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In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system

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BACKGROUND: Deficiencies remain in the ability of *in vitro*-matured human oocytes to acquire full developmental competence and give rise to a healthy pregnancy. A clear deficiency of current systems utilizing human oocytes has been the absence of cumulus cells. In the present study, a three-dimensional (3D) co-culture system exploiting an extracellular matrix was developed and compared to conventional methods for its ability to support maturation of human oocytes. METHODS AND RESULTS: Cumulus cells were embedded into a 3D collagen gel matrix with individual oocytes added to each gel. Oocytes from the same patient cultured in the gel matrix matured to metaphase II at rates similar to those of cumulus-free oocytes cultured in individual microdrops. Following maturation of oocytes and fixation of intact gels, chromatin and cytoskeletal elements were assessed in oocytes and cumulus cells. The activities of the key cell cycle kinases, maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK), were compared in oocytes matured under the two culture conditions. Compared with denuded oocytes, co-cultured oocytes exhibited increased MAPK activity, but no difference in MPF levels. CONCLUSIONS: This work characterizes a novel and efficacious culture system that takes advantage of the unique properties of the extracellular matrix, a 3D microenvironment, and the presence of cumulus cells for maturing human oocytes *in vitro*.

Key words: co-culture/cumulus cells/*in vitro* maturation/oocyte/three-dimensional collagen gel

Introduction

The *in vitro* maturation (IVM) of human oocytes has the potential to afford certain patients the opportunity to undergo fertility treatment without the elevated cost, inconvenience and risks associated with hormonal stimulation. Furthermore, IVM offers an alternative for preserving the fertility of women undergoing cancer treatment. Nevertheless, conditions for the IVM of human oocytes still await significant refinements (Smitz, 2002; Chian *et al.*, 2004). Immature human oocytes exhibit acceptable meiotic competence to metaphase II (MII), but their subsequent developmental competence remains disappointingly low. Only 40–80% of fertilized IVM oocytes progress through early cleavage (Cha and Chian, 1998; Moor *et al.*, 1998; Hardy *et al.*, 2000), and of those that do cleave and that are transferred, $<15\%$ implant to form a viable fetus (Chian *et al.*, 2004). Beyond the long-term goals and clear applications of IVM in fertility treatment is also the fundamental need to augment our understanding of oocyte maturation processes in the human. Therefore, an appropriate culture system designed specifically for use with human oocytes needs to be developed.

Although a variety of culture media have been utilized for human IVM, no single system has been shown to be clearly superior for the production of developmentally competent oocytes. Culture conditions, including the formulation of the base medium, supplementations, and the *in vitro* physical environment (such as the oxygen tension and presence of cumulus cells) all influence multiple events that are paramount to oocyte maturation and subsequent embryonic development (Van de Sandt *et al.*, 1990; Sutton *et al.*, 2003). The culture medium has been shown to modulate not only the metabolism of human oocytes (Roberts *et al.*, 2002), but also the maturation to MII, the kinetics of cell cycle progression, and spindle/chromatin organization (Cekleniak *et al.*, 2001; Trounson *et al.*, 2001).

The majority of human IVM studies have utilized immature oocytes denuded of surrounding cumulus cells that were obtained after ovarian stimulation with gonadotrophins (Cha and Chian, 1998; Trounson *et al.*, 2001). We have shown

previously that these denuded human oocytes exhibit accelerated meiotic resumption *in vitro*, a deficiency in the ability of the cytoplasm to maintain M-phase characteristics while meiosis is progressing, a propensity to activate spontaneously after M-phase arrest, and a lack of coordination between nuclear and cytoplasmic maturation (Combelles *et al.*, 2002). What contributes to these deficiencies remains to be determined, but given the interdependence of oocytes and cumulus cells for their normal development and function (Eppig, 1991), an immediate shortcoming in the type of culture system used to date has been the absence of somatic cell support.

Co-cultures of oocytes and cumulus cells have been employed *in vitro* to restore support from the surrounding cumulus cells to the oocyte and/or to probe interactions between the two cell compartments. Oocytes were cultured either over monolayers of cumulus or granulosa cells or along with (in suspension or not) pieces of mural granulosa cells or follicular shells in several species, including the mouse (Eppig, 1979; Herlands and Schultz, 1984; Cecconi *et al.*, 1991; Downs and Mastropolo, 1994), cow (Sirard and Bilodeau, 1990; Osaki *et al.*, 1997) and pig (Moor *et al.*, 1990; Motlik *et al.*, 1996). While preliminary reports exist for the co-culture of human oocytes with different cell types (Dandekar *et al.*, 1991; Janssenswillen *et al.*, 1995; Coskun *et al.*, 1998; Haberle *et al.*, 1999), to our knowledge, there has been no previous description of an efficacious co-culture system of human denuded oocytes and cumulus cells for IVM.

The objective of the current work was to develop a co-culture system that will favour the optimal development and function of not only the oocyte but also the cumulus cells. Of relevance in designing such a system is the well-established importance of the extracellular matrix (ECM) and a three-dimensional (3D) environment when studying cell behaviour and function *in vitro* (Abbott, 2003). Indeed, the ECM influences a multitude of cell functions, including morphogenesis, survival, migration, proliferation, communication, metabolism, and response to external stimuli (Weaver *et al.*, 1997; Bissell, 1998). In addition, it is equally important to culture cells in a dimension that mirrors, as closely as possible, the 3D environment *in vivo*. Indeed, in comparison with a two-dimensional (2D) environment, a 3D environment results in cell behaviour, signalling and gene expression profiles most resembling those observed in living tissues (Cukierman *et al.*, 2001, 2002). Granulosa cells also behave and function in distinct ways when cultured in the presence or absence of ECM (Aharoni *et al.*, 1996; Hwang *et al.*, 2000; Richardson *et al.*, 2000; Huet *et al.*, 2001). In addition, follicles and intact cumulus–oocyte complexes from several species have been cultured embedded in a 3D collagen gel (mouse: Torrance *et al.*, 1989; Gomes *et al.*, 1999; pig: Hirao *et al.*, 1994; cow: Osaki *et al.*, 1997; human: Abir *et al.*, 1999). However, a 3D system making use of an ECM has not been applied to the co-culture of oocytes and cumulus cells in any species to date.

The present study was designed to develop and characterize a novel co-culture 3D system for IVM of immature denuded human oocytes. We compared oocytes matured in microdrop or in co-culture with respect to their ability to progress to metaphase II (MII) and with regard to the organization of their cytoskeleton and chromatin. Lastly, given the importance of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) in the regulation of oocyte maturation (Heikinheimo and Gibbons, 1998; Trounson *et al.*, 2001) and the paucity of reports on MPF and MAP kinase activities in human oocytes (Pal *et al.*, 1994; Sun *et al.*, 1999; Anderiesz *et al.*, 2000), we investigated the dynamics of cell cycle kinases during human IVM.

Materials and methods

All reagents were obtained from Sigma (USA) unless noted otherwise.

Source of oocytes and cumulus cells

Immature human oocytes were aspirated from ovaries of women undergoing ovarian stimulation for ICSI. These immature oocytes were donated to research given their otherwise clinically-useless nature. Complete institutional review board approval (protocol #02-001986) and written consents were obtained prior to placing oocytes in culture. After enzymatic and mechanical removal of the cumulus and corona cells, the meiotic status of oocytes was determined, and immature oocytes [germinal vesicle (GV) or metaphase I (MI)] were used for IVM experiments. GV oocytes had an intact nucleus, and MI oocytes were defined by the absence of a nucleus and polar body. A total of 231 oocytes retrieved from 44 patients (35.2 ± 4.5 years; mean ± SD) were included in this study. *In vivo*-matured MII oocytes ($n = 13$) were donated from three patients (37.0 ± 3.8 years) who elected to have only a limited number of their oocytes inseminated. Oocytes were photographed prior to culture and the vitelline diameters (not including the zona pellucida) of fresh oocytes were determined from the maximum and minimum diameters of each oocyte.

Autologous cumulus cells were collected for use in co-cultures after their removal from cumulus–oocyte complexes in preparation for ICSI. All cumulus cells were pooled together within each patient regardless of the meiotic state of the associated oocytes.

Culture systems

Immature oocytes were matured *in vitro* in either a microdrop (C^-) or co-culture (CC) system. For the C^- group, denuded oocytes were placed in 25 µl microdrops of culture medium (see composition below) overlaid with embryo-tested light mineral oil (Irvine Scientific, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. After corona–cumulus cell removal, the medium (Ham's F-10 with 5% human serum albumin (HSA); InVitroCare Inc., USA) containing the removed cells was collected into a conical tube with an equal volume of warm (37 °C) HEPES-buffered Hanks' balanced salt solution (HBSS) without magnesium and calcium supplemented with 5% HSA. Following centrifugation at 200 *g* for 5 min, the cell pellet was resuspended in 1.5 ml of warm HBSS with 5% HSA and laid gently over a density gradient consisting of 1.5 ml of 90% Isolate (Irvine Scientific) and 1.5 ml of HBSS with 5% HSA. After centrifugation for 10 min to pellet red blood cells, purified cumulus cells were aspirated from the interface and washed with HBSS with 5% HSA prior to use. Cells were counted using a haemacytometer, and only cell preparations with a viability ≥90% as assessed by Trypan Blue exclusion were used. For the co-culture experiments, the cell pellet was resuspended in neutralized 1% collagen (4.12 mg/ml rat tail collagen, type I; BD Biosciences, USA) kept at 4 °C. Aliquots of

4 μl each were seeded in 4-well Nunc tissue culture dishes (Nunclon, Denmark) at a cell density of 1×10^6 cells/ml, creating gels that contained ~4000 cumulus cells each. A single immature oocyte was added carefully to each gel, which was then allowed to polymerize at 37 °C for 10 min before adding 400 μl of culture medium to each well. The culture medium used for all IVM studies consisted of M-199 with Earle's salts (Invitrogen Life Technologies, USA) supplemented with 5% HSA; 0.075 IU/ml recombinant FSH (Gonal-F; Serono Laboratories, USA); 0.075 IU/ml hCG (Profasi; Serono Laboratories); 1 μg/ml estradiol; 0.30 mmol/l sodium pyruvate; 1 mmol/l glutamine; 0.032 mg/ml penicillin; and 0.050 mg/ml streptomycin. This supplemented tissue culture medium (M199-S) supports the maturation of denuded human oocytes to MII (Cekleniak *et al.*, 2001; Chian and Tan, 2002), and it was chosen because of the need to support two distinct cell compartments, the somatic and germ cells.

Comparison of oocytes matured either in microdrops or co-cultures was performed within a single patient to account for potential inter-patient variability. Only patients with at least four immature oocytes and 10 cumulus–oocyte complexes on the day of retrieval were included. Also, only oocytes with a diameter of ≥110 μm (116.1 ± 4.7 μm, mean ± SD; $n = 135$) were placed in culture given that these oocytes are known to represent meiotically competent oocytes (McNatty *et al.*, 1979; Durinzi *et al.*, 1995; Combelles *et al.*, 2002). Oocytes were randomly allocated within each patient to the two culture groups.

In an initial study aimed at evaluating the influence of the environment, cumulus cells from each of seven patients were cultured either on a 2D surface (with or without collagen) or in a 3D collagen gel. Cell handling for 3D culture was done as described previously. For cells cultured on a 2D surface, purified cell preparations were resuspended in M199-S and plated at a concentration of 1×10^6 cells/ml. After allowing cell attachment to the culture surface (12 mm sterile glass coverslips), culture wells were flooded with M199-S. For 2D cultures in the presence of collagen, coverslips were coated with 50 μg/ml of collagen type I for 2 h at room temperature, followed by three washes with phosphate-buffered saline and air-drying before use.

Processing of samples for immunofluorescence analysis

Cumulus cell cultures on glass coverslips and collagen gels containing cells with or without oocytes were fixed and extracted for 15 min at 37 °C in a microtubule stabilizing buffer (0.1 mol/l PIPES, pH 6.9, 5 mmol/l $MgCl_2 \cdot 6H_2O$, 2.5 mmol/l EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 μmol/l taxol, 10 IU/ml aprotinin, and 50% deuterium oxide. Samples were washed and stored at 4 °C in a blocking solution of phosphate-buffered saline containing 2% bovine serum albumin, 2% powdered milk, 2% normal goat serum, 0.1 mol/l glycine and 0.01% Triton X-100 containing 0.2% sodium azide. Prior to processing, collagen gels were extracted with 0.5% Triton X-100 for 20 min at 37 °C with shaking. Microtubules were detected using a 1:1 mixture of monoclonal anti-α-tubulin and anti-β-tubulin antibodies (1:250) for 2 h at 37 °C with shaking, followed by three 15 min washes and Alexa-fluor 488 goat anti-mouse IgG for 2 h (1:600; Molecular Probes, USA). All samples were subsequently washed and stained with 1 μg/ml Hoechst 33258 (Polysciences Inc., USA) for 30 min to label chromatin prior to mounting in 50% glycerol and phosphate-buffered saline solution containing 25 mg/ml sodium azide. To preserve the three-dimensional integrity, each collagen gel was mounted uncompressed using wax recess. Samples were analysed using an Axiovert 200 inverted microscope (Zeiss, USA) or a TCS SP2 laser scanning confocal microscope (Leica Microsystems Inc., USA). Digital images were collected with an ORCA ER digital camera (Hamamatsu Corp., USA) and the Metamorph image analysis software (Universal Imaging Corp., USA). The pyknotic index, as defined by the percentage of cells with condensed or fragmented chromatin, was calculated from random fields (three per culture condition for each patient) that were imaged with a ×40 objective under both phase and fluorescent modes.

In vitro protein kinase assays

MPF and MAP kinase activities were measured simultaneously using histone 1 (H1) and myelin basic protein (MBP) as their respective substrates. Lysates of single human oocytes were prepared by six cycles of freezing and thawing in 4.5 μl of kinase lysis buffer consisting of 80 mmol/l glycerophosphate (pH 7.3), 20 mmol/l EGTA, 15 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 500 nmol/l cAMP-dependent protein kinase inhibitor. Lysates were stored at −80 °C before use. Kinase reactions were started by adding 5 μl of substrate buffer containing 1.98 mg/ml histone H1 (type III-S), 1.0 mg/ml MBP, 0.9 mmol/l ATP, and 25 μCi of [γ-^{32}P]ATP (Amersham Biosciences, USA). The kinase reaction was conducted for 30 min at room temperature and stopped by the addition of 5 μl of double-strength electrophoresis sample buffer (Laemmli, 1970). After boiling for 3 min, samples were separated by standard polyacrylamide gel electrophoresis (15% sodium dodecyl sulphate–polyacrylamide gels). Detection of phosphorylation levels was done by autoradiography at −80 °C with several film exposures of the same gel performed until quantification of activities was obtained in the linear range and without saturation of the counting system for each kinase. The mean pixel intensity of a preselected, set area was quantified using Adobe Photoshop (Adobe Systems, USA). Kinase activities were expressed relative to phosphorylation levels, which were set arbitrarily at a value of 1 in control samples consisting of lysis buffer alone without oocyte.

Statistical analysis

Proportions and relative kinase activity levels were analysed by χ^2 and one-way ANOVA respectively (SPSS 10.0; Statistics Package for Social Sciences, USA). $P < 0.05$ was considered significant.

Results

Influence of the environment on cumulus cell behaviour, morphology and viability

Cumulus cells grown on an uncoated glass surface appeared to migrate and formed separate cell aggregates, with elongated cells occasionally observed extending between adjacent cell aggregates (Figure 1A). In contrast, cell bodies were spread evenly over the ECM when cumulus cells were grown on collagen-coated coverslips (Figure 1B). Cumulus cells cultured in a restrained 3D collagen microenvironment were rounded in morphology and tightly organized, occupying all spaces in the gel (Figure 1C). The incidence of pyknotic nuclei with condensed or fragmented chromatin differed between the three culture groups (Figure 1a, b, c; arrows) with indices of 39, 21 and 16% observed in the uncoated 2D ($n = 658$ cells), collagen-coated 2D ($n = 784$) and collagen 3D ($n = 746$) groups respectively. As shown by both differential interference contrast microscopy (Figure 1B)

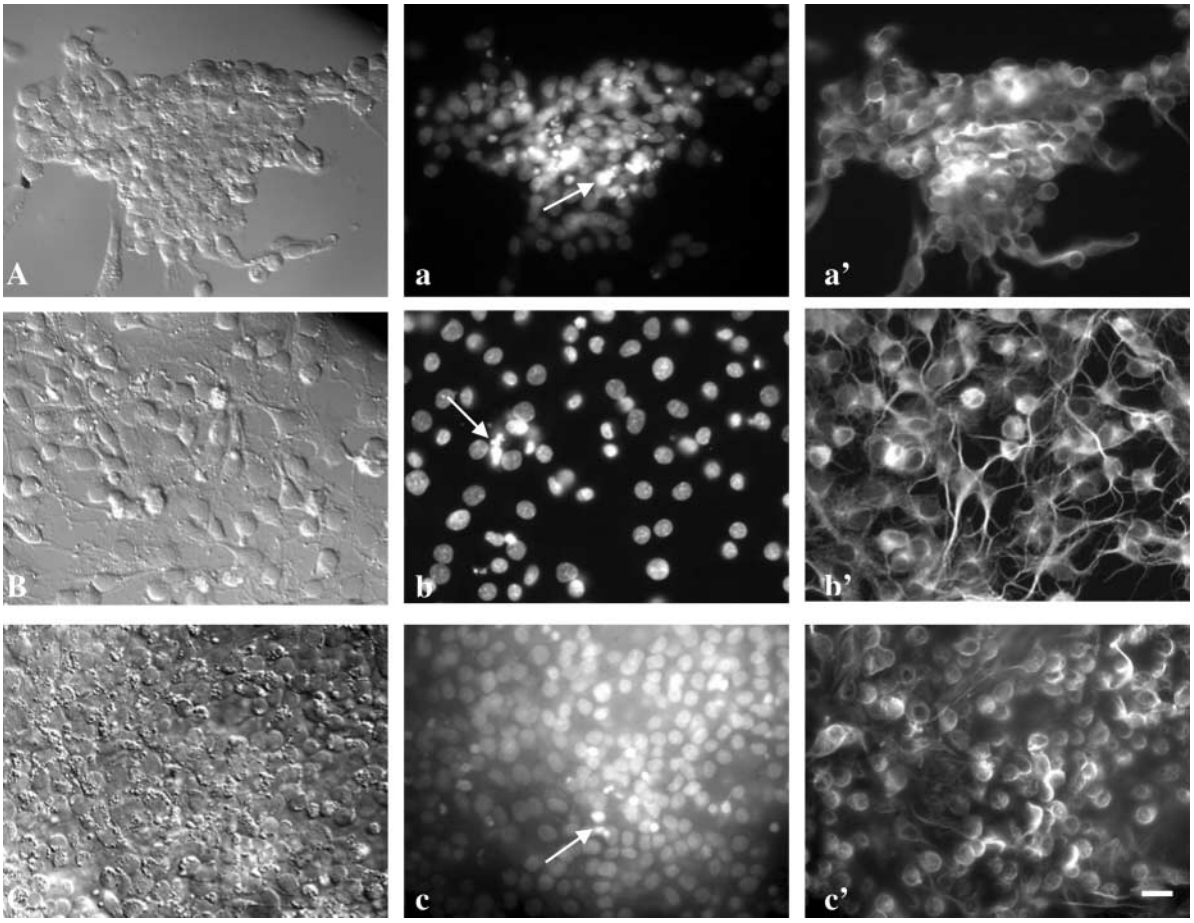

Figure 1. Correlative differential interference microscopy (**A**, **B**, **C**), Hoechst (**a**, **b**, **c**), and total α/β-tubulin (**a′**, **b′**, **c′**) patterns for human cumulus cells cultured in a two-dimensional environment without (**A**, **a**, **a′**) or with (**B**, **b**, **b′**) collagen, or in a three-dimensional collagen gel (**C**, **c**, **c′**). Arrows denote condensed and fragmented nuclei. Scale bar = 10 μm.

and microtubule staining patterns (Figure 1b′), long neuronal-like outgrowths, which developed into a net-like configuration, were characteristic of cumulus cells grown in the presence of collagen. No such arrangement was evident in cultures in the absence of collagen (Figure 1A,a) with cells characterized by a reduced cytoplasmic volume. Dendritic-like processes were also evident in cells grown in 3D collagen gel (Figure 1c′).

Table I. Incidence of metaphase II (MII) oocytes following *in vitro* maturation either in microdrop or in co-culture with cumulus cells using a three-dimensional collagen gel

Meiotic stage at time 0	Microdrop		Co-culture		Patients
	(*n*)	MII (%)	(*n*)	MII (%)	(*n*)
GV	51	33 (64.7)	67	45 (67.2)	30
MI	39	30 (76.9)	35	27 (77.1)	23
GV and MI	90	63 (70.0)	102	72 (70.6)	33

There was no statistical difference in the incidence of MII oocytes between the two culture groups (χ^2).
Mean patient age ± SD: 34.7 ± 4.2.
Oocyte diameters: 116.5 ± 4.5 and 115.8 ± 4.9 μm for the microdrop and co-culture groups respectively.
GV and MI (metaphase I) denote oocytes at the start of culture (time 0) with an intact germinal vesicle nucleus and the absence of a polar body respectively.

Maturation of oocytes to MII in the microdrop versus co-culture system

Table I shows the percentage of oocytes that matured to MII when cultured alone in microdrop or in co-culture with cumulus cells in a 3D collagen gel. When considering only immature oocytes that were at the GV stage at the start of culture, a comparable percentage of oocytes reached MII (microdrop: 64.7%; co-culture: 67.2%). Similarly, no difference was observed in the incidence of MII oocytes after IVM in either microdrop or co-culture when MI oocytes were used on the day of retrieval (microdrop: 76.9%; co-culture: 77.1%). When combining the GV and MI oocytes together, the overall incidence of oocytes maturing to MII was 70.0 and 70.6% for the microdrop or 3D collagen gel systems respectively.

Evaluation of chromatin and microtubules of oocytes matured in co-culture or microdrop

Chromatin and microtubule organization was analysed in either oocytes matured in microdrop ($n = 61$ oocytes from 22 patients) or in intact collagen gels containing individual oocytes with autologous cumulus cells (Figure 2A; $n = 55$ gels from 22 patients). Spontaneously activated oocytes, as

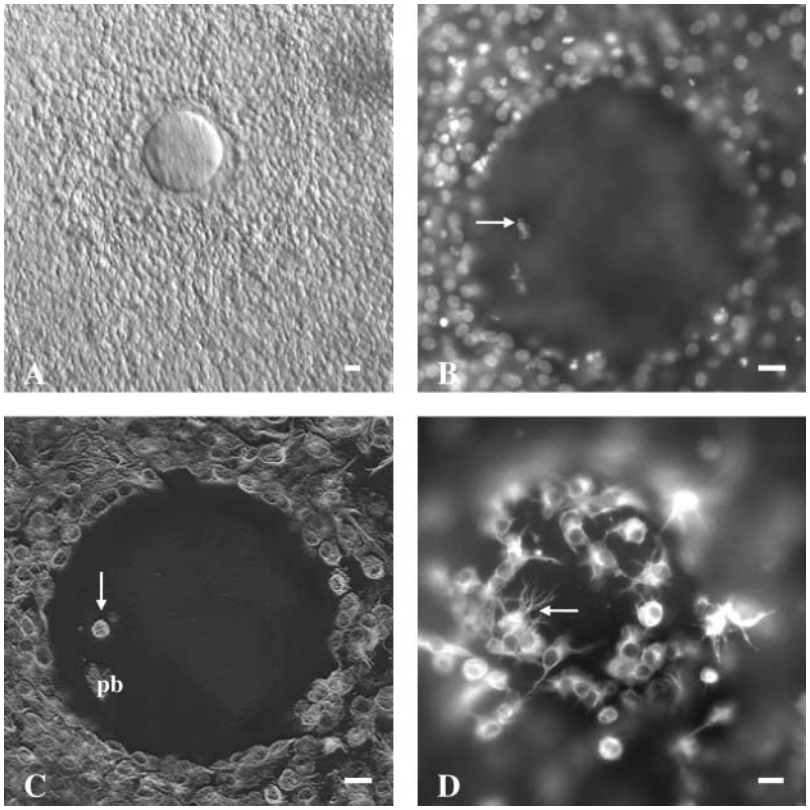

Figure 2. Analysis of intact three-dimensional co-culture collagen gels after culture under phase contrast (**A**) and fluorescence (**B**, **C**, **D**) microscopy. A representative metaphase II (MII) oocyte after in vitro maturation is shown embedded in a collagen gel with autologous cumulus cells (**A**). A three-dimensional confocal reconstruction through only the oocyte demonstrates a bipolar organization of the MII spindle (**C**, arrow; pb = polar body). Note the aligned chromosomes with Hoechst staining (**B**, arrow). (**D**) Microtubule-rich cell processes (arrow) extend from the surrounding cumulus cells toward the oocyte. Scale bars = 20 μm (**A**), 10 μm (**B**, **C**, **D**).

defined by the presence of a polar body, the presence of a pronucleus, and interphase microtubules were observed more frequently in oocytes cultured in microdrops (19.6%, $n = 46$) when compared to co-cultured oocytes (6.8%; $n = 44$; $P = 0.07$). Based on visualization in 3D using optical sections generated by confocal microscopy, MII oocytes matured in co-culture with cumulus cells exhibited normal bipolar spindles with aligned chromosomes (Figure 2B, C) at a comparable incidence to oocytes matured in microdrops (co-culture: 71%, $n = 41$ versus microdrop: 65%, $n = 37$). Microtubules were observed solely in association with condensed chromatin, whether the oocytes were co-cultured (Figure 2C) or cultured in the denuded state in microdrops. When focusing on the interface between the two cell compartments, multiple cell processes, which contained microtubules, typically extended from the surrounding cumulus cells towards the oocyte (Figure 2D).

Evaluation of cell cycle kinase activities in oocytes after maturation in vitro or in vivo

MPF and MAP kinase activities were measured simultaneously in single human oocytes at distinct meiotic stages, including GV stage oocytes on the day of retrieval, and MI and MII oocytes following maturation *in vitro* using the novel co-culture system described therein (Figure 3). Levels of MPF activities were significantly higher in MI oocytes when compared to GV oocytes, with no additional significant difference between MI and MII oocytes (Figure 3B). In contrast, MAPK activity levels were significantly higher in MII oocytes than MI oocytes that, in turn, exhibited higher activity than GV oocytes (Figure 3B).

MPF and MAP kinase activities were also measured in MII oocytes following maturation *in vivo*, and these levels were compared with those in MII oocytes matured *in vitro*, using either the microdrop or collagen co-culture system (Figure 4). There was no difference in MPF activity between oocytes matured in microdrops or in co-culture with cumulus cells (Figure 4C). For MAPK activity, levels were significantly greater in co-cultured oocytes when compared to oocytes matured in microdrops (Figure 4C). Notably, MAPK levels were significantly higher in *in vivo*-matured oocytes than for the other two culture groups; no differences were observed in MPF activity (Figure 4B, C). Kinase activities shown in Figure 4C are for all oocytes combined across the entire patient population; similar trends were observed within each patient as illustrated in Figure 4B. For a single patient, there were no differences in MPF levels across all MII oocytes analysed whereas higher MAPK levels were apparent in co-cultured oocytes with a further increase in *in vivo*-matured oocytes when compared to denuded oocytes in microdrops.

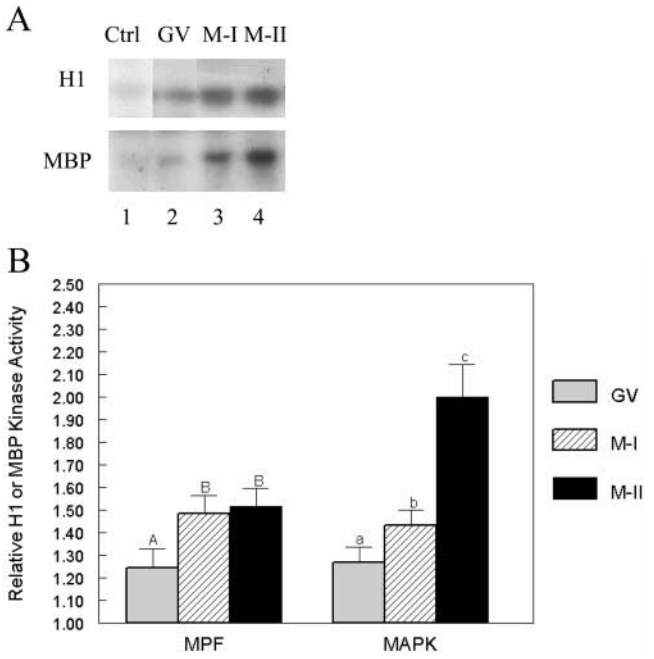

Figure 3. Analysis of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activity in single human oocytes at distinct meiotic stages after in vitro maturation using a three-dimensional collagen gel co-culture system. (**A**) Representative profiles of histone 1 (H1) and myelin basic protein (MBP) activity in a germinal vesicle (GV) (lane 2), metaphase I (MI) (lane 3), and metaphase II (MII) (lane 4) oocyte in comparison to levels in a control sample with kinase lysis buffer alone and no oocyte (lane 1). (**B**) Quantification by densitometry of MPF and MAP kinase activity shown as a fold increase over values in control samples that were arbitrarily given the value of 1. Different letters indicate significant differences in the activity of each kinase among GV, MI and MII oocytes. Twelve GV, 11 MI and 28 MII oocytes were analysed.

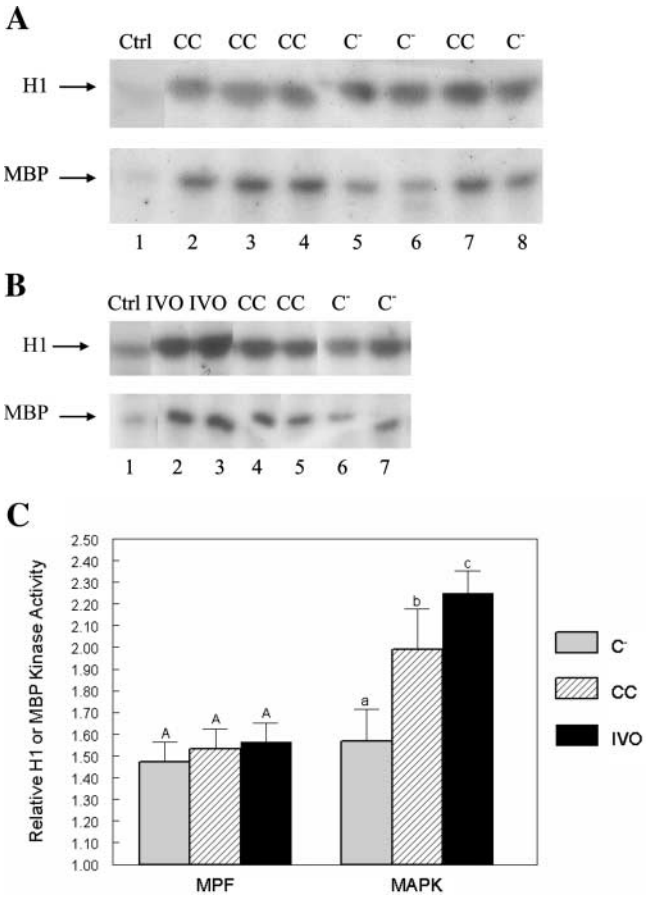

Figure 4. Analysis of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) kinase activity in single human metaphase II (MII) oocytes after in vivo or in vitro maturation, using either a microdrop or three-dimensional (3D) collagen gel co-culture system. (**A**) Representative profiles of histone 1 (H1) and myelin basic protein (MBP) activity in MII oocytes matured *in vitro* in co-culture (CC; lanes 2, 3, 4 and 7) or in microdrop (C^-; lanes 5, 6 and 8) for two patients (with lanes 2–6 representing one patient and lanes 7–8 another patient). (**B**) Representative profiles of H1 and MBP activity in MII oocytes matured *in vivo* (IVO; lanes 2 and 3), *in vitro* in a 3D collagen co-culture system (CC; lanes 4 and 5), or *in vitro* in microdrops (C^-; lanes 6 and 7) within a single patient. Lanes 1 in both (**A**) and (**B**) represent control samples consisting of kinase lysis buffer alone with no oocyte. (**C**) Quantification by densitometry of MPF and MAP kinase activity shown as a fold increase over intensity levels in controls that were arbitrarily given the value of 1. Different letters indicate significant differences in the activity of each kinase between the three groups. Twenty-five C^- (11 patients), 25 CC (11 patients) and 13 IVO (three patients) oocytes were included in this analysis.

Discussion

The present study defines a novel and efficient co-culture system for human oocytes and cumulus cells for the purpose of IVM. We report comparable rates of maturation to MII and similar organization of spindles and chromatin between oocytes matured in microdrops or in a collagen co-culture system. We also analysed, to our knowledge for the first time in single human oocytes, MPF and MAP kinase activity levels, which varied according to the oocyte meiotic stage and the maturation condition. Although a comparison of ocyte maturation under co-culture conditions in either the presence or absence of ECM remains to be performed, this work provides a stepping-stone for future efforts aimed at optimizing IVM culture conditions by utilizing a co-culture collagen 3D system. This innovative system affords several advantages, among which are the ability to reconstitute interactions between isolated cell populations, the use of a 3D microenvironment that mimics physiological conditions and 3D architecture *in vivo*, and, last but not least, the absence of any losses either at the time of oocyte embedding or recovery, together with the significant ease of protocol.

As demonstrated using other cell types, cumulus cells appear to behave differently and display distinct morphologies whether cultured with or without collagen, and whether in a 2D or a 3D environment. Cell viability, based on the presence of homogeneous chromatin in nuclei, is highest in a 3D collagen gel and lowest in cultures on a flat surface with an intermediate pyknotic index if cultured in a 2D environment with matrix. While cell survival and apoptosis remain to be specifically assayed, of relevance here are previous reports that apoptosis is more elevated when cells are

cultured on plastic without matrix and in serum-free media (Frisch and Francis, 1994; Aharoni *et al.*, 1996). The use of an ECM during IVM is all the more significant when considering the impetus to design a chemically defined culture medium for human IVM and notably serum-free conditions as were used here. Cumulus cells allowed to interact with a matrix displayed multiple neuronal-like processes (Figure 1), which may reflect cells exploring their surrounding environment and thereby becoming interconnected to each other in a 3D space, as demonstrated for fibroblasts grown in a 3D collagen gel (Grinnell *et al.*, 2003). Further studies need to assess metabolic coupling between cumulus cells under different culture conditions. In addition, given the close relationship between shape and function of both granulosa and cumulus cells (Aharoni *et al.*, 1996; Bar-Ami *et al.*, 1997; Huet *et al.*, 2001), future analysis of cumulus cell function under 2D or 3D cultures in the presence or absence of ECM should include not only the release and use of metabolites, but also the production of steroid hormones, growth factors, and ECM components.

Influences between the two cell populations used here may be exerted by junctional and/or paracrine actions. Whether gap junctional communication exists between cumulus cells and oocytes in the present co-culture system remains to be determined. Notably, dynamics of gap junctional communication between oocyte and cumulus cells are unknown in the human, and it is conceivable that these are absent or are highly dynamic at such an advanced stage of oocyte maturation. Also, the current supplementation of the culture medium may not favour, or may even inhibit, gap junction communication. A number of paracrine factors with regulatory local roles within COC are produced during oogenesis, including kit ligand, GDF-9, BMP-15, and other members of the transforming growth factor superfamily (Eppig, 1991; Elvin and Matzuk, 1998), and paracrine signalling is likely to take place when co-culturing oocytes with cumulus cells. Furthermore, culturing cumulus cells in a 3D ECM environment may permit the mobilization and modulate the activity of growth factors as demonstrated for other cell types (Rosso *et al.*, 2004). Future investigation into this possibility must include a comparison of oocyte–cumulus cells co-cultured in the presence (2D versus 3D) or absence of collagen. It is noteworthy that microtubule-rich processes resembling transzonal projections previously described in human cumulus–oocyte complexes (Motta *et al.*, 1994) were abundant at the oocyte–cumulus cell interface (Figure 2). The possibility that these transzonal projections serve as conduits for the transport of paracrine factors (Albertini *et al.*, 2001) remains to be determined in the co-culture system used here.

MPF and MAPK regulate cell cycle progression and a number of events during oocyte maturation (Heikinheimo and Gibbons, 1998). While the dynamics of these kinase activities has been extensively studied in oocytes from other species, very limited attention has been given to their analysis in human oocytes (Pal *et al.*, 1994; Sun *et al.*, 1999; Anderiesz *et al.*, 2000; Trounson *et al.*, 2001)—this is despite the fact that the kinetics of cell cycle progression are known to differ significantly among studies of human IVM (Cha and Chian, 1998). In our study, MAPK levels continued to rise from the GV stage through meiosis I until arrest at metaphase of meiosis II (Figure 3); this pattern is similar to previous findings in other species (Verlhac *et al.*, 1994; Heikinheimo and Gibbons, 1988; Wehrend and Meinecke, 2001). Also, MPF activity increased from the GV stage to MI as described previously in oocytes of several species (Hashimoto and Kishimoto, 1988; Wu *et al.*, 1997; Wehrend and Meinecke, 2001); no further significant increase in MPF level was observed between MI and MII human oocytes (Figure 3).

While no differences were observed in MPF levels at MII between oocytes matured *in vitro* (in microdrop or collagen co-culture) or *in vivo*, enhanced MAPK activity was detected in MII oocytes matured in collagen co-culture versus microdrops and a further increase was detectable in *in vivo*-matured oocytes (Figure 4). Here again, there is an immediate need to perform an internal comparison of oocytes and cumulus cells co-cultured with or without collagen in a 2D or 3D environment. Culture media and maturation conditions can influence cell cycle kinase activities as documented in pig (Naito *et al.*, 1992; Motlik *et al.*, 1996), horse (Goudet *et al.*, 1998), and cow (Sakaguchi *et al.*, 2002) oocytes. A hypothesis to explain why MAPK levels are reduced in denuded oocytes cultured in microdrop may be that by abrogating support from the surrounding somatic cells, phosphorylation events and more generally the activation of signalling pathways known to regulate kinase activity may be deficient. Indeed, cumulus cells modulate protein synthesis and phosphorylation levels in the oocyte (Colonna *et al.*, 1989; Cecconi *et al.*, 1991; Trounson *et al.*, 2001). In addition, Su *et al.* (2002) demonstrated further the influence of somatic cells on oocyte cell cycle kinases, with meiotic resumption requiring the participation of MAPK in the cumulus cells. It is interesting to note that denuded human oocytes matured *in vitro* exhibit a recurrent incidence of microtubule arrays typical of interphase in the oocyte cytoplasm with clear M-phase progression (Combelles *et al.*, 2002). Therefore, given the involvement of MAPK in regulating microtubule dynamics in mouse oocytes (Verlhac *et al.*, 1994, 1996), one may speculate that a causal relationship exists between compromised MAPK activity and interphase microtubules in denuded human oocytes matured *in vitro* in microdrops. Also, oocytes cultured in microdrops exhibited a greater incidence of spontaneous activation after MII arrest (Combelles *et al.*, 2002; data presented herein); in contrast, oocytes co-cultured with cumulus cells in 3D collagen gels rarely exited MII arrest. Therefore, a certain threshold of MAPK activity may be required to maintain M-phase arrest after polar body extrusion in human oocytes; this is not surprising given the interplay between the Mos–MAPK pathway and the cytostatic factor (CSF)-induced arrest in MII (Kosako *et al.*, 1994; Fan and Sun, 2004). Interplays are known to exist between MAPK and MPF activity in mammalian oocytes (Motlik *et al.*, 1998; Gordo *et al.*, 2001), a possibility that warrants further investigation in human oocytes. It is worth noting that diminished MAPK activity may be associated with an increased predisposition of oocytes to apoptosis; indeed, apoptosis may be regulated by MPF and MAPK levels

(Fissore *et al.*, 2002) and furthermore, the role of the Mos–MAPK pathway in inhibiting apoptotic events was demonstrated in *Xenopus* egg extracts (Tashker *et al.*, 2002).

The kinetics of maturation also remains to be assessed in relation to cell cycle kinase activities using the novel co-culture system defined here for human IVM. It may be speculated that the pace of maturation is slowed down in the co-culture when compared to the microdrop system, although the exact influence of cumulus cells on the progression of oocyte maturation is unknown. Interestingly, previous studies in bovine oocytes indicate that the kinetics of maturation is faster when kinase levels are elevated (Fissore *et al.*, 1996; Sakaguchi *et al.*, 2002).

One cannot disregard the inherent limitations of utilizing immature oocytes that failed to resume meiosis *in vivo* following gonadotrophin stimulation and retrieved from patients undergoing fertility treatment. Therefore, similar analyses must be undertaken with not only human oocytes from alternate sources but also oocytes from other animals, which may also provide the opportunity to compare mechanisms *in vivo*. In addition, while efforts were made in the design of the present co-culture system to mirror the physiological situation, the fact that MAPK levels were lower in oocytes matured in this system as compared to *in vivo*-matured oocytes indicates that deficiencies still remain. Given that diminished MAPK activity may be associated with normal microtubule organization and spindle function (Verlhac *et al.*, 1996), spontaneous activation (Kosako *et al.*, 1994; Fan and Sun, 2004; data presented herein), and/or apoptosis (Fissore *et al.*, 2002; Tashker *et al.*, 2002), all of which result in compromised oocyte developmental competence, the utilization of *in vitro*-matured human oocytes should not be encouraged until conditions are thoroughly tested.

Although additional studies first need to confirm a direct beneficial influence of ECM and a 3D environment on oocyte quality when using a co-culture approach, there is undoubtedly a need to continue optimizing culture conditions for human IVM. Additional improvements for the co-culture of cumulus cells and oocytes may include the use of extracellular matrices other than simple collagen gels. Indeed, the exact composition of the matrix influences cell function (Abbott, 2003), and while commercial ECM preparations, such as Matrigel, are readily available, the concept of using matrix components normally found in the cell system of interest in the body is gaining increased interest in other fields (Rosso *et al.*, 2004). A diversity of ECM molecules is present in ovarian follicles (Rodgers *et al.*, 2003), and proteoglycans, laminin, collagen IV and fibronectin are all present in the hyaluronan-rich matrix characteristic of cumulus–oocyte complexes (Zhuo and Kimata, 2001). With the technical challenges associated with designing and/or extracting the natural ECM of cumulus–oocyte complexes, synthetic 3D products may represent another attractive alternative. Artificial 3D scaffolds are receiving a great deal of attention in tissue engineering applications, thereby permitting a superior level of control over the use of natural ECM components (Bouhadir and Mooney, 1998). Alginate-based hydrogels have been applied to the culture of intact mouse granulosa cell–oocyte complexes (Pangas *et al.*, 2003), granulosa cells (Kreeger *et al.*, 2003a) and follicles (Kreeger *et al.*, 2003b). Indeed, one may envision constructing a 3D-engineered follicle *in vitro* with its multiple compartments by taking advantage of powerful and novel approaches now available.

Clinical IVM still awaits a breakthrough, and while the developmental competence of *in vitro*-matured oocytes using our 3D collagen co-culture system ultimately remains to be tested, we believe that this work opens exciting new avenues of research. This system may also prove valuable for use in the arrest of oocyte maturation with type-specific phosphodiesterase inhibitors (Nogueira *et al.*, 2003); as a result, immature oocytes may be 'prepared' and undergo prematuration events (Hendriksen *et al.*, 2000) in a controlled microenvironment that simulates physiological conditions, so that their subsequent developmental competence is improved.

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