min. The cumulus cells were injected one by one into oocytes with minimal accompanying medium. During the same period, control oocytes were also placed on the dish. They were then transferred back to CZB culture medium.

Fluorescent staining of chromosomes and spindles
In the first experiment, the morphologically surviving oocytes were examined for chromosomes and spindles immediately, and at 30 min, 1 h or 2 h after activation. Some injected oocytes were incubated for 2 h and then activated. At 3 h after activation, they were examined for the status of chromosomes and microtubules. Fixation and staining has been described in detail previously (Chen et al., 2000). Briefly, oocytes were fixed in 2% formaldehyde (Merck, Darmstadt, Germany) with 0.02% Triton X-100 (Merck) in Dulbecco’s phosphate-buffered saline (DPBS) at 37°C for 30 min. The oocytes were then incubated in

Figure 1. Photographic illustrations of injection of a cumulus cell into a mature mouse oocyte. (A) Prior to injection, a cumulus cell was sucked in and pushed out of the injection pipette to break the cytoplasmic membrane. (B) The chromosome–spindle complex of the oocyte was recognized as a translucent spot (arrow) in the ooplasm. It was placed in the 6 or 12 o’clock position to prevent injury to the oocyte nucleus during injection. The zona was penetrated by several pulses of piezo. (C) We advanced the injection needle into the ooplasm close to the 9 o’clock position. The cumulus cell was placed into the pipette tip. One piezo pulse was applied to break the oolemma. (D) The cumulus cell (arrow) was injected into the oocyte, and the needle was withdrawn. Scale bar = 50 μm.
anti-α-tubulin monoclonal antibody (Sigma) in DPBS with 0.5% bovine serum albumin (BSA) for 45 min. They were washed in 0.01% Tween-20 (Merck) for 15 min. The oocytes were incubated in fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) for 45 min together with Hoechst 33258 (20 μg/ml) (Sigma). Excess antibody and dye were washed for 15 min. The oocytes were transferred into DPBS with 0.5% BSA for 60 min and then wet mounted on depressed glass slides.

Fluorescence was observed using an Optiphot microscope with a magnification of 400× (Nikon, Tokyo, Japan). A Nikon UV-2A filter for the wavelength of 330–380 nm was used to search for the Hoechst 33258 blue fluorescence of chromosomes. A filter of set B-2A for the wavelength of 450–490 nm was employed for the FITC green signal of the spindle. The microscope was equipped with a digital imaging camera, and pictures were acquired and images processed as described (Chen et al., 2000).

**Activation of oocytes with Sr²⁺**

In the second experiment, oocytes were activated immediately, and at 30 min, 1 h or 2 h after injection using Ca²⁺- and Mg²⁺-free CZB medium containing 10 mM Sr²⁺ (Sigma) for 6 h. Some mature oocytes without injection were exposed to the above medium with 5 μg/ml cytochalasin B (Sigma) as parthenogenetic controls. After activation, they were washed and transferred to CZB medium.

**Definition of normal activation**

The presence of two pronuclei with a second polar body and a pseudo-second polar body in oocytes was defined as normal activation (Figure 2A and B). Those with one or three pronuclei as well as two pronuclei with one second polar body were thought to be abnormal (Figure 2C and D). For the parthenogenetic control specimens, the oocytes with two pronuclei without extrusion of the second polar body were selected for further culture.

**Development in vitro**

We observed the normally activated oocytes and parthenogenetic controls daily for 5 days. Development from the 2-cell stage to blastocysts was recorded. Blastocysts in each group were then treated with a hypotonic solution (0.9% sodium citrate and 3% fetal calf serum) for 30 min. They were fixed on slides individually with several droplets of fixative (methanol:acetic acid, 3:1) according to Tarkowski (1966). After staining with Hoechst 33258, the number of nuclei was calculated as the total cell counts under the fluorescent microscope.

**Transfer to pseudopregnant female mice**

In the third experiment, oocytes were activated at 2 h after injection. Embryos at the 2-cell stage from normally activated oocytes were transferred to oviducts of ICR females which had been mated with vasectomized ICR males 1 day previously. Recipient females were Caesarean sectioned on the 20th day post-coitum and their uteri were examined for implantation sites or fetuses.

**Cytogenetic analysis of embryos**

For the fourth experiment, some normally activated oocytes were incubated in CZB medium with 1 μg/ml colchicine (Sigma) for 13–14 h to enforce arrest at the metaphase of the first mitotic division. The zona pellucida was then removed using acidic Tyrode's solution (Sigma). The oocytes were then transferred to the hypotonic solution for 30 min. Each oocyte was fixed on a slide according to the method described above. Some zygotes from natural conception or natural activation were defined as normally activated since each exhibited two polar bodies and two pronuclei (two arrows). (B) Fluorescent stain with Hoechst 33258 demonstrated that both of the two polar bodies contained a nucleus. One was considered to have come from the oocyte genome and the other from the cumulus cell genome. (C) These two zygotes were thought to be abnormally activated. One zygote (left) had two polar bodies and three pronuclei (three white arrows). The other zygote had one polar body (black arrow) and two pronuclei. (D) Fluorescent stain verified one zygote (left) with three pronuclei and the other zygote containing one polar body and two pronuclei. Scale bar = 50 μm.

**Figure 2.** Zygote formation after activation treatment of injected oocytes. (A) These two zygotes were defined as normally activated since each exhibited two polar bodies and two pronuclei (two arrows). (B) Fluorescent stain with Hoechst 33258 demonstrated that both of the two polar bodies contained a nucleus. One was considered to have come from the oocyte genome and the other from the cumulus cell genome. (C) These two zygotes were thought to be abnormally activated. One zygote (left) had two polar bodies and three pronuclei (three white arrows). The other zygote had one polar body (black arrow) and two pronuclei. (D) Fluorescent stain verified one zygote (left) with three pronuclei and the other zygote containing one polar body and two pronuclei. Scale bar = 50 μm.
parthenogenesis were treated as described for controls. Chromosome numbers were counted after the Wright’s stain using the microscope with a magnification of 1000×.

### Micronuclei assessment

Some of the normally activated oocytes were cultured in CZB medium to the 2-cell stage, and then transferred to medium containing Hoechst 33258 (20 μg/ml) for 1 h. They were mounted between a glass slide and a coverslip supported by four dots of a vaseline:parafin (9:1) mixture (Kamiguchi et al., 1991). They were observed for the presence or absence of micronuclei under the fluorescent microscope, according to the criteria suggested by Tienko-Holland et al. (1998), i.e. (i) micronuclei were counted only if they were one-third or less the diameter of the main nucleus; (ii) fluorescence of micronuclei was similar to that of the main nucleus; and (iii) micronuclei were clearly separated from the main nucleus. Some 2-cell embryos from natural conception or parthenogenesis were examined as the control specimens.

### Statistics

The presence of premature chromosome condensation (PCC) and spindle formation, induced by injection of cumulus cell nuclei into oocytes, was examined at various times after injection. The percentages of normal activation, cleavage and blastocyst formation were calculated for injected oocytes with activation and parthenogenetic controls. The rates of normal chromosome number were calculated for embryos obtained by injection of cumulus cell nuclei, natural conception and parthenogenesis. A contingency table analysis was performed with several rows and columns for overall difference. If it was significant, a χ² test or Fisher’s exact test was then carried out for comparisons of groups two by two. The number of nuclei of blastocysts among various groups was compared using a one-way analysis of variance. The between-group differences were evaluated with the Student t-test. A P-value <0.05 was considered significantly different.

### Results

In the first experiment, 356 (85%) of 419 oocytes survived from injection of cumulus cells. The morphological changes of the chromosomes and spindle of injected nuclei in mouse oocytes at various incubation time are shown in Table I. Immediately after injection, the cumulus cell nucleus was still in interphase without spindle formation (Figure 3A). The percentage of PCC with a spindle at 1 h after injection was significantly greater than at 30 min. There was no difference in the rate of PCC between 1 and 2 h after injection. The prematurely condensed chromosomes with a spindle of all 176 oocytes observed at various incubation times displayed disorderly arrangements (Figure 3B and C). At 3 h after activation, the nuclei of both the oocyte and the cumulus cell were in the process of division in the presence of microtubules in 24 of 40 injected oocytes (Figure 3D). Forty-eight mature oocytes were activated only after 3 h. Although we injected cumulus cells into these oocytes, the interphase nuclei of cumulus cells remained unchanged without spindle formation (0/48) 2 h after injection.

In the second experiment, we injected 1094 oocytes, of which 952 (87%) survived. The outcome of activation and development of cumulus cell-injected oocytes activated at various incubation times or parthenogenetically are presented in Table II. The percentage of normal activation was significantly increased for those injected oocytes activated after 1–2 h of incubation compared with those activated immediately or at 30 min. The differences were not significant for the former two groups. The cleaving capability of semi-cloned embryos to the 2-cell stage was not different from parthenogenetic control specimens. However, development to 4-cell, 8-cell, morula and blastocyst stages was significantly impaired for semi-cloned embryos, compared with the parthenogenetic control specimens. Cleavage of semi-cloned embryos was not different among the four activated groups. The mean number of nuclei of blastocysts was not different among the 30 min (47 ± 11, n = 6), 1 h (49 ± 10, n = 22) and 2 h (50 ± 12, n = 24) groups, but that was significantly lower than control specimens (104 ± 12, n = 55).

In the third experiment, we injected 642 oocytes and activated them after 2 h incubation. In the 578 (90%) surviving oocytes, 341 (59%) reached normal activation and 324 (95%) cleaved. After transferring the 2-cell semi-cloned embryos to foster ICR mothers, no implantations or live births were achieved.

For the fourth experiment, 467 oocytes were injected and activated after 2 h of incubation. A total of 280 normal zygotes were attained. One hundred and seventy-two were examined for chromosomes at the metaphase of first mitosis. One hundred and fifty zygotes from natural conception and 179 from parthenogenesis were used as the control specimens. Among the embryos examined, 112 (65%), 105 (70%) and 118 (66%) of each group were analysed successfully (Table III). The percentage of semi-cloned embryos with a normal number of chromosomes was significantly lower than for embryos from natural conception and parthenogenesis (Table III, Figure 4A–C). There were no distinct differences between the latter two groups. Chromosome breakage (Figure 4D) was found in 10% of semi-cloned embryos, but the breakage was not detected in embryos from natural conception or parthenogenesis.

One hundred and two embryos at interphase of the 2-cell stage, which developed from 108 normally activated oocytes, were examined for micronucleation. The percentage of embryos with micronuclei in semi-cloned embryos (15%, 15/102) was significantly higher than embryos from natural conception (0%, 0/98) and parthenogenesis (0%, 0/97).

### Discussion

The results of our study demonstrated that the cytoplasm of mature oocytes triggered PCC and construction of a microtubular spindle for injected somatic cell nuclei in a time-
dependent way. The high level of maturation-promoting factor (MPF), a cell cycle regulator maintaining mature oocytes at MII, may be responsible for these events (Heikinheimo and Gibbons, 1998; Combelles et al., 2002; Tesarik, 2002). After activation, elevation of intracellular calcium induces a decline in the activity of MPF (Edwards and Brody, 1995). We saw that both the somatic cell nuclei and maternal genome underwent reduction segregation through the action of their spindles. When cumulus cells were injected into activated oocytes, their interphase nuclei remained unchanged. The ooplasm with low MPF did not force PCC or spindle formation for somatic cell nuclei.

The percentages of normal activation for injection of cumulus cells into mature oocytes increased with the incubation period before activation. Immediately after injection, the cumulus cell nuclei were at interphase and there was no spindle formation. Activation in this situation failed to induce haploidization of somatic cell nuclei. The percentages of PCC with a spindle of cumulus cell nuclei and normal activation were significantly greater for injected oocytes with 1 h of incubation than those with 30 min of incubation. The percentages were not different between those activated 1 and 2 h after incubation. However, the prematurely condensed chromosomes with a spindle derived by injected cumulus cell

### Table II. Activation of injected oocytes after various incubation periods and their development in vitro, compared with parthenogenetic control samples

<table>
<thead>
<tr>
<th>Development</th>
<th>Immediately (n = 224)</th>
<th>30 min (n = 237)</th>
<th>1 h (n = 235)</th>
<th>2 h (n = 256)</th>
<th>Controls (n = 366)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal zygotes</td>
<td>9 (4)</td>
<td>59 (25)</td>
<td>136 (58)(f)</td>
<td>159 (62)(f)</td>
<td>348 (95)(f)</td>
</tr>
<tr>
<td>2-cell stage</td>
<td>9 (100)</td>
<td>53 (90)</td>
<td>124 (91)</td>
<td>146 (92)</td>
<td>330 (95)</td>
</tr>
<tr>
<td>4-cell stage</td>
<td>7 (78)</td>
<td>44 (75)</td>
<td>112 (82)</td>
<td>134 (84)</td>
<td>313 (90)</td>
</tr>
<tr>
<td>8-cell to morula stage</td>
<td>2 (22)</td>
<td>22 (37)</td>
<td>54 (40)</td>
<td>70 (44)</td>
<td>296 (85)(f)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>0 (0)</td>
<td>6 (10)</td>
<td>22 (16)</td>
<td>24 (15)</td>
<td>285 (82)(f)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

\(a, b, c, d p < 0.05\) compared with injected oocytes of the four groups.

\(e p < 0.05\) compared with injected oocytes that were activated immediately or after 30 min incubation.

![Figure 3. The changes in chromosomes and spindle of cumulus cell nuclei after injection into mature oocytes.](image)
nuclei exhibited a chaotic array. The gross morphology of normal activation for these oocytes was not equal to normal complements of chromosomes.

In studies using meiotic cells, Sasagawa et al. (1998) injected mouse primary spermatocytes into mature oocytes, and verified that the ooplasm had the ability to separate the bivalent homologous chromosomes of primary spermatocytes and the sister chromatids of oocytes simultaneously. The reduction of chromosome number in meiosis was thought to be determined by properties built into the chromosomes (Paliulis and Nicklas, 2000). Kimura and Yanagimachi (1995) introduced mouse secondary spermatocytes into mature oocytes, and the derived embryos resulted in a pregnancy rate of 24% (28/128). The imprinting of male gametes was accomplished in advance of the second meiotic division (Shamanski et al., 1999).

We examined the chromosomes of semi-cloned embryos before the first mitotic division that reflected the status after haploidization of somatic cell nuclei and the second meiosis of oocytes. Judging from the numerical data alone, the incidence of chromosomal aberrations was high. As FISH analysis was not used to study the chromosome pairs, it is possible that not a single case of haploidization was successful.

In normal meiosis I of mammals, replicated homologous chromosomes physically attach by chiasmata at sites of chromatin exchange and segregate from each other to the two opposite spindle poles (Hodges et al., 2002; Eichenlaub-Ritter, 2003). In meiosis II, the sister chromatids stay affixed at their centromeres until anaphase II. This mediates orientation of kinetochores to the two opposite spindle poles and ensures high fidelity of chromosome segregation at the second meiosis (Sandalinas et al., 2002). In contrast, the injected G0/G1 diploid somatic cell consisted of two sets of physically unattached chromosomes. There was no pairing, recombination or cohesion of centromeres that were essential for correct reduction segregation between the homologous chromosomes (Paliulis and Nicklas, 2000). PCC of somatic cell nuclei randomly attached to spindle fibres and a spindle pole, and might move to a pole irrespective of the other parental copy. Therefore, the likelihood of correct haploidization is expected to be very small. Our result is consistent with that reported by Fulka et al. (2002) using somatic cell nucleus transfer into immature oocytes.

In this study, some chromosome breakage and micronucleation were observed for embryos derived from artificial

Table III. Cytogenetic analysis of semi-cloned embryos, compared with embryos from natural conception and parthenogenesis

<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>Semi-cloned embryos (n = 112)</th>
<th>Conception embryos (n = 105)</th>
<th>Parthenogenetic embryos (n = 118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 40</td>
<td>7 (6)</td>
<td>97 (92)</td>
<td>112 (95)</td>
</tr>
<tr>
<td>n &gt; 40</td>
<td>51 (46)</td>
<td>2 (2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>n &lt; 40</td>
<td>43 (38)</td>
<td>6 (6)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Chromosome breakage</td>
<td>11 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. 

$^{a,b,c,d}$P < 0.05 compared with embryos from natural conception or parthenogenesis.

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![Figure 4](image-url)

**Figure 4.** Cytogenetic studies of semi-cloned embryos and controls. (A) A control embryo from natural conception had a normal chromosome number of 40. The arrow indicates the chromatin mass of the second polar body. (B) A control embryo from parthenogenetic activation displays a normal chromosome count of 40. (C) A semi-cloned embryo shows numerical chromosomal aberration with 49 chromosomes. (D) A semi-cloned embryo exhibits chromosome breakage (arrow) possibly derived by chromatin from the injected cumulus cell.
haploidization of somatic cell nuclei in non-enucleated mature oocytes. Micronuclei of blastomeres may represent chromosome fragments or chromosomes that were not incorporated into daughter nuclei during mitosis (Kamiguchi et al., 1991; Milosevic-Dordevic et al., 2003; Norppa and Falck, 2003). Chromosome breakage was also reported for the reduction division of primary spermatoocyte nuclei in the non-enucleated mature oocytes (Kimura et al., 1998) and of the cumulus cell nuclei in enucleated immature oocytes (Palermo et al., 2002). The mechanisms were unclear, but may be due to injection of S-phase cells. Although a majority of cumulus cells surrounding recently ovulated oocytes were in G0/G1 phase of the cell cycle, some cells of S phase or G2/M phase might exist (Schuetz et al., 1996). If the injected nucleus was in the S phase and was driven into premature M phase by the MPF of oocytes, it could result in broken chromosomes after reduction segregation (Alberts et al., 2002). This catastrophe is precluded in normal cell cycles by a DNA replication checkpoint mechanism, which ensures that the initiation of M phase could not occur until the completion of S phase. Another reason for chromosome breakage may be severe anaphase lagging (Norppa and Falck, 2003).

Recently, Tateno et al. (2003a) injected mouse cumulus cells into enucleated mature oocytes. After activation as well as extrusion of the pseudo-polar body, they found that the percentage of chromosomes with the expected outcome of a haploid set of 20 was only 8.9% (15/168). Furthermore, none had a normal complement of chromosomes on karyotyping in the model of hamster cumulus cell injection (0/128). In contrast, some FISH analyses of pseudo-polar bodies from cumulus cell nuclei haploidization in mature human oocytes revealed normal haploid chromatids (Tesarik et al., 2001). This discrepancy may be due to limited information of FISH and the small number of cases in the latter study.

In early embryos of mammals, with the exception of rodents, the formation of the mitotic spindle is related to the sperm-derived centrosome (Rawe et al., 2002). For the semi-cloned embryos, the role of an introduced centriolar microtubule-organizing centre of somatic cells in the subsequent mitotic divisions regarding chromosome segregation at anaphase and ploidy of the daughter cells deserves further investigation (Tesarik, 2002). Development of semi-cloned embryos was poor in vitro, and no procreation of pups occurred after transfer of 324 embryos in our study. One of the causes was the serious chromosome abnormalities. Correct imprinting during gametogenesis and epigenetic reprogramming during the the preimplantation period are essential for normal developmental potential. They involve DNA methylation, modification of histones, and chromatin remodelling that regulates gene expression of early embryos, cell cleavage and cell determination (De Rycke et al., 2002). The epigenetic status of the donor nucleus is different from that of the gametes. Therefore, the other major determinant of success would hinge on the degree of epigenetic modification of somatic DNA in the cytoplasm of oocytes (Wakayama et al., 1998). In conventional cloning, reprogramming led to low efficiency and high rates of embryonic arrest, fetal death and anomalies (Humpherys et al., 2001). For the semi-cloning, embryonic nuclei were constructed by the genomes of oocytes and somatic cells. The reprogramming of somatic cell nuclei in non-enucleated oocytes deserves further study.

Acknowledgements

The authors are grateful to Ms Yi-Yi Tsai and Ms Li-Rong Chang for their technical assistance. This study was supported in part by grants from the National Science Council (NSC 91-2314-B002-368) and the National Taiwan University Hospital (92A06-6), Taipei, Taiwan.

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Submitted on November 7, 2003; accepted on January 8, 2004