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# *In vitro* development of non-enucleated rat oocytes following microinjection of a cumulus nucleus and chemical activation

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## Summary

The present study examined *in vitro* development and the cytological status of non-enucleated rat oocytes after microinjection of cumulus nuclei and chemical activation. Oocyte–cumulus complexes were collected from gonadotropin-treated prepubertal female Wistar rats 14 h after human chorionic gonadotropin (hCG) injection. Cumulus nuclei were injected into ovulated oocytes and then stimulated in the presence of 5 mM $SrCl_2$ for 20 min at various time points (0–3.5 h) after injection. Some of the reconstituted eggs were cultured to observe the pronuclear formation, cleavage, and blastocyst formation. The incidences of eggs forming at least one pronucleus or containing two pronuclei were not significantly different among the periods (82.4–83.5% and 43.4–51.9%, respectively). Nor did the incidences of eggs cleaving (86.7–97.7%) and developing to the blastocyst stage (0–3.5%) differ depending on when, after injection, stimulation began. When some of the reconstituted eggs were observed for cytological morphology 1–1.5 h after injection, 71.7% of the eggs caused premature chromatin condensation, but only 46.2% of them formed two spindles around each of maternal and somatic chromatins. However, the morphology of the somatic spindles differed from that of the spindles, which formed around the oocyte chromatins. Only 7.5% of the eggs contained the normal chromosomal number. In many reconstituted oocytes, before activation, an abnormal spindle formation was observed in the somatic chromatins. In conclusion, these results show that non-enucleated rat oocytes injected with cumulus nuclei can form pronuclei and cleave following chemical activation, whereas blastocyst formation is very limited, probably caused by abnormalities in the spindle formation and distribution of somatic chromatids.

Keywords: Cumulus cell, *In vitro* development, Microinjection, Oocytes, Rat

## Introduction

If diploid zygotes constituted with haploid somatic and oocyte genomes are able to successfully develop to term, we may have discovered a new method of reproduction, utilizing somatic cells as male or female gametes for animal production (Kubelka & Moor, 1997). Some attempts to haploidize the somatic genome have been reported in humans (Tesarik *et al.*, 2001; Palermo *et al.*, 2002), mice (Lacham-Kaplan *et al.*, 2001; Palermo *et al.*, 2002) and rabbits (Zhang *et al.*, 2005). Sperm injection into the ooplasm of human oocytes that were injected somatic nuclei after enucleation succeeded in causing the oocyte to extrude a pseudo-polar body, forming a male and a somatic pronucleus (Tesarik *et al.*, 2001). It has been shown that reconstituted zygotes, produced by injecting a somatic cell into the oocyte, formed two pronuclei with two additional polar bodies (Lacham-Kaplan *et al.*, 2001). Microinjection of somatic cells into oocytes at the germinal vesicle (GV) stage has also been examined as a way to haploidize the somatic genome (Palermo *et al.*, 2002; Zhang *et al.*, 2005).

It has been recognized that a chromosomal multi-protein complex, cohesin, establishes and maintains the cohesion between sister chromatids (Uhlmann, 2003). During mitosis, cohesin is removed from chromosomes in two steps: along the chromosome

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arms during condensation in prophase and then from the centrosomes preserved by the action of shugoshin (Watanabe & Watanabe, 2005) at the transition from metaphase to anaphase (Hauf *et al.*, 2001), caused by the activity of separase. However, cohesin rebinds chromosomes soon thereafter in telophase (Waizenegger *et al.*, 2000), whereas it is not known if chromatin-bound cohesin plays a role during the following $G_1$ period, before the synthesis of sister chromatids during S phase. During meiosis, on the other hand, cohesin along chromosome arms is dissolved in the first, reductional division to allow homologue segregation, and then cohesin left at the centrosomes appears to be cleaved by separase, allowing separation of sister chromatids in the second, equational division (Uhlmann, 2003). Although two core subunits of cohesin, Scc1 and Scc3, are replaced in meiosis by their homologues, Rec8 and SA3, respectively (Watanabe *et al.*, 1999; Losada *et al.*, 2000), cohesin appears to be removed by a pathway that probably involves the phosphorylation of cohesion subunits and cleaving by separase (Uhlmann, 2003). However, when somatic cells at the $G_1$ phase are microinjected into oocytes at the metaphase II stage and then activated, it is not known yet if cohesion rebound chromosomes are affected by the cytoplasmic factors of oocytes, during premature chromosome condensation (PCC), and then become helpful to distribute the somatic chromatids at oocyte activation.

Rats have been used widely in various fields of biomedical research as a model animal for human diseases and functional analysis (Abbott, 2004). To our knowledge, however, although some investigations have sought to determine the morphological changes, such as premature chromosome condensation, of somatic chromatins in rat oocytes (Ito *et al.*, 2005; Hirabayashi *et al.*, 2003), no investigations have been done on the haploidization of somatic genomes in this species.

In the present study, therefore, cumulus nuclei were microinjected into non-enucleated rat oocytes, the reconstituted oocytes were chemically activated, and the pronuclear formation and *in vitro* development of the embryos were observed. Cytological status, such as premature chromatin condensation and spindle formation after activation of the reconstituted eggs, and the chromosomal number at the first mitosis were also observed.

## Materials and methods

### Chemicals and culture media

NaCl, KCl and $KH_2PO_4$ were purchased from NACALAI TESQUE, Inc. $NaHCO_3$ and $CaCl_2$ were purchased from ISHIZU SEIYAKU LTD. MEM Amino Acids Solution and MEM Non-Essential Amino Acids Solution were purchased from GIBCO (Invitrogen Corp.). Other chemicals were purchased from Sigma-Aldrich. The medium used for collection of oocyte–cumulus complexes and for microinjection was BSA-free HEPES-buffered modified Krebs-Ringers bicarbonate solution (HEPES mKRB-PVA), composed of 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM $KH_2PO_4$, 1.19 mM $MgSO_4$, 5 mM $NaHCO_3$, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 20 mM HEPES, 0.1% polyvinyl alcohol (w/v, PVA), 50 $\mu$g/ml streptomycin sulphate and 75 $\mu$g/ml potassium penicillin. The basic embryo culture medium was modified 1-cell rat embryo culture medium (mR1ECM) (Miyoshi *et al.*, 1994)). The medium used for handling cumulus cells to determine the DNA content was phosphate-buffered saline (PBS).

### Collection of oocytes and cumulus cells

Following the guidelines for animal experiments at Okayama University, sexually immature female Wistar rats (21–28 days old) were superovulated by intraperitoneal (i.p.) injection of 15 IU hCG following 15 IU eCG at an interval of 48 h. Fourteen hours after hCG injection, ovulated oocyte–cumulus complexes were flushed with HEPES mKRB-PVA containing 0.1% hyaluronidase at 37°C, and incubated for 5 min. Both cumulus cells and oocytes were washed three times and collected in HEPES mKRB-PVA.

### Determination of DNA content in cumulus cells

To evaluate the ploidy of cumulus cells, the DNA content of each one was examined by flow cytometry. Collected cumulus cells were suspended in PBS, filtered through a 44 $\mu$m nylon mesh and then fixed with 70% ethanol. The cells were washed twice and treated with 0.5 $\mu$g/ml RNase A at 37°C for 30 min. The treated cells were stained with 50 $\mu$g/ml propidium iodide at room temperature. Fluorescence was measured using a flow cytometer (EPICS XL, Beckman Coulter), and data were analysed using the accessory software (SYSTEM II, Beckman Coulter).

### Microinjection of cumulus nucleus

Denuded oocytes and cumulus cells were placed in a droplet of 5 $\mu$l HEPES mKRB-PVA at room temperature. A cumulus cell was aspirated in and out by blunt-ended injection pipette with an inner diameter of 4–7 $\mu$m to break the cell membrane. The cumulus nucleus was injected into the ooplasm. Reconstituted oocytes were transferred to mKRB and cultured in the same medium at 37°C in an atmosphere of 5% $CO_2$ in air.

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### Chemical stimulation and *in vitro* culture of reconstituted oocytes

Reconstituted oocytes and the intact controls were cultured for 20 min in $Ca^{2+}$-free mKRB containing various concentrations (0, 1.25, 2.5, 5, and 10 vmM) of $SrCl_2$. The oocytes were then cultured in mKRB for 9–12 h to observe for survival and pronuclear formation. Some of the reconstituted eggs formed two pronuclei and were transferred into mR1ECM and cultured at 37 °C in an atmosphere of 5% $CO_2$ in air. Cleavage and blastocyst formation were examined 24 and 120 h after the start of culture, respectively.

### Analysis of spindle formation

Some of the reconstituted oocytes were fixed with 4% paraformaldehyde at room temperature for 30 min. After treatment with blocking medium containing 150 mM glycine and 10% fetal bovine serum for 15 min, the oocytes were then incubated in anti-tubulin monoclonal antibody in PBS PVA at 4 °C overnight. The oocytes were incubated in fluorescein isothiocyanate (FITC-)conjugated antimouse IgG at 39°C for 30 min. Following incubation in 20 $\mu$g/ml propidium iodide for 40 min, the oocytes were then mounted on glass slides, observed using a confocal laser scanning microscope (FV300, Olympus), and analyzed using the accessory software (FLUOVIEW, Olympus).

### Analysis of chromosomal aberration in reconstituted eggs

Nine to 12 h after chemical stimulation, some of the reconstituted eggs with two pronuclei were treated with 40 ng/ml demecolcine in mKRB for 13–15 h. The zona pellucida of the eggs, in which pronuclei had already vanished, were removed by acidified Tyrode's solution. The zona-free oocytes were transferred to a hypotonic solution (4% fetal bovine serum and 0.9% sodium citrate) for 10 min and then fixed on a slide with Karnoa solution (methanol:acetic acid, 3:1). The specimen was stained with Giemsa stain, and the chromosomal number was determined at 1000× magnification.

### Experimental design

Experiment 1: To determine the ploidy of cumulus cells, the DNA content of each cumulus cell was examined by flow cytometry.

Experiment 2: To examine the optimal $SrCl_2$ concentration for activation of rat oocytes, denuded oocytes were exposed to 0, 1.25, 2.5, 5 and 10 mM $SrCl_2$ for 20 min. Following culture in mKRB for 9–12 h, the oocytes were observed for survival, as determined by the normality of the plasma membrane and pronuclear formation, under an inverted microscope.

Experiment 3: The oocytes, after being injected with cumulus nuclei, were cultured for various durations (0–0.5, 1–1.5 or 3–3.5 h) in mKRB and were then treated for 20 min in $Ca^{2+}$-free mKRB containing 5 mM $SrCl_2$. For controls, we treated intact, mature oocytes with 5 $\mu$g/ml cytochalasin B in mKRB for 2 h after culturing them for 20 min in $Ca^{2+}$-free mKRB containing 5 mM $SrCl_2$. Survival and pronuclear formation were examined after culture in mKRB for 9–12 h. To examine the early development, some of the reconstituted eggs that formed two pronuclei were cultured in mR1ECM for 120 h.

Experiment 4: To examine the PCC of the nuclei and spindle formation, oocytes were cultured for 1–1.5 h in mKRB after injection of cumulus nuclei, and were fixed and processed as described above. To determine the chromosomal number, the reconstituted oocytes were chemically stimulated 1–1.5 h following injection of cumulus nuclei and then processed to observe the ploidy after being cultured for 13–15 h in the presence of demecolcine.

### Statistical analysis

The data from replicated experiments were analysed by one-way ANOVA and Bonferroni/Dunn's post hoc test, using the StatView program (Abacus Concepts, Inc.). All data were expressed as means ± S.E.M. A value of $p < 0.05$ was considered to be statistically significant.

## Results

### DNA content in a cumulus cell (Exp. 1)

DNA content in cumulus cells was evaluated by flow cytometry. Standard ploidies were set up by using testicular cells. Of cumulus cells derived from ovulated oocyte–cumulus complexes (OCCs), 90.0 ± 1.1% and 9.5 ± 1.0% were 2C and 4C DNA contents, respectively (Fig. 1).

### Optimal condition of chemical stimulation (Exp. 2)

When oocytes were cultured for 20 min in $Ca^{2+}$-free mKRB containing 0, 1.25, 2.5, 5 and 10 mM $SrCl_2$, survival rates did not differ in oocytes stimulated with between 0 and 5 mM $SrCl_2$ (96.7–100%), but did in those stimulated with between 1.25 and 10 mM (100% and 72.9%, respectively). Oocyte activation rates increased when oocytes were exposed to higher $SrCl_2$ concentrations and were higher at 5 and 10 mM (92.6 and 98.5%, respectively) as compared with other concentrations (Table 1).

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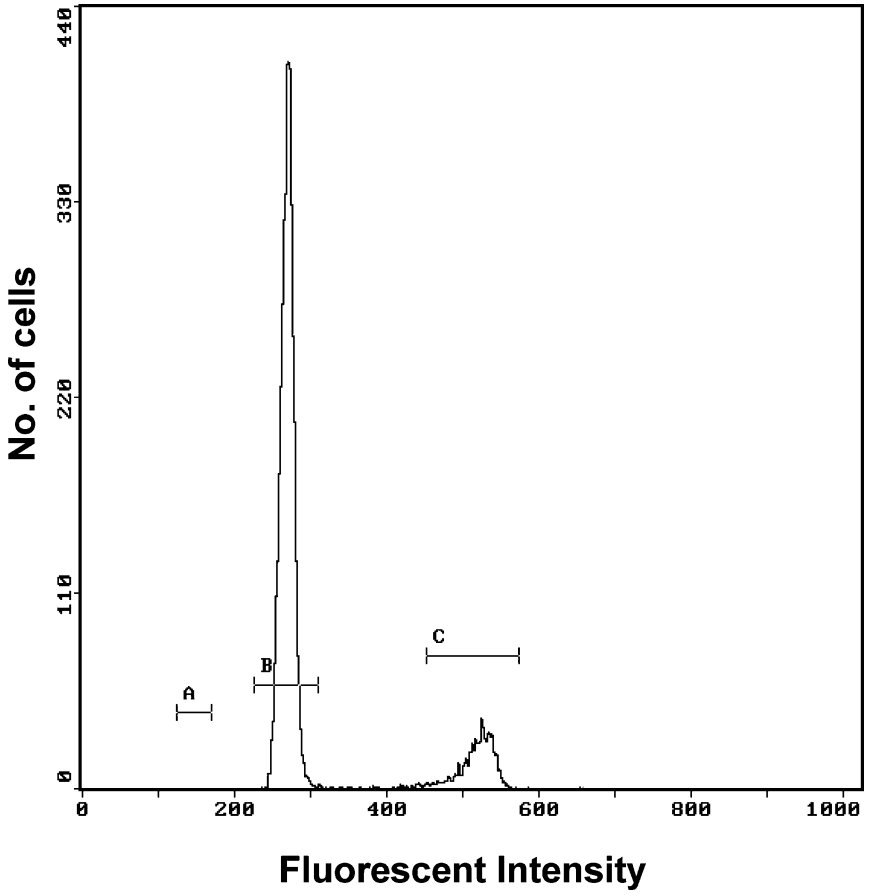


**Figure 1** Flow cytometric analysis of DNA content in cumulus cells. To set up the standard ploidies testicular cells of (a) 124–170, (b) 226–310, (c) 452–574 nm fluorescence intensities were used.

**Table 1** Effect of $SrCl_2$ concentrations on survival and activation of rat oocytes

| $SrCl_2$ (mM) | No. of oocytes examined | No. (%) of oocytes survived (day)[d] | No. (%) of oocytes activated[e] |
|---|---|---|---|
| 0 | 183 | 183 (99.5 ± 0.5)[a,b] | 6 (4.3 ± 4.3)[a] |
| 1.25 | 145 | 145 (100 ± 0)[a] | 11 (6.8 ± 3.0)[a] |
| 2.5 | 146 | 145 (99.3 ± 0.7)[a,b] | 66 (45.5 ± 9.1)[b] |
| 5 | 138 | 133 (96.7 ± 1.2)[a,b] | 122 (92.6 ± 3.8)[c] |
| 10 | 157 | 114 (72.9 ± 3.6)[b] | 112 (98.5 ± 1.5)[c] |

Rat oocytes were exposed to $SrCl_2$ for 20 min and then cultured in mKRB for 9–12 h.
Data are given as means ± S.E.M. from four replicated trials.
[a–c]Value with different superscripts within row are significantly different ($p < 0.05$).
[d]Percentage of the number of oocytes examined.
[e]Percentage of the number of surviving oocytes.

### *In vitro* development of reconstituted oocytes (Exp. 3)

When oocytes were activated 0–0.5, 1–1.5, or 3–3.5 h after injection with cumulus nuclei, the incidence of eggs forming at least one pronucleus was not different among the periods or as compared with control parthenogenetic eggs, whereas the survival rate was lower in eggs activated within 30 min after injection (Table 2). However, the incidence of eggs forming two pronuclei did not differ among the periods, but was lower than controls. When the reconstituted embryos forming two pronuclei were cultured in mR1ECM, the incidences of cleavage and blastocyst formation did not differ among the periods (Table 3). However, the blastocyst formation rates were much lower than parthenogenetic controls. Morphologies of the blastocysts seemed to be normal with 17–19 blastomeres (Fig. 2).

### Morphology of injected cumulus chromatin (Exp. 4)

When reconstituted oocytes were observed 1–1.5 h after injection, 71.7 ± 3.8% of the oocytes had already caused PCC in both chromatins derived from oocyte and somatic cell. Of those oocytes, 46.2 ± 4.6% formed two spindles. In a majority of oocytes, however, an abnormal spindle formation was observed around the somatic chromatins (Fig. 3*a*), whereas at least 44% of

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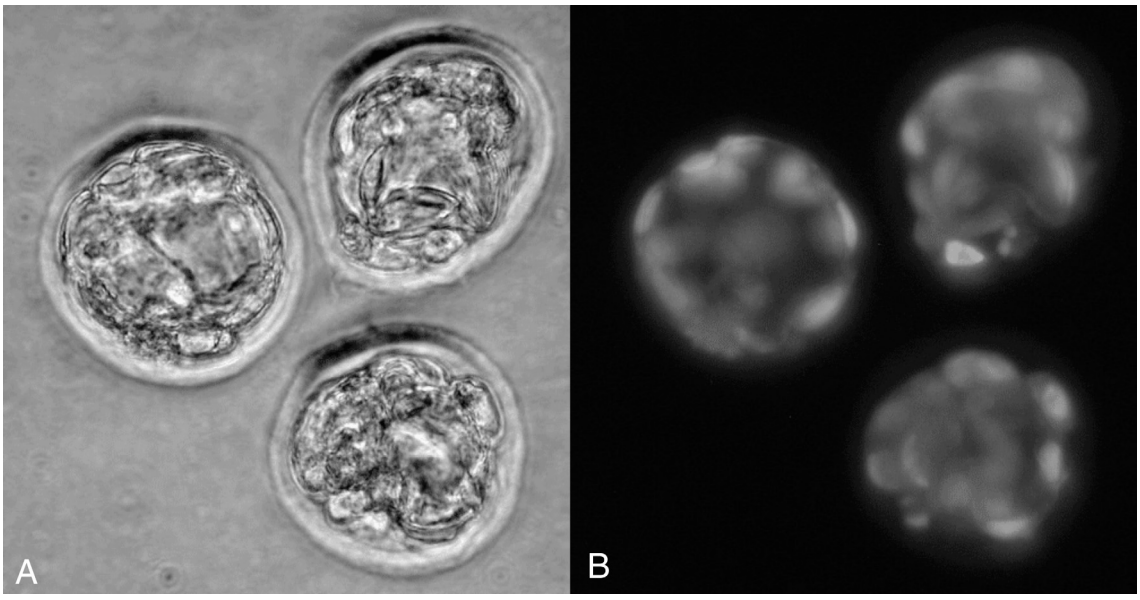


**Figure 2** Morphology of blastocysts developed from reconstituted eggs following injection of cumulus nucleus. (*a*) Image under phase contrast microscopy; (*b*) Image under fluorescence microscopy after staining with Sybr Green.

**Table 2** Pronuclear formation of reconstituted oocytes activated at different times after microinjection

| Duration between microinjection and activation (h) | No. of oocytes examined | No. of surviving oocytes (%) | No. of oocytes formed PN (%) | No. of oocytes with 2PN (%) |
|---|---|---|---|---|
| Control[d] | 218 | 167 (75.8 ± 8.8)[b] | 143 (87.2 ± 5.1) | 112 (68.7 ± 6.6)[b] |
| 0–0.5 | 353 | 202 (56.3 ± 4.0)[c] | 170 (83.5 ± 2.9) | 88 (43.4 ± 3.8)[c] |
| 1–1.5 | 353 | 229 (62.2 ± 3.3)[b,c] | 195 (82.5 ± 4.8) | 118 (49.7 ± 3.9)[c] |
| 3–3.5 | 302 | 202 (66.9 ± 3.7)[b,c] | 170 (82.4 ± 3.7) | 106 (51.9 ± 2.2)[c] |

Data are given as means ± S.E.M. from 4–11 replicates.
PN: pronuclei.
[a]Percentage of the number of surviving oocytes.
[b, c]Value with different superscripts within row are significantly different ($p < 0.05$).
[d]Controls were oocytes treated with 5 mM $SrCl_2$ for 20 min and then 5 $\mu$g/ml cytochalasin B for 2 h.

**Table 3** *In vitro* development of reconstituted eggs that formed 2PN after microinjection and chemical activation

| Duration between microinjection and activation (h) | No. of embryos examined[a] | No. (%) of embryos | No. (%) of embryos that developed to cleaved blastocyst stage |
|---|---|---|---|
| Control[d] | 112 | 108 (95.7 ± 2.2) | 49 (43.5 ± 11.2)[b] |
| 0–0.5 | 62 | 51 (86.7 ± 5.4) | 0 (0.0 ± 0.0)[c] |
| 1–1.5 | 93 | 85 (91.9 ± 2.6) | 4 (3.5 ± 2.3)[c] |
| 3–3.5 | 84 | 68 (97.7 ± 1.2) | 1 (0.7 ± 0.7)[c] |

Data are given as means ± S.E.M. from 4–8 replicated trials.
[a]Only embryos that formed two pronuclei following microinjection and activation were examined.
[b, c]Value with different superscripts within row are significantly different ($p < 0.05$).
[d]Controls were oocytes treated with 5 mM $SrCl_2$ for 20 min and then 5 $\mu$g/ml cytochalasin B for 2 h.

them had already progressed after anaphase (Fig. 3*b*). When the reconstituted eggs that formed two pronuclei after activation were fixed during the first mitosis, the incidence of eggs containing the correct chromosomal number was significantly lower than for eggs fertilized *in vivo* (7.5% and 76.9%, respectively; Fig. 4).

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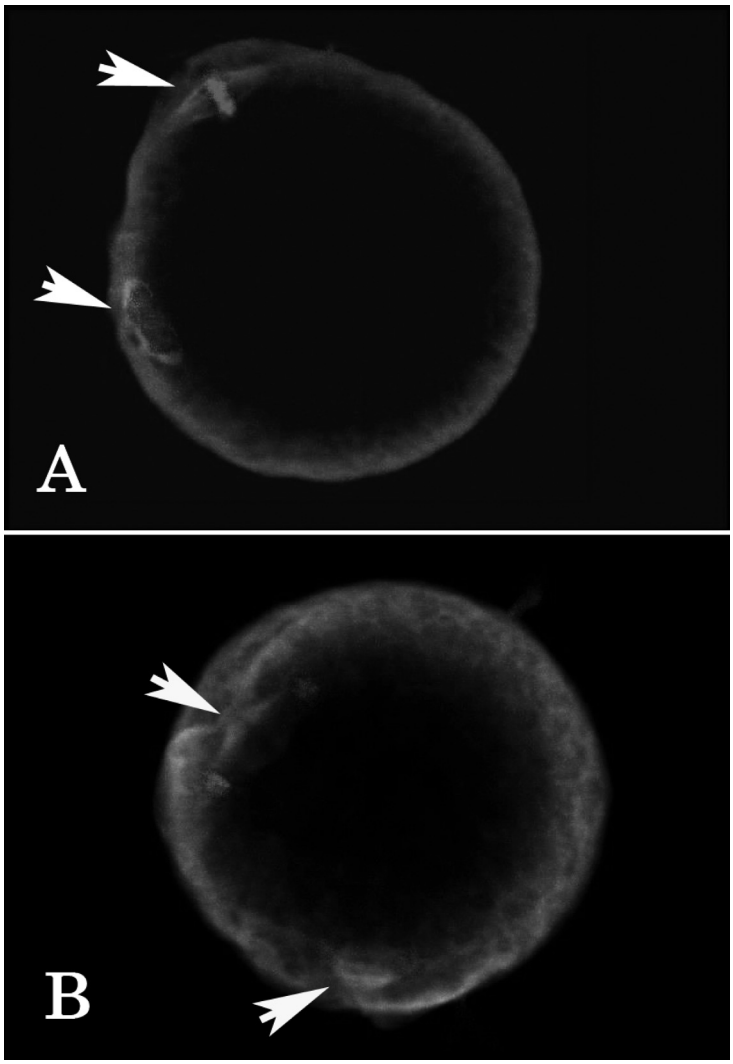


**Figure 3** PCC and spindle formation in a reconstituted egg at 1–1.5 h after injection of cumulus nucleus. (*a*) both oocyte and somatic chromatins caused PCC and formed spindles, whereas an abnormal spindle formation was observed in the somatic chromatin. Note abnormal microtubule organization of the lower (somatic) spindle. (*b*) Although at least 44.4% of the reconstituted oocytes resulted in spontaneous activation, note the different microtubule distribution (especially at the equatorial plate) in the lower (somatic) spindle, as compared with the meiotic one.

## Discussion

To obtain the successful development of reconstituted eggs by microinjection of somatic nuclei into intact rat oocytes should require: successful reprogramming of the somatic genome by exposure to the ooplasm (Wakayama *et al.*, 1998; Alberio *et al.*, 2006), efficient activating of oocytes, and normal reduction and distribution of both somatic and oocyte genomes to the reconstituted eggs. However, because few reports in the literature provide reliable information about what is normal for these steps, this study was undertaken to fill this gap in knowledge.

Mouse oocytes have been generally activated in strontium-containing medium (Wakayama *et al.*, 1998). Although some reports have demonstrated strontium-induced parthenogenetic activation of rat oocytes, researchers have not yet reached agreement about optimal condition for doing so (Kato *et al.*, 2001; Roh *et al.*, 2003; Tomashov-Matar *et al.*, 2005; Hirabayashi *et al.*, 2002). In a $Ca^{2+}$- and $Mg^{2+}$-free culture medium, treatment with 2 mM $SrCl_2$ has been reported as optimal to induce the first $[Ca^{2+}]_i$ transient, but the frequency and duration of the oscillations differed from sperm-activated eggs (Tomashov-Matar *et al.*, 2005). In a previous report (Hirabayashi *et al.*, 2002), rat oocytes injected with a round spermatid were successfully activated when placed for 20 vmin in $Ca^{2+}$-free mKRB containing 1.25 mM $SrCl_2$, whereas rates of pronuclear formation and first cleavage were 50 and 40%,

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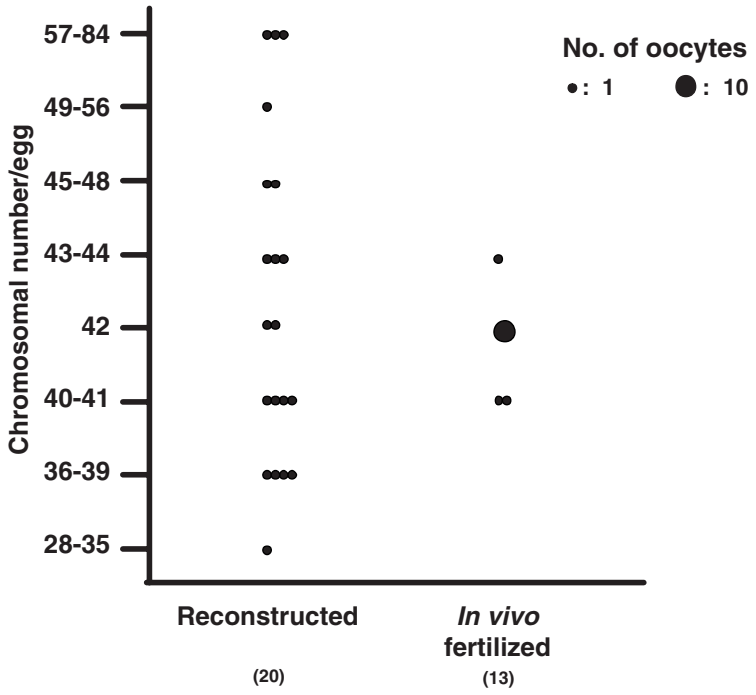


**Figure 4** Distribution of chromosomal number in reconstituted and *in vivo* fertilized embryos. Numbers in parentheses indicate the number of embryos examined.

respectively (Hirabayashi *et al.*, 2002). In the present study, however, treatment for 20 min with 1.25 mM $SrCl_2$ in $Ca^{2+}$-free mKRB did not activate oocytes well. A higher oocyte activation rate was observed at 5 and 10 mM, as compared with other concentrations, whereas survival rate was lower at 10 mM, as compared with 1.25 vmM. Furthermore, we observed that 95.7% and 43.5% of oocytes cleaved and developed to the blastocyst stage, respectively, after treatment for 20 min with 5 mM $SrCl_2$ for oocyte activation and then treatment for 2 h with 5 $\mu$g/ml cytochalasin B to prevent the extrusion of the second polar body, which occurred 1–1.5 h after activation (Roh *et al.*, 2003). These developmental rates of oocytes activated in the current conditions were much higher than previously reported for rats (Kato *et al.*, 2001; Krivokharchenko *et al.*, 2003; Roh *et al.*, 2003). From these results, we determined that the optimal concentration of $SrCl_2$ was 5 mM for chemical activation of rat oocytes in our conditions; this concentration was used in the following experiments.

Generally, a somatic cell is diploid (2N) with two identical pairs of sister chromatids (4C) during the $G_2$ phase, whereas the DNA content (the number of chromatids) reduces to the half (2C) after cell division ($G_1$ phase). In the current study, we expected cumulus cells, as a candidate cell type, to be incorporated into oocytes, since they were convenient to collect. In our experiments, we found that about 90% of cumulus cells derived from ovulated OCCs contain 2C DNA contents. Therefore, a majority of cumulus cells that we used for incorporation into oocytes by microinjection were at the $G_1$ phase (2N, 2C). On the other hand, germ cells become haploid (1N, 2C) after the first meiotic division, and then each of the remaining chromatids will be segregated to the daughter cells (1N, 1C) at the second meiotic division. When a somatic cell is microinjected into an intact oocyte, the somatic nuclear membrane should disappear, and the chromosomes should exhibit PCC and then form a spindle. As each identical pair of sister chromatids is distributed into each daughter cell during the first meiotic cleavage, we expected that, during PCC and the following meiosis II, each of the paternal and maternal chromatids of somatic cells would be divided equally caused by the ooplasmic factor and that the reconstituted eggs could develop normally. However, our results did not support our hypothesis. The number of chromosome in reconstituted eggs at the first cleavage varied from 28–84, whereas a majority of cells in the control embryos had an average of 42 chromosomes, varying from 40–44. In the current study, only 7.5% of reconstituted eggs contained the correct number of chromosomes (42). The current result shows that somatic chromatids are distributed at random into the reconstituted eggs at the second and pseudo-polar body extrusion in the rat. Previous reports on the mouse also showed a similarly low percentage of reconstituted eggs with normal numbers of chromosomes (Chen *et al.*, 2004) or relatively lower incidences (Tateno *et al.*, 2003; Heindryckx *et al.*, 2004). Therefore, this abnormality in

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the distribution of chromatids appears to be a common phenomenon when a $G_1$ somatic nucleus is introduced into an intact oocyte.

Furthermore, because the range of morphological differences were observed among exposure periods we examined here, this abnormal incidence seems unlikely to be improved by exposing somatic chromatins to ooplasmic factors for a longer duration. From S phase to prophase of the cell cycle, cohesion was established between sister chromatids, and then the cohesions were removed completely at the metaphase to anaphase transition (Uhlmann, 2003). Some factors required to distribute chromosomes equally – such as monopolin, a protein complex required for the segregation of homologous centromeres to opposite poles of a dividing cell during meiosis I of cell division – may not be available in oocytes at metaphase II. In this condition, chromosomes derived from the $G_1$ somatic cell, which would exist individually without any cohesion between paternal and maternal chromatids, would probably be distributed at random to the opposite poles of a reconstituted egg at the extrusion of the second and pseudo-polar body.

In the present study, furthermore, the injected cumulus nuclei had already changed to condensed chromatin in >70% of oocytes 1–1.5 h after the injection. However, we found abnormalities in the spindle formation, especially as to microtubule morphology in the reconstituted eggs. In mice, abnormality has been observed in spindle formation after somatic cell nuclear transplantation (Van Thuan *et al.*, 2006). A deficit of some factors associated with the segregation of homologous centromeres to opposite poles of a dividing cell may affect this abnormal microtubule distribution of the spindle. Regardless of the duration of the exposure of somatic nuclei to the cytoplasmic factors (the amount of time between microinjection of the somatic nucleus and egg activation), the incidence of reconstituted eggs with two pronuclei was not different. However, the incidence was lower than for parthenogenetic controls, even although the activation rate did not differ. Our results suggest that the reformation of the somatic pronucleus is somehow inhibited or delayed, caused by abnormal spindle formation in somatic chromatin in rat oocytes.

When reconstituted eggs were cultured for 120 h following injection of the cumulus nuclei and, after an interval of 1–1.5 h, chemical activation, a very low rate of blastocyst formation was observed (3.5%). This percentage was much lower than for parthenogenetic diploid controls (43.5%) and was also slightly lower than for mouse eggs injected with cumulus nuclei and then activated (10–15%) (Chen *et al.*, 2004). We consider this unsuccessful development of reconstituted eggs to be due mainly to abnormal spindle formation and/or unequal distribution of chromatids, as described above. In the current study, furthermore, we found that >40% of oocytes had already spontaneously resumed meiotic arrest at the metaphase II stage, 1–1.5 h after injection of the cumulus nuclei. In rats, matured oocytes are spontaneously activated *in vitro*. This abnormality has been pointed out as a major obstacle to succeeding in somatic cell nuclear transplantation (Zhou *et al.*, 2003; Mullins *et al.*, 2004). This abnormal spontaneous activation could be another possible cause of the reduction in the developmental competence of the reconstituted eggs to reach the blastocyst stage.

In the current study, cumulus nuclei had caused PCC, which may be essential for nuclear reprogramming (Collas & Robl, 1991), in a majority of manipulated eggs 1–1.5 h after injection. It is now clear that the epigenetic control is an essential feature of normal development (Reik *et al.*, 2001). The inefficient rate for producing live offspring by somatic cell nuclear transfer is believed to be associated with epigenetic errors of incorporated somatic genome, whereas the mechanism of reprogramming is still unclear (Rideout *et al.*, 2001; Wakayama, 2007). Insufficient reprogramming of somatic genome may be additional possible cause hidden behind abnormalities in chromosome distribution.

In conclusion, we demonstrate here that reconstituted rat eggs derived from microinjection of cumulus nuclei into intact oocytes have very limited developmental competence, caused by abnormal spindle formation, irregular chromosome distribution, and/or spontaneous oocyte activation. We did not observe any differences in the developmental competence of reconstituted eggs. When somatic nuclei are introduced into intact oocytes and then activated, abnormalities in chromosome distribution may be a more serious problem than the unsuccessful reprogramming of the somatic genome.

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