Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence

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Abstract

Purpose To investigate the role of cumulus cell contact during oocyte maturation on meiotic spindle assembly and the acquisition of developmental competence.

Methods Cumulus oocyte complexes isolated from mouse ovaries subjected to in vitro or in vivo maturation were analyzed by confocal microscopy with respect to oocyte somatic cell contacts and for their ability to develop after parthenogenic activation during embryo culture.

Results Cell contact is maintained during maturation in vivo, predisposing oocytes to cortical meiotic spindle assembly and developmental competence acquisition. In contrast, oocytes matured in vitro lose cell contact

coincident with central meiotic spindle assembly that results in cleavage delays upon egg activation and failure to form blastocysts. Experimental disruption of cell contact by the actin-depolymerizing agent latrunculin B results in the formation of enlarged meiotic spindles with dispersed chromosomes unlike the compact ordering of chromosomes observed on spindles formed after in vivo maturation, suggesting a link between cell contact and the acquisition of developmental competence.

Conclusions Somatic cell contact optimizes oocyte quality during meiotic maturation by regulating the spatial organization and function of the meiotic spindle through actin-dependent mechanisms that enhance development.

Capsule In vitro maturation of mouse COCs requires the maintenance of cumulus cell contact to achieve developmental competence through an actin dependent mechanism that mediates meiotic spindle anchoring to the oocyte cortex.

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Abbreviations

TZP	transzonal projections
GV	germinal vesicle
COC	cumulus oocyte complex
IVM	<i>in vitro</i> maturation
IVO	<i>in vivo</i> maturation
LatB	Latrunculin B
MT	microtubules
ICM	inner cell mass

Introduction

Oocytes acquire developmental competence through the progressive and protracted stages of oogenesis. Those that survive this process are ovulated and undergo the final

phases of maturation that prepare the nucleus and cytoplasm for embryogenesis. Throughout oogenesis in mammals, oocytes maintain physical contact with follicular somatic cells via granulosa cell extensions known as transzonal projections (TZPs) [1, 6, 23, 33, 43, 44]. Despite the recognized functions of TZPs in gap junction mediated metabolite exchange, heterocellular adhesion, cell cycle regulation and bidirectional signaling [10, 18, 40, 47], the precise role these structures play in the acquisition of developmental competence during oocyte maturation is unknown. It is known that the majority of mammalian oocytes develop *in situ* with an eccentrically positioned germinal vesicle (GV) that may facilitate retention of maternal transcripts and essential organelles by excluding these components from the developing spindle [2]. Interestingly, in rodent species, the GV has been shown to lose an eccentric position after culture [38, 39] and

That has been proposed to be a result of a loss in cortical stability secondary to precocious TZP retraction. At ovulation, oocyte derived signaling molecules such as GDF9 and BMP-15 effect expansion of the compact cumulus mass through a process that confers enhanced developmental competence in oocytes of certain mammals [21, 46]. Gonadotropins like follicle stimulating hormone (FSH) have profound effects on the stability and cytoskeletal composition of TZPs in the developing mammalian follicle [12]. TZPs are dynamic and undergo alterations composition and function at various stages of oogenesis [2] but exactly how cell contact or culture conditions impact nuclear and cytoplasmic maturation remains unclear [29]. With the need for *in vitro* maturation of human oocytes in ARTs growing, there is an increased demand for optimizing this technology in clinical settings.

With respect to oocyte maturation, meiosis-regulating molecules like cAMP is thought to be sourced from both the oocyte and the surrounding cumulus cells [28]. However, cAMP from the somatic cells relies upon gap junctions for gaining access to the ooplasm and in rodents. Upon the LH surge, MAP kinase-dependent phosphorylation of the gap junction protein connexin 43 blocks the transport of cAMP into the oocyte, which is also coincident with TZP retraction, and is believed to initiate oocyte maturation [41, 42]. Recent studies suggest that gap junctions are spatially regulated by functional closure between cumulus cells and mural granulosa thus limiting access of molecules like cAMP into the oocyte [30]. While TZP retraction has been documented in many models of oocyte maturation *in vitro* (IVM) [22, 32, 43], the persistence and/or amplification of TZPs noted in many non-rodent species [5] warrants further examination of the dynamics of TZPs in relation to the regulation of oocyte maturation and the acquisition of developmental competence as it may pertain to human ARTs [3].

Here an analysis of the influence of cell contact is undertaken during *in vitro* and *in vivo* oocyte maturation in mice to better define the role of TZP integrity on meiotic spindle morphogenesis and developmental competence acquisition. Besides revealing an adverse effect of *in vitro* maturation on TZP integrity, it is apparent that maintenance of cell contact during the earliest phases of oocyte maturation has a strong and lasting influence on oocyte developmental potential.

Materials and methods

Collection and maturation of oocytes

Experiments were performed using 21-day old CF-1 mice (Charles River). Animals were handled according to the *Guide for Care and Use of Laboratory Animals* (National Academy of Science, 1996) and maintained in a 14 L:10D photoperiod under constant temperature and relative humidity. Food and water were provided *ad libitum*. Ovaries were removed from animals primed 48 h earlier with 5 IU/100 μ l eCG. Cumulus enclosed oocytes were collected in Hepes buffered Eagle minimum essential medium (EMEM) with Hanks salts supplemented with 50 μ g/ml Gentamicin and 0.3% bovine serum albumin (BSA). Spontaneous maturation of cumulus enclosed oocytes (COCs) and manually denuded COCs was conducted in a basal maturation medium (IVMb), consisting of EMEM, 2 mM glutamine, 0.23 mM pyruvate, 0.3% BSA and 50 μ g/ml Gentamicin whereas induced maturation was performed in an enriched medium (IVM+), consisting of 2 mM glutamine, 0.23 mM pyruvate, 10% fetal bovine serum (FBS), 0.6 mM L-cysteine, 0.5 mg/ml D-glucosamine, 0.02 μ M ascorbate, 1% insulin transferring selenium (ITS), 50 μ g/ml Gentamicin, and 0.2 IU/ml recombinant human FSH (Serono Reproductive Biological Institute). IVM+ conditions have been shown to support cumulus expansion and improve oocyte quality [8]. COCs were cultured for 16 h (20–30 COCs/ml) in a humidified atmosphere of 5% CO₂ and air at 37°C and were imaged at various times during maturation. IVO oocytes were collected from either ovaries or oviducts from animals 2–16 h after receiving 5 IU hCG.

Experimental manipulation

Actin filament integrity was analyzed using the f-actin depolymerizing agent Latrunculin B (LatB) [36]. In all experiments, Lat B was used at a final concentration of 0.6 μ M (0.63 mM DMSO stock, Sigma L5288) with the same volume of DMSO (1 μ L) added to 1 ml of culture medium. Lat B or DMSO treatments were continuous for 0–6 h. To test the reversibility of LatB, COCs were washed

free drug or DMSO vehicle and maintained in control medium for 10 h. In some experiments TZPs were removed by stripping cumulus cells before culturing denuded oocytes in IVM+medium (IVM+denuded). For all experiments, 20–30 COCs per treatment group were cultured in 1 ml of maturation medium and each experiment was done in triplicate. Experiments were terminated by fixing samples in 2% PFA supplemented with the phosphatase inhibitor phenylarsine oxide (PAO, 40 μ M) for 30 min and samples were processed as described before [8].

Imaging of oocytes

Live imaging Transzonal projections were imaged in freshly isolated cumulus oocyte complexes labeled with 1 μ g/ml DiOC₁₆(3) (Invitrogen D1125) for 1 h at 37 °C. Z-series data sets were collected on a Noran Odyssey XL slit-scan confocal microscope (Noran Instruments, Inc., Middleton, WI; Nikon Diaphot, 60X oil objective, 1.3NA) that were deconvolved and reconstructed using Metamorph software (Universal Imaging). Live COC differential interference contrast (DIC) imaging was conducted in an environmental chamber (Zeiss CTI-controller 37°C, 5% CO₂/air mixture) in 100 μ l medium (Delta T dishes, Fisher Scientific) overlaid with sterile mineral oil (Sigma). 10 oocytes/dish were imaged on a Zeiss Axiovert 200 inverted microscope (20x Plan-Apo objective NA=0.75) to collect timelapse data sets of GVBD and PB extrusion (3–5 min interval image capture, 5–20 h culture interval). GV position and size were analyzed with Zeiss LSM software from a total of 64 COCs. Live imaging experiments were repeated 3–5 times for each treatment group.

Developmental competence assessment

Diploid (digynic) parthenogenetic embryos were generated to study cleavage kinetics and blastocyst development after activation of oocytes matured *in vitro* or *in vivo*. Oocytes were washed in Ca²⁺ free KSOM (3X, 5 min each) and activated with 10 μ M SrCl₂ and 5 mM Cytochalasin B in Ca²⁺ free KSOM for 6 h. At 6 h, parthenotes were washed in KSOM+sucrose (3×5 min) and cultured in KSOM+sucrose for 96 h. Blastomere number was determined at 24-hour intervals. At 96 h, all embryos were fixed in 2% paraformaldehyde (37°C, 30 min) and stored in wash buffer as described previously [8].

Confocal imaging of fixed samples

A Zeiss LSM 510 laser scanning confocal microscope and was used to collect Z-axis data sets from uncompressed COCs (1–2 μ m step size, 63x Plan-Apo objective, NA=1.4)

from 3 channels (HeNe 548 nm excitation, rhodamine-phalloidin; Argon 488 nm excitation, tubulin; and Diode 405 nm excitation, Hoechst33258) under constant gain, offset, and image averaging conditions. Linescan profiles were used to measure the frequency and distribution of TZPs in 10 μ m thick projections ($n=5$ z-axis images) using a single 5 μ m wide perimeter line superimposed on the zona pellucida; wavelength specific peak height and spacing was measured from five line scans of different oocytes for each treatment group. GV and spindle locations were measured from object centroids in the spindle that were most proximate to the oolemma. Inter-chromosome spacing measurements were made similarly on 5 or more oocytes from each treatment group. At various points during the maturation process, the location of the meiotic spindle was determined after the 488 signal was amplified and as a result increased signal became apparent in the zona, due to non-specific antibody binding.

Statistical analysis

GV position and measurements along with MI spindle size were subjected to one-way analysis of variance followed by Bonferroni's multiple comparison post hoc-test to determine the significance of each treatment group using Prism 4 (GraphPad Software, Inc.). Calculated values are shown $\pm$ SEM with a significance level of $P<0.05$ being considered significant.

Results

Relationship between TZP patterning and spindle position during *in vivo* and *in vitro* oocyte maturation

To first establish the native organization of TZPs in living COCs, ovaries from eCG-primed mice were expressed into collection medium containing 10 μ g/ml DiO₁₆(3) to label plasma membranes (Fig. 1). As shown in Fig. 1A, a 20 μ m projection of a representative COC ($n=5$), cumulus cells attached to the zona pellucida emit 8–10 TZPs from each cell. TZPs are of two kinds, made primarily from actin (A-TZPs) or microtubules (MT-TZPs), and either extend horizontally into the zona (green) or project perpendicular to the oocyte plasma membrane (blue). Freshly isolated and fixed COCs processed for detection of f-actin using rhodamine phalloidin show foci of f-actin at the outer edge of the zona pellucida (Fig. 1B). The zona is occupied by a dense network of TZPs oriented at various angles relative to the brightly stained oocyte cortex. Thus, individual cumulus cells in intact COCs emit multiple actin-rich projections that appear to anchor the cell body to the zona surface and oolemma. The acute lability of these TZPs next prompted

Fig. 1 Comparison of TZP organization in living or fixed oocytes. **A** Confocal image of vitally stained cumulus oocyte interface [DiO16 (3)] shows TZPs extending horizontally (green) or vertically (blue) TZPs relative to the surface of the zona pellucida; projections were pseudocolored using image processing. In contrast, confocal images of fixed oocytes **B** stained with rhodamine phalloidin (red) shows predominantly vertically-oriented TZPs at the oocyte-cumulus cell interface. Arrows depict individual TZPs. Scale bar=10 μ m

an analysis of naturally and artificially matured mouse oocytes given known developmental deficiencies in IVM mouse oocytes.

TZP integrity was determined after delivering an ovulatory stimulus of hCG. Digital imaging was used to ascertain the frequency and distribution of TZPs based upon fluorescence intensity of actin in COCs fixed at 0 h, 2 h, 4 h, or 6 h post hCG (Fig. 2). At time 0, compact COCs exhibited a uniform density of A-TZPs throughout the zona except for an increase detected overlying the GV. Linescan optical density profiles for actin labeling confirmed TZP abundance at the site of GV localization relative to the remaining oocyte cortex in 18 of 20 COCs examined. At 2 h post hCG ($n=10$), no significant changes in TZP density were noted. These patterns changed dramatically between 4 and 6 h post hCG. At 4 h, A-TZPs were enriched where the spindle is anchored at the oocyte cortex ($n=10$). Moreover, MT-TZPs become apparent in total linescans at 4 and 6 h post hCG and the spindle retains cortical localization through 6 h post hCG ($n=10$). Thus, under *in vivo* conditions, TZPs are retained and remodeled in approximation to the cortically positioned GV and forming spindle. We next determined the effect of IVM conditions on TZP integrity and GV positioning.

COCs were cultured under conditions that either promote cumulus expansion, by adding hormone supplement-

tation as well as the addition of FBS (IVM+), or not (IVMb) [8, 39]. In IVMb (basal conditions, $n=30$), oocytes undergo GVBD within 2 h and chromosome condensation proceeds within a centrally positioned GV (Fig. 3A). By 4 h, the central spindle acquires definitive poles and migrates back to the cortex at 6 h (Fig. 3B and C). IVMb is characterized by a progressive decrease in TZP density at 2 and 4 h and by 6 h they are undetectable (Fig. 3C). Interestingly, TZPs were not apparent in juxtaposition to the GV or spindle between 2, 4 or 6 h in IVMb (bottom Fig. 3A, B, C) in sharp contrast to oocytes matured in IVM+ ($n=30$; Fig. 3D–F). IVM+ conditions resulted in an overall increase and persistence of A-TZPs. Moreover, the MI spindle retains a cortical position between 2–6 h of culture (Fig. 3D and E) and while few MT-TZPs are evident at 6 h in IVM+ numerous A-TZPs persist overlying the MI spindle (Fig. 3F). Thus, conditions supporting cumulus expansion (IVM+) result in TZP retention and cortical spindle positioning similar to IVO conditions but distinct from IVMb conditions. That IVMb results in loss of TZP integrity is further evidenced by the appearance of numerous f-actin foci at the outer margin of the zona (see Fig. 3; 4 and 6 h, IVMb). To better assess GV behavior under IVMb and IVM+ conditions, we next evaluated GV positioning using time-lapse digital microscopy.

Imaging of GV positioning during meiotic progression

GV positioning was established using differential interference contrast (DIC) timelapse microscopy between 0 and 4 h (GVBD), 4–6 h, (M1 spindle formation and 10–12 h (PB extrusion). Timing of these events was relative to the time of COC isolation and COC culture in IVMb and IVM+. Figure 4 illustrates the two patterns of GV positioning observed in living COCs ($n=64$). In one case, a central GV is observed that remains in this location throughout GVBD and M1 spindle assembly (Fig. 4A and B); all oocytes in this category exhibited spindle migration to the cortex where first PB extrusion occurred and this behavior was typically found in IVMb COCs (73%, $n=34$). In IVM+ COCs (Fig. 4C and D) the most commonly observed behavior was characterized by retention of cortically positioned GVs where GVBD, M1 spindle assembly, and PB extrusion took place at the original site of the GV attachment. Thus, conditions that promote retention of TZPs stabilized cortical GV positioning (IVM+) whereas those that compromise TZP integrity are more likely to result in central displacement of the GV with subsequent spindle migration to the cortex (IVMb). It was also noticed that the size of the GV prior to GVBD may have been altered depending on the original location of the GV. To this end, measurements of GV diameter were made from digital images of living oocytes (Fig. 5) that showed either

Fig. 2 Persistence of TZPs during oocyte maturation *in vivo*. COCs were isolated at T=0, 2, 4 and 6 h following hCG and stained for detection of f-actin (red), microtubules (green) and DNA (blue) as described in text. Channel specific linescans below each image correspond to intrazonal optical density profile for TZPs and solid

bar denotes position of GV or spindle. Note actin TZP density remains elevated and concentrated near the GV (0, 2, and 4 h) and by 4 h post microtubule-rich TZPs become apparent. Insets show enlarged area of zona containing TZPs. Scalebar=10 μm

Fig. 3 TZP retraction and GV centration occur during *in vitro* maturation. Confocal projections of COCs at 2(A and D), 4(B and E) and 6(C and F) hours of *in vitro* maturation in IVMb (A–C) or IVM+medium (D–F). Linescans below each image denote position of GV or spindle with a horizontal bar. In IVMb medium, GV are centrally located ($n=30$) and low levels of actin TZPs are evident (A–C; and compare to Fig. 2). In contrast, oocytes matured in IVM+medium display cortical GV or spindles ($n=30$) and maintenance of actin TZPs (D–E). Insets demonstrates TZPs. Scalebar=10 μm

Fig. 4 GV positioning during *in vitro* maturation in living oocytes. DIC time-lapse imaging was used to map GV position during *in vitro* maturation in either IVMb or IVM+ media. Left panel (A and C) shows GV position (black circles) at the start of imaging ($n=20\text{--}24$ oocytes in each treatment group) and GV position is shown just prior to GVBD on the right for the same oocyte. Central (A,B) or cortical (C,D) patterns were observed in IVMb or IVM+ respectively. Scale bar (A and B) represents $30\text{ }\mu\text{m}$. Scale bar (C and D) represents $20\text{ }\mu\text{m}$

persistent central or cortical GVs that resulted in GVBD at either of these locations.

Oocytes containing GVs that were consistently anchored to the cortex exhibited a mean diameter of $18\text{ }\mu\text{m}$ (± 0.34) whereas those retaining a central location had a mean diameter of $28\text{ }\mu\text{m}$ (± 0.78). Interestingly, denuded oocytes (C-) that were subsequently cultured under either IVM+ ($27\text{ }\mu\text{m} \pm 0.52$) or IVMb ($27\text{ }\mu\text{m} \pm 0.53$) conditions consistently contained central GVs with diameter indistinguishable from IVMb COCs. Thus, oocytes with central

GVs undergo GV enlargement prior to GVBD consistent with a dependence on cell contact for maintaining both the size and location of the GV.

Actin regulates TZP integrity and GV positioning

To more directly test the role of A-TZPs in GV positioning, as suggested here and elsewhere [1, 34], we undertook a series of experiments using the f-actin depolymerizing agent Latrunculin B (LatB). Intact COCs were cultured in IVMb or IVM+ medium containing $0.6\text{ }\mu\text{M}$ (LatB). We first asked whether LatB-induced TZP severing would affect GV and spindle positioning as shown previously in mouse oocytes treated with cytochalasin B [26, 49]. We also wanted to know whether TZP disruption was reversible, and if so, would spindles reposition and reattach to the oocyte cortex after LatB removal from culture medium. Confocal image Z-stacks on individual oocytes were used to measure TZP integrity, spindle size, spindle to cortex distance, and inter-chromosome spacing. COCs cultured for 6 h in IVM+ without LatB exhibited A-TZPs in the zona and MI spindles located at the cortex (Fig. 6A); the mean distance between the spindle pole and cortex of controls was $3.1\text{ }\mu\text{m}$ (± 0.52 , $n=5$) and this was increased in IVMb COCs ($4.7 \pm 0.47\text{ }\mu\text{m}$, $n=5$). After a 6-hour exposure to LatB, all COCs lacked intact A-TZPs and displayed numerous phalloidin labeled foci indicative of TZP disruption.

Fig. 5 GV enlargement accompanies centration during IVM. Mean GV diameters were calculated for 64 COCs during IVM and are plotted as a function of IVM conditions (IVMb, IVM+) and as to their original GV location (stayed in cortex; migrated from cortex to center; or stayed in center. Note that a fixed cortical position occurs only under IVM+ conditions and GVs remain smaller in diameter whereas in all other cases, GV centration results in larger GVs. Asterisks (*) denote statistical significance between treatment groups at $P < 0.01$

tion. Moreover, all LatB exposed COCs contained oocytes with enlarged and centrally located MI spindles (Fig. 6C). The mean distance between the cortex and proximate spindle pole was 18 μm in both groups (± 2.0 , $n=5$, IVM+; ± 3.3 , $n=5$ for IVMb). Thus, constant exposure to LatB from 0 to 6 h permits meiotic progression and results in central spindle assembly independent of culture conditions.

To test for reversibility, COCs were washed free of drug and oocytes were allowed to mature an additional 10 h in the absence of LatB. In control COCs, A- and MT-TZPs remained intact throughout the course of maturation as mentioned earlier for IVM+ conditions (Fig. 6B). However, oocytes exposed to LatB were unable to re-establish TZPs following drug removal (Fig. 6D). Oocytes from all treatment groups underwent spindle migration to the cortex and first PB extrusion ($n=35/35$) showing that upon recovery of actin function, meiotic progression was unimpaired.

Spindle hypertrophy has previously been shown to result from IVM in mouse oocytes [8, 38] but the impact of this on chromosome spacing and TZP integrity has not yet been investigated. Towards this end, inter-chromosomal spacing of bivalents was determined from z-axis data sets collected from 6 h cultures of cumulus-enclosed or naked oocytes (C+, C-), COCs cultured in either the presence or absence of LatB, or COCs matured under IVO, IVMb, or IVM+

conditions (Fig. 7). A nearly 2-fold increase in bivalent spacing in naked oocytes (C-, $> 2.0 \mu\text{m}$) was observed relative to conditions in which cumulus integrity was maintained (IVO, 0.65 μm ; IVM+, 1.2 μm ; IVMb, 1.1 μm). All conditions in which LatB was used resulted in a statistically significant increase bivalent spacing; interestingly, LatB resulted in inter-chromosome spacing distances comparable to those observed in naked oocytes (C-). Since the naked oocytes used in these studies were obtained by removing cumulus cells from COCs, these findings emphasize the importance of TZP integrity, whether mechanically or pharmacologically disrupted, on the regulation of spindle size and chromosome spacing. Given the impact of actin integrity on spindle formation, we next sought to test the developmental competence of oocytes matured under various conditions.

TZP integrity during IVM affects embryo developmental competence

We first determined the effect of TZP integrity on the rate of cleavage and development to the blastocyst stage using parthenogenetically activated (SrCl₂ +/- cytochalasin B) oocytes [9, 25, 37]. Diploid parthenotes cultured to blastocyst stage were used to assess the maternal contribu-

Fig. 6 Latrunculin B severs TZPs but does not impair meiotic progression. COCs incubated in control (IVM+) medium (A,B) or medium supplemented with Latrunculin B for 6 h were fixed at either 6 (A,C) or 16 (B,D) hours and analyzed by confocal microscopy. Representative images are shown from 10 oocytes per treatment group and time point and in D, the effects of washing out Latrunculin show that the ability to extrude the first polar body has not been impaired due to prior drug exposure. Scalebar=25 μm and magnification is same for A/C and B/D

Fig. 7 Cumulus cell contact and actin integrity influences inter-chromosomal spacing in MI. 10–15 Distances between adjacent chromosomes were calculated from confocal z-stacks of MI oocytes matured under IVO or IVM conditions as described before. Furthermore, oocytes were subdivided a priori with respect to whether cumulus cells were retained (C+) or not (C-). At least 10 oocytes from each group were measured (~ 10 chromosomes/group) and inter-

chromosome spacing is plotted in microns. Note that Coocytes exhibit a 2–3 fold increase in chromosome spacing and that latrunculin B treatment caused an increase independent of maturation conditions or cumulus cell attachment. *, Denotes statistical significance compared to all groups, $P < 0.01$, **, Denotes statistical significance compared to matched LatB group, $P < 0.01$

tion to embryonic potential. Parthenotes made from IVMb, IVM+, IVO, IVM+denuded oocytes and IVM+LatB treated oocytes were assayed for blastomere number at 24-hour intervals up to 96 h (Table 1). By 24-hours, 70–80% of parthenotes in IVMb, IVM+ and IVO treatment groups reached the 2-cell stage whereas denuded and LatB embryos exhibited a slight delay in completion of the first cell cycle (Table 1). IVM+ and IVO parthenotes readily progressed to the 4-cell stage by 48-hours, were compacted by 72-hours, and developed into blastocysts by 96 h; blastocyst efficiency was 48% in the IVO group and 20.8% for IVM+parthenotes. While 22% of IVMb embryos were able to compact, development to this stage was delayed and few of these embryos went on to form blastocysts (top line, Table 1). Importantly, a similar pattern of developmental delay and failure to compact and cavitate was observed for embryos made from denuded oocytes or those matured in the presence of LatB (lines 4 and 5). In addition to this effect of TZP integrity on developmental potential, a further analysis of cell numbers in blastocysts suggested more subtle defects

as well. Few blastocysts were obtained from the LatB ($n=1$) and denuded groups ($n=1$) but assessment of both total cell and inner cell mass (ICM) numbers revealed comparable total cells (denuded=48 cells; LatB-IVM+=54 cells) and of these only 8.3% (denuded) or 9.3% (LatB-IVM+) were clearly identifiable as inner cells (ICM). Importantly, while IVO embryos displayed mean cell numbers of 57 ± 4.0 ($n=14$), with 33% of these assigned to the ICM, IVM+ ($n=11$, 45 ± 4.7 , 21% ICM) and IVMb ($n=5$, 51 ± 4.2 , 10.6% ICM) had comparable total cell numbers in blastocysts but reduced allocations to the ICM. Thus, both rates of development and blastocyst quality are adversely affected under conditions that compromise the integrity of TZPs in oocytes while they are undergoing maturation.

Discussion

The properties of *in vitro* matured mouse oocytes are distinct from those of *in vivo* matured counterparts with

Table 1 Developmental competence of *in vivo* and *in vitro* matured oocytes

Treatment	24h	48h		72h		96h	
	%2-Cell	%2-Cell	%4-Cell	%4-Cell	%Compacted	%Compacted	%Blastocyst
IVMb ($n=210$)	69.8 (± 1.7)	40.7 (± 1.7)*	29.6 (± 9.7)**	28.0 (± 1.1)**	2.9 (± 4.5)*	21.7 (± 4.3)*	4.5 (± 5.9)*
IVM+ ($n=248$)	81.6 (± 3.0)	18.4 (± 2.4)	46.6 (± 10.2)	6.2 (± 1.4)	51.8 (± 8.5)	61.9 (± 6.0)	20.8 (± 5.6)**
IVO ($n=316$)	77.3 (± 6.1)	7.2 (± 9.6)	59.7 (± 9.6)	12.7 (± 0.1)	53.9 (± 1.5)	52.0 (± 3.5)	48.0 (± 4.4)
IVM+Denuded ($n=55$)	64.0 (± 5.4)	20.9 (± 10.6)	40.5 (± 14.8)	17.5 (± 8.9)	14.3 (± 2.9)**	7.4 (± 4.0)*	1.7 (± 1.7)***
IVM+LatB ($n=43$)	51.2 (± 22.6)	22.2 (± 6.4)	44.0 (± 29.7)	21.0 (± 6.7)	12.4 (± 1.9)**	11.5 (± 4.3)*	2.65 (± 2.65)***

Values are mean $\pm$ SEM

*Indicates significance of $P \leq 0.001$ compared to IVO control

**Indicates significance of $P \leq 0.01$ compared to IVO control

***Indicates significance of $P \leq 0.05$ compared to IVO control

respect to meiotic cell cycle progression, meiotic spindle assembly, polar body extrusion, and developmental competence [8, 46]. The present studies focused on cumulus cell TZP-based contact with the oocyte with respect to the topography of meiotic progression and how this may influence the acquisition of developmental competence. Our major findings are that (1) the position of the GV is fixed at the oocyte cortex determining the site of meiotic spindle assembly during maturation *in vivo*; (2) COCs undergo rapid TZP retraction and centripetal displacement of the GV upon release from follicles; (3) during IVM, hypertrophied meiotic spindles form in the oocyte center that increases inter-chromosome spacing prior to actin-dependent migration of the spindle back to the oocyte cortex; (4) cortical positioning of the GV and spindle depends on the integrity of TZPs and optimizes developmental potential. These findings highlight the importance of structural integrity within the COC with regard to the production of developmentally competent oocytes and illustrate the importance of maintaining cumulus cell contact with the oocyte during IVM. Our results bear directly on the mechanisms that are believed to integrate oocyte and cumulus cell physiology and draw attention to the need to optimize IVM for clinical applications.

Significant advances in our understanding of the signaling mechanisms deployed during ovulation in rodents have recently been made [13, 14, 31, 35]. The facts that complex signaling cascades are elicited within hours of the LH surge from mural granulosa to cumulus cells, and that reciprocal signals of oocyte origin modulate cumulus expansion serve to re-emphasize the importance of oocyte-granulosa interactions during ovulation. As an obvious substrate for cell interactions, it is somewhat surprising that so little is known about TZP remodeling especially with respect to the acquisition of developmental competence after IVM. TZPs are ubiquitous amongst mammals [1–3] and have been widely implicated in paracrine signaling within the COC [12, 17, 19, 20, 45]. However, the fate and function of TZPs during oocyte maturation under *in vivo* or *in vitro* conditions has evaded definition for signaling mechanisms other than those of paracrine or junctional varieties [15, 22, 29, 32].

Here, we document rapid loss of TZP integrity when COCs are removed from the follicle and placed into basal medium that can be partially prevented if COCs are maintained in an enriched medium. Moreover, IVM affects GV positioning, size and the spacing between homologous bivalents on the metaphase-1 spindle. To our knowledge, this is the first study on COCs retrieved after an induced LH surge linking the structural integrity of TZPs with the cortical localization of the GV during meiotic resumption and progression. Given the demands for metabolic cooperation and/or contact-based signaling that are likely to occur within

the COC, we suggest that the persistence of TZP contacts seen *in vivo* is required for metabolic loading of the oocyte and establishing a structural asymmetry that economizes the metabolic demands imposed by spindle morphogenesis [2, 14]. This idea receives support from several recent studies. Maintaining a structural interaction during the earliest stages of meiotic resumption between the GV and oocyte cortex is likely to impact spindle morphogenesis at later stages of meiotic maturation as well as over-all developmental competence of the egg. *In vivo* matured oocytes assemble spindles within a contracted nuclear matrix comprised of lamin B that was proposed to limit MT assembly and MTOC involvement thus yielding diminutive spindles [39] compared to their *in vitro* matured counter. The notion of conserving resources at the protein level has also been studied in relation to spindle and/or polar body size. Smaller spindles and first polar bodies typify *in vivo* matured mouse oocytes and these findings were consistent with limited recruitment and incorporation of γ -tubulin into the forming spindle [8]. Thus, under physiological conditions, the ability to minimize spindle size ensures chromosome approximation with attendant proximity to the oocyte cortex. To determine whether TZP integrity actively maintains this cortical asymmetry in the mouse oocyte was a goal for these studies and is shown to confer selective advantages in terms of developmental competence.

Actin inhibitors cause meiotic spindles to form in a central location [11, 26]. LatB is expected to affect oocyte cortical actin which plays a role in asymmetric cytokinesis [7]. While the present study addresses the role of TZPs in stabilizing the oocyte cortex by pharmacologically depolymerizing these cellular extensions and their contacts with the oolemma, we cannot exclude direct effects on the oocyte cytoskeleton. We extend these findings by showing that the actin cytoskeleton stabilizes the oocyte cortex because under IVO or IVM+conditions TZP integrity is coincident with cortical GV positioning. Moreover, LatB induced a reversible change in GV position from a cortical to central location and targeted TZPs due to their f-actin content, suggesting a direct link between TZP integrity and oocyte polarity has been demonstrated. Hormone stimulation of the COC is likely to regulate TZP integrity [12] and gonadotropin supplementation to culture media enhanced cortical actin assembly and stabilized cortical GV positioning in hamster COCs [33]. Recently, several studies have addressed the mechanisms that regulate GV position and its relationship to spindle morphogenesis.

The classical studies of Longo and Chen first implicated actin in spindle positioning and asymmetric cytokinesis during polar body extrusion in mice [26]. Alexandre and colleagues [4] later showed that the tendency of the mouse oocyte GV to assume a central position was due to an actin-based contraction normally held in check by what they

termed a “centrifugal displacement property” (CDP) effected by a cortical microtubule complex. Notably, these studies were conducted on naked oocytes that therefore lacked cumulus cell contact. More recently, the actin binding protein formin-2 has been implicated in the process of GV anchoring to the cortex as deletion of this actin binding protein results in central spindle assembly and failure of the meiosis-1 spindle to migrate back to the cortex [16, 24]. Formin-2 deficits are linked directly to gross aneuploidy in embryos that fail to develop normally. It is interesting that the present studies have uncovered an association between TZP integrity, spindle position, and developmental failure, as this would be consistent with the suspected origins of a major cause of embryonic failure in mammals. Thus, while naked oocytes provide an experimentally tractable murine model system [48], designing experiments to optimize oocyte quality will require treatments that preserve the integrity of cumulus cell interactions with the oocyte.

Relevant to this point is the present demonstration that media composition predictably influences TZP integrity in cultured COCs and TZP integrity improves oocyte quality as measured by developmental performance following oocyte activation (Table 1). Specifically, growth factor-rich IVM+medium (containing rhFSH, FBS and ITS), supported development to the blastocyst stage comparable in incidence to that observed for oocytes matured *in vivo*. Importantly, early cleavage rates and compaction followed parallel courses when IVM+ and IVO oocytes were cultured after activation. In contrast, IVMb or IVM+/LatB oocytes exhibited deficiencies in cleavage, compaction and blastocyst potential. While it is tempting to speculate that the differences in TZP integrity underlie these distinctions in oocyte quality, the mechanistic forces that confer developmental potential on oocytes remain ill-defined. As noted earlier, these likely include junctional, paracrine, or other signaling pathways such as those recently described for Src kinases [27]. These findings emphasize the need to define culture conditions that maintain TZP integrity as a determinant of oocyte quality.

Collectively our studies reveal an important level of organization within intact COCs that bears directly on the control of oocyte quality and the use of assisted reproductive technologies. Maintaining a physical link between cumulus cells and the oocyte during and beyond ovulation has long been acknowledged and the importance of this interaction on overall oocyte organization should be a target for ongoing imperatives in the field of fertility preservation and other evolving assisted reproductive technologies such as IVM and oocyte cryopreservation.

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