Zygote **13** (May), pp. 109–114. © 2005 Cambridge University Press
doi:10.1017/S0967199405003199 Printed in the United Kingdom

Transplantation of somatic nuclei into oocyte cytoplasm reveals that the chromosome properties determine the chromosome separation fate in rabbit

Li-sheng Zhang, Li-Juan Yao, Yan Jiang, Man-Xi Jiang, Zi-Li Lei, Qing-Yuan Sun and Da-Yuan Chen
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, People's Republic of China

Date submitted: 08.10.04. Date accepted: 25.01.05

Summary

G_2/M somatic nuclei were introduced into enucleated meiotically competent oocytes and subsequently cultured in TCM199 plus 10% fetal calf serum (FCS). Pseudo-first polar bodies could be extruded, but the chromosomes failed to arrange normally. Kinetochores were traced with immunofluorescent microscopy using autoimmune sera from patients with CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasia) scleroderma. *In vitro* matured oocytes arrested at second meiotic metaphase and kinetochores were detectable as paired structures aligned at the spindle equator. At meiotic anaphase, present or past the kinetochores separated and remained aligned at the distal sides of the chromosomes until telophase, when their alignment perpendicular to the spindle axis was lost. Kinetochores failed to arrange normally after transferring somatic nuclei into oocytes. Our results suggest that somatic cell nuclei are unable to proceed normally through meiosis when introduced into oocyte meiotic cytoplasm.

Keywords: Chromosome separation, Kinetochore, Nuclear transfer, Oocyte, Rabbit, Somatic cell

Introduction

Primary spermatocytes are in the G_2 cell-cycle phase before the first meiotic division. Normal offspring have been born after microfertilization with round spermatids in mouse, rabbit and human, and with secondary spermatocytes in mouse (Ogura *et al*., 1998). The nucleus of primary spermatocytes can participate in normal fertilization and support full embryonic development (Ogura *et al*., 1998; Sasagawa *et al*., 1998). Can the G_2/M somatic nucleus chromosomes separate and complete the meiotic division when introduced into the cytoplasm of oocytes?

There are diverse scientific opinions on the possibility of constructing viable female gametes by transferring diploid somatic cell nuclei into enucleated oocytes. Chang's study demonstrated that a high proportion of G_2/M somatic nuclei appear to undergo meiosis-like division, in two successive steps, forming a pseudo-polar body and two separate pseudo-pronuclei upon *in vitro* maturation and activation treatment (Chang *et al*., 2002, 2004). Immature mouse ooplasm supported separation of somatic chromosomes to expected numbers, implying that haploidization may be occurring (Palermo *et al*., 2002). However, Fulka *et al*. (2002) reported that when embryonic cell nuclei were introduced into cytoplasts obtained from immature meiotically competent oocytes, polar bodies were extruded in about 75% of reconstructed cells but the metaphase plates were abnormal in almost all cases. When somatic cell nuclei were inserted into the above-mentioned cytoplasts, polar bodies were extruded only very exceptionally and the chromosomes were arranged in abortive metaphase plates. Tateno (2003) showed that in metaphase II oocytes extrusion of the polar body failed to leave a haploid number of chromosomes in the oocyte in almost all cases in mouse. In this study, somatic nuclei were transferred into prometaphase I cytoplasm to observe the changes in

All correspondence to: Da-Yuan Chen, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, #25 Bei Si Huan Xi Lu, Haidian, Beijing 100080, People's Republic of China. Tel: +86 10 62560528, 62562793. Fax: +86 10 62565689. e-mail: chendy@panda.ioz.ac.cn

nuclei and the organization of microtubules. Kinetochores were traced in *in vitro* spontaneously matured oocytes and somatic cell–oocyte reconstructed cells in order to elicit some clues regarding the separation of chromosomes and the possibility of producing haploidization by using somatic cells.

Materials and methods

Animals

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethics committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy Sciences. Female Japanese Big Eared white rabbits were housed in stainless steel cages, and were fed regular rabbit fodder and water ad libitum.

Cell lines

Fibroblast cells were obtained from ear skin tissue of a mature Female Japanese Big Eared white rabbit. Cell culture and assessment procedures have been described previously (Chen *et al.*, 2002; Yang *et al.*, 2003). Skin ear tissues were taken, cut into pieces and digested with 0.25% trypsin for 30 min at 37.5 °C. The digested cells and tissues were cultured in DMEM/F12 (1:1) supplemented with 20% fetal calf serum (FCS) in a 5% CO_2 in air incubator. Cells were passaged when they reached 70–80% confluence. After reaching 50% confluence, the cells were incubated with 0.3 μg/ml nocodazole for 12 h and disaggregated with 0.25% (w/v) trypsin and then resuspended in DMEM/F12 (1:1). Large cells were transferred into enucleated prometaphase I ooplasts by micromanipulation and electrofusion. Cells passaged for three to nine generations were used as donors.

Animal superovulation and oocyte collection

Rabbits were superovulated by PMSG. Female rabbits were injected intraperitoneally with 120–150 IU PMSG and killed 72 h after injection. Germinal vesicle (GV)-stage oocytes were recovered by aspiration of follicles >3 mm in diameter, using an 18-gauge needle and a 1 ml syringe. Cumulus cells of all oocytes were removed by exposure to M_2 medium containing 500 IU/ml hyaluronidase. GV-stage oocytes were cultured in TCTCM199 medium supplemented with 10% FCS and 50 μg/ml 3-isobutyl-1-methylxanthine (IBMX) for 2 h to prevent spontaneous GV breakdown (GVBD) and to develop a perivitelline space. In order to remove the prometaphase I (Pro-MI) karyoplast, first a slit was made in the zona just above the GV by pressing a glass micro-needle tangentially into the perivitelline space (after IBMX incubation as mentioned above), then culturing the oocytes in TCM199 supplemented with 10% FCS for 2–3 h (Yu *et al.*, 2002). Next, by increasing the pressure inside a holding pipette, the pro-MI karyoplast was expelled through the slit (Meng *et al.*, 1996; Li *et al.*, 2001).

Microinjection

The microinjection process was as described previously (Li *et al.*, 2002), The pipette was introduced through the same slit in the zona pellucida made during enucleation, and the cell was wedged between the zona and the cytoplast membrane contact for subsequent fusion.

Electrofusion and culture

The reconstructed oocytes were equilibrated in TCM199 plus 10% FCS medium for 10 min. and then transferred into a drop of fusion medium (0.27 M mannitol, 0.05 mM $MgSO_4$, 0.1 mmol/L HEPES and 0.1 mg/ml bovine serum albumin (BSA)). Electrofusion was stimulated with two electrical pulses (150 V/mm DC for 10 μs) delivered by a Kefa Electro Cell manipulator (Academia Sinica). The fusion was examined 30 min later. Then the reconstructed oocytes were transferred to TCM199 with 10% FCS.

Immunocytochemical staining of reconstructed oocytes

Samples were taken at 1 h intervals after fusion, and the immunocytochemical staining of microtubules was conducted by the method described previously (Zhu *et al.*, 2003). For CREST and nucleus staining, *in vitro* spontaneously matured and reconstructed oocytes were fixed in 4% paraformaldehyde/PHEM (60 mM Pipes, 25 mM Hepes at pH 6.9, 10 mM EGTA, 8 mM $MgSO_4$) for 20 min, and washed three times in PBS with 0.05% polyvinylpyrrolidone (PVP), then permeabilized in 1% Triton X-100/PHEM for 10 min, and washed three times in PBS with 0.05% PVP. After blocking by 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated with anti-CREST antibody (1:500 in 1% BSA/PHEM with 100 mM glycine) at 4 °C overnight. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-human IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min. Then the oocytes were further washed three times in PBS with 0.05% Tween 20 and stained with propidium iodide (PI) in PBS with 0.05% Tween 20 for 2–3 min. Finally the oocytes were mounted on glass slides and examined with a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany).

Table 1 Cell cycle of *Rasiir* adult fibroblasts incubated with nocodazole and at different confluence *in vitro* culture

	Cell cycle phase (mean ± SD)		
	$G_0 + G_1$ (%)	S (%)	$G_2 + M$ (%)
Cells treated with nocodazole (0.3 μg/ml, 50% confluence)	44.3 ± 4.2^a	6.2 ± 3.1^a	49.5 ± 5.5^a
Cells growing to 100% confluence	91.9 ± 1.2^b	1.0 ± 0.4^b	7.1 ± 1.1^b

Values with different superscripts within a column differ significantly ($p < 0.05$).

Results

Cell cycle analysis of donor cells

The cell cycle stage of ear fibroblasts reaching 100% confluence or 50% confluence treated with nocodazole (0.3 μg/ml for 12 h) was analysed using flow cytometry. Cells treated with nocodazole had a significantly increased proportion of cells in G_2/M phase compared with the cells at 100% confluence. Analysis of the cell cycle stages in confluent cells is shown in Table 1.

Fusion and the first polar body extrusion

The fusion rate was 91.5% when nocodazole-treated cells were transferred into Pro-MI ooplasts. The rate of pseudo-polar body extrusion was examined at 15 h after fusion (Table 2).

Table 2 Fusion and pseudo-polar body extrusion rate *in vitro* maturation

	Total no. of cells fused (%)	No. of oocytes with polar bodies (%)
Reconstructed oocytes	238/260 (91.5%)	$38/99$ (38.4%)^a
Control oocytes		$69/80$ (86.3%)^b

Percentages with different superscripts within a column differ significantly ($p < 0.05$).

Microtubule patterns and nuclear changes after nuclear transfer

Microtubule patterns and nucleus changes after transferring a fibroblast cell into enucleated Pro-MI stage cytoplasm are shown in Fig. 1. Nuclear swelling occurred after the somatic cell was fused into the oocyte cytoplasm. A spindle began to organize 2 h after fusion in pro-MI cytoplasm (Fig. 1A). The normal-appearing first meiotic spindle could be detected after 5 h of culture (Fig. 1B) (5/35). Some enucleated rabbit oocytes fused with somatic cells and thereafter cultured *in vitro* did not extrude the first polar body, and had chromosomes dispersed chaotically on the spindle (Fig. 1C). However, there was scarcely any normal second meiotic metaphase plate, though pseudo-first polar bodies were seen after *in vitro* culture (Fig. 1D).

Kinetochores

Kinetochore staining was seen in two rows on two opposite sides of aligned chromosomes in MI-stage oocytes (Fig. 2A). At meiotic anaphase, the kinetochores separated and remained aligned at the distal sides of the chromosomes until telophase, when their alignment perpendicular to the spindle axis was lost (Fig. 2B,C). Oocytes arrested at second meiotic metaphase and kinetochores were detectable as paired structures aligned at the spindle equator (Fig. 2D). Kinetochores could be detected but their arrangement was abnormal after transfer of a somatic nucleus into an oocytes (Fig. 2E,F,G,H).

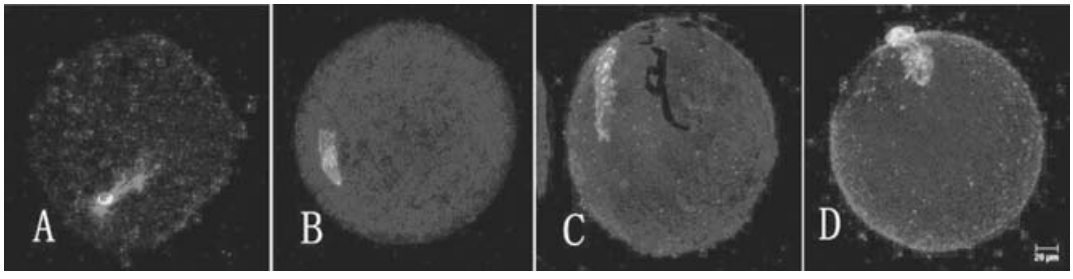

Figure 1 Laser scanning confocal microscopic images of microtubules and chromatin in reconstructed oocytes. Green, microtubule; red, chromatin. (*A*)–(*D*) Enucleated metaphase I oocytes fused with fibroblast cell nuclei. (*A*) Microtubules associated with swelling nuclei. (*B*) Normal-appearing metaphase I spindle was formed. (*C*) Chromosomes were distributed throughout the cytoplasm 4 h after fusion. (*D*) Pseudo-first polar body was extruded but the chromosomes could not align on the metaphase plate normally.

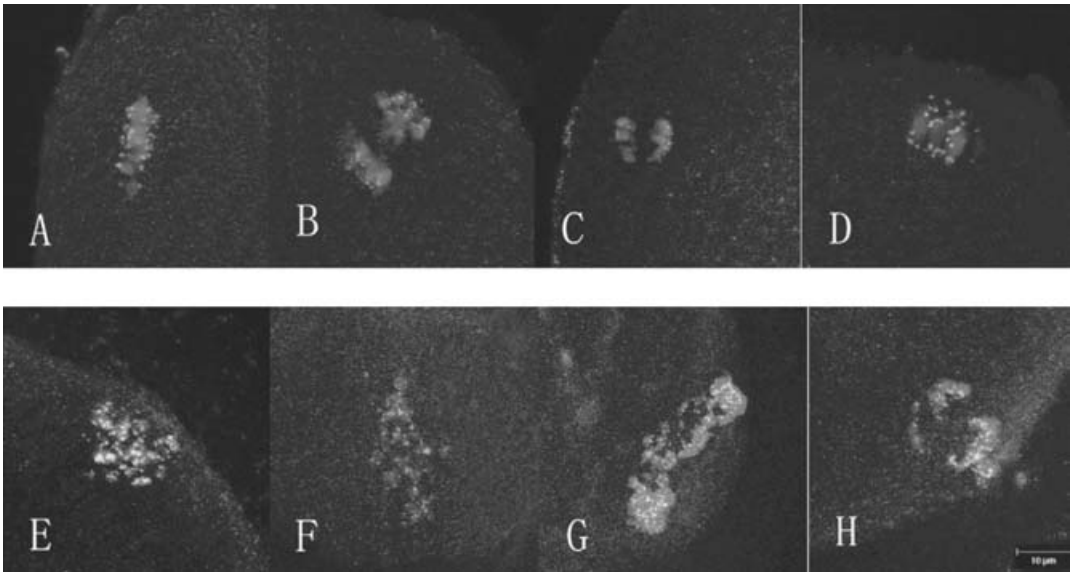

Figure 2 Localization of CREST in *in vitro* matured (*A–D*) and somatic–oocyte reconstructed oocytes (*E–H*). Green, CREST; red, nucleus. Kinetochore staining was seen in two rows on two opposite sites of aligned chromosomes at metaphase II stage (*A*). At meiotic anaphase, the kinetochores separated and remained aligned at the distal sides of the chromosomes until telophase, when their alignment perpendicular to the spindle axis was lost (*B*, *C*). Oocytes arrested at second meiotic metaphase and kinetochores were detectable as paired structures aligned at the spindle equator (*D*). Kinetochores could be detected but were arranged abnormally after a somatic nucleus was transferred into oocytes (*E–H*).

Discussion

Since the birth of the somatic cloned sheep, Dolly, reproductive cloning has been proposed. It is proposed that the haploidization of a patient's somatic cell diploid chromosome complement within enucleated donor oocytes may result in the production of cells with half the number of chromosomes, which could then be used as gametes with their own genetic identity for the treatment of certain forms of infertility (Kubiak *et al*., 2001; Fulka *et al*., 2002).

In our experiments most of the microtubules failed to arrange normally though a few normal-appearing pseudo-first meiotic spindles could be detected when G_2/M phase fibroblasts were transferred into Pro-MI ooplasts. And there was scarcely any normal second meiotic metaphase plate though pseudo-first polar bodies were seen after *in vitro* culture. Ooplasm has an amazing capacity to organize bipolar spindles, even in the absence of chromosomes, which requires expression of microtubule motor proteins, tubulin, and cell extracts with active maturation promotion factor and cytostatic factor (Heald *et al*., 1996; Tateno *et al*., 2003). However, absence of bivalents impairs the formation of a normal bipolar spindle in mammalian ooplasm entirely (Woods *et al*., 1999), and this may contribute to the aberrant spindles and uncontrolled chromosome segregation in reconstituted oocytes.

Though immature mouse ooplasm supported separation of somatic chromosomes to the expected numbers (Palermo *et al*., 2002), the roles of genetic imprinting and fidelity of chromosome segregation are unknown. Nonetheless, the way somatic nucleus haploidization is proposed to take place in ooplasm is quite different from what happens during meiosis in the germ line.

Kinetochore orientation and exposure is critical for the correct spatial and temporal segregation and union of the chromosomes, and appears to be tightly regulated during mammalian meiosis, fertilization and mitosis (Schatten *et al*., 1988). The meiotic reduction in chromosome number depends on a distinctive attachment of chromosomes to the spindle as well as distinctive regulation of the cohesion between sister chromatids. The pattern of attachment in the first meiotic division is different from attachment in somatic mitosis. In mitosis, sister kinetochores lie back to back and capture microtubules from opposite poles; as a result, sister chromatids move to opposite poles in anaphase. In the first meiotic division, however, sister chromatid kinetochores lie side by side, and capture microtubules from the same spindle pole; as a result, sister kinetochores move to the same pole in anaphase I. The meiosis II chromosome behaves like a mitotic chromosome; sister kinetochores are back to back in metaphase II, and they capture microtubules from opposite poles and move to opposite poles in anaphase II (Moore & Orr-Weaver, 1998; Paliulis *et al*., 2000).

Kinetochores could be detected when somatic nuclei were transferred into the ooplasm; however, their

arrangement was quite abnormal. This may be due to the orientation of the kinetochores. The somatic chromosomes consist of two sister chromatids. One kinetochore faces one pole while its sister kinetochore faces the opposite pole and they move to opposite poles in anaphase. In metaphase, sister chromatids are held together by cohesion along chromosome arms and between centromeres. In anaphase both centromere and arm cohesion are released (Paliulis *et al*., 2000).

The ability to induce attachment to opposite poles in metaphase I is correlated with a change in kinetochore structure. In prophase I bivalents the kinetochores are not visibly double, but by metaphase I, two sister kinetochores are evident (Goldstein, 1981; Lin & Church 1982). Only at this time is it possible, though with great difficulty, to induce the sister kinetochores to attach to opposite spindle poles, by repeatedly detaching chromosomes from the spindle and placing the kinetochores so that they do not face either pole directly.

We assume that the kinetochores of transferred somatic cells may have a tendency to shift from mitosis type to meiosis type, that is, kinetochores that are back-to-back capturing surfaces turn to side-by-side capturing surfaces. However, due to the properties of the chromosomes, the chromosomes cannot complete the separation cleanly and the centromere cohesion cannot be removed properly.

Dysfunctional kinetochores or depletion of some CENPs may cause premature anaphase and then induce unequal distribution of sister chromatids during cell division, yielding aneuploidy, and consequently resulting in tumor or severe congenital syndromes (Pennisi, 1998; Cimini *et al*., 2001; Ma *et al*., 2003).

The reductional segregation of parental chromosomes, originally derived from the father and mother, usually requires a physical connection between homologous chromosomes. Physical association is mediated by presence of at least one chiasm at a site of genetic exchange on all chromosomes in male and female meiosis in mammals and cohesion between sister chromatids of homologues within each bivalent. Failures in recombination greatly increase the risk for random segregation of univalents (Eichenlaub-Ritter, 2003). Properties built into the chromosomes and not the cytoplasm or spindles determine the behaviour of chromosomes as Paliulis & Nicklas (2000) reported. Our results showed that kinetochores of somatic chromosomes in ooplasm could not behave like those of meiotic cells.

Genomic imprinting status is also vital for normal development. Genomic imprints appear to be established very early during oogenesis, and imprinting processes associate with chromatin remodelling in a gradual, stepwise fashion during the entire period of oocyte growth and folliculogenesis (Obata & Kono, 2002). It is unlikely that GV, Pro-MI or MII cytoplasm would be able to erase and re-establish imprinting information.

The fertilization of metaphase I oocytes with primary spermatocytes (Ogura *et al*., 1998; Sasagawa *et al*., 1998) provides direct evidence that the nuclei of male germ cells acquire the ability to fertilize oocytes before the first meiotic division. Although chromosome structure peculiarities are the immediate cause of the distinctive behaviour of chromosome in meiosis, these chromosomal properties must arise from earlier events in the differentiation of meiotic cells, as Paliulis & Nicklas (2000) reported.

In conclusion, somatic cell nuclei are unable to go through meiotic division when introduced into oocyte meiotic cytoplasm, partly due to the abnormal kinetochore distribution.

Acknowledgement

This research was supported by Climbing Special Grant 08 from the Ministry of Science and Technology of China and by grant KSCX1-05-01 from the Knowledge Innovation Project of the Chinese Academy of Sciences.

References

Brinkley, B.R., Brenner, S.L., Hall, J.M., Tousson, A., Balczon, R.D. & Valdivia, M.M. (1986). Arrangements of kinetochores in mouse cells during meiosis and spermiogenesis. *Chromosoma* **94**, 309–17.

Chang, C., Tian, X.C., Nagy, Z.P., Abdelmassih, R. & Yang, X. (2002). Cell cycle synchronization of mouse skin fibroblasts with nocodazole to obtain G_2/M phase rich somatic cell population for nuclear transfer procedure. *Fertil. Steril*. **78** (3S), 284 (abstract P506).

Chang, C.C., Nagy, Z.P., Abdelmassih, R., Yang, X. & Tian, X.C. (2004). Nuclear and microtubule dynamics of G_2/M somatic nuclei during haploidization in germinal vesicle-stage mouse oocytes. *Biol. Reprod*. **70**, 752–8.

Chen, D.Y., Wen, D.C., Zhang, Y.P., Sun, Q.Y., Han, Z.M., Liu, Z.H., Shi, P., Li, J.S., Xiangyu, J.G., Lian, L., Kou, Z.H., Wu, Y.Q., Chen, Y.C., Wang, P.Y. & Zhang, H.M. (2002). Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. *Biol. Reprod*. **67**, 637–42.

Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F. & Salmon, E.D. (2001). Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *J. Cell Biol*. **153**, 517–27.

Eichenlaub-Ritter, U. (2003). Reproductive semi-cloning respecting biparental origin. Reconstitution of gametes for assisted reproduction. *Hum. Reprod*. **18**, 473–5.

Fulka, J. Jr, Martinez, F., Tepla, O., Mrazek, M. & Tesarik, J. (2002). Somatic and embryonic cell nucleus transfer into intact and enucleated immature mouse oocytes. *Hum. Reprod*. **17**, 2160–4.

Goldstein, L.S. (1981). Kinetochore structure and its role in chromosome orientation during the first meiotic division in male *D. melanogaster*. *Cell* **25**, 591–602.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A. & Karsenti, E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature* **382**, 420–5.

Kubiak, J.Z. & Johnson, M.H. (2001). Human infertility, reproductive cloning and nuclear transfer: a confusion of meanings. *Bioessays* **23**, 359–64.

Li, G.P., Chen, D.Y., Lian, L., Sun, Q.Y., Wang, M.K., Liu, J.L., Li, J.S. & Han, Z.M. (2001). Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). *Mol. Reprod. Dev*. **58**, 180–5.

Li, G.P., Chen, D.Y., Lian, L., Han, Z.M., Zhu, Z.Y. & Seidel, G.E. Jr (2002). Rabbit cloning: improved fusion rates using cytochalasin B in the fusion buffer. *Mol. Reprod. Dev*. **61**, 187–91.

Lin, H.P. & Church, K. (1982). Meiosis in *Drosophila melanogaster*. III. The effect of orientation disrupter (ord) on gonial mitotic and the meiotic divisions in males. *Genetics* **102**, 751–70.

Ma, W., Hou, Y., Sun, Q.Y., Sun, X.F. & Wang, W.H. (2003). Localization of centromere proteins and their association with chromosomes and microtubules during meiotic maturation in pig oocytes. *Reproduction* **126**, 731–8.

Meng, L., Rutlege, J., Kidder, G., Khamsi, F. & Armstrong, D.T. (1996). Influence of germinal vesicle on the variance of patterns of protein synthesis of rat oocytes during maturation *in vitro*. *Mol. Reprod. Dev*. **43**, 407–11.

Moore, D.P. & Orr-Weaver, T.L. (1998). Chromosome segregation during meiosis: building an unambivalent bivalent. *Curr. Top. Dev. Biol*. **37**, 263–99.

Obata, Y. & Kono, T. (2002). Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J. Biol. Chem*. **277**, 5285–9.

Ogura, A., Suzuki, O., Tanemura, K., Mochida, K., Kobayashi, Y. & Matsuda, J. (1998). Development of normal mice from metaphase I oocytes fertilized with primary spermatocytes. *Proc. Natl. Acad. Sci. USA* **95**, 5611–15.

Palermo, G.D., Takeuchi, T. & Rosenwaks, Z. (2002). Technical approaches to correction of oocyte aneuploidy. *Hum. Reprod*. **17**, 2165–73.

Paliulis, L.V. & Nicklas, R.B. (2000). The reduction of chromosome number in meiosis is determined by properties built into the chromosomes. *J. Cell Biol*. **150**, 1223–32.

Pennisi, E. (1998). Cell division gatekeepers identified. *Science* **279**, 477–8.

Sasagawa, I., Kuretake, S., Eppig, J.J. & Yanagimachi, R. (1998). Mouse primary spermatocytes can complete two meiotic divisions within the oocyte cytoplasm. *Biol. Reprod*. **58**, 248–54.

Schatten, G., Simerly, C., Palmer, D.K., Margolis, R.L., Maul, G., Andrews, B.S. & Schatten, H. (1988). Kinetochore appearance during meiosis, fertilization and mitosis in mouse oocytes and zygotes. *Chromosome* **96**, 341–52.

Tateno, H., Latham, K.E. & Yanagimachi, R. (2003). Reproductive semi-cloning respecting biparental origin. A biologically unsound principle. *Hum. Reprod*. **18**, 472–3.

Woods, L.M., Hodges, C.A., Baart, E., Baker, S.M., Liskay, M. & Hunt, P.A. (1999). Chromosomal influence on meiotic spindle assembly: abnormal meiosis I in female Mlh1 mutant mice. *J. Cell Biol*. **145**, 1395–406.

Yang, C.X., Han, Z.M., Wen, D.C., Sun, Q.Y., Zhang, K.Y., Zhang, L.S., Wu, Y.Q., Kou, Z.H. & Chen, D.Y. (2003). *In vitro* development and mitochondria fate of *Macaca*–rabbit cloned embryos. *Mol. Reprod. Dev*. **65**, 396–401.

Yu, H.Q., Bou, S., Chen, D.Y. & Sun, Q.Y. (2002). Phosphorylation of MAP kinase and p90rsk and its regulation during *in vitro* maturation of cumulus-enclosed rabbit oocytes. *Zygote* **10**, 311–16

Zhu, Z.Y., Chen, D.Y., Li, J.S., Lian, L., Lei, L., Han, Z.M. & Sun, Q.Y. (2003). Rotation of meiotic spindle is controlled by microfilaments in mouse oocytes. *Biol. Reprod*. **68**, 943–6.