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Reorganization of the Endoplasmic Reticulum and Development of Ca^{2+} Release Mechanisms During Meiotic Maturation of Human Oocytes[1]

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ABSTRACT

Oocyte maturation in rodents is characterized by a dramatic reorganization of the endoplasmic reticulum (ER) and an increase in the ability of an oocyte to release Ca^{2+} in response to fertilization or inositol 1,4,5-trisphosphate (IP_3). We examined if human oocytes undergo similar changes during cytoplasmic meiotic maturation both in vivo and in vitro. Immature, germinal vesicle (GV)-stage oocytes had a fine network of ER throughout the cortex and interior, whereas the ER in the in vivo-matured, metaphase II oocytes was organized in large (diameter, ~2–3 μm) accumulations throughout the cortex and interior. Likewise, oocytes matured in vitro exhibited cortical and interior clusters with no apparent polarity in regard to the meiotic spindle. In vivo-matured oocytes contained approximately 1.5-fold the amount of IP_3 receptor protein and released significantly more Ca^{2+} in response to IP_3 compared with GV-stage oocytes; however, oocytes matured in vitro did not contain more IP_3 receptor protein or release more Ca^{2+} in response to IP_3 compared with GV-stage oocytes. These results show that at least one cytoplasmic change occurs during in vitro maturation of human oocytes that might be important for fertilization and subsequent embryonic development, but they suggest that a low developmental competence of in vitro-matured oocytes could be the result of deficiencies in the ability to release Ca^{2+} at fertilization.

calcium, endoplasmic reticulum, human oocyte, IP_3, oocyte maturation

INTRODUCTION

A hallmark feature of fertilization is the release of Ca^{2+} from intracellular stores. Ca^{2+} is stored in the endoplasmic reticulum (ER) and is released into the cytoplasm following sperm-egg fusion by inositol 1,4,5-trisphosphate (IP_3), which is produced from cleavage of the membrane lipid phosphatidylinositol 4,5-bisphosphate [1]. In mammals, an initial release of Ca^{2+} occurs soon after sperm-egg fusion, and this is followed by a series of repetitive Ca^{2+} oscillations that last until pronuclear formation [2]. Intracellular Ca^{2+} release is essential for polyspermy prevention, egg activation, and recruitment of maternal RNAs that initiate protein synthesis after fertilization [3, 4].

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The ability to release Ca^{2+} at fertilization develops during oocyte maturation. Immature, prophase I oocytes (germinal vesicle [GV] stage) are less sensitive to IP_3-induced Ca^{2+} release than mature, metaphase II (MII) oocytes [5–7]. An increase in the sensitivity to IP_3 during oocyte maturation likely involves several modifications in the oocyte during maturation, including a reorganization of Ca^{2+} stores as well as increased numbers of IP_3 receptors [8, 9]. Indeed, the ER undergoes a dramatic reorganization during maturation of a diverse array of species (from marine worms to starfish, frogs, rodents, and cows [10–16]) as well as an increase in the amount of IP_3 receptor protein [8, 17]. In GV-stage mouse oocytes, the ER is continuous with the nuclear envelope and is present throughout the cytoplasm as well as in small accumulations throughout the oocyte interior. During maturation to MII, the ER changes such that clusters of ~1–2 μm appear in the cortex opposite the meiotic spindle [9, 12]. These changes parallel a rearrangement of IP_3 receptors, which also are generally absent from the cortex of immature oocytes but appear in clusters in the cortex of mature eggs [8, 18]. Both of these changes are thought to be involved in the increased sensitivity to IP_3 in the mature egg as opposed to the immature oocyte.

Human oocytes undergo repetitive Ca^{2+} oscillations at fertilization [19], and the ability to release Ca^{2+} has been reported to develop, as it does in other species, during oocyte maturation [20]. The distribution of IP_3 receptors has also been reported to change during oocyte maturation into an organization that is consistent with the structure of the ER in the cortex of the mouse oocyte [21]. However, it is not known if these changes are accompanied by a reorganization of the ER. In vitro-matured human oocytes have reportedly been unable to exhibit the same ability to release Ca^{2+} in response to the sulfhydryl agent thimerosal, which sensitizes the IP_3 receptor as it does in vivo-matured oocytes, suggesting that these changes do not develop properly in oocytes matured in vitro [20]. In vitro-matured oocytes can be fertilized, but they have low developmental competence [22, 23]. Therefore, it is possible that Ca^{2+}-releasing ability is deficient in oocytes matured in vitro. Because the ability to mature oocytes in vitro has important clinical applications, it is of interest to examine various components of normal cytoplasmic maturation to identify where oocytes matured in vitro might be deficient.

In the present study, we examined the ER distribution, the relative amount of IP_3 receptor protein, and the ability to release Ca^{2+} in response to IP_3 in GV-stage as well as in vivo- and in vitro-matured, MII oocytes. Our results show that in human oocytes, the ER undergoes a dramatic reorganization during maturation and that oocytes matured in vitro exhibit the same reorganization. Oocytes matured in vivo contain approximately 1.5-fold as much IP_3 receptor protein as GV-stage oocytes; however, oocytes matured in vitro do not exhibit this increase. Likewise, the ability to release Ca^{2+} in response to IP_3 increases during maturation in vivo but not in vitro.

MATERIALS AND METHODS

Source and Culture of Oocytes

The present study was approved by the Institutional Review Board at the University of Connecticut Health Center (IRB 06–125). All patients gave informed consent to donate oocytes before participation in the study. Immature, GV-stage oocytes were retrieved from the ovaries of women aged 21–44 yr who were undergoing standard in vitro fertilization procedures using intracytoplasmic sperm injection. All patients underwent pituitary suppression using a gonadotropin-releasing hormone agonist or antagonist. Controlled ovarian stimulation was performed by injecting 150–450 IU of recombinant follicle-stimulating hormone (Gonal-F [EMD Serono] or Follistim [Schering Plough]) daily with or without 75–150 IU of human menopausal gonadotropin (Menopur or Repronex; Ferring Pharmaceuticals). Doses were adjusted based on follicular response as evidenced by serial transvaginal ultrasounds and serum estradiol levels. A subcutaneous injection of 3300–10 000 IU of human chorionic gonadotropin was administered when three or more follicles reached a mean diameter of 18 mm. In selected cases, final oocyte maturation was achieved with 20 IU of leuprolide acetate (Lupron; TAP Pharmaceuticals) [24]. Transvaginal ultrasound-guided oocyte retrieval was performed 35 h after human chorionic gonadotropin or Lupron injection.

Oocytes were aspirated from follicles with a diameter of ~14–22 mm. Most oocytes retrieved from such follicles are at MII (mature eggs). However, a small percentage of oocytes are at prophase I; these oocytes are identified by the presence of a GV. Because these immature oocytes are not used clinically at our center, they are routinely discarded. In some cases, we obtained MII oocytes that were leftover from women who chose to expose only a few oocytes to sperm and that would have otherwise been discarded. Cumulus-oocyte complexes were aspirated into culture medium containing 5% human serum albumin (Global SL Behring) in Quinn Advantage Fertilization Medium (SAGE; Cooper Surgical) and incubated in an atmosphere of 5% CO_2/95% at 37°C for 3–5 h. Cumulus cells were stripped enzymatically with hyaluronidase type VIII from bovine testes (catalog no. H-3757; Sigma-Aldrich) and mechanically by pipetting up and down through a small-bore pipette. Following cumulus removal, oocytes that had a GV were placed into blastocyst medium (SAGE) containing 10% serum protein substitute (Cooper Surgical) and 10 μM cilostamide (Calbiochem), a phosphodiesterase 3A-specific inhibitor that prevents spontaneous meiotic resumption. Oocytes retrieved from all patients on a given day were pooled.

We selected GV-stage oocytes that had a readily discernible GV following an overnight culture in medium containing 10 μM cilostamide except for some of the immunoblotting experiments, in which GV-stage oocytes were frozen on the day of retrieval. To obtain in vitro-matured, MII oocytes, we selected oocytes that were GV-intact after an overnight culture and transferred them to blastocyst medium that did not contain cilostamide. Oocytes were cultured in a humidified atmosphere of 5% CO2/95%, and oocytes that underwent GV breakdown and formed a first polar body within 24 h were selected for further experimentation.

Experiments using mice were performed in accordance with the Center for Laboratory Animal Care at the University of Connecticut Health Center. GV-stage mouse oocytes were retrieved from the ovaries of mice that had been injected 42–44 h previously with 10 IU of equine chorionic gonadotropin. Oocytes were retrieved in culture medium containing 10 μM milrinone to prevent spontaneous oocyte maturation and were transferred to medium that did not contain milrinone to initiate oocyte maturation. Zonae pellucidae were removed by pipetting the oocytes through a small-bore pipette in the presence of 10 μg/ml α-chymotrypsin.

Oocyte Microinjection, DiI, and Ca^{2+} Imaging

Oocytes were microinjected with DiI (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) as previously described [25]. Briefly, a saturated solution of DiI (Invitrogen) was prepared in soybean oil (Wesson Oil; ConAgra Foods, Inc.) and stored at 4°C. The DiI solution was front-loaded into a beveled, mercury-filled pipette connected to a micrometer syringe system filled with Fluorinert FC-70 (Sigma). The same pipette was used to inject several oocytes with ~7 pl of the DiI solution, which formed an oil droplet inside the oocytes. The volume injected was calculated based on the diameter of the sphere that forms in the oocyte cytoplasm during microinjection. DiI-labeled oocytes were observed with a confocal microscope (Zeiss Pascal; Carl Zeiss Microimaging, Inc.) ~1–2 h after microinjection. Fluorescence was excited with the 543-nm line of an HeNe laser and was detected using a 560-nm emission filter. Images were collected using a 40× NA 1.2 water-immersion objective (C-Apochromat; Carl Zeiss MicroImaging, Inc.).

Oocytes were microinjected with ~30 pl of a 400 μM stock of calcium green 10-kDa dextran (final concentration in the oocyte, ~13 μM; calculation based on an oocyte volume of 900 pl), and Ca^{2+} was imaged as previously described [26]. For IP_3 injections, a baseline measurement was obtained from each oocyte, then the recording was interrupted while a pipette loaded with a solution of IP_3 was inserted into the oocyte. After pipette insertion, the recording was started again. Following another baseline recording, 20 pl of a 5 μM solution of IP_3 (final concentration in the oocyte, 100 nM) were injected while simultaneously measuring intracellular Ca^{2+}. The amplitude of Ca^{2+} release was calculated based on the increase in the peak amplitude of the response to injection (F) subtracted by the baseline (F_0) and then divided by the baseline signal $[(F- F_0)/F_0]$. Figures were made using Adobe Photoshop after scanning Ca^{2+} records into a computer.

FM 1–43 Labeling

To label plasma membranes, zonae pellucidae were removed from oocytes in serum-free blastocyst medium containing 0.5% pronase (Calbiochem). Zona-free oocytes were incubated in 2 μM FM 1–43 (Invitrogen) diluted in serum-free Hank balanced salt solution. Oocytes were examined with a confocal microscope after ~1–2 h. Fluorescence was excited at 488 nm and was detected at 560 nm. Images were collected using a 40× NA 1.2 water-immersion objective.

Immunoblotting

The GV-stage, in vivo- and in vitro-matured oocytes were washed with PBS containing 0.1% polyvinyl alcohol and then frozen in liquid nitrogen in a minimal amount of medium and stored at −80°C until use. Two to seven oocytes were solubilized in Laemmli sample buffer, and proteins were separated on 4–15% gradient gels. IP_3 receptor protein was detected using an affinity-purified antibody kindly provided by Dr. Jim Watras (University of Connecticut Health Center, Farmington, CT) [27]. Blots were developed using enhanced chemiluminescence (ECLPlus; GE Healthcare). Densitometric analysis was carried out using ImageJ software (National Institutes of Health).

RESULTS

Distribution of ER in GV-Stage and MII Oocytes

The cortical ER in GV-stage and MII mouse oocytes differs considerably, with the immature oocyte having little ER membrane in the cortex and the mature egg having cortical ER that is organized into large, distinct clusters [9, 12]. To examine the ER in human oocytes, we looked at the cortices of GV-stage and MII oocytes with confocal microscopy after injection of the lipophilic, fluorescent dye DiI [9, 25, 28]. DiI was microinjected into oocytes, where it contacts intracellular membranes; because the ER is a continuous network, it spreads throughout the ER in the entire cell [28]. We found that human, GV-stage oocytes contained little cortical ER that was not organized into cortical clusters (n = 21) (Fig. 1A), although in some cases, small, punctate spots of ER were observed in the cortex (not shown). In contrast, 69% of in vivo-matured, MII oocytes contained ER membrane throughout the cortex that was organized into large (diameter, ~2–3 μm), distinct ER clusters (n = 16) (Fig. 1B). The cortical ER in human GV-stage and MII oocytes therefore undergoes changes during maturation similar to those in mouse oocytes [9, 12].

The ER in mature mouse oocytes is polarized such that the clusters of ER are localized in the cortex opposite the meiotic spindle, whereas immature oocytes contain smaller clusters of ER throughout the entire oocyte and surrounding the GV [9, 12]. Human oocytes differed in this regard. None of the GV-stage oocytes examined contained ER around the GV, and larger clusters of ER were absent from the oocyte interior (Fig. 2A). In MII oocytes, clusters of ER were present not only in the cortex but also throughout the entire oocyte (Fig. 2B). In addition, we generally observed no apparent polarity in relation to the meiotic spindle, although in one case, the clusters were concentrated in half of the oocyte at roughly a 45° angle to the spindle (Fig. 2B). This oocyte contained ER clusters throughout the rest of the oocyte as well.

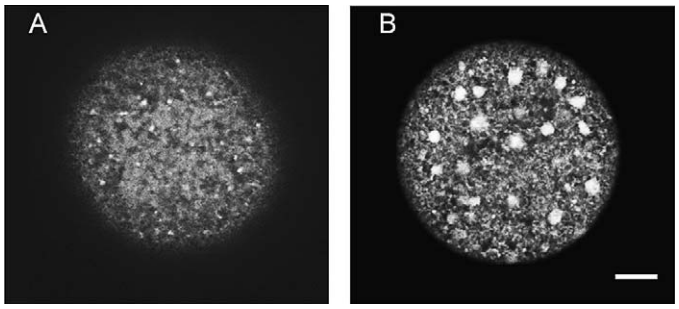

FIG. 1. Cortical ER in GV-stage (**A**) and MII (**B**) oocytes. The ER was labeled with the lipophilic, fluorescent dye DiI and imaged with a confocal microscope. The cortical ER in GV-stage oocytes is largely devoid of ER, whereas the ER in MII oocytes is characterized by large ER accumulations. Bar = 10 μm.

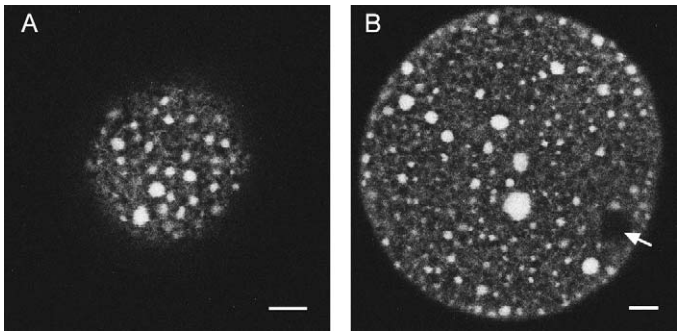

FIG. 3. ER distribution in an in vitro-matured oocyte at the MII stage. **A**) ER in the cortex just under the plasma membrane. **B**) Equatorial view showing cortical and cytoplasmic ER accumulations. Arrow in **B** points to the meiotic spindle. Bar = 10 μm.

Distribution of ER in In Vitro-Matured Human Oocytes

One issue with obtaining immature human oocytes and maturing them in vitro is that although these oocytes develop to MII, they have low developmental competence after fertilization [22, 23]. We sought to determine if oocytes matured in vitro were able to develop cortical ER accumulations following in vitro maturation to MII. We found that 79% of in vitro-matured oocytes exhibited large clusters of ER in the cortex (n = 14), similar to those in the in vivo-matured human oocytes (Fig. 3A). In addition, ER was distributed throughout the cytoplasm as well as in the cortex, with no polarity in regard to the meiotic spindle, as in the in vivo-matured oocytes (Fig. 3B). These results demonstrate that in vitro-matured oocytes are capable of undergoing the changes in ER reorganization that occur normally in vivo. Because Ca^{2+} is stored in the ER, this in turn suggests that in vitro-matured oocytes are able to mount a sufficient Ca^{2+} response at fertilization. Taken together, these results suggest that Ca^{2+}-releasing ability in the in vitro-matured oocyte is not responsible for the developmental incompetence seen in the in vitro-matured human oocytes.

It has been reported that human oocytes contain no polarity with regard to the meiotic spindle and that sperm are able to fuse anywhere on the surface of the oocyte [29], whereas in mouse oocytes, a microvilli-free domain is present above the meiotic spindle that prevents the sperm from binding in this region [30, 31]. This might explain the lack of ER polarity with regard to the meiotic spindle in human oocytes, because Ca^{2+} stores would be expected to be present even if the sperm fuses in the region of the oocyte containing the meiotic spindle. To confirm the lack of polarity in the mature human oocyte, we used FM 1–43, a dye that is fluorescent when it contacts membranes. We found that in the in vitro-matured oocytes, the fluorescence intensity was the same around the entire surface of the oocyte, with no apparent polarity (Fig. 4A). To illustrate that this dye can, indeed, show polarity in an oocyte, we used mouse oocytes. Figure 4B shows the presence in a mouse oocyte of a microvilli-free domain in the region of the meiotic spindle.

Human Oocytes Synthesize IP_3 Receptor Protein During In Vivo, but Not In Vitro, Oocyte Maturation

In addition to changes in reorganization of ER during maturation in mouse oocytes, the amount of IP_3 receptor protein approximately doubles [8]. IP_3 receptors are present on the ER [18] and allow the oocyte to release Ca^{2+} at fertilization in response to sperm-egg fusion. Using Western blot analysis, we examined if human oocytes undergo such an increase in IP_3 receptor protein during maturation. We examined the type 1 IP_3 receptor, both because it has been shown previously to be present in human oocytes [21] and because it is the receptor responsible for Ca^{2+} release after fertilization or injection of IP_3 in rodent as well as in human oocytes [32–34]. The IP_3 receptor was abundant in the oocyte, because we were able to detect protein in a single oocyte. Compared with the amount of IP_3 receptor protein in GV-stage oocytes, the amount of receptor protein in the in vivo-matured, MII oocytes increased

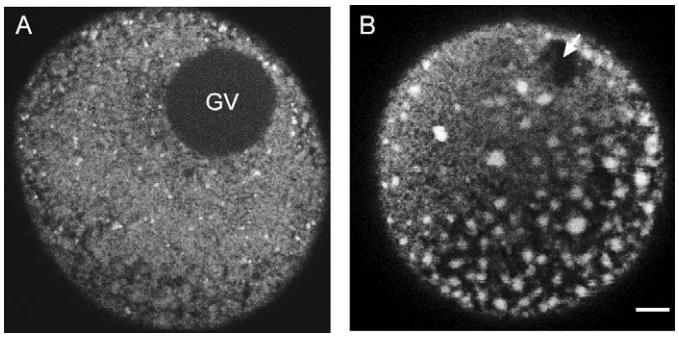

FIG. 2. Equatorial view of the ER in GV-stage (**A**) and MII (**B**) oocytes showing the lack of abundant ER in the immature oocyte and large ER accumulations throughout the cortex and inner cytoplasm of MII eggs. The arrow in **B** points to the meiotic spindle. Bar = 10 μm.

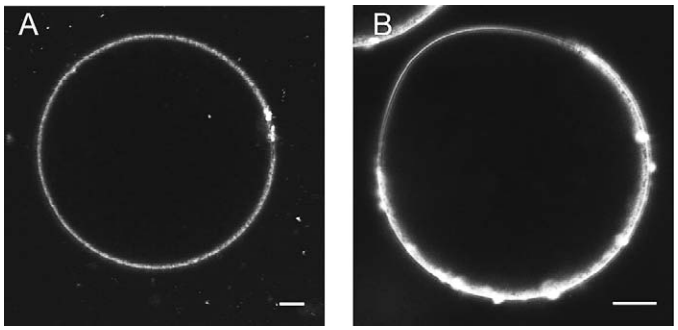

FIG. 4. The membrane of the mature, MII human oocyte does not exhibit polarity with regard to the meiotic spindle. **A**) Plasma membrane of an in vitro-matured human oocyte labeled with the fluorescent membrane dye FM 1–43. **B**) Polarization with regard to the meiotic spindle in an MII mouse oocyte. Bar = 10 μm.

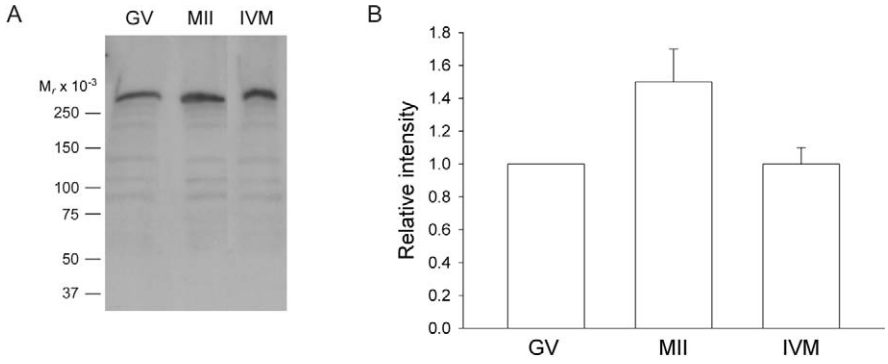

FIG. 5. The amount of IP_3 receptor protein increases during oocyte maturation in vivo, but not in vitro. **A**) Western blot showing IP_3 receptor protein in lysates from seven GV-stage, in vivo-matured (MII), and in vitro-matured (IVM) oocytes per lane. **B**) Densitometric analysis of IP_3 receptor protein amounts in in vivo-matured and in vitro-matured oocytes compared to GV-stage oocytes. Densitometric values were obtained from four separate experiments using in vivo-matured oocytes and from eight separate experiments using in vitro-matured oocytes each. Values are reported as the intensity of MII oocytes relative to that of GV-stage oocytes that were run on the same blot. Overall, the relative amount of IP_3 receptor protein increased by 1.5-fold in oocytes matured in vivo but showed no increase in oocytes matured in vitro. Data are presented as the mean ± SEM are shown. The amount of protein in in vivo-matured oocytes versus in vitro-matured oocytes did not quite reach statistical significance ($P = 0.05$).

by approximately 50% (Fig. 5). In contrast, oocytes matured in vitro did not synthesize IP_3 receptor protein to the same extent as oocytes matured in vivo (Fig. 5). Although the amount of protein in the in vivo- versus in vitro-matured oocytes did not quite reach statistical significance ($P = 0.05$), these results suggest that oocytes are deficient in their ability to synthesize protein during in vitro maturation.

Ability to Release Ca^{2+} Increases in Response to IP_3 During In Vivo, but Not In Vitro, Oocyte Maturation

It has been reported previously that human oocytes develop the ability to release Ca^{2+} in response to the sulfhydryl reagent thimerosal, which sensitizes the IP_3 receptor to low levels of IP_3 [35], during maturation in vivo [20]. This previous study [20] indicated that oocytes matured in vitro, however, were deficient in their ability to release Ca^{2+} following in vitro maturation. The oocytes used in that study were obtained from ovaries of unstimulated patients, so it differed from our study, which used oocytes following hormonal stimulation. Here, we chose to measure Ca^{2+} release in response to the more physiological Ca^{2+}-releasing agent IP_3. We found that oocytes matured in vivo exhibited a single Ca^{2+} transient in response to injection of 100 nM IP_3 (Fig. 6). This concentration was chosen because it is not saturating in mouse oocytes [7]. GV-stage oocytes also released a single Ca^{2+} transient in response to IP_3, but the amplitude of the Ca^{2+} transient was significantly lower than that in the in vivo-matured oocytes (Fig. 6). However, oocytes matured in vitro showed a single transient that did not differ in amplitude from that of GV-stage oocytes (Fig. 6). In all cases, the duration of the transient was the same. These results demonstrate that immature oocytes develop an increased ability to release Ca^{2+} in response to a physiological stimulus in vivo, but that the ability to release Ca^{2+} in response to IP_3 does not increase during in vitro maturation under our culture conditions.

DISCUSSION

The present study demonstrates that human oocytes undergo a dramatic reorganization of ER during meiotic maturation. GV-stage oocytes contain a fine ER network throughout the cortex and interior, whereas mature, MII oocytes contain striking, ~2- to 3-μm accumulations in both the cortex and throughout the cytoplasm, with no apparent polarity in regard to the meiotic spindle. The ER in oocytes matured in vitro is indistinguishable from the ER of in vivo-matured oocytes, with membrane accumulations of the same size located both in the cortex and throughout the cytoplasm. In addition, during in

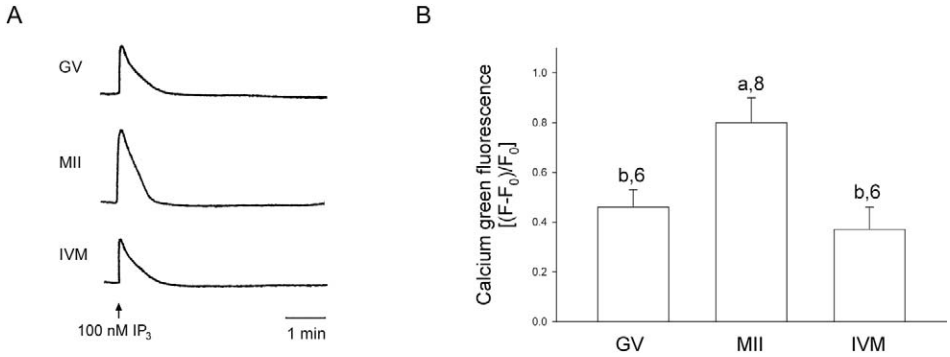

FIG. 6. Human oocytes increase their ability to release Ca^{2+} in response to injection of IP_3 during in vivo, but not in vitro, oocyte maturation. **A**) Pattern of Ca^{2+} release following injection of 100 nM IP_3. Oocytes were injected with the Ca^{2+} indicator dye calcium green dextran, and Ca^{2+} release was monitored during injection of IP_3. **B**) Quantification of the amplitude of Ca^{2+} release in response to IP_3 injection. Results shown are the average increase in calcium green fluorescence after subtracting the baseline fluorescence (F_0) by the peak amplitude (F) and then dividing by the baseline ($[F - F_0)/F_0]$). Average amplitude and SEM are shown. Different letters represent significant differences in amplitude ($P < 0.001$), and numbers are the number of oocytes tested.

vivo maturation, the amount of IP_3 receptor protein increases by approximately 1.5-fold, and oocytes develop an increased ability to release Ca^{2+} in response to IP_3. However, oocytes matured in vitro are deficient in their ability to synthesize IP_3 receptor protein and fail to develop an increased sensitivity to release Ca^{2+} in response to IP_3.

The ER is the major Ca^{2+} storage organelle in the oocyte [36]. As such, it plays an important role in Ca^{2+} release that occurs at fertilization in all species that have been studied [37, 38]. Ca^{2+} release is necessary for polyspermy prevention, egg activation, and recruitment of maternal RNAs that initiate protein synthesis following fertilization [3, 4]. In mature mouse oocytes, the ER is polarized such that clusters of ER are present in the cortex opposite the meiotic spindle [9]. This region of the oocyte corresponds to the region that contains both microvilli and cortical granules [30, 39, 40], is the region where sperm-egg fusion occurs [30, 31], and is the site for the generation of repetitive Ca^{2+} waves following fertilization [18]. We did not observe polarity of the ER in the human oocyte. Because the human oocyte does not contain a microvilli-free area over the meiotic spindle and, thus, the sperm is able to fuse anywhere over the oocyte surface [29], this is not unexpected. A previous study indicated that the IP_3 receptor is polarized in the human oocyte with regard to the meiotic spindle [21], as in mouse oocytes. Although we did not label the IP_3 receptor, it is likely that IP_3 receptors are located on the ER even in the region of the meiotic spindle, because IP_3 receptors have been shown previously to be localized to the ER in mouse oocytes [18].

A rearrangement of ER during meiotic maturation, including the development of cortical clusters, has been reported in other species, including the marine worm *Cerebratulus lacteus* [10] and the frog *Xenopus laevis* [16]. Among the mammals, mouse oocytes develop cortical clusters during maturation [9, 12], whereas hamster and bovine oocytes exhibit a change from cortical localization to the formation of loosely organized clusters of ER throughout the cytoplasm [14, 15]. Some of these species have been found to develop the ability to release Ca^{2+} during maturation [6, 7, 28], and the rearrangement of Ca^{2+} stores is thought to play a role in this ability. The arrangement of ER could also play a role in the ability of a mature oocyte to generate Ca^{2+} waves at fertilization, because immature *C. lacteus* and hamster oocytes do not generate Ca^{2+} waves at fertilization [14], nor do mouse oocytes generate Ca^{2+} waves in response to PLCζ [41], a sperm-specific phospholipase C thought to initiate Ca^{2+} oscillations at fertilization [42]. In the present study, we found that in vitro-matured human oocytes undergo a reorganization of ER but do not develop the ability to release Ca^{2+} in response to IP_3, suggesting there is more to increasing IP_3-induced sensitivity than simply a rearrangement of ER structure.

The amount of IP_3 receptor protein increased by approximately 50% during meiotic maturation in vivo. These mature oocytes also released significantly more Ca^{2+} in response to IP_3 injection compared with GV-stage oocytes. However, we found that oocytes matured in vitro possessed similar amounts of IP_3 receptor protein and released Ca^{2+} in response to IP_3 injection to the same extent as immature oocytes, demonstrating that they did not increase their sensitivity to IP_3 during maturation. These results are in agreement with those of a previous study showing that the human oocyte develops an increased Ca^{2+}-releasing ability during oocyte maturation and that oocytes matured in vitro do not have the same ability to release Ca^{2+} in response to the sulfhydryl reagent thimerosal compared with oocytes matured in vivo [20]. The finding that GV-stage and in vitro-matured oocytes had a completely different ER arrangement yet released comparable amounts of Ca^{2+} in response to IP_3 suggests that the amount of IP_3 receptor protein or other factors, such as an increase in Ca^{2+} stores, is perhaps as important as the presence of ER clusters.

The finding that oocytes matured in vitro do not release as much Ca^{2+} as those matured in vivo in response to IP_3 suggests this could contribute to the lower developmental competence seen after in vitro maturation. Other factors could also contribute to this lower developmental competence. For example, microtubules or microfilaments could be disrupted during maturation in vitro. The cytoskeleton has been shown to be necessary for reorganization of the ER during both GV breakdown and development of clusters afterward [12]. Our results showing that ER clusters form during in vitro maturation suggest that microtubules and microfilaments function normally, at least during this process, but they could potentially affect other aspects of cytoplasmic maturation. Another contributor to low developmental competence could be the particular culture medium used, because this has been shown to be important for proper ER reorganization during in vitro maturation of mouse oocytes [25]. Our results demonstrate that the culture medium used in the present study supports ER reorganization. It would be interesting to test a variety of maturation media to see if they have positive or negative effects on the ability of IP_3-induced Ca^{2+} release to increase during maturation.

Despite our inability to detect a differential development of Ca^{2+} signals during in vitro maturation, our study nonetheless shows that at least one important event during cytoplasmic maturation occurs during oocyte maturation in vitro. Other cytoplasmic changes, such as changes in IP_3 receptor phosphorylation during maturation, also likely are involved. For example, the type I IP_3 receptor becomes phosphorylated early after the onset of maturation by Polo-like kinase 1 (PLK1) and mitogen-activated protein kinase in mouse and pig oocytes [43–45], and this phosphorylation is associated with the ability of the oocyte to form cortical IP_3 clusters during maturation and to mount a full Ca^{2+} response following IP_3 stimulation or in response to other agonists known to cause Ca^{2+} release [43, 46]. Further studies will be needed to determine if cell-cycle regulatory proteins likewise contribute to the increased sensitivity that has reportedly been seen during maturation of human oocytes [20]. Indeed, mitogen-activated protein kinase activity has been shown to be lower in human oocytes matured in vitro than in those matured in vivo [47]. Because we did not observe a noticeable increase in the ability of in vitro-matured oocytes to release Ca^{2+} in response to IP_3, such oocytes possibly are deficient in other, as-yet-unidentified factors that change during cytoplasmic maturation.

In summary, we have shown that human oocytes undergo a dramatic reorganization of the ER during progression from the GV stage to MII and develop an increased sensitivity to IP_3-induced Ca^{2+} release during maturation. Whereas oocytes matured in vitro retain their ability to reorganize the ER, they do not release as much Ca^{2+} in response to IP_3 as oocytes matured in vivo, and this likely results, at least in part, from their inability to synthesize IP_3 receptor protein during in vitro maturation. The ability of an oocyte to release Ca^{2+} could potentially be used as an endpoint to examine proper events of cytoplasmic maturation in oocytes matured in vitro using different culture conditions.

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