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Oocyte nucleus controls progression through meiotic maturation

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Abstract

We analyzed progression through the meiotic maturation in oocytes manipulated to replace the prophase oocyte nucleus with the nucleus from a cumulus cell, a pachytene spermatocyte or the pronucleus from a fertilized egg. Removal of the oocyte nucleus led to a significant reduction in histone H1 kinase activity. Replacement of the oocyte nucleus by a pronucleus followed by culture resulted in premature pseudomeiotic division and occasional abnormal cytokinesis; however, histone H1 kinase activity was rescued, microtubules formed a bipolar spindle, and chromosomes were condensed. In addition to the anomalies observed after pronuclear transfer, those after transfer of the nucleus from a cumulus cell or spermatocyte included a dramatically impaired ability to form the bipolar spindle or to condense chromosomes, and histone H1 kinase activity was not rescued. Expression of a cyclin B-YFP in enucleated oocytes receiving the cumulus cell nucleus rescued histone H1 kinase activity, but spindle formation and chromosome condensation remained impaired, indicating a pleiotropic effect of oocyte nucleus removal. However, when the cumulus cell nucleus was first transformed into pronuclei (transfer into a metaphase II oocyte followed by activation), such pronuclei supported maturation after transfer into the oocyte in a manner similar to that of normal pronuclei. These results show that the oocyte nucleus contains specific components required for the control of progression through the meiotic maturation and that some of these components are also present in pronuclei.
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Keywords: Prophase oocyte; Oocyte nucleus; Pronucleus; Meiotic maturation; Nuclear transfer; MPF

Introduction

Although cell cycle progression in mitotic and meiotic cells involves some common mechanisms, such as oscillations in cdc2 kinase activity, numerous differences exist in the control of these processes. In oocytes, the unique parameters of meiosis include transient arrests during meiotic progression, lack of interphase between two meiotic metaphases and involvement of the Mos/MAPK kinase pathway. The enormous length of the first meiotic M-phase in the mouse oocyte (Polanski, 1986, 1997) as compared to first mitosis of the fertilized egg (Ciemerych et al., 1999; Howlett and Bolton, 1985) or of a typical somatic cell might reflect the complexity of meiosis, which requires: proper positioning of the meiotic spindle to ensure asymmetric division (Maro and Verlhac, 2002); maintenance of bivalents during condensation; and establishment of mechanisms that prevent sister-chromatid separation during first meiosis, including suppression of biorientation of sister kinetochores (Petronczki et al., 2003) and controlled biphasic destruction of cohesins (Kitajima et al., 2004).

In recent years, interest has grown in the use of nuclear transfer into prophase oocytes as an approach to studying cell cycle control during oocyte meiosis, with the goal of applying this information to the treatment of reproductive disorders or to somatic cloning. Some investigators initially proposed the possibility that division of the somatic nucleus in the oocyte cytoplasm in order to achieve a haploid number of chromosomes might yield a cell, rather than a gamete, suitable for treatment of infertility. However, such an approach is contingent on the prevention of random segregation of donor chromosomes (Kubiak and Johnson, 2001), a crucial requirement that cannot be met at present

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(Eichenlaub-Ritter, 2003; Tateno et al., 2003a,b). Another perhaps more promising approach involves the use of a prophase oocyte as a recipient in the cloning by nuclear transfer. The success of mammalian cloning (Wilmut et al., 1997) shows that at least limited reprogramming of the donor nucleus in the cytoplasm of the metaphase II oocyte is possible, and it has been suggested that passing the donor nucleus through the cytoplasm of the oocyte at earlier stages of oogenesis may further improve reprogramming (Santos et al., 2002), since at least some aspects of reprogramming occur in prophase oocytes (Bao et al., 2000). Recent evidence that factors present in the prophase nucleus of *Xenopus* oocytes activate expression of Oct4 in nuclei of fully differentiated human cells (Byrne et al., 2003) supports this concept. Exploration of this possibility requires studies of the behavior of donor nuclei after transfer into immature oocytes.

Initial attempts to obtain mature mouse oocytes after replacement of the prophase oocyte nucleus (germinal vesicle, GV) by nuclei from somatic cells have been unsuccessful (Fulka et al., 2002; Kubelka and Moor, 1997). In those studies, enucleated oocytes that received nuclei from primary spermatogonia, fibroblasts or cumulus cells did not divide during subsequent culture. However, a recent report describes the maturation, although associated with some anomalies, of the enucleated prophase oocytes after nuclear transfer of nuclei from somatic cells (Chang et al., 2004). Our present experiments demonstrating maturation after nuclear transfer associated with abnormalities in cytokinesis and chromosome condensation as well as premature division are consistent with those results. Moreover, we show that enucleation of the prophase oocyte results in decreased activity of Maturation Promoting Factor (MPF), and that different types of donor nuclei vary in their ability to restore normal levels of MPF and to support maturation. The anomalies displayed by oocytes after nuclear transfer are not due to decreased MPF activity, indicating that removal of the oocyte nucleus exerts a pleiotropic effect. Transformation of a somatic cell nucleus into the pronucleus before transfer into the enucleated oocyte substantially improves maturation.

Materials and methods

Mice, recipient oocytes and donor nuclei

Mice from OF1 outbred stock and (C57/Bl × DBA)F1 hybrids were used throughout the experiments. Recipient prophase oocytes (with clearly visible GV) were isolated from ovaries of OF1 mice and maintained in M2 culture medium supplemented with dbcAMP. Isolated oocytes were transferred to medium containing cytochalasin D (1 μg/ml) for at least 30 min before starting micromanipulation. Recipient metaphase II oocytes were recovered after superovulation (Nagy et al., 2003) of F1 females sacrificed 16–18 h after hCG injection and handled in M2 medium supplemented with cytochalasin D.

Cumulus cells, pachytene spermatocytes and pronuclei were used as donors. Cumulus cells were removed from superovulated hybrid females (Nagy et al., 2003) and kept in M2 medium at 4°C. To obtain pachytene spermatocytes, F1 males were sacrificed, their testes dissected and the seminiferous tubules placed into M2 medium and squeezed to expel cellular contents into the medium. The cell suspension was centrifuged for 5 min at low speed (500 rpm) to separate the larger cells (pellet) from most of the small cells, spermatozoa and cytoplasmic bodies (discarded supernatant). The pellet was resuspended in M2 medium mixed (2:5) with M2 containing 12% PVP (Sigma) and kept at 4°C until transfer. The cell suspension was placed in the micromanipulation chamber, and primary spermatocytes were selected based on size (15–20 μm) and morphology under an inverted microscope equipped with differential interference contrast.

Fertilized eggs obtained from superovulated F1 females mated with F1 males served as a source of pronuclei. Zygotes were collected (Nagy et al., 2003) into M2 medium 21–25 h after hCG injection and incubated in M2 medium at 37°C until 27–30 h after hCG injection to allow them to proceed to the G2 phase of the first mitotic cycle.

Nuclear transfer, parthenogenetic activation and meiotic maturation in vitro

To remove the nucleus from donor GV-stage oocytes, an opening in the zona pellucida was made with a glass needle (Tsunoda et al., 1986), and the karyoplast containing GV was pinched out according to the procedure of McGrath and Solter (1983). The amount of cytoplasm removed in the karyoplast did not exceed half the volume of the GV (Fig. 1). Assuming diameters of GV and the prophase oocyte as

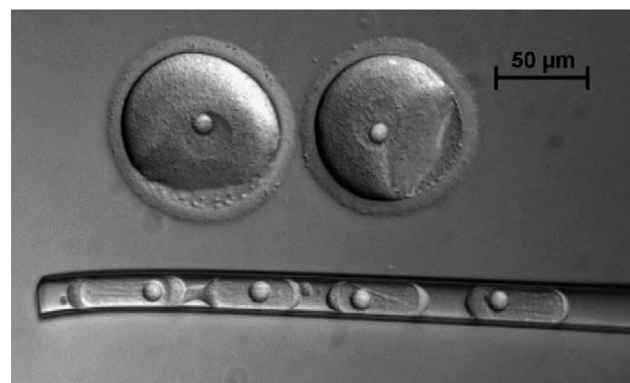

Fig. 1. Typical karyoplasts removed from recipient prophase oocytes. Note that the volume of the nucleus greatly exceeds the volume of cytoplasm (cytoplasm is seen as a rim around the GV). As a reference, two intact GV-stage oocytes are shown. The concavity seen in these oocytes is a normal feature of GV oocytes treated with cytochalasin D.

25 μm and 75 μm, respectively, no more than 2% of the total amount of cytoplasm was removed along with the GV.

Transfer of donor pronuclei (pronuclear transfer, PT) into enucleated prophase oocytes was based on Sendai virus-mediated fusion of the donor karyoplast with recipient oocytes as described (McGrath and Solter, 1983). The same method was used for control transfers in which the GV removed from one prophase oocyte was introduced into another enucleated prophase oocyte (referred to hereafter as "oocyte reconstruction").

Primary spermatocyte nuclei (spermatocyte transfer, ST) and cumulus cell nuclei (cumulus cell transfer, CT) were injected immediately into the cytoplasm of recipient oocytes using piezoinjection (Nagy et al., 2003). Briefly, donor nuclei were separated from cytoplasm by pipetting the cells in M2PVP solution using injection pipettes (5–7 μm and 8–10 μm inner diameter for CT and ST, respectively). Application of appropriate piezo pulses enabled first drilling through the zona pellucida and then rupturing of the oolemma followed by injection of the donor nucleus into the cytoplasm. As the injection pipette was retracted, the small portion of recipient cytoplasm aspirated served to seal the oolemma ("hole removal"), thus greatly increasing survival of injected oocytes (Zhou et al., 2001). Donor nucleus injection was performed first and, after 1–2 h of recovery in culture, the GV was removed from surviving oocytes. In pilot experiments, ST and CT were achieved by fusion of whole donor cells with recipient prophase oocytes using Sendai virus. In those pilot experiments, enucleation was performed first.

For enucleation of recipient MII oocytes, a pipette of 8 μm diameter attached to a piezoinjector (PrimeTech, Japan) was used to drill the opening in the zona pellucida. The karyoplast containing the spindle was subsequently removed using the same pipette (Nagy et al., 2003).

In serial transfer experiments, enucleated MII oocytes were injected with cumulus cell nuclei (first transfer). "Hole removal" was not necessary and injection was carried out in cytochalasin D-free medium. After 1–3 h of recovery in culture, injected oocytes were activated by a 1-h treatment with strontium (Wakayama et al., 1998) and cultured for an additional 5–6 h in M2-cytochalasin D. During this time, the majority of such oocytes developed two pronuclei. Both pronuclei were transferred into a single enucleated GV-stage oocyte as described above (second transfer).

To allow meiotic maturation, manipulated and intact oocytes were rinsed carefully in M2 medium to wash away dbcAMP and cytochalasin D, and cultured further for 16–20 h at 37°C in 5% CO_2. At the end of culture, the oocytes which extruded first polar body were fixed or processed for the measurement of histone H1 kinase activity (see below).

In some experiments, nuclear transfer into zygotes was performed. Fertilized eggs were collected as described above. After removal of both pronuclei, a cumulus cell nucleus or GV was introduced into an enucleated zygote by means of piezoinjection or Sendai-induced fusion, respectively.

Plasmid construction and injection

The cyclin B-YFP cDNA was subcloned from pEFpl cyclin B-YFP (gift from S. Geley) into the *Nco*I and *Xba*I sites of pBS31 (gift from R. Graeser), and ligated into pRN3 (gift from M-H. Verlhac) using *Eco*RI and *Not*I sites. In vitro transcription and purification of RNA was performed as described (Brunet et al., 1998). The final concentration of RNA was 3.5 μg/ml.

Cyclin B-YFP RNA was microinjected simultaneously with piezoinjection of a cumulus cell nucleus. Briefly, the cumulus cell nucleus was isolated and placed close to the tip of the injection pipette. The pipette was then moved into the drop containing RNA. The cumulus cell nucleus was aspirated back into the pipette followed by the RNA solution. After moving the pipette into the drop containing the oocyte, slight positive pressure was applied to allow slow outflow of RNA from the pipette to avoid mixing the RNA with the medium. After zona drilling, excess RNA was released, leaving the desired volume (calculated based on the distance from the pipette tip to the position of the donor nucleus). The pipette was introduced deep into the oocyte and, after rupturing the oolemma with a piezoelectric pulse, the RNA followed by the donor nucleus were injected into the cytoplasm. Assuming that RNA filled the pipette from its tip to the position of the donor nucleus, approximately 6 pl of cyclin B-YFP RNA was injected into a single oocyte.

Kinase assay

For combined histone H1 kinase/myelin basic protein (MBP) kinase assay, oocytes were collected into 1 μl of distilled water containing 20 mg/ml BSA, frozen immediately in liquid nitrogen and stored at −70°C until use. To each sample, 2.5 μl of lysis buffer (40 mM Tris–HCl pH 7.5, 160 mM β-glycerolphosphate, 40 mM EGTA, 30 mM $MgCl_2$, 2 mM DTT, 30 μg/ml leupeptin and pepstatin, 2 mM PMSF) was added. Samples were kept on ice for 5 min before starting the reaction by adding 1.25 μl of reaction buffer [7.5 mg/ml histone H1, 6 mg/ml MBP, 16 mM $MgCl_2$, 2 mM DTT, 0.32 mM ATP, 0.6 mCi/ml [32P]ATP (from 10 mCi/ml stock, Amersham)]. After 30-min incubation at 37°C, the reaction was stopped by adding 4 μl of 2× Laemmli buffer and putting the samples into boiling water for 2.5 min. Samples were resolved by SDS-PAGE and kinase activities were quantitated using PhosphorImager and NIH Image software (version 1.62).

Immunofluorescence and chromosome analysis

Oocytes were fixed in 2.5% paraformaldehyde/0.1% Triton X, rinsed with PBS and blocked with 3% BSA in PBS. Primary antibodies used were rat monoclonal antibody YL1/2, which recognizes tyrosinated α-tubulin (Chemicon, dilution 1:1500) or rabbit polyclonal to γ-tubulin (Abcam, ab11317, dilution 1:2000). As secondary antibodies, we

used goat anti-rat FITC-conjugated (Santa Cruz Biotechnology) or goat anti-rat TRITC-conjugated (Jackson Immunoresearch) as well as goat anti-rabbit FITC-conjugated (Santa Cruz Biotechnology) antibodies, all at concentration 1:250–500. DNA was stained by 5-min treatment with 5 μg/ml propidium iodide (Molecular Probes) or DAPI (2 μg/ml; Sigma).

Chromosomes were prepared according to Tarkowski (1966) and stained with 2% Giemsa stain.

Results

We performed more than 1300 successful nuclear transfers into enucleated prophase oocytes using different donor nuclei, namely pronuclei (pronuclear transfer-PT), spermatocyte nuclei (ST) and cumulus cell nuclei (CT).Whereas the success rate of fusion-mediated PT and of control GV transfer (oocyte reconstruction) was very high, ST and CT were less successful (Table 1). In each of the latter, piezoinjection of donor nuclei was found to be advantageous over the fusion (shortest time required for micromanipulation, better survival of ST oocytes) and, after pilot experiments, this procedure was used in majority of experiments.

An overall goal in these studies is to determine the suitability of prophase oocytes as recipients in somatic cloning, an application that requires inhibition of one or both meiotic divisions (depending on whether the donor nucleus is in G2/M or G0/G1 stage, respectively) to secure the correct ploidy of the cloned embryos. However, we did not block the first meiotic division in our studies because the ability to undergo this process was used as an indicator of maturation "quality" after nuclear transfer.

Table 1
Meiotic maturation after nuclear transfer into prophase oocytes

Donor nucleus	Method	No. of manipulated oocytes	No. (%) of successful transfers*	% of manipulated oocytes undergoing first meiotic division
GV (reconstructed)	Fusion	186	181 (97)	75
Cumulus cell	Fusion	133	61 (46)	54
Cumulus cell	Piezo	600	291 (49)	52
Cumulus cell	Total	733	352 (48)	52[a]
Spermatocyte	Fusion	180	53 (29)	69
Spermatocyte	Piezo	686	299 (44)	67
Spermatocyte	Total	866	352 (41)	67
Maternal pronucleus	Fusion	134	132 (98)	90
Paternal pronucleus	Fusion	186	184 (99)	86
Pronucleus (total)		320	316 (99)	88[a]

[a] Significantly different from control (χ^2 test).
* Depending on method—fusion of karyoplast or survival after injection.

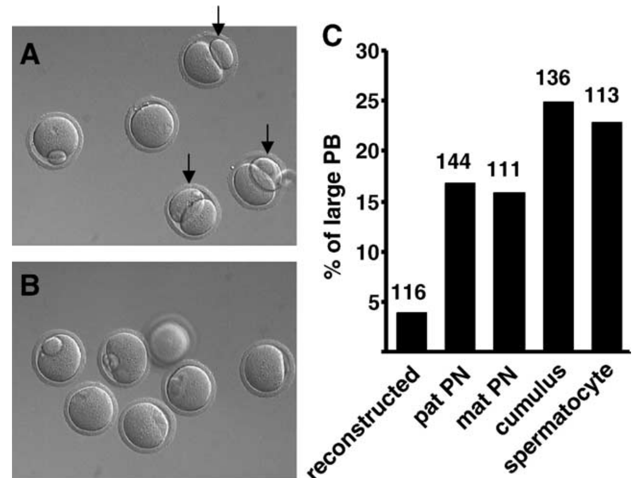

Fig. 2. Anomalies in cytokinesis after nuclear transfer. (A and B) Large polar bodies (arrows) in ST oocytes and normal-size polar bodies in reconstructed oocytes, respectively. (C) Frequency of large polar body extrusion among oocytes undergoing first meiotic division after nuclear transfer. Number of oocytes analyzed is indicated.

The donor nucleus determines the type of anomalies during meiotic maturation in recipient prophase oocytes

Among reconstructed oocytes (control GV transfer), 75% extruded the first polar bodies during in vitro culture, indicating that micromanipulation per se does not have a major impact on progression through meiotic maturation. Surprisingly, even a higher percentage of PT oocytes underwent first "meiotic" division in vitro. Most of the ST and CT oocytes also divided in culture, although at lower frequency (especially for CT; Table 1), and in many cases, exhibited abnormal cytokinesis; instead of forming the small polar body, oocytes tended to extrude large polar bodies or even divided into equal-sized daughter cells (Fig. 2). This anomaly in cytokinesis also occurred to a lesser extent among PT oocytes but was very rare in control GV-transfer oocytes.

Morphological analysis of the second metaphase spindle in oocytes that progressed through maturation revealed microtubules arranged in a typical bipolar structure in control and PT oocytes (Figs. 3A, B and F). As expected, the chromosomes in PT oocytes were scattered throughout the spindle rather than aligned in the equatorial plane, since the chromosomes separated into single chromatids in the course of first division so that the individual chromatids in the resulting second metaphase spindle were pulled toward the spindle pole to which the kinetochore is attached.

Among ST oocytes, 39% did not form the spindle and exhibited a different profile of anomalies. Often, randomly aligned microtubules were associated with chromatin, or telophase-like figures were observed. In the latter cases, chromatin bridging was occasionally present (Fig. 3D), suggesting impaired resolution of bivalents into homologs during anaphase. The unequal distribution of chromatin

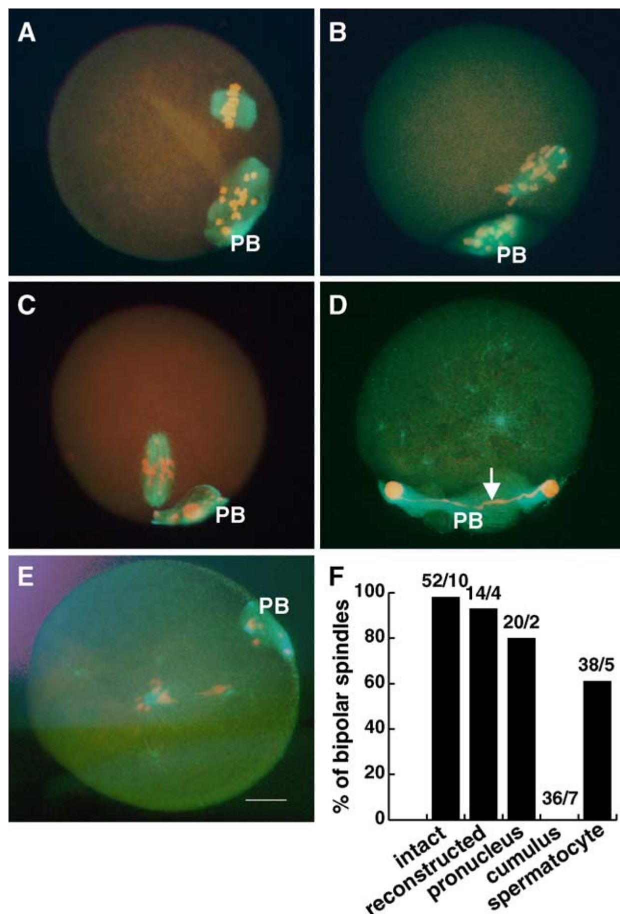

Fig. 3. Spindle appearance in oocytes undergoing meiotic maturation after nuclear transfer. (A) Normal metaphase II spindle in reconstructed oocyte. (B and C) Bipolar spindle with misaligned chromosomes in PT and ST oocytes, respectively. (D) Arrested telophase in ST oocyte. Chromatin bridging (arrow) indicates impaired separation of chromatin into two sets. (E) Scarce microtubules connecting chromatin clumps in CT oocyte. PB—first polar body; DNA—red; microtubules—green. Scale bar = 20 μm. (F) Frequency of bipolar spindle formation (number of oocytes analyzed/ number of experiments is indicated).

between the oocyte and polar body in ST oocytes (in extreme cases, with all of the chromatin present in only one of the daughter cells) supports this suggestion. The chromatin often appeared incompletely condensed into individual chromosomes and instead formed one or several clumps.

In 61% of ST oocytes, microtubules aligned into a spindle (Figs. 3C, F) but the chromosomes were usually scattered throughout it. This would not be expected if separation of bivalents in the course of first division occurred normally because resulting bichromatid chromosomes would attach to both poles of the second metaphase spindle and thus align in the equatorial plane, analogous to GV-transfer or intact oocytes. Apparently, the segregation of chromosomes in these ST oocytes was also abnormal.

None of the analyzed CT oocytes revealed microtubules arranged into the spindle (Fig. 3F). Most often, the scarce and randomly aligned microtubules were associated with a chromatin clump(s) (Fig. 3E), and sometimes no apparent microtubules were visible. In most cases, the distribution of the chromatin between the two daughter cells was clearly unequal. The same anomalies were observed, regardless the CT oocytes were obtained by piezoinjection of the nucleus (16 oocytes) or fusion of the whole cell (20 oocytes).

It is possible that the inability to assemble spindle by CT oocytes (and to lesser extent by ST oocytes) could result from enucleation due to the removal of microtubule organizing centers (MTOCs) and microtubule asters along with GV. Therefore, some enucleated prophase oocytes were checked for the presence of γ-tubulin (marker of MTOCs) and microtubule arrangement. The number of distinct γ-tubulin foci was reduced in enucleated oocytes (2.6 foci/egg compared to 3.5 foci/egg in intact oocytes, $P < 0.01$; Fig. 4). All enucleated oocytes had, however, normal interphase microtubule network similar to the network observed in intact oocytes (Fig. 4). In both groups, in some cases (12% of enucleated oocytes and 6% of intact oocytes) this typical

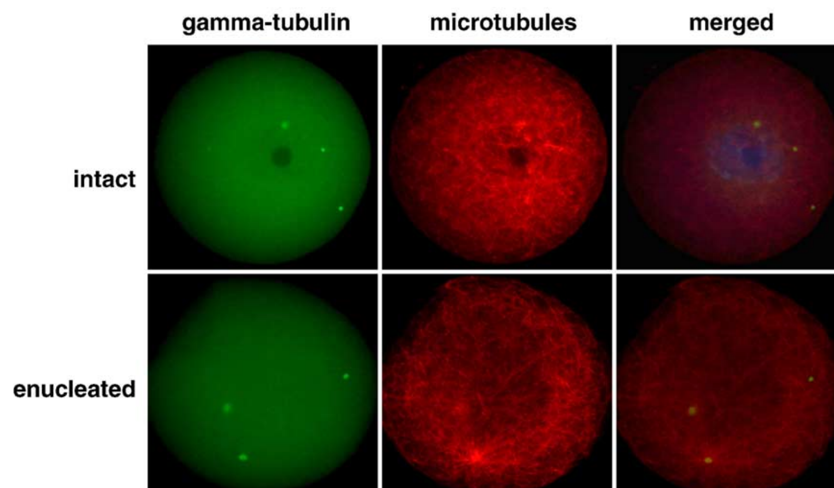

Fig. 4. γ-Tubulin foci and microtubule arrangement in prophase oocytes after enucleation. Distinct γ-tubulin foci (green) are visible both in intact as well as in enucleated oocyte. Note that removal of GV does not affect the arrangement of microtubules (red). In intact oocyte the GV is also shown (blue).

interphase network was present in the absence of any distinct γ-tubulin foci.

Detailed chromosome analysis showed that, in contrast to control and PT oocytes, most ST and CT oocytes did not condense chromatin into individual chromosomes and only pulverized chromatin was observed (Figs. 5D, E). Normal condensation of chromosomes was observed only in 12% of ST oocytes and in 18% of CT oocytes (Fig. 5F).

PT did not cause major anomalies in spindle formation or chromosome condensation, but progression through maturation was significantly accelerated in these oocytes (Fig. 6). The microsurgical procedure itself resulted in some acceleration, since reconstructed oocytes extruded the first polar body ~1 h earlier than did intact oocytes. However, PT caused further acceleration by 2–3 h. On average, ST and CT oocytes extruded the polar body in time periods similar to those of reconstructed oocytes but even in these groups, extrusion of polar bodies started earlier (first oocytes with polar bodies appeared after 8 h of maturation in CT and ST,

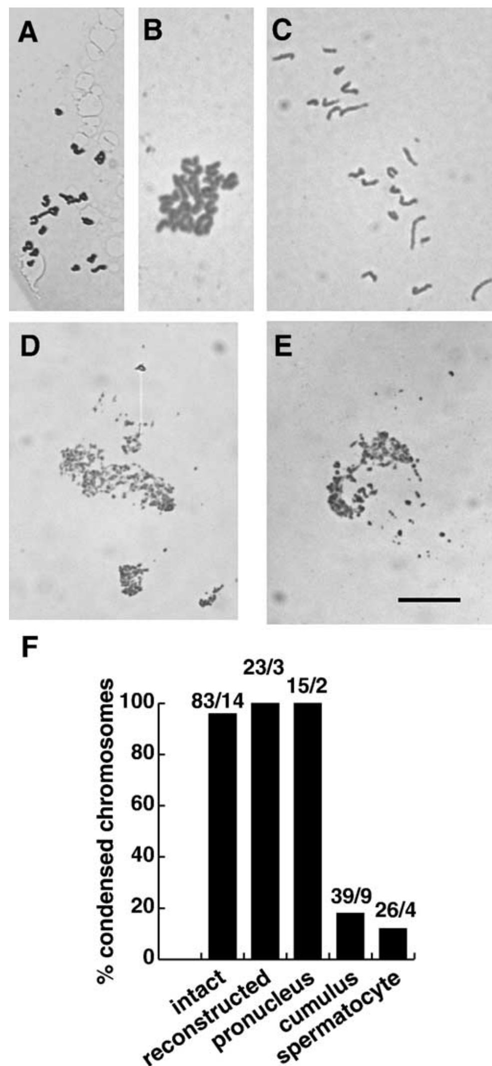

Fig. 5. Chromosome condensation in oocytes undergoing meiotic maturation after nuclear transfer. Note the condensed chromosomes in intact (A), reconstructed (B) and PT oocytes (C) but the pulverized chromatin in CT (D) and ST oocytes (E). (F) Frequency of metaphase chromosomes (number of oocytes analyzed/number of experiments). Scale bar = 20 μm.

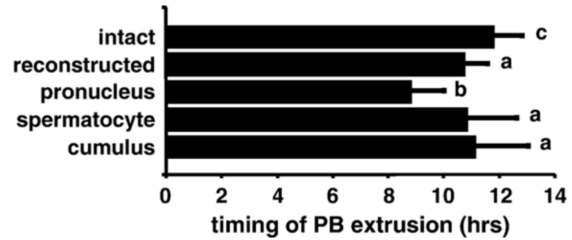

Fig. 6. Timing of polar body extrusion during meiotic maturation of oocytes receiving nuclear transfer. Bars represent mean ± SEM. Different letters indicate statistically significant differences (*t* test).

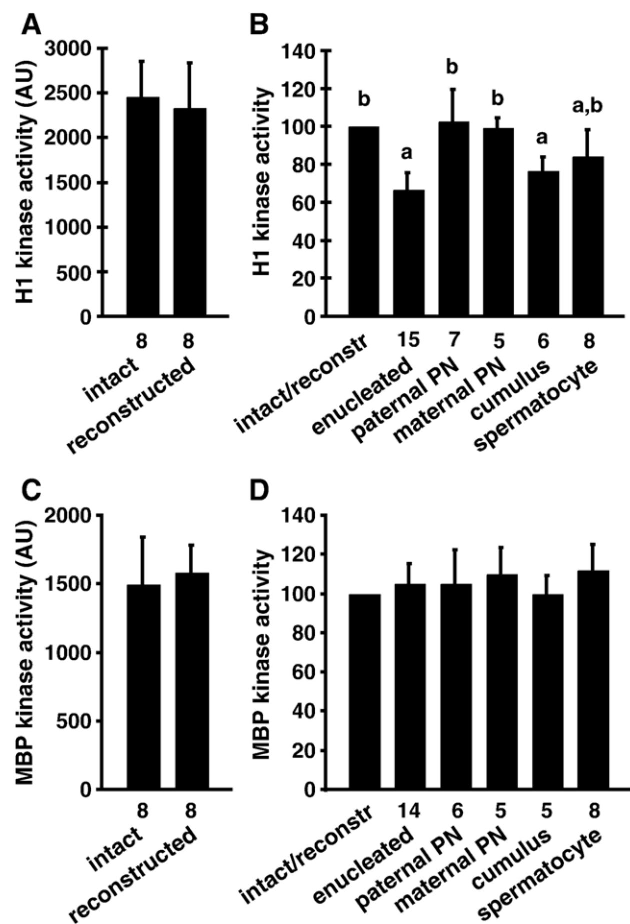

Fig. 7. Histone H1 kinase activity but not MBP kinase activity is affected after nuclear transfer into prophase oocytes. (A and C) No significant difference between intact and reconstructed oocytes in either H1 kinase or MBP kinase activity. (B) Significantly reduced activity of histone H1 kinase after enucleation, which is not fully rescued by CT or ST. (D) Enucleation does not influence MBP kinase activity (no significant difference between groups). In B and D, values shown are relative to the activity scored in intact or reconstructed oocytes (designated as 100). Letters in B denote statistical significance (*t* test): a—significantly different from intact/reconstructed oocytes; b—significantly different from enucleated oocytes. In B and D, a single-sample *t* test was used when the intact/reconstructed group was compared to other groups (hypothetical mean for intact/reconstructed = 100). Mean ± SEM is shown, with number of assays given below each bar.

but after 9 h of maturation in reconstructed oocytes). These results also point to the tendency for CT and ST oocytes to undergo premature polar body extrusion. Most likely, the severe anomalies in the spindle formation and in chromosome condensation cause that premature polar body extrusion is not as clearly manifested in these oocytes as in PT oocytes.

PT but not CT or ST fully restores histone H1 kinase activity depleted after removal of the oocyte nucleus

Analysis of histone H1 kinase activity (reflecting activity of the major cell cycle regulator MPF) and MBP kinase activity (reflecting activity of another regulator of meiotic maturation, namely MAPK kinase) at the end of the culture period (16–20 h after start of maturation) revealed no difference between intact and reconstructed oocytes in either activity (Figs. 7A and C), indicating that the microsurgical procedure did not affect these kinases. In enucleated oocytes, histone H1 kinase activity was reduced by 1/3 (65%) as compared to that in intact/reconstructed oocytes (Fig. 7B). Replacement of the oocyte prophase nucleus with a pronucleus led to complete restoration of the activity, whereas the cumulus cell nucleus did not substantially elevate histone H1 kinase activity, and the spermatocyte nucleus restored it only partially (Fig. 7B). Neither enucleation nor introduction of foreign nuclei affected the activity of MBP kinase (Fig. 7D).

Injection of cyclin B RNA into CT oocytes rescues histone H1 kinase activity but does not correct other anomalies

CT and ST oocytes displayed anomalies related to chromosome condensation and spindle formation, as well as decreased levels of MPF (as revealed by histone H1 kinase activity) compared to intact or reconstructed oocytes. In cells entering M-phase, both microtubule- and chromatin-associated proteins are modified directly or indirectly by activated MPF, thus enabling proper spindle formation and chromatin condensation (reviewed in Nigg, 2001). We recently found that a slow rate of cyclin B synthesis underlies the low MPF activity in enucleated oocytes (Tsurumi et al., manuscript in preparation). To test whether the spindle and chromatin anomalies after CT and ST are directly related to the low MPF activity, we injected cyclin B-YFP RNA together with a cumulus cell nucleus into an enucleated oocyte in attempt to increase MPF activity in CT oocytes. In mouse oocytes starting from the time point around GVBD cyclin B is intensively synthesized throughout maturation. In agree-

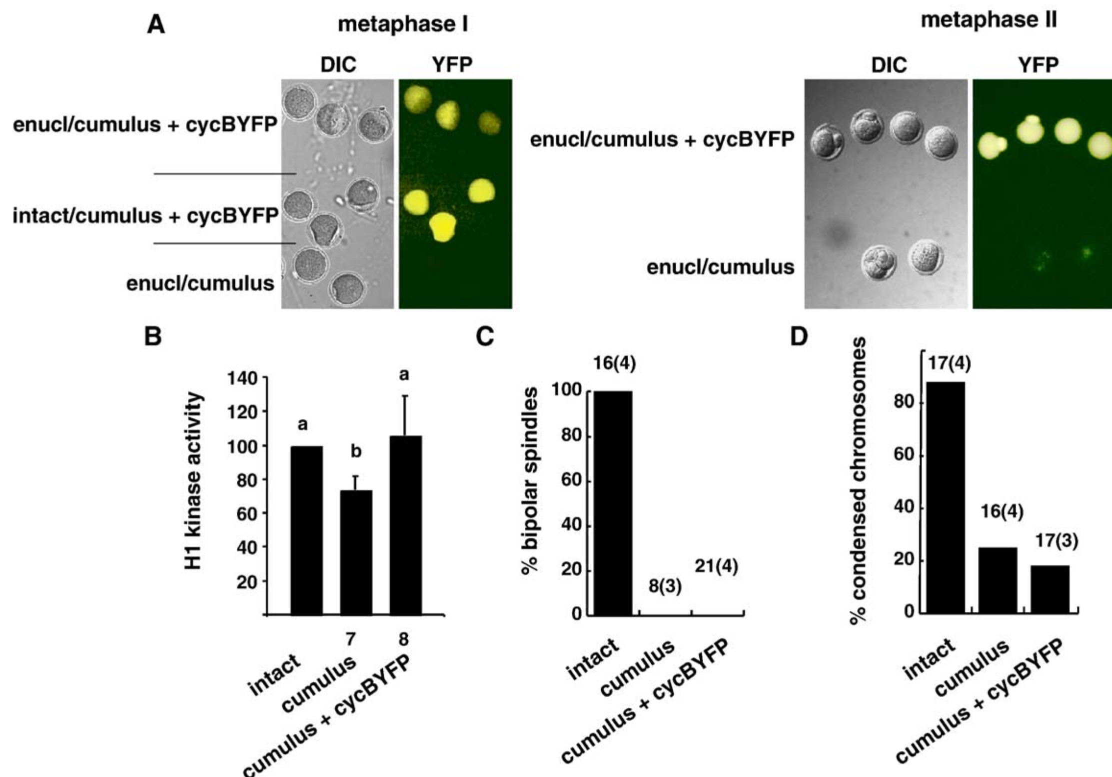

Fig. 8. Cyclin B-YFP RNA injected into CT oocytes undergoes expression during maturation (A) and rescues histone H1 kinase activity (B) but does not improve spindle formation (C) or chromosome condensation (D). In B, mean ± SEM is shown. Different letters indicate significant difference (*t* test or single-sample *t* test; see Fig. 7 legend); number of assays is given below each bar. (C, D) Number of oocytes analyzed/number of experiments is shown above the bars. (A) The oocytes double injected with cumulus cell nucleus/cyclin B-YFP RNA in which GV was left intact are also shown (metaphase I, middle group).

ment with previous studies using cyclin B-GFP (Ledan et al., 2001: Hyslop et al., 2004), we have observed distinct expression of our cyclin B-YFP construct in majority of injected oocytes both at MI as well as at MII (Fig. 8A) which shows that the synthesis of injected cyclin B-YFP resembles the pattern of cyclin B synthesis in intact oocytes. Accordingly, histone H1 kinase activity in second metaphase CT oocytes expressing injected cyclin B-YFP shifted to the level comparable to intact oocytes (Fig. 8B) showing that the injected construct was functional. The chromosome and spindle anomalies, however, remained in these oocytes uncorrected (Figs. 8C, D).

Transformation of a cumulus cell nucleus into the pronucleus before transfer into the prophase oocyte improves maturation

We performed serial transfer experiments in which cumulus cell nuclei were transferred first into enucleated MII eggs, followed by transfer of the two pronuclei formed after parthogenetic activation into enucleated prophase oocytes. Upon release from cAMP arrest, such oocytes underwent accelerated meiotic maturation and, in most cases, revealed a maturation phenotype like that of PT oocytes, that is, well-condensed chromosomes and microtubules arranged into a bipolar structure (Fig. 9), as well as a significantly greater efficiency as compared with controls in progressing through the first meiotic division (88/90; 98% for serial CT vs. 116/155; 75% for reconstructed oocytes; $P < 0.0001$, χ^2 test). These results suggest that: (1) GV and pronuclei, regardless of the origin of the latter, share some common factors necessary to promote normal features of meiotic maturation; and (2) the inability of cumulus cell nuclei to support normal maturation is not related to the cumulus chromatin per se but to the lack of such putative factors.

GV but not cumulus cell nuclei can substitute for pronuclei to promote chromosome condensation in the zygote

Assuming that the GV and the pronucleus share some components required for cell cycle progression, the

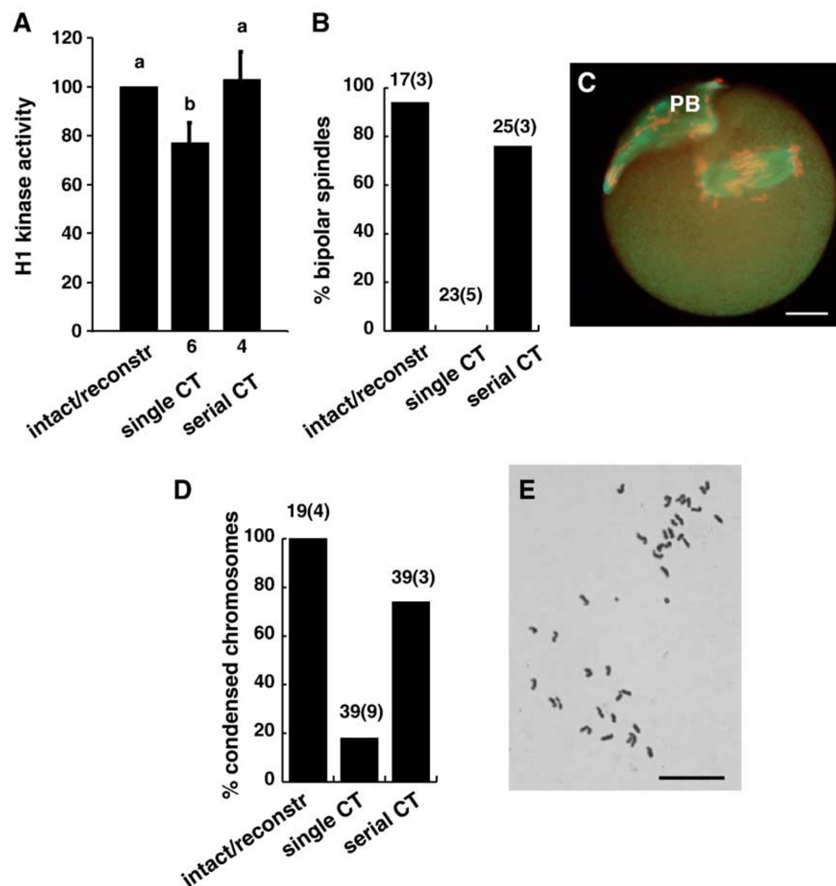

Fig. 9. Transformation of a cumulus cell nucleus into a pronucleus promotes high activity of histone H1 kinase (A), formation of bipolar spindle (B, C) and chromosome condensation (D, E) after serial CT. A cumulus cell nucleus was injected into an enucleated metaphase II oocyte (first transfer), which was then activated parthenogenetically. The two pronuclei formed from cumulus chromatin were introduced into enucleated prophase oocyte (second transfer) followed by culture. In A, B and D, the value from a single CT is also shown for comparison (taken from Figs. 6, 3, and 4, respectively). (A) Mean ± SEM is shown. Different letters denote statistically significant difference (*t* test or single-sample *t* test; see Fig. 7 legend). Number of assays is given below the bars. (B, D) Number of analyzed oocytes/experiments is above bars. (C, E) Scale bar = 20 μm.

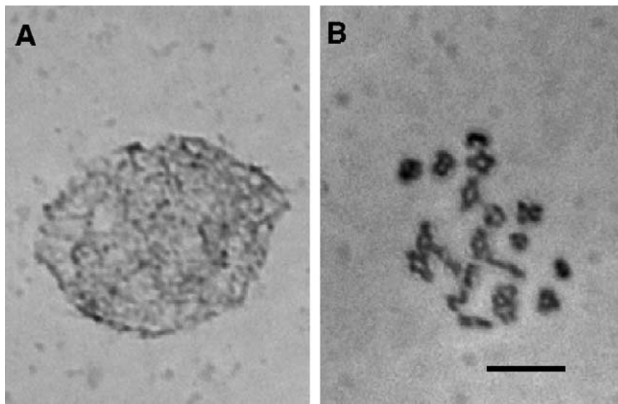

Fig. 10. Chromosome condensation after transfer of foreign nuclei into enucleated zygote. (A) Decondensed chromatin after transfer of cumulus cell nucleus. (B) Condensed chromosomes (MI bivalents) after GV transfer. Scale bar = 20 μm.

replacement of the pronuclei with GV in the zygote should result in normal condensation of the transferred chromatin. We performed GV transfer into zygotes from which the pronuclei were removed. Because zygotes, unlike maturing oocytes, display a relatively short M-phase (Ciemerych et al., 1999), the status of chromatin was assessed after blocking the eggs at metaphase by incubation in the presence of nocodazole. After culture in these conditions, all intact (control) zygotes revealed condensed mitotic chromosomes. Upon GV transfer into enucleated zygotes, 41% of eggs (7/17; 3 experiments) showed chromatin condensed into MI bivalents (Fig. 10B) and 53% had partially condensed chromosomes. In contrast, under the same experimental conditions, most zygotes that received a cumulus cell nucleus showed decondensed chromatin (18/21; 5 experiments, Fig. 10A), and condensation into mitotic chromosomes occurred only in one case.

Discussion

We tested three types of foreign donor nuclei for their ability to substitute for the nucleus of the prophase oocyte to achieve meiotic maturation. To our knowledge, three studies on the meiotic maturation after nuclear transfer into prophase oocytes were published previously. In these studies, the ability to extrude first polar body was used as an indicator of successful progression through meiotic maturation. The data presented in these papers, however, differ significantly rising the concern to what extent they are influenced by technical problems (e.g., the type of micromanipulation techniques used by different authors) and experimental design (e.g., type of donor nucleus used). Kubelka and Moor (1997) reported the apparent inability of somatic nuclei to support the extrusion of polar body after the transfer into enucleated prophase oocytes. Fulka et al. (2002) reported that embryonic, but not somatic nuclei, supported the polar body extrusion after NT. In most recent study, Chang et al. (2004) showed for the first time that first polar body was extruded after transfer of somatic (fibroblast) nuclei into prophase oocytes. Importantly, in the present study, we show that nuclear transfer of cumulus cell nuclei result with the extrusion of the first polar bodies in contrast to previous data in which the same donor nuclei were used (Fulka et al., 2002). Altogether, these data (Chang et al., 2004; our study) present evidence that the lack of success reported in earliest attempts (Fulka et al., 2002; Kubelka and Moor, 1997) was not related to the type of the donor nuclei but rather to some technical problems.

In our study, transfer of the cumulus cell nucleus led to highly abnormal maturation, while use of the primary spermatocyte nucleus, which exhibits a stage of meiotic progression analogous to that of GV, resulted in only slight improvement. Thus, in contrast to an earlier suggestion (Fulka et al., 2002), the impaired cell cycle progression of donor chromatin in the maturing oocyte does not appear to reflect any intrinsic properties of somatic chromosomes (as opposed to meiotic chromosomes normally present in the oocyte). Another factor that might influence meiotic maturation after nuclear transfer is donor-recipient incompatibility of the cell cycle stage, as in the case of CT where the donor nucleus is at G0/G1 stage instead of G2/M stage of the prophase oocyte. In two nuclear transfer studies by others, care was taken to synchronize the cell cycle stages of donor somatic nuclei and prophase oocytes. In one study (Fulka et al., 2002), oocytes receiving a cumulus cell nucleus were blocked at the prophase stage for 24 h, leading to DNA synthesis and progression to the G2 stage of the donor nucleus in the recipient cytoplasm; however, after release from the prophase block, the oocytes were unable to progress through maturation. In the second study (Chang et al., 2004), fibroblasts synchronized in G2/M in cell culture served as donors, and half of the recipient oocytes underwent accelerated first meiotic division but often developed large polar bodies and failed to condense chromosomes. Thus, the maturation phenotype using a donor nucleus at G2/M phase was very similar to that observed after CT in our study. Moreover, maturation after ST using a donor and recipient in the same phase showed similar anomalies. Finally, in serial CT, the pronuclei originating from cumulus cell chromatin were removed 6–7 h after MII oocyte activation and were presumably still in G1 phase of the first mitotic cell cycle (Howlett and Bolton, 1985); however, despite the lack of cell cycle stage synchronization, such pronuclei supported maturation when transferred into prophase oocytes.

Possibly the enucleation of the prophase oocytes and zygotes may result with removal of microtubule organizing centers (MTOCs) and microtubule asters associated with GV or pronuclei. This could cause the inability to assemble spindle observed in CT oocytes (and to some extent in ST oocytes) obtained after piezoinjection of the cumulus cell nuclei from which the major portion of the cytoplasm is

removed before injection. However, in CT oocytes obtained by fusion of the whole cumulus cells with enucleated oocytes, the spindles did not form as well, although the donor cells apparently supplied their own MTOCs and asters. Moreover, enucleated oocytes preserve typical microtubule network and γ-tubulin foci (marker of MTOCs) are only reduced in these oocytes by one third, instead of being completely removed. We should also to stress that in some enucleated and intact oocytes, we were unable to distinguish γ-tubulin foci, although the normal microtubule network was present in such oocytes. These data indicate that the presence of distinct γ-tubulin foci in the GV stage oocyte is not a prerequisite for the proper assembly of the spindle. Instead, it seems that MTOC material is diffused throughout the cytoplasm of the GV oocyte with only a minor accumulation as clear foci close to GV or in cytoplasm. Thus, unless the ability to promote spindle formation is exclusively restricted to GV-associated MTOCs/asters, the MTOCs/asters present in oocyte cytoplasm (and/or introduced with fused cumulus cell) seem likely to be able to organize microtubules in the meiotic spindle following GVBD. This suggests that the problem with spindle assembly in CT oocytes is not related to depletion of MTOCs and asters but rather some other factor(s).

As discussed above, neither the state of the chromosomes (mitotic vs. meiotic) nor the cell cycle stage of the donor nucleus appears to affect maturation after nuclear transfer. Instead, we postulate that removal of GV deprives the oocyte of some crucial components, localized in the GV nucleoplasm that control MPF activity as well as spindle assembly and chromatin condensation upon dissolution of nuclear envelope and mixing of the GV contents with the cytoplasm. This view is supported by studies in which the donor nucleus matures in the presence of the oocyte nucleus or in which the latter is removed after GVBD; the progression through maturation was essentially normal when either primary spermatocytes (Ogura et al., 1997; Sasagawa et al., 1998) or ES cells (Gao et al., 2002) were used as donors. Our serial CT experiment shows that while the cumulus cell nucleus is unable to support meiotic maturation, its transfer into the pronucleus substantially improves maturation. Thus, the impaired control of progression through meiotic maturation after nuclear transfer into prophase oocytes cannot be attributed to the chromosome status of the donor nuclei but rather to their non-chromosomal components.

In the present study, we demonstrate that MPF activity in the mouse oocyte is controlled by some GV-associated factor(s), as shown previously in *Xenopus* oocytes (Iwashita et al., 1998). However, the mouse oocyte exerts its control on this activity by regulating cyclin B synthesis (Tsurumi et al., manuscript in preparation), whereas *Xenopus* GV rely on regulation of cdc2 kinase phosphorylation (Iwashita et al., 1998). We detected no dependence of MAP kinase on the GV material, consistent with previous results in porcine oocytes (Sugiura et al., 2001).

Based on the reported direct link between MPF activity and the behavior of the microtubules and chromatin condensation (reviewed by Nigg, 2001), we tested whether the abnormalities in chromatin condensation and spindle formation in CT and ST oocytes result directly from decreased MPF levels. However, restoration of normal MPF levels (after injection of cyclin B-YFP RNA) did not correct these abnormalities, suggesting that withdrawal of the oocyte nucleus has broad pleiotropic effects independent of MPF levels. The existence of GV-associated factors controlling the timing of the first meiotic division (Polanski et al., 1998) or the formation per se of the pronuclei (Balakier and Tarkowski, 1980; Borsuk, 1991; Gao et al., 2002) has been reported. Our present study shows that GV-associated components control not only MPF activity, but also spindle formation and chromatin condensation during meiotic maturation in a manner independent of MPF. After release into the oocyte cytoplasm upon GVBD, at least some of these factors appear to be sequestered back into the pronuclei after egg activation, as evidenced by PT and serial CT experiments. Support for the notion that pronuclei and GV share common components necessary to control cell cycle progression also comes from observations on the behavior of donor chromatin after nuclear transfer into enucleated zygotes. In the absence of pronuclei, cumulus cell chromatin cannot properly condense (Wakayama et al., 2000, the present study), but the oocyte chromatin condenses into bivalents in a considerable number of cases (present study). On the other hand, the premature PB extrusion and cytokinesis abnormalities observed in PT oocytes, as well as the reduced ability to condense chromosomes after GV transfer to zygotes, shows that the prophase nucleus of the oocyte and the pronucleus are not fully equivalent.

The nature of the putative GV/pronucleus-associated factors controlling chromatin condensation/spindle assembly remains to be determined. Among the numerous molecules involved in these processes, NuMA (nuclear-mitotic apparatus), a nuclear protein that controls spindle formation (Cleveland, 1995), could be considered as a likely candidate. In a primate nuclear transfer study, removal of this protein from the oocyte along with the second metaphase spindle resulted in malformations of the spindle formed around donor chromatin and misalignment of the chromosomes (Simerly et al., 2003). However, in fertilized mouse eggs, NuMA is present only in the female pronucleus (Moreira et al., 2003), and in our studies, both female and male pronuclei supported meiotic maturation equally well in the absence of the oocyte nucleus, suggesting that NuMA is not a major candidate as an essential control factor.

Consistent with a recent report (Chang et al., 2004), we observed accelerated extrusion of the first polar body after nuclear transfer. This acceleration was clearly evidenced in PT and in serial CT (where donor pronuclei were formed from cumulus cell chromatin), and even in ST and single

CT, oocytes began to extrude first polar bodies earlier than in control reconstituted oocytes. Acceleration of the first meiotic cycle in single CT and ST may be masked in many of these oocytes by the opposing effects of impaired spindle formation and chromosome condensation. Premature extrusion of the first polar body after nuclear transfer might reflect disruption of the spindle assembly checkpoint (SAC) pathway, which has been shown to function during first meiosis of mouse oocytes (Brunet et al., 2003; Homer et al., 2005; Tsurumi et al., 2004; Wassmann et al., 2003; Woods et al., 1999) and, when disrupted, results in premature anaphase in mitotic cells (Geley et al., 2001; Gorbsky et al., 1998) and in maturing oocytes (Homer et al., 2005; Tsurumi et al., 2004). After CT and ST, extrusion of polar bodies associated with abnormal segregation of chromatin also suggests inactivation of SAC control, which should block division entirely in order to prevent abnormal segregation. Impaired SAC control would explain the higher efficiency of meiotic division after transfer of pronuclei (both upon PT and serial CT) compared to control reconstructed oocytes, since an intact checkpoint in the control group, but not in the PT group, should block a fraction of control oocytes at metaphase I in which spindle anomalies sensed by the checkpoint occur (the lower efficiencies after single CT and ST might reflect anomalies of the division apparatus that are sufficiently severe to disable division regardless of SAC status). Since the SAC pathway differs between meiosis and mitosis (Kallio et al., 2000; Yu et al., 1999), our results raise the possibility that components of SAC in donor nuclei are incompatible with meiotic elements of this pathway remaining in the cytoplasm of the oocyte.

In contrast to CT and ST, the transfer of pronuclei resulted in minor anomalies, consistent with previous findings that the nuclei from 2-cell stage embryos, but not from cumulus cells, can support maturation of enucleated mouse oocytes (Fulka et al., 2002). Thus, nuclei from cells developmentally “closer” to the oocyte and presumably sharing some common components are better donors than nuclei from differentiated cells. It has been suggested that the program of cell cycle control in the egg changes gradually from the meiotic to the mitotic in the course of the first mitotic cell cycles of the dividing embryo (Ciemerych et al., 1999). Such a transition might rely on gradual degradation of maternal transcripts occurring during early cleavage divisions, since such degradation would progressively eliminate proteins involved in a specific manner during cell cycle control of the oocyte.

In conclusion, our study shows that the nuclei of prophase oocytes and the pronuclei of fertilized/activated eggs contain factors crucial for controlling some events of progression through meiosis and the first mitotic cycle. Removal of such nucleus-associated components might underlie, at least in part, the lack of cloning success after nuclear transfer into zygotes (McGrath and Solter, 1984; Wakayama et al., 2000), and may be the major obstacle to using the prophase oocyte as a recipient in somatic cloning.

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