

Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes

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BACKGROUND: With improved prospects for the use of human oocyte in-vitro maturation in assisted reproductive technologies, the need to define more clearly the coordination of nuclear and cytoplasmic maturation has arisen. **METHODS:** Immunofluorescence and confocal microscopy were used to evaluate cell cycle-dependent modifications in chromatin and microtubules in human germinal vesicle oocytes ($n = 455$) undergoing in-vitro maturation. **RESULTS:** Four distinct classes of germinal vesicle stage oocytes were identified based on the expression of G2/interphase characteristics, but, of these, only one class of oocytes was competent to complete meiotic progression to metaphase-II *in vitro*. The majority of germinal vesicle stage oocytes resumed meiosis within 6 h (88.9%) of culture and exhibited an accelerated pace of progression to metaphase-II (66.7%) over 24 h, but in general were unable to maintain meiotic arrest and defaulted into interphase within 24 h of polar body emission. Characterization of microtubule dynamics and chromatin phosphorylation demonstrates specific cell cycle deficiencies in in-vitro matured human oocytes. **CONCLUSION:** This work forms a basis for future studies aimed at optimizing nuclear and cytoplasmic maturation during in-vitro maturation.

Key words: chromatin/human/maturation/microtubule/oocyte

Introduction

Current programmes in assisted reproductive technology routinely use ovarian stimulation for ovulation induction. Although an increased number of oocytes can be obtained using this technique, possible health risks to the patient, high cost, and modest success rates remain of concern. Despite its clinical utility, in-vitro maturation (IVM) of human oocytes remains an experimental approach deserving further attention and awaiting improvement before becoming a routine and practical assisted reproductive procedure (Cha and Chian, 1998; Moor *et al.*, 1998; Trounson *et al.*, 2001). Early attempts of IVM in human oocytes date back to 1965 (Edwards *et al.*, 1965), with the first successful birth reported in 1991 (Cha *et al.*, 1991). To date, several births have been achieved from in-vitro matured human oocytes, with patient history, oocyte source, and culture media utilized varying between studies (Cha *et al.*, 1991, 2000; Trounson *et al.*, 1994; Barnes *et al.*, 1995, 1996; Cha and Chian, 1998; Kim *et al.*, 2000; Smith *et al.*, 2000). Therefore, pregnancy and live birth success rates remain low following transfer of embryos derived from in-vitro matured oocytes when compared with in-vivo matured oocytes.

Likely reasons for the low efficacy of IVM in assisted reproduction include inadequacies of the culture media used.

In addition, heterogeneity in reported maturation rates are probably due to intrinsic differences in oocytes recovered after ovarian stimulation from variously sized follicles *in vivo* that vary in their developmental capacity due to incomplete or abnormal growth. Meiotic competence acquisition and expression has been related to changes in oocyte growth, germinal vesicle chromatin organization, meiotic cell cycle status, and transcriptional activity in oocytes of several mammalian species (McGaughey *et al.*, 1979; Wickramasinghe *et al.*, 1991; Schramm *et al.*, 1993; Fair *et al.*, 1995, 1996). Markers of oocyte differentiation and meiotic cell cycle state include germinal vesicle chromatin patterns, mitotic phosphoprotein monoclonal-2 reactive protein (MPM-2) phosphorylation, and cytoplasmic microtubule organization (Wickramasinghe *et al.*, 1991; Wickramasinghe and Albertini, 1992). Phosphorylation of histone-3, characteristic of metaphase (M-phase), is also a useful indicator of chromatin condensation in mitotic and meiotic cells (Hendzel *et al.*, 1997; Wei *et al.*, 1999; Carabatsos *et al.*, 2000). In rodent oocytes, the cell cycle expression of these markers is as follows: the G2 phase of the cell cycle, specifically the dictyate stage of prophase-1, is characterized by lack of phosphohistone 3 (PH3) reactivity, nuclear MPM-2, and interphase arrays of microtubules. In contrast,

following formal entry into M-phase of the cell cycle, histone-3 is phosphorylated, MPM-2 becomes cytoplasmic, and microtubules are converted from a stable interphase state into dynamic polymers associated with condensed chromosomes (Wickramasinghe and Albertini, 1992; Carabatsos *et al.*, 2000). Microtubule reorganization and stability is influenced at the transition of interphase to M-phase by several factors, including protein kinase activity, centrosome-based microtubule nucleation, and post-translational modifications of tubulin (Albertini, 1992). The relative contribution of each of these factors has not been defined in human oocytes, despite the possibility that disruption of microtubule patterning might underlie failures in chromosome segregation or organelle allocation during later development (Van Blerkom *et al.*, 1995, 2000). That human female gametes present an elevated risk of aneuploidy when compared with oocytes from other mammalian species further buttresses the need for additional study into the cell cycle-dependent changes in microtubule organization in human oocytes. To our knowledge, the baseline M-phase markers defined above (MPM-2, PH3, and microtubules) have not been analysed in human oocytes during meiotic competence acquisition and expression.

Available evidence indicates that culture systems adequately support nuclear maturation in human oocytes but fail to produce oocytes with cytoplasmic competency, thereby resulting in embryos with reduced developmental potential (Cha and Chian, 1998; Moor *et al.*, 1998; Trounson *et al.*, 2001). Cytoplasmic maturation encompasses a wide array of metabolic and structural modifications, including events that ensure the occurrence of normal fertilization, meiotic to mitotic cell cycle progression, and activation of pathways required for genetic and epigenetic programmes of preimplantation embryonic development (Eppig *et al.*, 1994; Eppig, 1996; Heikinheimo and Gibbons, 1998; Moor *et al.*, 1998; Trounson *et al.*, 2001). Therefore, three possibilities might underlie the limited success of human IVM: culture conditions, to date, are not supportive of expression of intrinsic developmental competency of oocytes; current IVM systems induce an asynchrony in the progression of nuclear and cytoplasmic maturation; or the oocytes utilized lack one or more of the components necessary for nuclear and cytoplasmic maturation and later embryonic development. The present study was designed to investigate the last possibility, which is that cytoplasmic maturity is deficient in in-vitro matured human oocytes. Specifically, cell cycle-dependent modifications in chromatin and microtubule patterning were studied in immature oocytes and during IVM in a defined culture medium.

Materials and methods

Source of oocytes and use in experiments

Immature human oocytes were aspirated from ovaries of women undergoing ovarian stimulation for ICSI. Complete institutional review board approval (protocol #97-08750) and written consent was obtained from all patients prior to placing oocytes in culture. Following removal of corona-cumulus cells with hyaluronidase and mechanical pipetting, the meiotic status of oocytes was assessed. Immature oocytes were defined as either germinal vesicle stage (GV), repres-

enting oocytes arrested at prophase of meiosis-I, or as meiosis-I (M-I) as evidenced by the absence of a polar body and no discernable germinal vesicle nucleus. A total of 455 oocytes retrieved from 131 patients (35.54 ± 4.07 years; mean \pm SD) in 137 ovarian stimulation cycles were included in this study. Following retrieval, seven M-II oocytes were donated from a patient because no sperm were available for ICSI. After corona-cumulus cell removal, oocytes were photographed at $\times 400$ magnification, and the vitelline diameters (not including the zona pellucida) of fresh oocytes were determined from the maximum and minimum diameters of each oocyte.

Five experiments were evaluated: (i) GV chromatin patterning to identify what association, if any, the GV chromatin might have to meiotic competence; (ii) kinetic experiment to characterize the timing of meiotic progression; (iii) study of microtubules, with respect to their post-translational modification (acetylation) and the presence of phosphoproteins, to evaluate nuclear and cytoplasmic maturation during IVM; (iv) activation incidence to assess the ability of M-II oocytes to maintain meiotic arrest *in vitro*; and (v) taxol exposure to analyse microtubule dynamics in in-vivo matured human oocytes. The number of oocytes employed in each of these studies is shown in Table I; note that some oocytes were used in more than one study.

Culture system (Experiments 2, 3 and 4)

GV stage oocytes were collected into standard P-1 (Preimplantation Stage One; Irvine Scientific, Santa Ana, CA, USA) medium and placed in our defined IVM medium (P1-S) within 5 h of retrieval. In the P1-S IVM medium, 60.6% of GV oocytes progress to M-II within 48 h (Cekleniak *et al.*, 2001). Oocytes were cultured in 25 μ l microdrops of medium overlaid with embryo-tested light mineral oil in a humidified atmosphere of 5% CO₂ at 37°C. Oocytes were randomly allocated at the beginning of culture and fixed at various time points.

Taxol exposure (Experiment 5)

Five to seven hours after retrieval, oocytes at various cell cycle stages were exposed to the microtubule stabilizing agent, taxol. A taxol solution (final concentration of 10 μ mol/l) was prepared from a 1 mmol/l dimethylsulphoxide (DMSO) stock (stored at -20°C) in human tubal fluid medium (Irvine Scientific). Denuded oocytes were treated for 10 min at 37°C in taxol- or DMSO (control)-containing medium, prior to fixation and immunofluorescence analysis.

Processing of oocytes for immunofluorescence analysis (Experiments 1-5)

Oocytes were fixed and processed for microtubule detection, chromatin organization, and the presence of phosphoproteins as previously described (Messinger and Albertini, 1991; Cekleniak *et al.*, 2001). Microtubules were labelled using either a monoclonal anti- α -tubulin and anti- α -tubulin mixture (Sigma Biosciences, St Louis, MO, USA), a rat monoclonal antibody against α -tubulin (YOL 34) (Kilmartin *et al.*, 1982), or a monoclonal anti-acetylated α -tubulin (Sigma Biosciences) at 1:100 final dilutions. Chromatin was detected using either a mouse monoclonal anti-histone H1 IgG (Leinco Technologies Inc., St Louis, MO, USA), or a rabbit polyclonal antibody directed against the PH3 mitosis marker (Upstate Biotechnology, Lake Placid, NY, USA), or Hoechst 33258 (Polysciences Inc., Warrington, PA, USA). To detect phosphoproteins, an MPM-2 antibody was used (Upstate Biotechnology). Affinity-purified fluoresceinated, Texas Red, or Cy-5 donkey anti-mouse, rat or rabbit IgG were used at a 1:500 final dilution (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Processed oocytes were mounted and analysed by conventional fluorescence or laser scanning confocal microscopy as previ-

Table I. Experiments and number of oocytes utilized

Type of oocytes ^a	<i>n</i>	Experiment				
		1 GV chromatin pattern	2 Kinetic	3 Nuclear and cytoplasmic markers	4 Activation	5 Taxol exposure
IVM	262	133	64	90	74	—
IVO	193	—	—	69	—	186

A total of 455 oocytes was analysed from 131 patients (137 stimulation cycles) with a mean age \pm SEM of 35.54 ± 4.07 years.

^aFor in-vitro maturation (IVM; Experiments 1–4), only germinal vesicle (GV) stage oocytes were placed in culture, while in-vivo matured (IVO) oocytes were at cell cycle stages between GV and M-II (Experiments 3 and 5). Note that some oocytes were used for more than one experiment.

ously described (Messinger and Albertini, 1991; Cekleniak *et al.*, 2001).

Statistical analysis

For the kinetic analysis (Experiment 2), all oocytes obtained from a given patient were treated as one experiment to eliminate interpatient variability. For every experiment, the average percentage of oocytes displaying a particular meiotic stage was measured and an overall mean percentage \pm SEM was derived for each time point across all experiments. When comparing oocyte diameters across germinal vesicle chromatin patterns (Experiment 1), the mean \pm SEM were reported; a non-parametric Mann–Whitney test was used to determine statistical differences between groups. For the GV chromatin pattern (Experiment 1) and activation studies (Experiment 4), proportions were compared using a two-tailed Z-test. $P < 0.05$ was considered significant.

Results

Experiment 1: Germinal vesicle chromatin configurations

To define chromatin patterns in relation to meiotic competence acquisition in human oocytes, chromatin configurations were characterized in GV stage oocytes using immunofluorescence analysis following Hoechst 33258 and/or histone H1 immunolabelling; identical results were obtained with both chromatin stains, and oocytes incubated only with secondary antibodies showed no staining. Four distinct patterns were identified (Figure 1). The first pattern ('A') exhibited a nucleolus partially surrounded by chromatin and fibrillar chromatin distributed throughout the nucleus (Figure 1A). In the second pattern ('B'), all the chromatin surrounded a large nucleolus, with no evidence of chromatin throughout the remainder of the nucleoplasm (Figure 1B). The third pattern ('C') was characterized by chromatin surrounding the nucleolus and masses of condensed chromatin distributed throughout the nucleus (Figure 1C). The last pattern ('D') exhibited a nucleolus surrounded by chromatin and threads of dispersed chromatin throughout the nucleoplasm without any evidence of fibrillar chromatin patterning (Figure 1D). Identification of the incidence of each GV pattern in oocytes before culture ($n = 92$) and in those after culture ($n = 41$) showed that comparable proportions of GV stage oocytes exhibited chromatin pattern A before (13.0%) and after (14.6%) culture (Figure 2). In

contrast, oocytes with chromatin patterns B–D were observed in significantly different frequencies when compared before and after culture (Figure 2). After culture, the majority of GV oocytes showed pattern B (43.9%) while only 25.0% of oocytes exhibited pattern B before culture ($P < 0.05$). Similarly, the fraction of oocytes with pattern D was significantly greater after culture (31.7%) when compared with before culture (2.2%; $P < 0.0001$). Lastly, pattern C was the predominant chromatin configuration in GV stage oocytes before culture (59.8%) but $<10\%$ of oocytes exhibited this pattern after culture ($P < 0.0001$).

To ascertain whether changes in GV chromatin configuration were correlated with oocyte size, oocyte diameter data were stratified according to the four chromatin patterns described above (Figure 2; A–D). Diameters of oocytes with either chromatin pattern B ($113.25 \pm 0.55 \mu\text{m}$; $n = 37$) or C ($115.53 \pm 0.49 \mu\text{m}$; $n = 51$) were significantly greater ($P < 0.0005$) than oocytes with either pattern A ($106.64 \pm 0.84 \mu\text{m}$; $n = 17$) or D ($108.39 \pm 0.96 \mu\text{m}$; $n = 14$) (Figure 2).

To characterize the expression and to establish baseline cell cycle markers for meiotic arrest as defined above, histone-3 phosphorylation, M-phase phosphoproteins (detected by the MPM-2 monoclonal antibody) and microtubules were evaluated relative to chromatin patterns. PH3 reactivity was not associated with human oocytes arrested at prophase of meiosis-I, regardless of the GV chromatin pattern (Figure 1E, inset; $n = 29$). In all GV chromatin patterns examined, MPM-2 reactivity was confined to the nucleoplasm with prominent foci distributed throughout the nucleus (Figure 1E; $n = 36$). Investigation of microtubule patterning showed that for all chromatin patterns, GV stage oocytes exhibited a dense interphase subcortical microtubule array (Figure 1F; $n = 133$), consistent with an interphase cell cycle state. Under no circumstances were focused microtubule arrays observed. Indeed, all GV oocytes that failed to resume meiosis in culture retained these cell cycle properties.

Experiment 2: Kinetic analysis of IVM

To determine the rate of meiotic progression in P-1, meiotic stages were classified during IVM over a 24 h culture period. At the start of culture, the majority of oocytes were at the GV

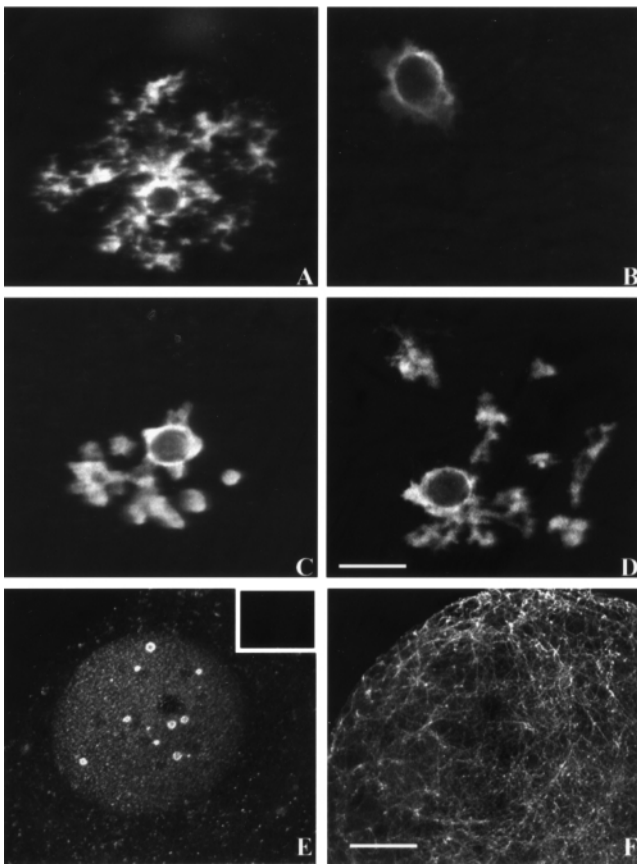


Figure 1. Chromatin (A–D), mitotic phosphoprotein monoclonal-II reactive protein (MPM-2) (E), and microtubule (F) patterns in germinal vesicle (GV) stage human oocytes. Four distinct patterns of GV chromatin organization were identified before and after culture (Table II), and are represented here as three-dimensional confocal reconstructions of histone H1-labelled preparations (A–D). In pattern 'A', a nucleolus partially surrounded by chromatin is observed along with fibrillar chromatin distributed throughout the nucleoplasm (A). Pattern 'B' exhibits a large nucleolus completely surrounded by chromatin, with no chromatin distributed throughout the nucleus (B). A chromatin-surrounded nucleolus with masses of condensed chromosomes in the nucleus is characteristic of pattern 'C' (C). Pattern 'D' displays a chromatin-surrounded nucleolus and threads of chromatin dispersed throughout the nucleoplasm (D). MPM-2 reactivity is illustrated in (E) where a diffuse nucleoplasmic staining and multiple foci are evident for all patterns of GV chromatin organization identified ($n = 36$). PH3 epitope was not detected in any GV stage oocytes analysed ($n = 29$; E, inset). Interphase-like arrays of microtubules were observed throughout the oocyte cytoplasm of for all GV types (F; $n = 133$). (E) and (F) represent a single oocyte that had GV type 'C'. Scale bar = 10 μ m.

stage ($97.6 \pm 2.4\%$; mean \pm SEM) with a minor fraction (one out of 15 oocytes) exhibiting signs of germinal vesicle breakdown (GVBD) (Figure 3). After 6 h in culture, 88.9% of oocytes had resumed meiosis with $61.1 \pm 15.3\%$ at GVBD and $27.8 \pm 16.5\%$ in prometaphase (PM)-1. At 12 h, 78.5% of oocytes were maturing with $19.0 \pm 14.3\%$ having undergone GVBD and $59.5 \pm 17\%$ being at PM-I/M-I. At 18 h, an approximately equivalent proportion of oocytes were in M-I ($44.5 \pm 22.2\%$) and telophase (T)-1 ($49.9 \pm 25.5\%$), with $66.7\% (\pm 19.23)$ progressing to M-II by 24 h.

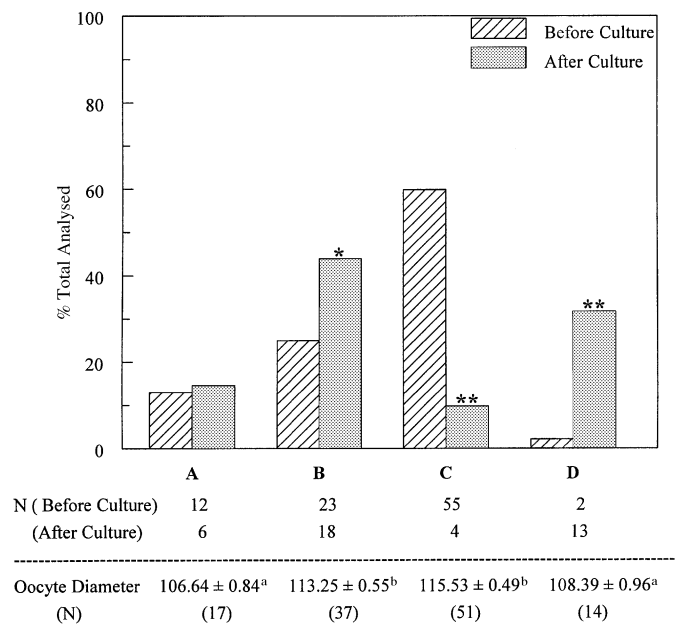


Figure 2. Incidence of chromatin patterns in germinal vesicle stage human oocytes before and after in-vitro maturation. Patterns A–D represent the four characteristics chromatin configurations observed and illustrated in Figure 1. Bars represent percentage of total number of oocytes analysed, and a two-tailed Z-test was used to compare before and after culture groups (* $P < 0.05$, ** $P < 0.0001$). Numbers of oocytes (N) are indicated below the graph for each chromatin pattern, with 92 oocytes analysed before culture (following retrieval), and 41 after culture (48 h IVM). For each chromatin pattern, oocyte diameters (mean \pm SEM) were determined; Mann–Whitney test: $^a b P < 0.0005$.

Experiment 3: Markers of nuclear and cytoplasmic remodeling during meiotic maturation of human oocytes

To further address whether synchrony between nuclear and cytoplasmic maturation occurs under these conditions, we monitored those changes in microtubule and chromatin organization known to take place during the transition between interphase and M-phase of the cell cycle. Accordingly, we determined whether maturing oocytes exhibited PH3 reactivity and microtubules in restricted association with chromatin, typical of oocytes in M-phase. In addition, we assayed oocytes ($n = 113$) for microtubule acetylation, a post-translational modification of α -tubulin known to confer stability to microtubules (Webster and Borisy, 1989).

The staining patterns for pH3 and microtubules for in-vitro matured human oocytes are shown in Figures 4 and 5. PH3 reactivity, first detected at GVBD (Figure 4A), was maintained throughout meiotic progression until M-II arrest (Figures 4–5, insets). However, the staining intensities for oocytes at GVBD and telophase I (T-I) were lower relative to those for other stages of meiotic progression. In GV and GVBD stage oocytes, interphase-like microtubule arrays were observed throughout the oocyte cortex (Figures 1F and 4A). In contrast to the clear persistence of microtubule acetylation in cumulus cell microtubule-rich projections traversing the zona pellucida (Albertini *et al.*, 2001; Figure 4B, arrows), GV and GVBD stage oocytes lacked detectable reactivity to antibodies specific for acetylated α -tubulin (Figure 4B; GV, $n = 39$ and GVBD,

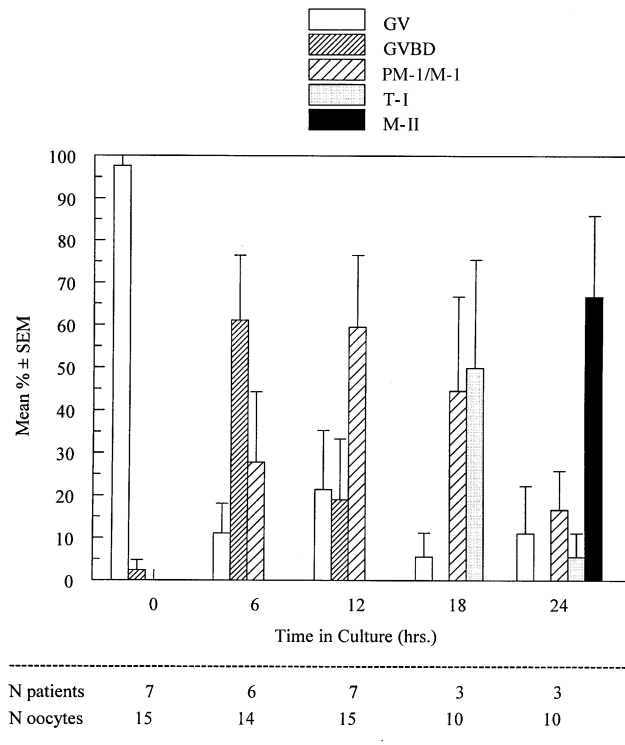


Figure 3. Kinetic analysis of in-vitro maturation of germinal vesicle human oocytes. Bar graph illustrates the mean percentage of oocytes in a given meiotic state (\pm SEM, error bars) across patients ($n = 7$, 64 oocytes) at 6 h intervals over the first 24 h of culture in P-1 medium. Following analysis of chromatin and microtubule organization, oocytes were classified at each time point as: germinal vesicle (GV), germinal vesicle breakdown (GVBD), prometaphase to metaphase of meiosis-I (PM-I/M-I), telophase-I (T-I), or metaphase-II (M-II). Under the present culture conditions, the majority of oocytes resumed meiosis by 6 h in culture (88.9%, $n = 14$), 59.5% ($n = 15$) were in PM-I/M-I at 12 h, 49.9% ($n = 10$) in T-I at 18 h, and by 24 h 66.7% of oocytes reached M-II. The number (N) of patients and oocytes analysed for each time point are indicated below the graph.

$n = 18$). Between prometaphase and anaphase of meiosis-I, individual chromosome bivalents were located within the meiotic spindle, and no microtubules were observed in the ooplasm (Figure 4C, E). Microtubules lacked acetylation at M-I (Figure 4D; $n = 17$) while at anaphase-I, spindle microtubules were acetylated (Figure 4F; $n = 3$). At T-I, oocytes displayed long microtubules, albeit in low densities, throughout the oocyte cortex alongside typical midbodies and spindle structures (Figure 5A); only spindle microtubules were acetylated (Figure 5B; $n = 12$). M-II oocytes possessed spindle-associated microtubules with chromosomes aligned at the spindle equator and no acetylated α -tubulin was detectable (Figure 5C, D, inset; $n = 16$). In an effort to compare our in-vitro findings using IVM with in-vivo matured oocytes, microtubule acetylation was analysed in seven M-II oocytes fixed immediately at retrieval. None of these seven oocytes exhibited acetylated microtubules, consistent with the results reported above for in-vitro matured M-II oocytes. Also, oocytes that were in various stages of meiotic maturation following retrieval ($n = 62$; see Table I, IVO) revealed total and acetylated microtubule staining patterns similar to those of in-

vitro matured oocytes. In spontaneously activated oocytes (see below), only a small subset of cytoplasmic microtubules was acetylated (Figure 5E, F).

Experiment 4: Incidence of spontaneous activation

Following meiotic maturation, mammalian oocytes arrest at metaphase of meiosis-2 until fertilization, at which time meiotic resumption is initiated. To assess further whether the present culture conditions appropriately maintain oocytes in M-phase arrest, we determined the incidence of spontaneous activation among oocytes that had extruded a polar body following IVM of GV stage oocytes (Table II). The presence of one polar body, a dense array of interphase microtubules, a single pronucleus, and the loss of pH3 reactivity were taken as indicators of M-phase exit. Oocytes exhibiting these characteristics were designated as activated (Figure 5E,F, insets). At 24 h in culture, 12.8% of oocytes ($n = 47$) were activated while the majority of oocytes were in M-II (87.8%, $n = 47$). However, by 48 h in culture a significant proportion of oocytes were spontaneously activated (48.1%, $n = 27$; Table II, $P < 0.0005$).

Experiment 5: Factors regulating microtubule patterning during meiotic maturation in vivo

To investigate the competency of microtubules to assemble at specific cell cycle stages, the microtubule stabilizing agent taxol was used to evaluate spatial and temporal differences in microtubule patterning in in-vivo maturing oocytes ($n = 186$; see Table I, IVO). Exposure of GV stage oocytes ($n = 19$) to taxol caused the appearance of a dense array of interphase microtubules throughout the cytoplasm that did not differ significantly from untreated oocytes (Figure 1F). In GVBD oocytes ($n = 34$) exposed to taxol, three distinct microtubule patterns were observed in association with chromatin: microtubule bundles forming a cage around the chromatin (55.9%), an astral arrangement of microtubules (11.7%), or no microtubules associated with chromatin (32.4%). The configuration consisting of microtubule bundles forming a cage is shown in Figure 6A. The percentage of oocytes in this pattern did not differ from untreated oocytes (55.9%, $n = 34$ versus 57.5%, $n = 42$ respectively). We note that cytoplasmic interphase-like microtubules were evident in all taxol-treated and untreated GVBD oocytes. Taxol treatment of oocytes in prometaphase to metaphase of meiosis-I ($n = 38$) consistently caused spindle enlargement but the degree of cytoplasmic sites of assembly response varied from either a complete absence of microtubules (Figure 6B; 36.8%, Table III) to an array of cortically situated short microtubule bundles (Figure 6C; 63.2%, Table III). Control, untreated in-vivo matured M-I oocytes lacked cytoplasmic microtubules ($n = 16$), and are thus comparable with in-vitro matured oocytes (Figure 4C,E). At T-I ($n = 4$), taxol exposure resulted in an increased density of interphase microtubules in the oocyte cortex with no significant enlargement of the spindle (Figure 6D). Following taxol exposure, M-II oocytes ($n = 28$) displayed a similar response as M-I oocytes, which resulted in a significant increase in meiotic spindle size (both width and length). Again, the response was mixed in M-II oocytes exposed to taxol with either no

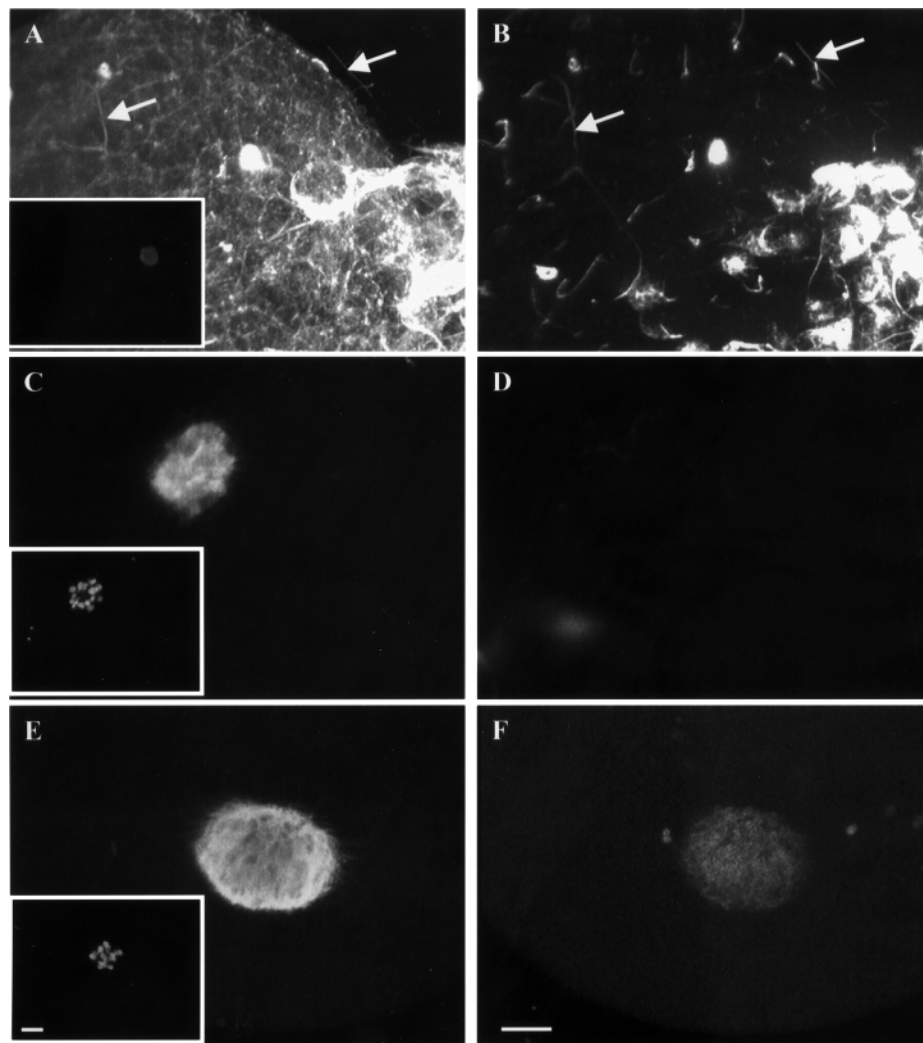


Figure 4. Analysis of chromatin and microtubule modifications during meiotic maturation of human oocytes, from germinal vesicle breakdown to anaphase of meiosis-I. Correlative total α/β -tubulin (A, C, E) and acetylated α -tubulin (B, D, F) staining patterns for oocytes at germinal vesicle/germinal vesicle breakdown (GV/GVBD; A, B), metaphase-I (M-I; C, D), and anaphase-I (A-I; E, F). Insets in (A, C, E) illustrate representative PH3 reactivity for each meiotic stage. All images shown here are three-dimensional confocal reconstructions. In GV and GVBD stage oocytes, interphase microtubules were observed throughout the oocyte cytoplasm (A), with a condensed mass of chromatin, as illustrated by PH3 epitope (A, inset), located in the oocyte cortex. During the PM-I/M-I to A-I transition, microtubules were associated solely with the forming spindle within which condensed chromosome bivalents were distributed (C, inset; E). At GVBD, note the intense anti-acetylated α -tubulin labelling of cumulus cell microtubule transzonal projections (arrows; A and B) and distinct absence of acetylated microtubules in microtubule-rich cytoplasm of oocyte (A, B). No microtubule acetylation was revealed during the PM-I/M-I transition (D), in contrast with A-I where acetylated microtubules were observed in the meiotic spindle (F). Scale bar = 10 μ m.

cytoplasmic microtubules (57.1 %; Table III) or an array of short microtubules throughout the oocyte cortex (42.9%; Table III). Control M-II oocytes revealed the complete absence of cytoplasmic microtubules ($n = 5$). Untreated and taxol-treated oocytes were analysed for markers of microtubule organizing centres or centrosomes (pericentrin, γ -tubulin, and kendrin) and failed to reveal organized structures reminiscent of the disposition of these proteins in typical somatic cell (including granulosa cell) centrosomes. Following taxol exposure of M-I to M-II oocytes, γ -tubulin staining was associated solely with spindle microtubules, in accordance with previous reports of γ -tubulin spindle distribution in untreated human oocytes (Van Blerkom *et al.*, 1995; George *et al.*, 1996). Thus, taxol exposure failed to reveal the existence of pericentrin/ γ -tubulin foci at any meiotic stage, in contrast to other species,

where increased microtubule stability causes enhancement of these structures.

Discussion

The present study defines several novel aspects of nuclear and cytoplasmic maturation in GV stage human oocytes both prior to and following completion of IVM in a defined culture system (Cekleniak *et al.*, 2001). Our main findings are: (i) establishment of germinal vesicle chromatin patterns that identify meiotically competent oocytes *in vitro* in relation to G2/M cell cycle transition markers; (ii) the rapid resumption and completion of maturation of GV stage oocytes in the P1 system as compared with previous reports using conventional IVM systems; (iii) deficiencies in the ability of mature oocytes

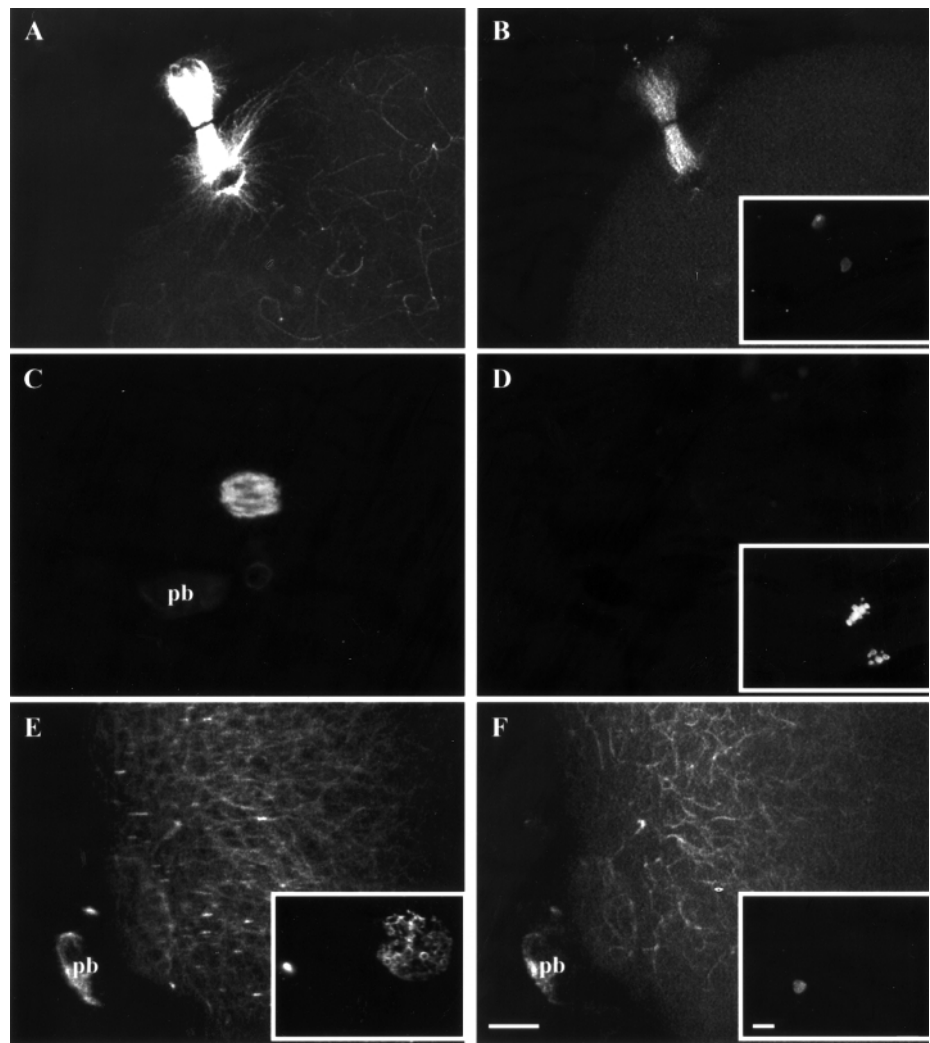


Figure 5. Analysis of chromatin and microtubule modifications during meiotic maturation of human oocytes, in telophase-I (T-I), metaphase-II (M-II), and spontaneously activated oocytes. Correlative total α/β -tubulin (A, C, E) and acetylated α -tubulin (B, D, F) staining patterns for oocytes at T-I (A, B), M-II (C, D), and following spontaneous activation (E, F). Insets in (B, D, F) illustrate representative PH3 reactivity for each meiotic stage, while inset in (E) represents histone H-1 staining. In addition to spindle microtubules, T-I oocytes displayed long microtubules in the oocyte cortex (A); two chromatin masses were detected with PH3 antibody, one in the forming polar body and the other in the oocyte (inset in B). At M-II, microtubules were found solely in association with the bipolar spindle in proximity to the first polar body (C, pb); condensed chromosomes were aligned at the spindle equator (inset in D). Following spontaneous activation, oocytes exhibited a single polar body (pb), interphase-like arrays of microtubules (E), a single pronucleus in the oocyte (inset in E; note lack of PH3 epitope in the oocyte in contrast to in the polar body, pb, inset in F). Microtubule acetylation was observed only at T-I (B), and in activated oocytes (F), while M-II oocytes failed to exhibit detectable acetylated microtubules (D). Scale bar = 10 μ m.

Table II. Analysis of oocytes that have extruded a polar body following in-vitro maturation (IVM) of germinal vesicle stage oocytes for 24 and 48 h

IVM	<i>n</i>	Metaphase-II	Activated
24 h	47	41 (87.2)	6 (12.8)
48 h	27	14 (51.9)	13 (48.1)*

Spontaneously activated oocytes displayed a polar body, a single pronucleus, no PH-3 reactivity and interphase microtubules (see Figure 5E, F).
* $P < 0.0005$.

to maintain cytoplasmic M-phase based upon spontaneously occurring and taxol-induced microtubule patterns; and (iv) a restricted expression of microtubule acetylation (anaphase-I, T-I) during maturation despite the premature onset of

acetylation in spontaneously activated oocytes following maturation to M-II. Together, these findings reveal anomalies in nuclear and cytoplasmic maturation, consistent with cell cycle deficiencies that would compromise both the meiotic and developmental competencies of in-vitro matured human oocytes. As the search for markers indicative of quality oocytes continues, particularly in reference to the use of in-vitro matured human oocytes, these studies establish important baseline information for future efforts aimed at understanding primary determinants of oocyte maturation.

Germinal vesicle chromatin patterns predict meiotic competency

Ovarian stimulation protocols generate predominantly mature M-II oocytes (Cha and Chian, 1998; Trounson *et al.*, 2001).

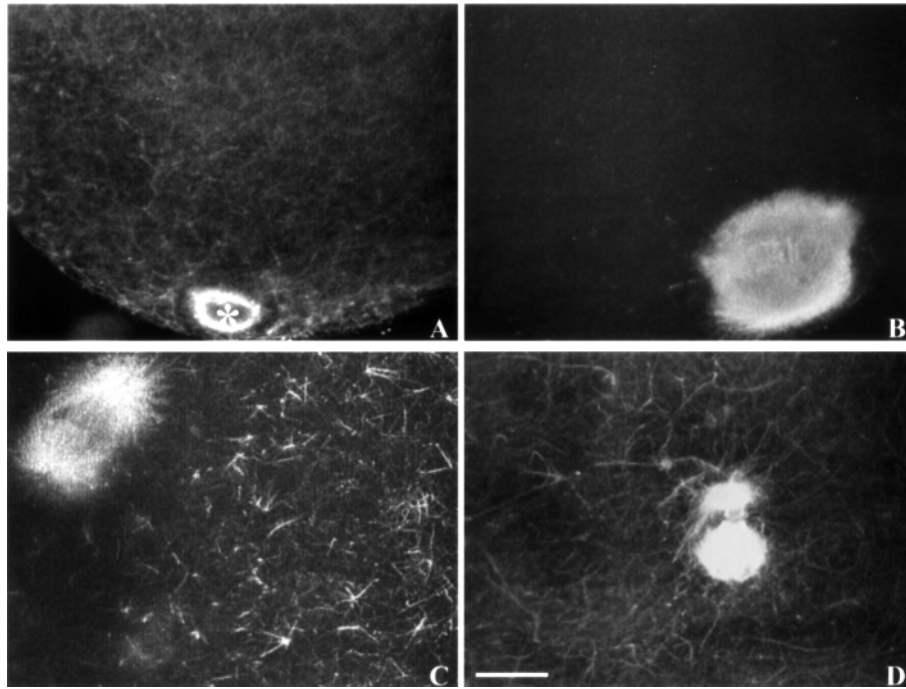


Figure 6. Effects of brief taxol exposures on microtubule patterning in human oocytes. Following retrieval, denuded oocytes were exposed to taxol (10 $\mu\text{mol/l}$, 10 min) before immunofluorescence analysis of microtubule organization by confocal microscopy (**A–D**). Germinal vesicle breakdown oocytes exposed to taxol displayed intense interphase microtubule arrays in the cortex, and a fraction of oocytes (55.9%, $n = 34$) displayed microtubule bundles circularly arranged around the chromatin (**A**, asterisk marking chromosome position). Around metaphase of meiosis-I (M-I), two distinct microtubule patterns were observed: either no microtubules (**B**) or arrays of short microtubules (**C**) were detected in the cytoplasm. For all M-I oocytes exposed to taxol, spindles became significantly enlarged (**B**, **C**). At telophase-I, taxol treatment resulted in enhanced microtubule assembly in the cytoplasm while spindle morphology was comparable with that in untreated telophase oocytes (**D**; see Figure 5A). M-II oocytes exposed to taxol exhibited microtubule patterns similar to those of M-I oocytes (see **B** and **C**; Table III). Scale bar = 10 μm .

Table III. Cytoplasmic response of human metaphase-I and metaphase-II oocytes following taxol exposure (10 $\mu\text{mol/l}$, 10 min)

	<i>n</i>	Microtubule assembly in the cytoplasm	
		+	–
Metaphase-1	38	24 (63.2)	14 (36.8)
Metaphase-2	28	12 (42.9)	16 (57.1)

The number of oocytes (%) exhibiting microtubule assembly (+) or not (–) in the cytoplasm in response to taxol are reported. All control metaphase-I or metaphase-II oocytes not exposed to taxol lacked microtubules in the cytoplasm ($n = 21$).

However, germinal vesicle stage oocytes are frequently obtained, which, when fully stripped of cumulus cells and cultured in appropriate media, show a high incidence of resumption and completion of nuclear maturation, giving rise to M-II spindles that appear normal. Despite the many reports documenting meiotic competency, and even developmental potential of these cells (Cha and Chian, 1998; Trounson *et al.*, 2001), no systematic effort has been undertaken to determine whether markers of cytoplasmic or nuclear maturation could predict the meiotic cell cycle efficiency of human GV stage oocytes retrieved during the course of ovarian stimulation. Developmental modification in chromatin organization, from a decondensed to a condensed perinucleolar disposition, has been reported in many mammals and is believed to correspond

to a transition from a transcriptionally active to an inactive state near the end of the growth phase of oogenesis (Mattson and Albertini, 1990; Zuccotti *et al.*, 1995; Bouniol-Baly *et al.*, 1999). In rodent oocytes, this modification occurs coincident with genomic imprinting (Kono *et al.*, 1996), transcriptional repression (Bouniol-Baly *et al.*, 1999; De La Fuente and Eppig, 2001), and the acquisition of meiotic and embryonic competency (McGaughey *et al.*, 1979; Wickramasinghe *et al.*, 1991; Zuccotti *et al.*, 1998). Similar alterations in chromatin have been reported in oocytes of both primates (Schramm *et al.*, 1993) and bovine (Fair *et al.*, 1996). Our studies are the first to define the states of chromatin in human oocytes. Among the four classes of GV oocytes identified, three (B, C and D) were characterized by perinucleolar condensed chromatin (Figure 1) with the type B pattern most closely resembling the 'karyosphere structure' (Parfenov *et al.*, 1989). That type B (and perhaps others) represents a chromatin organization in a transcriptionally repressed state would be expected given the diminished [^3H]uridine incorporation noted by one study (Parfenov *et al.*, 1989). Most striking, however, were changes observed in the distribution of GV classes prior to and following IVM (Figure 2). While class B and D GV were consistently observed at the time of oocyte retrieval (25.0 and 2.2% respectively, $n = 92$), their incidence was significantly increased to 43.9 and 31.7% ($n = 41$) amongst the subpopulation of oocytes that failed to resume meiosis after 48 h *in vitro* (in a previous sample, 39.4% of oocytes remained as

GV, $n = 256$) (Cekleniak *et al.*, 2001). Consistent with the idea that class B and D oocytes represent meiotically incompetent and cell cycle-arrested (G2) GV is the finding that these oocytes retain an extensive interphase network of microtubules with no PH3 reactivity following prolonged culture, further indicative of the failure to mount an MPF (M-phase or maturation promoting factor) response sufficient to depolymerize microtubules and condense chromosomes in anticipation of meiotic spindle assembly. To our knowledge, this is the first experimental demonstration that class C oocytes, the predominant type observed following retrieval, exhibit meiotic competence following culture; this conclusion is based on the observation that 59.8% of oocytes are type C before culture, 9.8% after culture, and from previous studies 60.6% of oocytes matured to M-II by 48 h (Figure 2; Cekleniak *et al.*, 2001). Further support for the idea that class C oocytes represent the meiotically competent fraction of oocytes is derived from our observation that these oocytes were larger in diameter than those in the other GV classes (Figure 2), and therefore probably correspond to oocytes that achieved an advanced stage of growth during folliculogenesis. Conversely, class A GV oocytes were smallest in diameter and represented in similar proportions prior to and following culture, attesting to their classification as incompetent (13.0 and 14.6%, Figure 2). This finding thus supports work in the bovine showing a relationship between oocyte diameter, follicle size, and IVM rates (Fair *et al.* 1995). Two studies (McNatty *et al.*, 1979; Durinzi *et al.*, 1995) were consistent with the diameter of human oocytes reflecting their meiotic potential *in vitro*.

Unlike the chromatin state, evaluation of the other nuclear (MPM-2 positive foci, lack of pH3 epitope) and cytoplasmic (interphase microtubules) markers of G2 failed to demonstrate a relationship with meiotic competency, suggesting that this important aspect of nuclear remodelling deserves further study. The fact that nuclear state can be reversibly controlled with respect to meiotic competence expression makes this an attractive marker for manipulating human oocytes in culture.

Kinetic analysis of IVM

Given previous reports that in-vitro matured human oocytes are compromised in their ability to fertilize and support embryonic development, we additionally explored the kinetics and coordination of nuclear and cytoplasmic maturation in GV stage oocytes that exhibited meiotic progression under our culture conditions. Our analysis of 64 competent oocytes revealed a striking capacity to reinitiate and complete maturation to M-II (Figure 3). The culture system used in this study supported rapid progression of GV stage human oocytes to M-II by 24 h. Relative to previous in-vivo estimates (Edwards, 1965) and studies from other laboratories (Cha and Chian, 1998; Trounson *et al.*, 2001), the accelerated pace of meiotic progression was largely attributable to an enhancement in germinal vesicle breakdown since 88.9% of oocytes advanced through M-I by 6 h in culture compared with ~20% of oocytes over the same time interval (Cha and Chian, 1998). Differing reports of rates of IVM are probably due to many factors including media composition, hormone/growth factor supplementation, the source of oocytes (unstimulated versus

stimulated cycles), and whether or not cumulus cells are retained with the oocyte (Prins *et al.*, 1987; Gunnala *et al.*, 1993; Cha and Chian, 1998; Goud *et al.*, 1998; Anderiesz *et al.*, 2000). Absence of cumulus cells and exposure to gonadotrophins have both been documented to accelerate meiotic maturation *in vitro* (Gomez *et al.*, 1993; Cha and Chian, 1998; Goud *et al.*, 1998; Wynn *et al.*, 1998; Trounson *et al.*, 2001). Both of these variables are relevant to the culture conditions utilized here since we stripped cumulus cells prior to culture and added recombinant FSH and HCG (P-1) (Cekleniak *et al.*, 2001). In addition, P-1 medium is a simplified, glucose-free medium that could influence oocyte metabolism and other related processes. For example, removal of cumulus may compromise metabolic support required for the sustained activation of MPF that could alter microtubule dynamics and/or chromatin stability. Therefore, factors important in regulating the onset of oocyte maturation, the temporal aspects of meiotic progression, the completion (polar body extrusion) and maintenance of M-II state are not clearly defined and merit further attention given the propensity of human oocytes to become aneuploid.

M-Phase deficiencies in in-vitro matured human oocytes

Having documented enhanced nuclear progression in the above experiment, we next asked whether characteristic changes in microtubule dynamics were in any way compromised as a potential contributing factor to cytoplasmic immaturity. Activation of MPF, the driving force for progression through M-phase of animal cell cycles, results in significant post-translational modifications in histone-3, to ensure maintenance of compacted chromatin, and the presence of proteins that alter microtubule stability (Murray and Hunt, 1993; Hendzel *et al.*, 1997; Wei *et al.*, 1999). Deficiencies in the activation, amplification, or inactivation of MPF would be expected to offset the temporal and/or spatial parameters of cell cycle control that underlie the co-ordination of nuclear and cytoplasmic maturation of oocytes (Albertini and Carabatsos, 1998; Pines, 1999). Using immunodetection of phosphorylated histone-3 with an epitope-specific antibody (Wei *et al.*, 1999), we show timely modifications in histones commencing with diakinesis and ending with arrest at M-II (Figures 4 and 5). However, this analysis revealed two critical junctures when loss of PH3 epitope occurred. At telophase-I and during parthenogenetic activation, we observed, respectively, partial reduction and complete loss of PH3. Thus, changes in H3 phosphorylation were correlated with chromatin decondensation of varying degrees. This chromatin-based indicator of failure to maintain M-phase could be explained by the transient inactivation of MPF known to occur during telophase of meiosis-I as documented in other mammalian oocytes (Hashimoto and Kishimoto, 1988; Fulka *et al.*, 1992; Wu *et al.*, 1997). In addition, the rapid and total loss of PH3 in in-vitro matured M-II oocytes that failed to maintain M-phase arrest could be due to an impaired c-mos/MAP kinase influence (Sagata, 1997).

Further support for M-phase deficiencies derives from our analysis of microtubule patterns throughout the course of IVM. Again, at both telophase-I and in activated oocytes, we observed

expression of prominent cytoplasmic microtubule arrays that would be an expected outcome from failure to sustain adequate levels of MPF or c-mos/MAP kinase. Whether such changes are a direct response to degradation of the cyclin B component of MPF, or a failure to sustain adequate levels of ATP to maintain cell cycle kinase activities, remain to be established. However, besides the appearance of interphase microtubules, this work also exemplified a direct post-translational modification of α -tubulin in anaphase-I, telophase-I, and activated oocytes. The appearance of immunodetectable acetylated α -tubulin subunits within both spindle (Figures 4F and 5B) and cytoplasmic (Figure 5F) microtubules further attests to the transient, yet specific, nature of a biochemical modification in microtubules known to confer polymer stability (Webster and Borisy, 1989; Bulinski and Gundersen, 1991). It is intriguing to note that a persistent expression of acetylated microtubules has been observed throughout the course of meiotic maturation in mouse (de Pennart *et al.*, 1988; Can and Albertini, 1997) and yet, as reported here, microtubule acetylation is limited to discrete stages of meiosis (anaphase-I, telophase-I) in human. Although microtubule acetylation patterns were consistent between our analysis of human oocytes matured under in-vivo and in-vitro conditions, it is difficult to ascertain the significance of restricted microtubule stability through acetylation. However, its limited occurrence would be a primary contributor to meiotic spindle defects predisposing human oocytes to meiotic non-disjunction. Further support for the notion that human oocytes lack those spindle-stabilizing forces expressed in species less prone to meiotic aneuploidy, is the apparent lack of spindle pole protein complexes (pericentrin, γ -tubulin) observed in the present study.

It should be emphasized that the deficiencies alluded to above are representative of oocytes that failed to mature *in vivo* under standard conditions of ovarian stimulation. Alterations in cell cycle progression for both in-vitro and in-vivo (see below) matured human oocytes provides a baseline for future studies aimed at optimizing culture conditions to support maturation of oocytes obtained after reduced ovarian stimulation protocols. Finally, our studies have made rather novel use of the microtubule-stabilizing agent, taxol, to explore further the question of microtubule dynamics in in-vivo matured human oocytes. The application of exceedingly brief pulses of taxol revealed significant heterogeneity in the spatial and temporal responsiveness of oocytes to this test of tubulin assembly status (Figure 6, Table III). While some M-I and M-II oocytes exhibited no response, others were readily coaxed into elaborating a cytoplasmic network that is most often associated with the oocyte cortex. This heterogeneity argues strongly for variability in individual oocytes to maintain an MPF-driven restriction of microtubule assembly to the spindle and generally lends further credence to the idea of M-phase deficiencies in human oocytes matured under in-vivo or in-vitro conditions.

Given the significant risk factors in human oocytes associated with the age-related increase in meiotic aneuploidy (Hunt and LeMaire-Atkins, 1998; Volarcik *et al.*, 1998), coupled with the use of experimental culture conditions for IVM and IVF, and the need to develop adequate cryopreservation methods,

further evaluations of human oocyte quality must focus on the coordination of nuclear and cytoplasmic maturation. These studies add yet another note of caution with respect to the use of human oocyte IVM, although they do offer direction for further studies involved with the manipulation and optimization of IVM for use in assisted reproduction.

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