

Meiotic spindle morphogenesis in *in vivo* and *in vitro* matured mouse oocytes: insights into the relationship between nuclear and cytoplasmic quality

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BACKGROUND: This work addresses the hypothesis that events occurring within the follicle soon after the LH surge are essential for coordinating morphogenesis of the spindle and cytoplasm in mouse oocytes matured *in vivo* (IVO); we further tested whether *in vitro* maturation (IVM) fails to support these events. **METHODS:** Oocytes collected at 1, 2, 3, 4 and 5 h post-hCG or after IVM were analyzed for chromatin, nuclear lamina, microtubules (MTs) and centrosomal proteins by conventional fluorescence and confocal microscopy. In addition, these parameters were monitored in oocytes maintained in 50 μ M roscovitine, followed by IVM, or in oocytes retrieved at 1.5 and 5 h post-hCG *in vivo* and cultured up to 16 h. **RESULTS:** A G2/M delay was observed in IVO oocytes based upon persistence of cytoplasmic MTs, nuclear lamina and centrosomes at the cortex; rapid meiotic progression in IVM oocytes was related to loss of these markers, indicating that a global activation of MPF occurred in culture. Also, maturing-promoting factor (MPF) inactivation resulted in cultured oocytes that exhibited IVO characteristics after drug removal. IVO-like characteristics were also exhibited by oocytes retrieved at 5 but not at 1.5 h after hCG treatment, even though these oocytes were subsequently cultured. **CONCLUSIONS:** The results emphasize the importance of coupling MT remodeling and cell cycle components during oocyte maturation to achieve a balanced coordination of nuclear and cytoplasmic maturation that under physiological conditions occurs within the first 5 h of LH stimulation.

Key words: centrosome/*in vivo* or *in vitro* maturation/microtubules/MTOCs/nuclear lamina

Introduction

Oocyte maturation involves a complex series of molecular and organellar remodeling events between resumption of the first meiotic cell cycle and cell cycle arrest at metaphase of meiosis II (M-II). This stepwise process requires coordination of events in both nuclear and cytoplasmic compartments that ensures the production of oocytes capable of supporting the early stages of embryonic development (Albertini *et al.*, 2003). The maternal *in vivo* environment enlists a diverse array of hormonal and paracrine signals that operate in the follicle to temporally coordinate oocyte maturation with cumulus expansion and ovulation (Richards *et al.*, 2002). If removed from the follicular environment, mammalian oocytes spontaneously resume meiosis (Edwards, 1965). However, despite the occurrence of nuclear maturation

in vitro these oocytes frequently do not acquire cytoplasmic maturation or terminal differentiation, in part because the culture environment fails to recapitulate the complex signaling and communication between somatic and gametic components of the ovulatory follicle (Eppig *et al.*, 1996). Although efforts to define optimal *in vitro* maturation conditions have been applied to *in vitro* embryo production in many mammalian species (Telfer, 1998; Sutton *et al.*, 2003), which have been notably successful in the bovine, it is clear that *in vivo* matured (IVO) oocytes have superior cytoplasmic quality and greater developmental potential than their *in vitro* (IVM) counterparts in most mammals, including the human (Leibfried-Rutledge *et al.*, 1987; Liu *et al.*, 2001; Trounson *et al.* 2001; Combelles *et al.*, 2002). Both positioning of mitochondria and ATP content are but two examples of

cytoplasmic properties linked to improvements in later development when *in vivo* and *in vitro* matured oocytes were compared in mice (Sun *et al.*, 2001; Combelles and Albertini, 2003; Nishi *et al.*, 2003).

Another aspect of oocyte maturation that may impinge upon oocyte quality and developmental competence is cytoskeletal remodeling. In mice, variations in microtubule remodeling during spindle morphogenesis were previously shown to underlie the distinct organization of meiotic spindles evident after either *in vivo* or *in vitro* maturation (Sanfins *et al.*, 2003). Earlier studies of IVM oocytes suggested that once removed from the follicle, mouse oocytes undergo dramatic changes in microtubule patterning and centrosome positioning during spindle morphogenesis (Messinger and Albertini, 1991; Combelles and Albertini, 2001). Although cell cycle specific rearrangements in oocyte microtubule organizing centers (MTOCs) and γ -tubulin have been defined during IVM in mouse oocytes (Messinger and Albertini, 1991; Combelles and Albertini, 2001), the overall significance of these cytoplasmic remodeling events to cell cycle control and oocyte nuclear maturation and developmental potential remains unknown. Accordingly, in an effort to better understand the importance of cell cycle markers during meiotic resumption and M-II arrest we have (i) analyzed cell cycle progression markers during the initial cytoskeletal remodeling in IVO and IVM oocytes, namely centrosome proteins (γ -tubulin and pericentrin), nuclear lamin integrity (lamin B) and microtubule patterning; (ii) assessed the *in vivo* window of oocytes removed from ovulatory follicles at specific times after exposure to hCG and (iii) evaluated the effect of imposing a G2 cell cycle delay with the maturation-promoting factor (MPF) inhibitor, roscovitine, on the overall quality of cytoplasm and spindles. Our results indicate that the timing and spatial patterning of spindle morphogenesis influences the allocation of maternal γ -tubulin stores during maturation such that both the physical properties of the meiotic spindle and the positioning of MTOCs are predictably distinct in IVM and IVO oocytes.

Materials and methods

Collection of *in vivo* and *in vitro* mouse oocytes

All experiments were performed using 7–8-week old CF-1 mice (Charles River). Animals were handled according to the Guide for Care and Use of Laboratory Animals (National Academy of Science, 1996) and maintained on a 14L:10D photoperiod under constant temperature and relative humidity conditions. Food and water were provided *ad libitum*. IVO oocytes were obtained from mice previously injected with 5 IU of equine chorionic gonadotropin (eCG, CalBiochem) to stimulate follicular development, followed 46 h later by 5 IU of human chorionic gonadotropin (hCG, Sigma). Oocytes were collected from Graafian follicles (at 1, 2, 3, 4 and 5 h post-hCG) or from the ampulla (16 h post-hCG) into medium consisting of Hepes-buffered Eagle's minimal essential medium (MEM) with Hanks' salts supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin and 0.3% bovine serum albumin (BSA). Following removal of cumulus cells by gentle pipetting (at 1, 2, 3 and 4 h post-hCG) or a brief treatment with hyaluronidase (190 IU/ml, Sigma, St Louis, MO) for 5 min at room temperature

(at 5 and 16 h post-hCG), oocytes were immediately fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer (0.1 M Pipes, pH 6.9, 5 mM MgCl₂·6H₂O and 2.5 mM EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 μ M taxol, 10 U/ml aprotinin and 50% deuterium oxide (Messinger and Albertini, 1991). Oocytes were stored at 4°C until further processing in a blocking solution of phosphate-buffered saline (PBS) containing 2% BSA, 2% powder milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100. *In vitro* matured oocytes (IVM) were obtained from mice injected 46 h earlier with 5 IU of eCG and cumulus-enclosed oocytes were collected by follicular puncture in collection medium and either immediately fixed or cultured for 1, 2, 3, 4, 5 and 16 h in *in vitro* maturation medium consisting of Eagle's MEM supplemented with Earle's salts, 2 mM glutamine, 0.23 mM pyruvate, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 0.3% BSA in a humidified atmosphere of 5% CO₂ in air. After removing the cumulus cells by gentle pipetting, oocytes were fixed and stored at 4°C until further processing.

The influence of intrafollicular residence time post-hCG on M-II phenotype

To address the influence of the follicular environment during early stages of oocyte maturation, mice were injected with 5 IU of eCG followed 46 h later by 5 IU of hCG. At 1.5 and 5 h post-hCG injection (GV-stage and prometaphase/M-I stage, respectively), mice were sacrificed, COCs collected by follicular puncture from Graafian follicles and immediately cultured in IVM medium up to 16 h. Therefore, for the group collected 1.5 h post-hCG, oocytes were cultured for 14.5 h in IVM medium; and for the group collected 5 h post-hCG, oocytes were cultured for 11 h in IVM medium. Following removal of cumulus cells by gentle pipetting (1.5 h group) or brief treatment with hyaluronidase (5 h group), oocytes were immediately fixed and stored at 4°C until further processing. IVO and IVM matured oocytes, obtained 16 h post-hCG or post-culture, respectively, were used as controls for these experiments.

Roscovitine treatment of GV-stage oocytes

COCs were collected from 7–8-week old CF-1 mice 46–48 h after injection with 5 IU eCG as described above. Oocytes were either exposed to 1% DMSO (control) or to 50 μ M roscovitine in 1% DMSO (Biomol, Pennsylvania, PA) in maturation medium for 3 and 6 h. Fifty micromolar roscovitine was determined to be the optimal concentration to use based on reversibility experiments. Following treatment, oocytes were washed three times in 100 \times volume of maturation medium without roscovitine and were subsequently matured *in vitro* for 16 h without drug. Oocytes were immediately fixed and stored at 4°C until further processing.

Processing of oocytes for immunofluorescence

All oocytes were processed in parallel for fluorescence microscopy in specific combinations to assess meiotic status (Hoechst 33258) relative to microtubule (MT) patterning (tubulin), nuclear lamina stability (lamin B), and MTOCs bearing constitutive (pericentrin) or regulative (γ -tubulin) markers. Oocytes were incubated sequentially with primary and secondary antibodies. Oocytes were separately processed for γ -tubulin (mouse) followed by anti-mouse secondary, then α -tubulin (rat) and anti-rat secondary. This sequence was followed for pericentrin (rabbit) and α -tubulin (rat); and γ -tubulin (mouse) and lamin B (rabbit), as shown in Table I. Each antibody was incubated for 1 h at 37°C with shaking followed by three 15 min washes in blocking solution. Oocytes were mounted using 1.5 μ l of a 50% glycerol/PBS containing sodium azide and Hoechst 33258

Table I. Labeling combination strategies used for this study

Combination 1	Combination 2	Combination 3
1st mouse monoclonal anti- γ -tubulin (1:100 dilution, Sigma)	1st rabbit polyclonal anti-pericentrin (1:200 dilution, Covance, CA)	1st mouse monoclonal anti- γ -tubulin (1:100 dilution, Sigma)
2nd Alexa-Fluor 568 goat anti-mouse IgG (1:800 dilution, Molecular Probes)	2nd Alexa-Fluor 568 goat anti-rabbit IgG (1:800 dilution, Molecular Probes)	2nd Alexa-Fluor 568 goat anti-mouse IgG (1:800 dilution, Molecular Probes)
3rd rabbit polyclonal nuclear lamin B (1:200 dilution, Gerace <i>et al.</i> , 1978)	3rd rat monoclonal α -tubulin YOL-34 (1:200 dilution, Kilmartin <i>et al.</i> , 1982)	3rd rat monoclonal α -tubulin YOL-34 (1:200 dilution, Kilmartin <i>et al.</i> , 1982)
4th Alexa-Fluor 488 goat anti-rabbit IgG (1:600 dilution, Molecular Probes)	4th Alexa-Fluor 488 goat anti-rat IgG (1:600 dilution, Molecular Probes)	4th Alexa-Fluor 488 goat anti-rat IgG (1:600 dilution, Molecular Probes)

(1 μ g/ml Polysciences Inc., Warrington, PA) to label chromatin. Incubation of oocytes in secondary antibodies alone failed to yield detectable staining either singly or in repeat sequence as above. Labeled oocytes were analyzed using a Zeiss IM-35 inverted microscope and a 50W mercury arc lamp using 40 \times and 63 \times Neofluor objectives. Digital images were collected with a Hamamatsu Orca ER digital camera (model #C4742-95) interfaced with a Meta Morph Imaging System. A triple band pass dichroic and automated excitation filter selection permitted collection of in-frame images with minimal magnification or spatial distortion.

Confocal microscopy

Triple labeled samples prepared as described above were analyzed by confocal microscopy using a Zeiss LSM Pascal system mounted on a Zeiss Axioscope II [63 \times plan Neofluor oil 1.4 numerical aperture (n.a.) or 40 \times water immersion objectives 1.2 n.a.]. LSM 5 Image Browser was used to analyze samples of at least 6 (*n*) oocytes per treatment group from which complete *z*-axis data sets of 30 sections per oocyte were collected at every 0.7 μ m. HeNe laser excitation for each probe permitted complete spatial restoration of Z-series or computed 3 D projections.

Statistical analysis

The number of MTOCs was represented by notched box and whisker plots (Slide Write Plus for Windows, Version 5.01; Advanced Graphics Software Inc., Encinitas, CA). Notched box plots display order statistics, and the notches of the box plots correspond to median confidence limits. Statistically, two medians are considered significantly different at the 0.05 level if their confidence limits do not overlap. Additionally, data were analyzed using SPSS 10.0 (Statistics Package for Social Sciences, Chicago, IL). Comparison of cytoplasmic MTOC numbers for each category of oocytes was evaluated using a nonparametric Kruskal-Wallis test followed by Mann-Whitney tests for two independent samples. Differences were considered significant at $P < 0.05$.

Results

Kinetic parameters of early meiotic progression in IVM and IVO oocytes

Simultaneous visualization of DNA and microtubules was used to assess the kinetics of early meiotic progression in IVO ($n = 364$) and IVM ($n = 266$) oocytes. IVO oocytes retain GVs for a longer period of time when compared to their IVM counterparts (Figure 1). Specifically, GVBD was observed at 3 h in IVO oocytes (26% diakinesis, 74%

prometaphase), while 67% of IVM oocytes at 2 h undergo GVBD and progress to diakinesis (37%) and prometaphase (30%). At 4 h, 90% of IVO oocytes reach prometaphase and by 5 h 20% of these oocytes progress to metaphase-I (M-I). In contrast, at 4 h most IVM oocytes reach prometaphase-I while the remainder are either in diakinesis or GVs. These results show that, although GVBD is delayed in IVO oocytes, the rate and extent of progression to metaphase-I is more

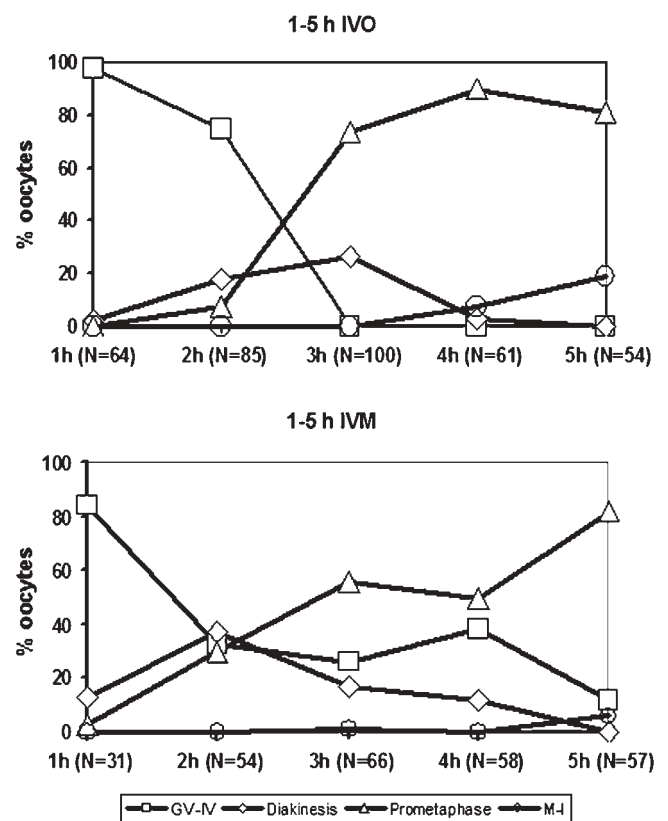


Figure 1. Kinetics of meiotic progression in IVM and IVO oocytes at 1, 2, 3, 4 and 5 h post-hCG (top) and post-culture (bottom). Sixty-seven percent of IVM oocytes have undergone GVBD by 2 h and show a gradual increase in the percentage of prometaphase stages by 5 h. In contrast, IVO oocytes retain GV stages at 2 h and GVBD only occurs at 3 h. Note that IVO oocytes exhibit greater synchrony, compared to IVM oocytes from GVBD (open squares) to prometaphase (open triangles). N represents number of oocytes analyzed at each time point.

rapid and synchronous during IVO maturation than during spontaneous maturation in culture. To more closely define the basis for these differences, we next analyzed nuclear lamina integrity and microtubule organization as markers of cell cycle progression during assembly of the M-I spindle *in vivo*.

Organization of chromatin, nuclear lamina, microtubules and centrosomes during IVO and IVM spindle morphogenesis

The distribution of centrosomal proteins γ -tubulin and pericentrin was analyzed with respect to nuclear lamina and microtubule organization in IVO and IVM oocytes at 1, 2, 3, 4 and 5 h of meiotic progression. As shown in Figures 2 and 3A, both IVO and IVM GV-stage oocytes are characterized by an intact and distended nuclear lamina with which are associated γ -tubulin and pericentrin containing MTOCs (Combelles and Albertini, 2001). IVO oocytes at 2 h retain an intact nuclear lamina with γ -tubulin foci associated with surface irregularities in nuclear lamina overlying the nucleolus (Figure 2B). When chromatin condensation is complete (3 h), the nuclear lamina collapses and γ -tubulin foci relocate from the external to internal surface of the lamina (see also Figure 4D and E), IVO oocytes exhibit characteristic γ -tubulin foci and usually the GV in close association with the nuclear lamina (Figures 2C and 4E). Interestingly, and in contrast to what happens in IVM oocytes (Figure 3D and E), IVO oocytes retain a collapsed nuclear lamina during spindle morphogenesis and within this structure two γ -tubulin foci appear, that define the poles of the first meiotic spindle (Figure 2D and E). Throughout meiotic progression, IVO oocytes exhibit numerous γ -tubulin foci in cortical cytoplasm, which increase in number (Figure 4) and show a diminished capacity to support microtubule nucleation as maturation proceeds. Parallel investigations of IVM oocytes extend earlier studies in showing that the initial collapse of the nuclear lamina is followed by gradual expansion coincident with dissolution of the nuclear lamina prior to spindle assembly. This secondary expansion coincides with increased microtubule assembly at the spindle. In addition, both γ -tubulin and pericentrin foci accumulate around the lamina and more pronounced incorporation of these proteins into the spindle is apparent. Strikingly, there is a clear absence of immunodetectable MTOCs and subcortical microtubules throughout the process of spindle formation.

Laser scanning confocal microscopy (Figure 4) further revealed that the size, number and microtubule nucleation capacity of MTOCs are dramatically altered from prometaphase (Figure 4B) to metaphase I (Figure 4C) ($n = 300$ oocytes). These studies show a gradual decrease in mean number and length of MTOC associated microtubules and the overall size of MTOCs decreased during meiotic progression (Figure 4A–C). IVM oocytes analyzed by confocal microscopy failed to reveal subcortical MTOCs except on rare occasions (1–3 unusually large and rarely with microtubules) (Mattson and Albertini, 1990). Finally, the relationship of γ -tubulin and nuclear lamina integrity consistently showed localization of γ -tubulin foci at nuclear lamina invaginations

spatially coincident with condensing chromatin (Figure 4D and E). At later stages, the nuclear lamina nearly completely encases condensed chromatin and large γ -tubulin foci are located at deep nuclear lamina invaginations (Figure 4E). In contrast, during IVM the nuclear lamina is diffuse and fibrillar with many small γ -tubulin foci located along the periphery of the nuclear lamina (Figure 4F).

These studies uncover basic distinctions in nuclear lamina integrity and MTOC behavior in IVM and IVO oocytes. Coupled with our earlier reports, it seems likely that containment of chromatin and limiting access to centrosome proteins may be linked to the smaller spindles and multiple MTOCs seen in IVO oocytes (Sanfins *et al.*, 2003).

Influence of the intrafollicular environment on the M-II phenotype

The above results suggest that events soon after hCG reception in the follicle may specify properties in the M-II oocyte previously shown to affect spindle shape, size and cytoplasmic MTOC number. How soon after hCG would oocytes be committed to the expression of an IVO character? To determine if early signaling affected the properties of oocytes at M-II, experiments were designed using COCs recovered from pre-ovulatory Graafian follicles at 1.5 and 5 h post-hCG. Meiotic progression of the oocytes to M-II was not affected by removal at either time (80% from 1.5 h versus 72.7% from 5 h reached M-II stage). In oocytes retrieved 1.5 h post-hCG, M-II matured oocytes displayed a barrel shaped spindle (Figure 5C) with γ -tubulin distributed throughout the spindle proper (Figure 5B), similar to the IVM control group (Figure 5J–M). Typically, the 1.5 h group exhibited large spindles, lacking bundled microtubules and was characterized by a diffuse γ -tubulin throughout (Figure 5C). In contrast, oocytes retrieved after 5 h exhibited pointed spindles containing distinct microtubule bundles (Figure 5F) and γ -tubulin staining was only seen at the spindle poles (Figure 5E). The spindles were more cortically positioned in oocytes retrieved at 5 h versus 1.5 h (30/33 versus 10/36). Consistent with this, as shown in Figure 6, the number of MTOCs was greater when the intrafollicular residence time was longer, although significantly different from the IVO group ($P < 0.05$). IVO oocytes (control) show the highest mean number of MTOCs/oocyte followed by oocytes collected 5 h post-hCG (27.3 ± 6.3 and 21.9 ± 6.48 , respectively). Moreover, M-II oocytes collected 1.5 h post-hCG also exhibit a higher number of MTOCs when compared to the IVM group, but these were considerably reduced in MTOC mean number when compared to the 5 h post-hCG group (16.7 ± 5.4 , 10.8 ± 3.1 and 21.9 ± 6.48 , respectively). Thus, between 1.5 and 5 h post-hCG, events have occurred that impact properties of M-II oocytes following retrieval and subsequent culture. These findings further support the idea that an initial delay in meiotic progression *in vivo* is due to resistance to effect a G2/M cell cycle transition.

Influence of roscovitine treatment on the M-II phenotype

Next we asked if delaying GVBD by preventing MPF activation might alter the acquisition of nuclear (spindle)

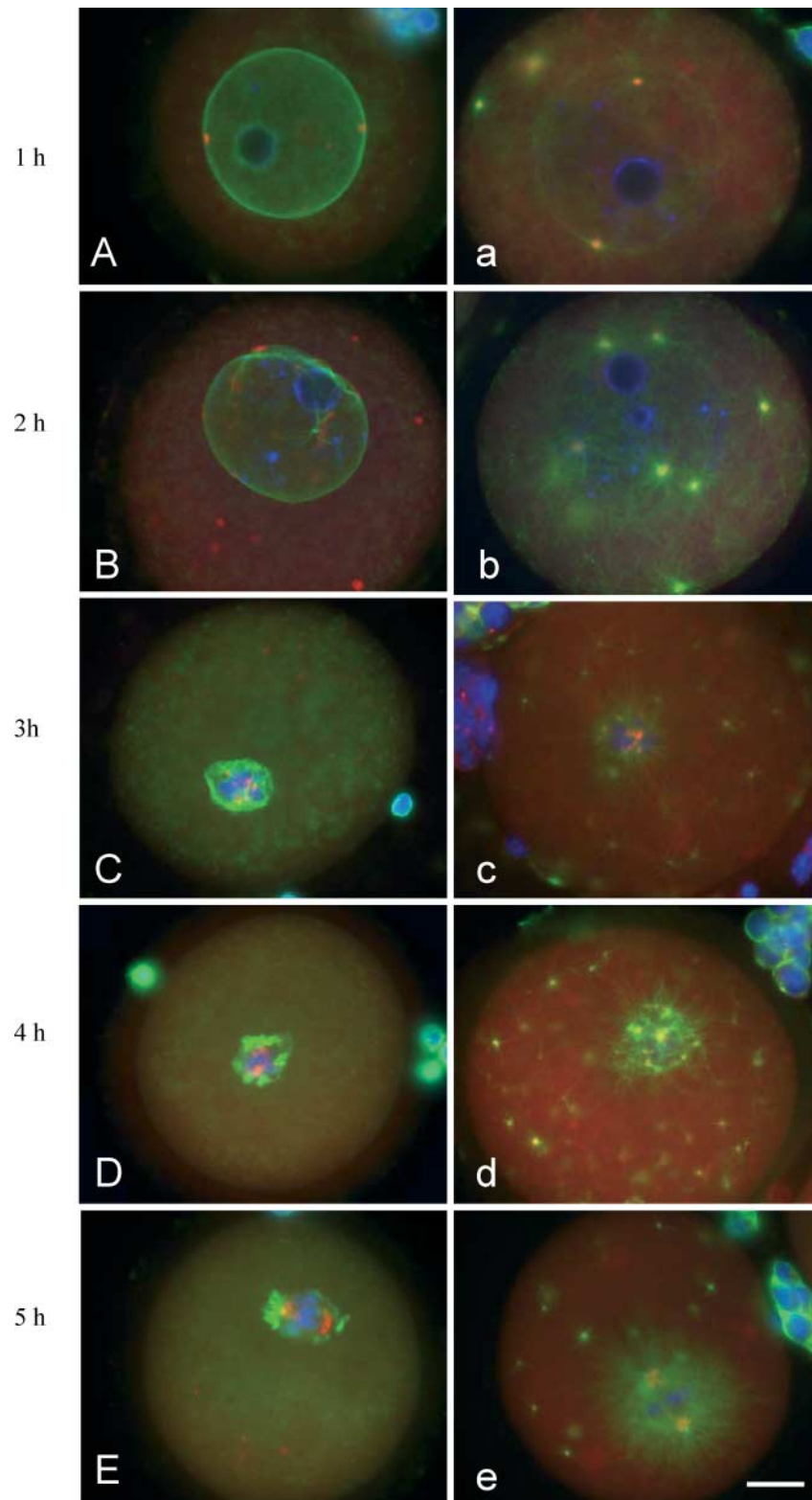


Figure 2. Representative examples of IVO oocytes 1 (**A, a**), 2 (**B, b**), 3 (**C, c**), 4 (**D, d**) and 5 h (**E, e**) post-hCG showing distribution of γ -tubulin (red), lamin B (green) and chromatin (blue) (left or A–E); pericentrin (red), tubulin (green) and chromatin (blue) (right or a–e). Note the restricted distribution of γ -tubulin (A–E) and pericentrin (a–e) with collapsed nuclear lamina (A–E) during the initial stages of meiotic progression in IVO oocytes. γ -tubulin and pericentrin foci form poles of the 1st meiotic spindle (E, e) in IVO oocytes which display increased number of MTOCs (a–e) that support microtubule nucleation (a–e). Bar represents 20 μm .

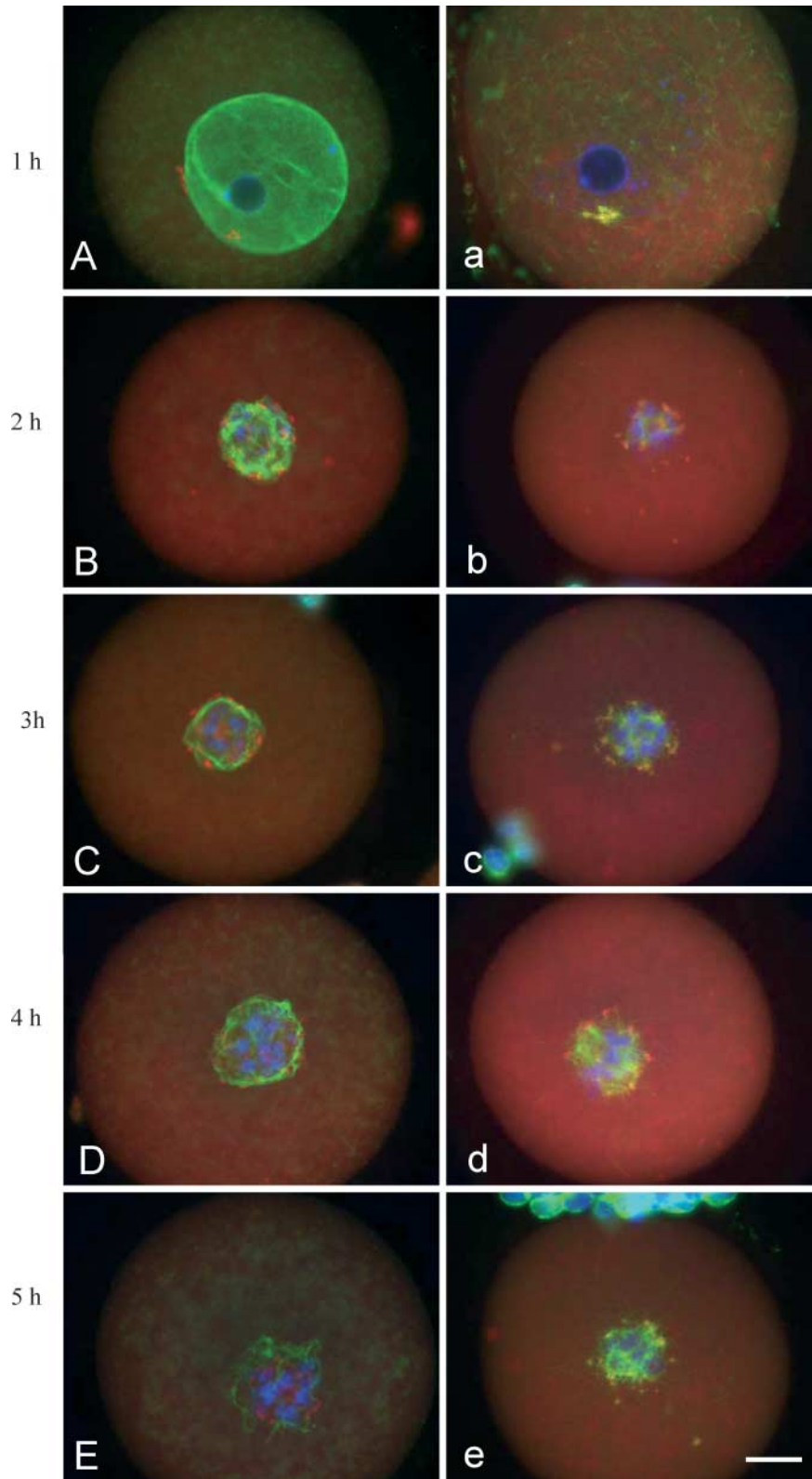


Figure 3. Representative examples of IVM oocytes 1 (A, a), 2 (B, b), 3 (C, c), 4 (D, d) and 5 h (E, e) in culture showing distribution of γ -tubulin (red), lamin B (green) and chromatin (blue) (left or A–E) and pericentrin (red), tubulin (green) and chromatin (blue) (right or a–e). Note perinuclear distribution of γ -tubulin around an expanded nuclear lamina (B–E) and pericentrin around forming spindle (b, e). Few cytoplasmic MTOCs are evident in IVM oocytes (b, e). Bar represents 20 μm .

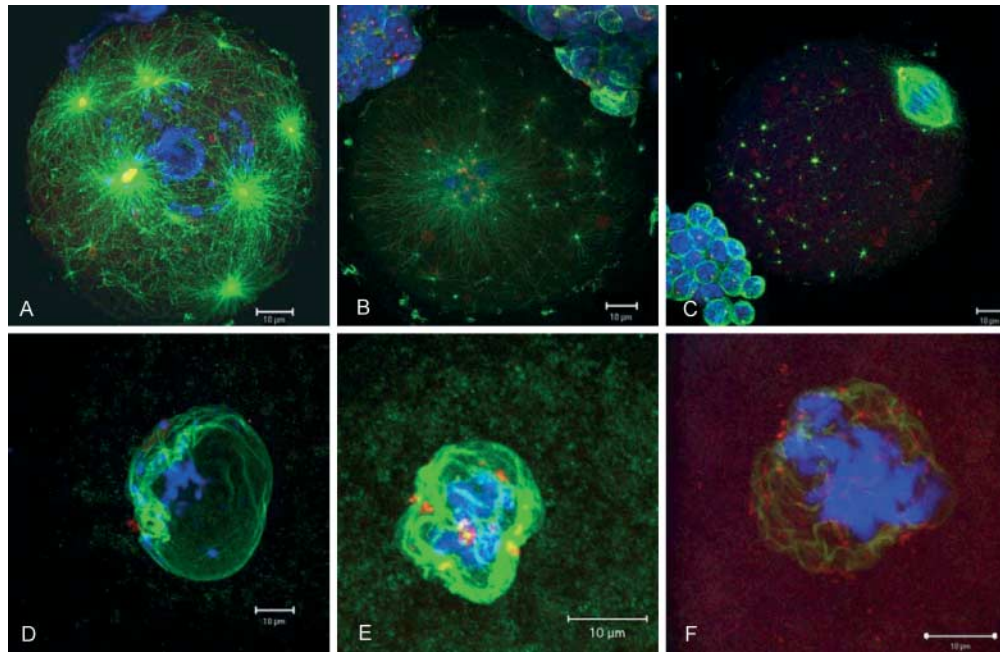


Figure 4. Confocal 3-D reconstructions of IVO GV-stage oocyte (A); prometaphase oocyte (B) and metaphase I oocyte (C) illustrating tubulin (YOL-34, green), MTOCs (pericentrin, yellow is due to overlap with tubulin signal) and chromatin (Hoechst 33258, blue). The increased number of MTOCs and apparent reduction in size suggests fragmentation during meiotic progression. IVO oocyte in prediakinesis (D), IVO prometaphase (E) and IVM prometaphase (F). (B–F) γ -tubulin (red), nuclear lamina (lamin B, green) interaction in IVO (D and E) and IVM (F) oocytes. Lamin B integrity is maintained in IVO oocytes at prometaphase where γ -tubulin foci are retained at the surface (E) whereas a more expanded and diffusely fibrillar lamina with multiple small γ -tubulin foci is seen at comparable stages in IVM oocytes (F). Bar represents 10 μ m.

and cytoplasmic (MTOCs) traits that distinguish oocytes matured in IVO and IVM conditions. The G2/M (GVBD) cell cycle transition was prevented for 3 or 6 h using 50 μ M roscovitine, a specific inhibitor of MPF (cdk1). Oocytes so treated have a surrounded-nucleolus (SN) chromatin configuration and interphase microtubule array characteristic of a G2 phenotype (Wickramasinghe *et al.*, 1991) and monoclonal antibody MPM-2 negative (data not shown). Oocytes progressed to M-II after removal of roscovitine and culture for an additional 16 h in the absence of the drug. Reversibility was more effective on the 3 h treatment group (74.8% of M-II stage oocytes at 3 h versus 48.8% at 6 h). These oocytes exhibit cortically localized (data not shown) and more compact spindles with γ -tubulin foci restricted to the spindle poles (Figure 7a). In both cases, an increased number of cytoplasmic centrosomes evidenced by γ -tubulin positive foci is observed in M-II oocytes subjected to either 3 or 6 h of roscovitine treatment, relative to their M-II IVM controls (Figure 7b). Thus, brief exposure to roscovitine yields oocytes with IVO characteristics, suggesting that delaying the G2/M transition by reversal inhibition of MPF confers upon IVM oocytes spindle and cytoplasmic properties resembling those of IVO oocytes.

Discussion

IVM remains a challenge in assisted reproductive technologies (ARTs). Understanding the relationship between cytoskeleton organization and cell cycle progression will be

central to designing efficient protocols for IVM. In human oocytes, the importance of this relationship has been emphasized by several reports showing the adverse consequences of IVM on the coordination of nuclear and cytoplasmic maturation (DeSisciolo *et al.*, 2000; Combelles *et al.*, 2002, 2003). Using a mouse model, the present studies aimed at understanding how and when conditions are established that lead to optimized nuclear and cytoplasmic maturation during induced ovulation. We focused on cell cycle and cytoskeleton variations in IVO and IVM oocytes in light of the metabolic demands of MPF activation and inactivation that ensure coordination of cytokinesis and karyokinesis. By tracking centrosome proteins (γ -tubulin and pericentrin), nuclear lamina integrity (lamin B) and microtubule patterning in response to hCG, we found that the unique attributes of both spindle (size) and cytoplasm (MTOC number) of IVO oocytes derives from a cell cycle delay favoring compartmental cell cycle control. We show further that between 1.5 and 5 h post-hCG, the intrafollicular signals necessary for coordination have been achieved and that the cdk1 inhibitor roscovitine imposes nuclear and cytoplasmic maturation by maintaining a G2 state prior to M-phase entry. This work raises several questions about the basic mechanisms that distinguish G2/M in somatic cell versus oocytes and how the use of M-phase inhibitors may optimize IVM protocols.

The distribution of centrosomal proteins (γ -tubulin and pericentrin) and the microtubule cytoskeleton during the early stages of meiotic maturation is clearly distinct between IVO and IVM oocytes. IVO oocytes retain centrosomal

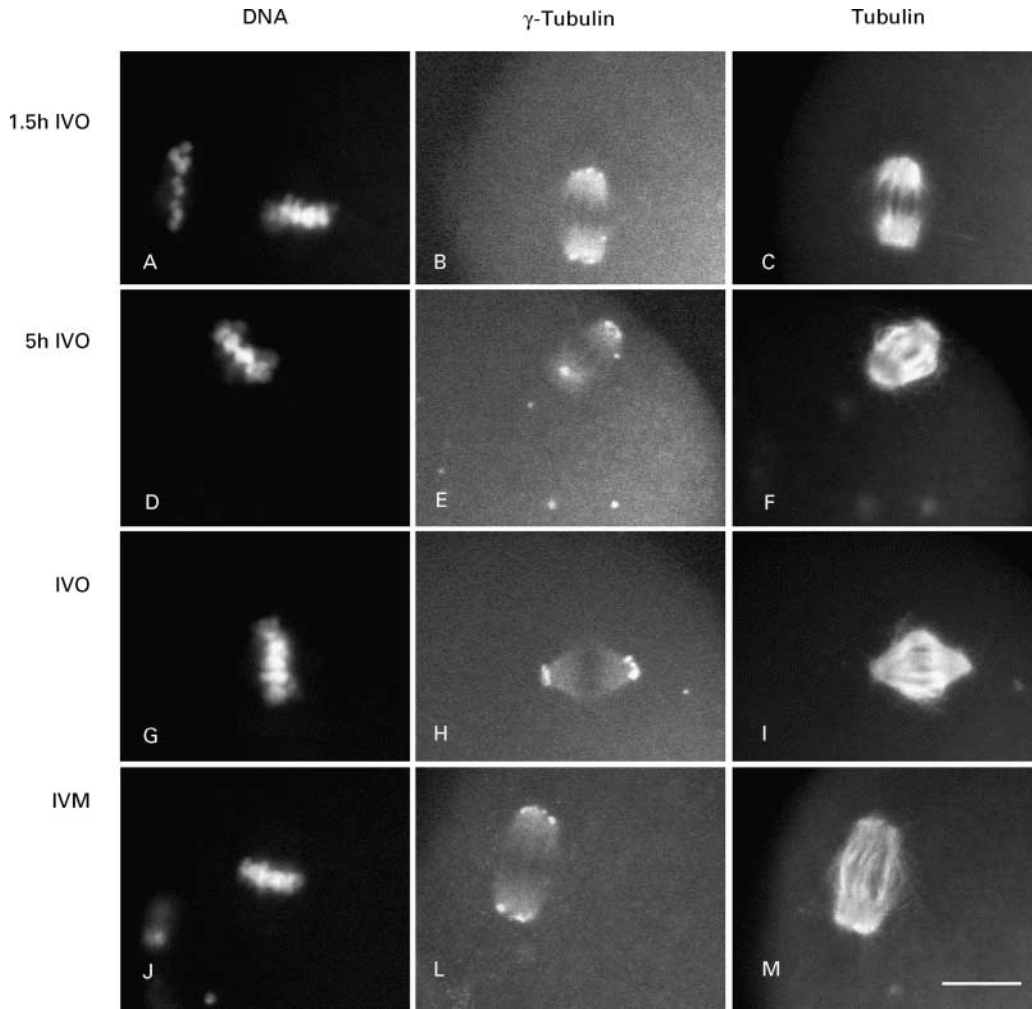


Figure 5. Influence of the intrafollicular environment on the M-II spindle. Correlative images of chromatin (A, D, G and J), γ -tubulin (B, E, H and L) and microtubules (C, F, I and M) of representative oocytes retrieved from follicles 1.5 h post-hCG (A–C), 5 h post-hCG (D–F), 16 h post-hCG (G–I), and IVM oocytes that have been cultured for 16 h (J–M). Oocytes collected from follicles 5 h post-hCG display tapered spindles with polar γ -tubulin foci (D–F) resembling IVO M-II oocytes (G–I) rather than IVM oocytes (J–M) or those retrieved 1.5 h post-hCG (A–C). Bar represents 20 μ m.

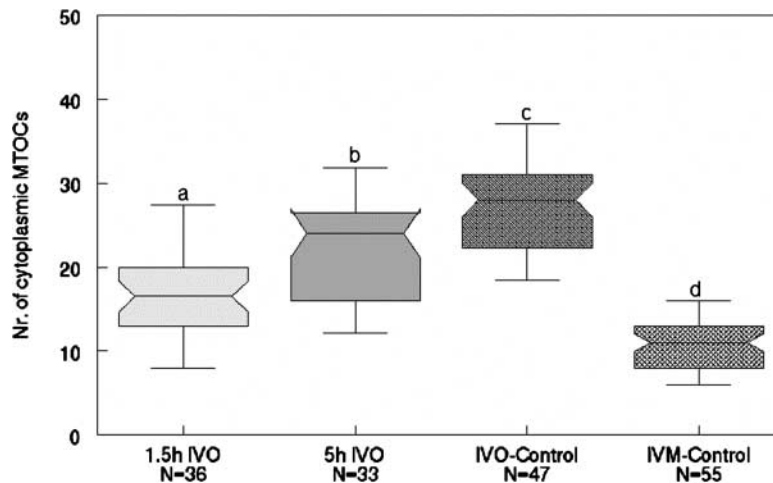


Figure 6. Notched box plots showing cytoplasmic MTOCs number in oocytes retrieved at 1.5 (1.5h IVO), 5 (5h IVO) or 16h post-hCG (IVO-control) and also oocytes cultured for 16h (IVM-control). The data suggests that cytoplasmic MTOC numbers are influenced by residence time within the follicular environment. N represents number of oocytes for each group in three representative experiments. Different letters represent significant differences obtained across oocyte categories using non-parametric Kruskal–Wallis and Mann–Whitney tests ($P < 0.05$).

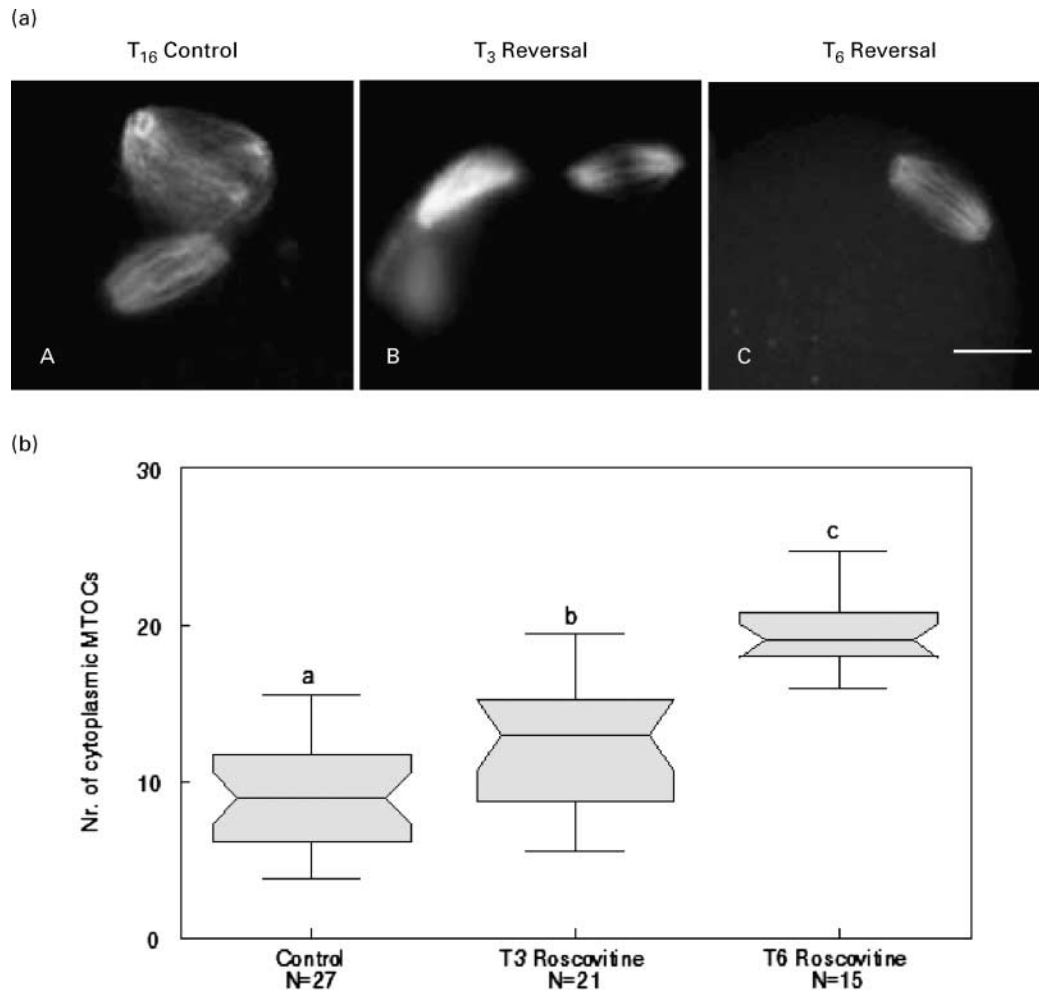


Figure 7. (a) M-II spindles of oocytes obtained in culture for 16 h (Control) (A) or after 3 h (B) and 6 h (C) of roscovitine treatment. Roscovitine treated oocytes exhibit more compact tapered spindles than control (B and A, respectively) and exhibit an increase in cytoplasmic MTOCs (b). N represents number of oocytes for each group in two representative experiments. Different letters in the notched box plots represent significant differences obtained across oocyte categories using non-parametric Kruskal–Wallis and Mann–Whitney tests ($P < 0.05$). Bar = 20 μm .

proteins subcortically within multiple MTOCs of enhanced microtubule nucleation capacity that increase in number from diakinesis to M-I. In contrast, fewer cytoplasmic MTOCs are present in IVM oocytes over the same cell cycle stages, presumably as a result of the dramatic redistribution of γ -tubulin and pericentrin to the nuclear lamina during spindle morphogenesis (Messinger and Albertini, 1991; Combelles and Albertini, 2001). While Can *et al.* (2004) proposed that the availability of γ -tubulin and pericentrin to the nuclear lamina during early stages of maturation in IVM oocytes reveals a shift of microtubules from stabilization of the cortex to morphogenesis of the spindle (Can *et al.*, 2004), our results on IVO oocytes add further insight. Centrosome positioning in somatic cells requires radial or astral arrays of cytoplasmic microtubules so that pushing and pulling forces involving MT mediated by motors can be anchored to and stabilize the overlying cortex (Burakov *et al.*, 2003). We suggest that IVM oocytes similarly exhibit more rapid and complete nuclear lamina disruption because of the rapid and nearly

total accumulation of centrosomes upon GVBD. Interestingly enough, the minus end focusing motor dynein was also shown to be a key factor during nuclear envelope breakdown in mitosis due to M-phase dynamics of perinuclear centrosomes and their associated MTs (Salina *et al.*, 2002). Conversely, the delayed GVBD and persistence of the nuclear lamina in IVO oocytes is consistent with the fact that increased number of MTOCs support microtubule nucleation, preventing cytoplasmic MTOCs from participating in nuclear lamina disruption. Thus, while preserving integrity of the cortical microtubule cytoskeleton, spindle morphogenesis will proceed with minimal centrosome involvement within a constrained space. While admittedly speculative, the potential advantages of compartmentalizing spindle morphogenesis might include favoring chromosome directed over centrosome forces to achieve proper metaphase alignment and limiting the utilization of maternal molecules like γ -tubulin in meiotic spindles so that adequate stores remain in the fertilized egg. Why would it be advantageous to limit centrosome

access to the forming spindle? Some insight into this problem comes from recent studies on the regulation of the G2/M cell cycle transition in somatic cells.

Unlike what we report here and elsewhere in mouse oocytes (Combelles and Albertini, 2001), somatic cells rapidly condense chromatin and depolymerize the nuclear lamina upon entry into M-phase. Moreover, it has been shown that active cyclin B1–Cdk1 first appears on centrosomes in prophase (Jackman *et al.*, 2003). The spatial segregation of cell cycle components appears to be central to achieving the correct temporal readout of checkpoint controls that allow somatic cells to reach and engage the chromosome alignment checkpoint at metaphase. While centrosomes can participate in mitotic spindle assembly, chromatin alone is necessary and sufficient to build a bipolar spindle as long as a minus end motor like dynein is present (Heald *et al.*, 1997). In fact, centrosomeless spindles function during mitosis and excess centrosomes can be deleterious to somatic cells, since they favor the formation of multipolar spindles that contribute to aneuploidy (Khodjakov and Rieder, 2001; Sluder and Nordberg, 2004). Given the large volume of the oocyte, and the requirement to retain maternal stores that likely include MTOC components needed for mitoses during embryogenesis, restricting MTOC access to the GV may serve to engage cortical anchoring of the spindle prior to first polar body extrusion and launch nuclear maturation within a nuclear lamina compartment favoring chromatin-directed spindle morphogenesis as noted above. Might this interplay between nuclear and cytoplasmic maturation also influence cell cycle progression?

The spatial distribution of cell cycle factors has been proposed to be a key factor for the coordination of nuclear and cytoplasmic maturation in mouse oocytes (Mitra and Schultz, 1996). As in somatic cells (Pines, 1999), relative protein and mRNA levels of key cell cycle factors cannot account for differences in the ability of mouse oocytes to initiate a G2 (incompetent) to M (competent) cell cycle transition (Kanatsu-Shinohara *et al.*, 2000). Also, since centrosome phosphorylation has been associated with increased microtubule dynamics (Messinger and Albertini, 1991) and meiotic competence acquisition (Wickramasinghe *et al.*, 1991), maintaining cortical centrosomes may stratify cell cycle machinery between the nucleus and cytoplasm to prevent precocious meiotic M-phase entry (Albertini and Carabatsos, 1998). Thus, the synchrony in the rate of meiotic progression observed in IVO oocytes may be due to the selective retention of centrosomal material or MPF to the oocyte cortex whilst restricting recruitment of centrosomes/MPF for spindle morphogenesis. During IVM, it appears that oocytes liberate these factors leading to precocious MPF activation, the consequences of which would include rapid and more extensive dissolution of the nuclear membrane and lamina, resulting in formation of larger spindles and fewer cytoplasmic MTOCs. Asynchrony in the processes of nuclear and cytoplasmic maturation during IVM may then lead to compromised oocyte quality that might be ameliorated by better management of the G2/M cell cycle transition. With this in mind, the specific MPF inhibitor,

roscovitine, was used to impose a delay on GVBD onset. Surprisingly, oocytes treated with roscovitine and allowed to re-initiate M-phase exhibited spindle and cytoplasmic properties resembling IVO oocytes (Figure 7). It has been reported that maintaining oocytes in a G2 cell cycle stage with roscovitine increases the developmental potential of porcine (Marchal *et al.*, 2001), bovine (Ponderato *et al.*, 2001; Lagutina *et al.*, 2002) and horse (Franz *et al.*, 2003) oocytes. Further studies are needed to elucidate the basis for oocyte quality improvement when a cell cycle delay is imposed prior to GVBD but our data with roscovitine imply that delaying activation of MPF and concurrent with microtubule stabilization may enhance the coordination of nuclear and cytoplasmic maturation and/or optimize oocyte metabolism as meiosis proceeds.

The metabolic and physiological complexity of the intra-follicular environment during *in vivo* ovulation must be addressed in any comparison with *in vitro* conditions (Sutton *et al.*, 2003). Specific changes occur within the follicular milieu, mediated by oocyte–granulosa cell contact and/or growth factors that coordinate oogenesis with folliculogenesis through ovulation (Albertini and Carabatsos, 1998; Carabatsos *et al.*, 2000; Richards *et al.*, 2002). Not surprisingly then IVO oocytes exhibit elevated ATP content (Combelles and Albertini, 2003), mitogen-activated protein (MAP) kinase activity (Su *et al.*, 2003) and characteristic mitochondrial distributions (Van Blerkom, 1991; Sun *et al.*, 2001; Nishi *et al.*, 2003) compared to IVM counterparts. In addition, EGF-related growth factors (Park *et al.*, 2004) and specific transcriptional profiles responsible for cumulus expansion (Su *et al.*, 2003) and presumably oocyte quality determination are different in IVM oocytes. We addressed this directly by collecting IVO oocytes at 1.5 and 5 h post-hCG injection and culturing up to 16 h in IVM medium that did not recapitulate cumulus expansion conditions. Despite the fact that basal conditions yield IVM properties, oocytes removed from the follicles at 5 h post-hCG exhibited striking similarities to IVO oocytes in spindle shape and a slightly increased MTOC number (although MTOC number is significantly different to the IVO oocytes, $P < 0.05$); oocytes removed from follicles at 1.5 h post-hCG more closely resembled IVM oocytes. These findings suggest that cumulus signaling and events occurring within the follicle in the first 5 h are critical to establish properties characteristic of IVO oocytes. This 5 h time interval is of interest because it coincides with the activation of cumulus MAPK and the production of EGF-like paracrine signals that mediate LH induced maturation (Su *et al.*, 2003; Park *et al.*, 2004).

Future efforts to optimize IVM should seek to recreate follicular changes in both cumulus and oocyte during the early events of oocyte maturation. Collectively these findings re-emphasize the importance of the early effects of LH on the process of oocyte maturation. Using markers for oocyte quality as have been identified here should help to understand the importance of maternal resource conservation mechanisms on embryonic developmental potential and give guidance to new protocols for *in vitro* maturation in humans.

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