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**Brief Report**

# Granulosa Cells Alone, Without Theca Cells, Can Mediate LH-induced Oocyte Meiotic Resumption

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## Abstract

Signaling in the granulosa cells of mammalian ovarian follicles is necessary for maintaining prophase arrest in the oocyte and for mediating the resumption of meiosis in response to luteinizing hormone (LH). However, the follicle also includes an outer layer of theca cells, some of which express receptors for LH. To investigate whether theca cells are required for maintaining meiotic arrest and reinitiating meiosis in response to LH, we mechanically separated the granulosa cells and oocyte from the theca and basal lamina. This was accomplished by cutting a slit in the outer surface of isolated follicles such that the mural granulosa cells and cumulus-oocyte complex were extruded from the theca shell, forming a lawn of cells on an organotypic membrane. The remnant of theca cells and basal lamina was then removed. The separation of the granulosa cells from the theca cells and basal lamina was demonstrated by immunofluorescence localization of endomucin (blood vessels of the theca) and laminin gamma (basal lamina). Cells comprising these granulosa cell-oocyte complexes expressed LH receptors and were connected by gap junctions. Oocytes within these granulosa cell complexes maintained meiotic arrest and resumed meiosis in response to LH, showing that the granulosa cells alone, without theca cells, transduce these signals. This semi-intact and mostly 2-dimensional preparation could facilitate imaging studies of follicle physiology.

**Key Words:** ovarian follicle, luteinizing hormone, theca, granulosa cells, meiotic resumption

**Abbreviations:** cGMP, cyclic guanosine monophosphate; HA, hemagglutinin; LH, luteinizing hormone; LHR, LH receptor.

Within a mammalian preovulatory follicle, the oocyte is surrounded by approximately 10 layers of granulosa cells connected to each other and to the oocyte by gap junctions. The approximately 3 layers of granulosa cell bodies closest to the oocyte, called cumulus cells, are partially separated from the outer layers of mural granulosa cells by an antral space. Outside of the granulosa compartment, separated by a basal lamina, are several layers of theca cells. The theca includes steroidogenic cells, smooth muscle cells, immune cells, and fibroblasts, as well as an extensive network of blood vessels (1).

The granulosa cells of preovulatory follicles of mice and rats, as well as several other mammalian species, maintain the oocyte in meiotic prophase arrest by providing cyclic guanosine monophosphate (cGMP) that diffuses into the oocyte through gap junctions, where it inhibits meiotic progression by inhibiting the breakdown of cyclic adenosine monophosphate (2-4). The preovulatory surge of luteinizing hormone (LH) then acts on its receptors located in a subset of the mural granulosa cells adjacent to the basal lamina (5) to cause meiotic resumption and ovulation. LH activation of its receptor leads to inactivation of the natriuretic peptide receptor 2 guanylyl cyclase, lowering cGMP in the granulosa compartment, and by way of gap junctions, cGMP subsequently decreases in the oocyte (4, 6, 7). This cGMP decrease releases meiotic arrest, with nuclear envelope breakdown occurring in the oocyte a few hours after a mouse follicle is exposed to LH.

Although the LH receptors (LHRs) in the mural granulosa cells appear to be solely responsible for mediating the reinitiation of meiosis, many of the cells in the theca also express LHRs (5). As with many aspects of theca biology (1), the possible role of theca cells in responding to LH surge remains incompletely understood. Bovine theca cells undergo changes in transcription in response to the LH surge (8), but the physiological consequences are unknown. Here, we investigate the possible function of the theca cells in LH signaling of meiotic resumption by physically separating granulosa cell-oocyte complexes from the theca cells of fully grown mouse follicles.

## Materials and Methods

### Mice

Protocols covering the maintenance and experimental use of mice were approved by the Institutional Animal Care Committee at the University of Connecticut Health Center. The studies were performed using wild-type C57BL/6J mice (originally obtained from The Jackson Laboratory, Bar Harbor, ME), B6SJLF1/J mice (The Jackson Laboratory), or C57BL/6J mice with a hemagglutinin (HA) epitope tag on the endogenous LHR (HA-LHR) (5). The HA-LHR mice are available from the Mutant Mouse Resource and Research

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Centers repository at The Jackson Laboratory (RRID: MMRRC_071301-JAX).

### Culture of Isolated Follicles and Granulosa Cell-Oocyte Complexes

Fully grown follicles, 320 to 400 µm in diameter, were dissected from ovaries of 23- to 26-day-old mice with no previous hormonal treatment. The follicles were cultured on optically clear organotypic membranes (Millicell-CM low height culture plate inserts an ~40-µm-thick membrane with 0.4-µm pores; MilliporeSigma, St. Louis, MO; PICMORG50) (9). The inserts were placed in 35-mm Petri dishes containing 1.6 mL of MEMα without phenol red (Gibco 41061-029), supplemented with 75 µg/mL penicillin G (Sigma P7794), 50 µg/mL streptomycin (Sigma S6501), 3 mg/mL BSA (MP Biomedicals, 103700), and a mixture of 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium (Sigma I1884). FSH (from ovine pituitary, National Hormone and Pituitary Program, Torrance, CA) was included in the medium (0.3 nM) to stimulate LHR expression. LHR expression occurs only in fully grown follicles, beginning approximately 12 hours after FSH receptor stimulation and continuing to increase up to 48 hours (5).

Twelve follicles were placed on each Millicell, using a mouth pipet. After a few hours, the follicles had flattened on the Millicell such that the oocytes with their prophase-arrested nuclei were visible (9). Any follicles in which the oocytes had resumed meiosis spontaneously were discarded. Follicles were then cut using two 30-gauge needles and cultured overnight to generate open follicles (see results). Four to 5 hours after removing theca remnants from the open follicles, LH (from ovine pituitary, National Hormone and Pituitary Program) was applied at a concentration of 10 or 300 nM, by transferring the Millicell to a new dish. A timeline of these procedures is shown in Fig. 1A. As controls, Millicells were transferred in parallel to dishes without LH. If these controls showed >25% spontaneous nuclear envelope breakdown at 5 hours, the experiment was excluded from the analysis of the time course of LH-induced nuclear envelope breakdown.

### Imaging of Granulosa Cell-oocyte Complex Formation and LH-induced Nuclear Envelope Breakdown

For visualizing the formation of the granulosa cell-oocyte complexes and for scoring of the time course of LH-induced nuclear envelope breakdown, follicles on the Millicell membranes were imaged using a Zeiss Standard upright microscope with a 20×/0.4 NA LD Acroplan objective and photographed using an iPhone camera (model SE, Apple, Cupertino CA) and a LabCam Microscope adapter (iDu Optics, New York, NY). For documenting the appearance of the oocyte and cumulus cells before and after applying LH, granulosa cell-oocyte complexes on the Millicell membrane were photographed using a Zeiss Axioskop upright microscope with a 10×/0.3 NA PlanNeofluar objective and a Zeiss Axiocam 208 camera.

### Immunofluorescence Labeling and Confocal Microscopy

For fixation and immunofluorescence labeling, Millicells with open follicles or granulosa cell-oocyte complexes were rinsed by exchanging the culture media under the membrane with 1× PBS. Millicells were then transferred to 35-mm dishes containing 4% paraformaldehyde (157-8, Electron Microscopy Sciences, Hatfield, PA) in PBS. The dishes were kept at 4 °C for 20 minutes, then rinsed 3 times with 1× PBS. Millicells were placed in 0.2% Triton-X 100 in PBS for 10 to 15 minutes, rinsed again with PBS, then incubated in 1 mL of blocking buffer (5% normal goat serum in 1% BSA in PBS) for 30 minutes. Millicells were then incubated overnight at 4 °C in 0.5 mL of diluted antibody, in 35-mm dishes wrapped with parafilm. The following antibodies were used: anti-endomucin (rat, 1:200, Santa Cruz Biotechnology sc65495, RRID:AB_2100037), anti-laminin gamma (rat, 1:100, Santa Cruz Biotechnology sc65643, RRID:AB_1123687), anti-HA (rabbit, 1:500, Cell Signaling Technology 3724, RRID: AB_1549585), and anti-connexin 43 (rabbit, 1:100 Cell Signaling Technology 3512, RRID:AB_2294590).

After rinsing in PBS, Millicells were incubated in 1% BSA in PBS for 10 minutes, then in 1 mL of secondary antibody diluted 1:500 to 1:1000 for 30 minutes, followed by a final rinse in PBS. Secondary antibodies were from ThermoFisher Scientific: Alexa fluor 488 goat-anti-rat (A48262, RRID: AB_2896330), Alexa fluor 488 goat-anti-rabbit (A11034, RRID:AB_2576217), and Alexa fluor 647 goat-anti-rabbit (A32733, RRID:AB_2633282).

For fluorescence imaging, Millicells were transferred to a 35-mm glass-bottom dish (Mattek, Ashland, MA; P35GINV-1.5-20-C) containing 1 mL of PBS. Fluorescence and scanning transmission images were collected using a laser scanning confocal system (LSM 980, Zeiss) on an inverted microscope, with a 10×, 0.5 NA Fluar objective.

## Results

### Generation of Granulosa Cell-oocyte Complexes From Antral Follicles

When placed in culture on Millicell membranes, the originally spherical ovarian follicles flattened to form disks ~500 µm in diameter, allowing visualization of the oocyte with its prophase-arrested nucleus and nucleolus (9). Approximately 3 hours after isolating the follicles, we cut a slit in the follicle surface, using two 30-gauge needles, one to hold the follicle in place and a second to make a small cut (Fig. 1A and 1B). This allowed the granulosa cells and oocyte to extrude, forming a lawn of granulosa cells surrounding the prophase-arrested oocyte (Fig. 1C). The extrusion of cells began immediately after cutting the follicle, and the lawn continued to expand over time. Approximately 80% of these "open follicles" maintained meiotic arrest in the oocyte for at least 20 hours. Approximately 20 hours after making the initial cut to release the granulosa cells and oocyte, we cut away the theca remnant (Fig. 1A and 1D) and removed it, leaving a lawn of granulosa cells, ~300 to 1000 µm in diameter, surrounding the oocyte (Fig. 1D). Approximately 60% of these "granulosa cell-oocyte complexes" maintained meiotic arrest in the oocyte when observed 4 hours after removing the theca remnant.

### Granulosa Cell-oocyte Complexes Are Theca Free

In open follicles, theca and basal lamina protein markers remained associated with the theca remnant, and not with the extruded granulosa cells. Little or no endomucin, a protein that is present in the endothelial cells of the theca layer (10), was detected in the extruded cells of open follicles (Fig. 2A).

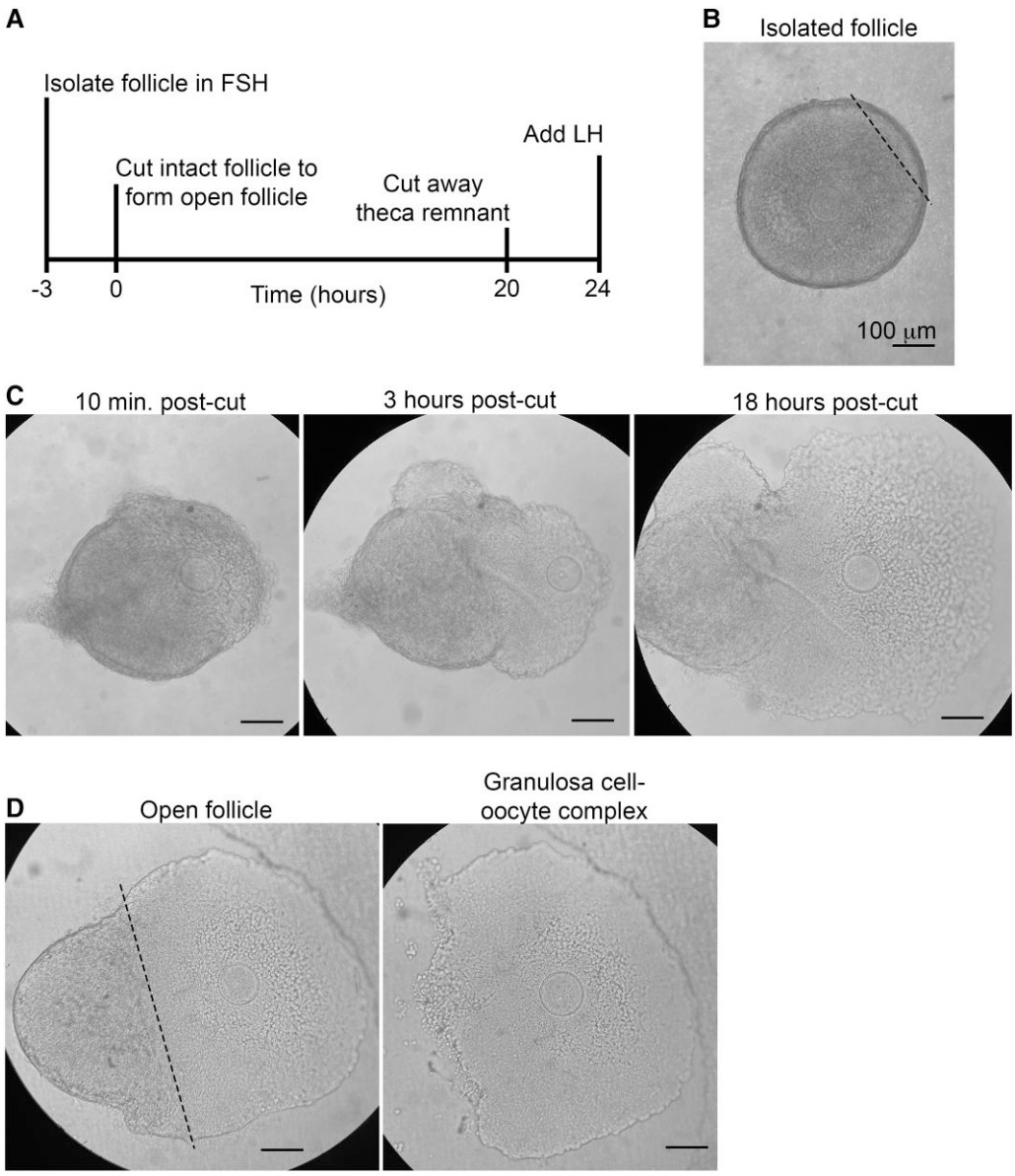


**Figure 1.** Formation of open follicles and granulosa cell-oocyte complexes. (A) Timeline of experimental procedures. (B) An intact follicle. The dashed line indicates the cut made to allow extrusion of the granulosa cells to form an open follicle. (C) A follicle at 10 minutes, 3 hours, and 18 hours after cutting to allow granulosa cell and oocyte extrusion. (D) An open follicle before and after removing the theca. The dashed line indicates the cut made to remove the theca remnant to form a granulosa cell-oocyte complex. All scale bars are 100 μm.

Likewise, laminin gamma, a marker for the basal lamina (11), remained associated with the theca cells, with little or none associated with the extruded granulosa cell-oocyte complexes (Fig. 2B).

## Localization of LH Receptors and Gap Junctions in Granulosa Cell-oocyte Complexes

To examine the localization of LHRs, granulosa cell-oocyte complexes were generated using mice with an HA epitope tag on the endogenous LHR, for immunofluorescence labeling with an HA tag antibody (5). As in intact follicles, LHRs were heterogeneously expressed in granulosa cells distant from the oocyte (Fig. 3A). Based on measurements from 4 images like that in Fig. 3A, the region containing LHRs occupied ~50% to 70% of the total area of the complex. The granulosa cells throughout the complex were connected by gap junctions, as indicated by immunofluorescence labeling of gap junction plaques using an antibody against connexin 43 (GJA1) (9) (Fig. 3B). This distribution is similar to that seen in intact follicles (9).

## LH Induces Nuclear Envelope Breakdown in Granulosa Cell-oocyte Complexes

To test the LH responsiveness of the granulosa cell-oocyte complexes, LH or control medium without LH was applied, and the cultures were observed hourly to score for the presence of an intact nuclear envelope and nucleolus in the oocyte. Almost all controls without LH maintained meiotic arrest over a 6-hour period (Fig. 4A and 4C). In contrast, 89% of oocytes in LH-treated granulosa cell-oocyte complexes underwent nuclear envelope breakdown ~2 to 5 hours after LH exposure (Fig. 4B and 4C), indicating the resumption of meiosis. This time course is similar to that of LH-induced nuclear envelope breakdown in intact isolated follicles (5, 12-14). These results

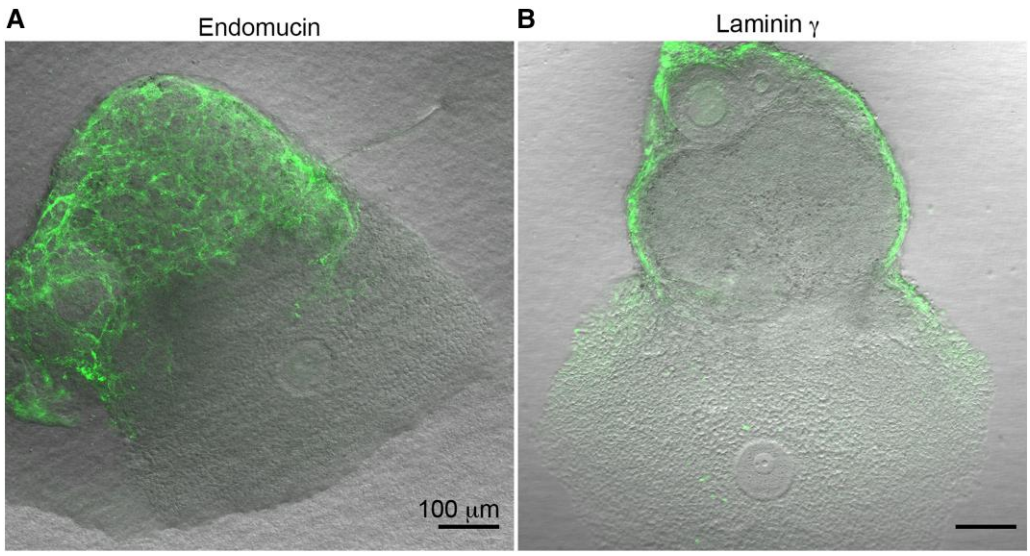


**Figure 2.** Immunofluorescence localization of theca and basal lamina markers, showing their separation from the granulosa cells in open follicles. (A) Transmitted light image and immunofluorescence of endomucin. (B) Transmitted light image and immunofluorescence of laminin gamma. Both scale bars are 100 μm.

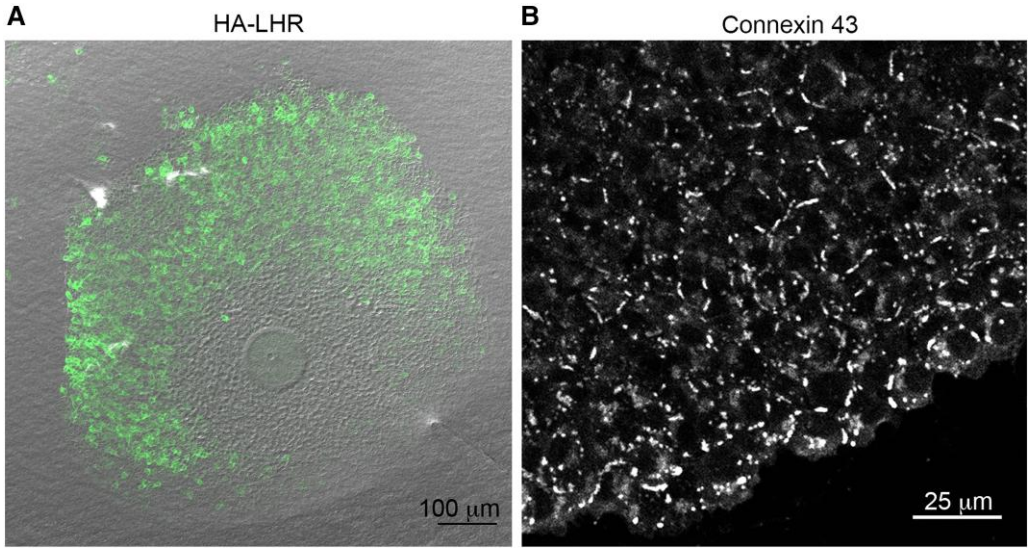


**Figure 3.** Immunofluorescence localization of HA-tagged LH receptor and connexin 43 (GJA1) in granulosa cell-oocyte complexes. (A) Transmitted light image and immunofluorescence of HA-tagged LH receptors. (B) Immunofluorescence of connexin 43.

showed that the granulosa cells alone, without theca cells, can transduce the LH signal that causes meiotic resumption.

In control complexes that were cultured for 6 hours without LH, the appearance of the ~3 layers of cell bodies closest to the oocyte, corresponding to the cumulus cells of an intact follicle, showed little or no change in 11/14 examples examined (Figs. 4A). In contrast, in 7/8 examples photographed at 6 hours after LH application, nuclear envelope breakdown was accompanied by the cumulus cells becoming rounder and less adherent to their neighbors (Fig. 4B), as occurs during cumulus expansion (15).

## Discussion

Our results show that the granulosa cells alone, without theca cells, can transduce the LH signal that reinitiates meiosis in the oocyte, at least in this in vitro system. These studies confirm and extend previous research that suggested this same conclusion (16). In previous experiments, granulosa-oocyte complexes were enzymatically isolated from ovaries of 12-day-old mice, removing almost all of the theca cells and basal lamina from the follicles, based on transmitted light observation. After a 10-day culture period in the presence of FSH, LH was found to induce nuclear envelope breakdown in oocytes within these complexes, although the kinetics were slower than reported in intact follicles (5, 12-14). LH also caused expansion of the cumulus cell mass. In the present study, we generated granulosa cell-oocyte complexes from follicles that grew to their full size in vivo, and demonstrated, using molecular markers, that the theca cells and basal lamina were removed. We showed that LH application to granulosa cell-oocyte complexes causes nuclear envelope breakdown

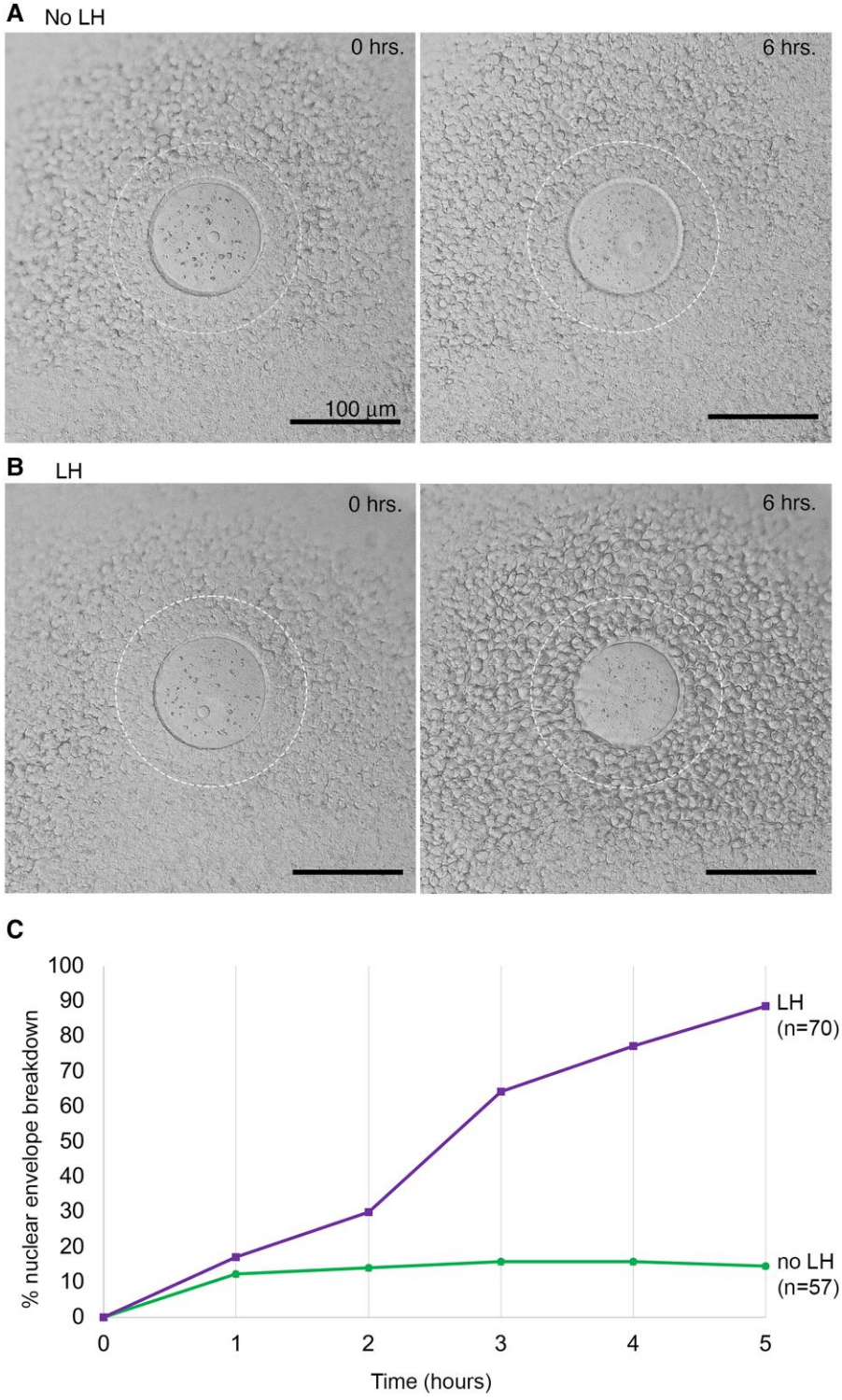


**Figure 4.** LH-induced resumption of meiosis in granulosa cell-oocyte complexes. (A) A granulosa cell-oocyte complex at 0 and 6 hours after applying a control solution, showing maintenance of meiotic arrest. (B) A granulosa cell-oocyte complex at 0 and 6 hours after applying LH, showing breakdown of the oocyte nuclear envelope and nucleolus, and rounder cumulus cells that are less adherent to their neighbors (right). Dashed circles depict the cumulus cell region. All scale bars are 100 µm. (C) Time course of nuclear envelope breakdown in granulosa cell-oocyte complexes after transfer of the Millicell to a dish containing LH or control media. n values indicate the total numbers of complexes analyzed for each condition. The graph combines the results of 6 experiments with either C57BL/6J or B6SJLF1/J mice, using either 10 or 300 nM LH. Similar results were obtained in each individual experiment. Nuclear envelope breakdown in a few of the controls without LH appeared to be due to mechanical disturbance during transfer of the Millicell.

to occur with kinetics similar to those in intact follicles, indicating that LHRs in the theca cells are not needed to transduce the signal that reinitiates meiosis. It remains to be determined whether the LHRs in the theca cells are required for other aspects of the follicle's response to the LH surge, including ovulation.

The granulosa cell-oocyte complexes preserve the gap junctions and LHRs that allow signaling from the granulosa cells to the oocyte in a mostly 2-dimensional structure. As in intact follicles (5, 17), there is a region of cells expressing LHRs and a region of cells closer to the oocyte that do not express LHRs (Fig. 3A). These 2 regions might correspond to the outer mural granulosa cells, where LHRs are present in an intact follicle, and a combination of the cumulus and inner mural granulosa cells, which lack LHRs in an intact follicle. The LHR-expressing region occupies ~50% to 70% of the total area of the granulosa cell-oocyte complex, consistent with previous measurements indicating that ~57% of the granulosa cells in an intact follicle are in the outer mural region where LHRs are expressed (5). Thus, although the LHR-expressing cells are rearranged after being extruded from the follicle, the fractional area that they occupy in the complex is similar to that occupied by outer mural cells in an intact follicle.

The sharp boundary seen between LHR-expressing and nonexpressing cells may be determined by a combination of inhibitory factors from the oocyte and interaction with the basal lamina. Signaling molecules from the oocyte, including GDF9 and BMP15, are thought to inhibit the expression of the LHRs in cumulus and inner mural cells (18, 19), and these may function in granulosa cell-oocyte complexes as well. Conversely, contact of the mural cells with the basal lamina acts together with FSH to induce LHR expression (18). Because the mural granulosa cells were in contact with the basal lamina and FSH together for more than 3 hours before the follicle was cut, these cells could have been determined to express LHRs while they were in contact with the basal lamina and remained as a population distinct from the cells that were originally in the inner mural and cumulus regions.

Because granulosa cell regulation of the oocyte is maintained with less surrounding tissue compared with an intact follicle, granulosa cell-oocyte complexes might be useful for live imaging of LH-induced events such as meiotic resumption in the oocyte (20), changes in the cell processes between cumulus cells and the oocyte (21), and granulosa cell migration (17, 22). The accessibility of the granulosa cell surface to the medium could also allow introduction of experimental reagents that cannot pass through the intact follicle tissue, facilitating studies of LH-signaling mechanisms. In addition, development of methods to form functional granulosa cell-oocyte complexes on a glass surface could facilitate imaging of processes such as oligomerization of LHR proteins (23) and LHR internalization (24).

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## Disclosures

The authors have nothing to disclose.

## Data Availability

All relevant data can be found within the article.

## References

1. Richards JS, Ren YA, Candelaria N, Adams JE, Rajkovic A. Ovarian follicular theca cell recruitment, differentiation, and impact on fertility: 2017 update. *Endocr Rev*. 2018;39(1):1-20.
2. Norris RP, Ratzan WJ, Freudzon M, *et al*. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development*. 2009;136(11):1869-1878.
3. Zhang M, Su Y-Q, Sugiura K, Xia G, Eppig JJ. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*. 2010;330(6002):366-369.
4. Jaffe LA, Egbert JR. Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Annu Rev Physiol*. 2017;79(1):237-260.
5. Baena V, Owen CM, Uliasz TF, *et al*. Cellular heterogeneity of the LH receptor and its significance for cyclic GMP signaling in mouse preovulatory follicles. *Endocrinology*. 2020;161(7):bqaa074.
6. Egbert JR, Shuhaibar LC, Edmund AB, *et al*. Dephosphorylation and inactivation of the NPR2 guanylyl cyclase in the granulosa cells contributes to the LH-induced cGMP decrease that causes resumption of meiosis in rat oocytes. *Development*. 2014;141(18):3594-3604.
7. Shuhaibar LC, Egbert JR, Norris RP, *et al*. Intercellular signaling via cyclic GMP diffusion through gap junctions in the mouse ovarian follicle. *Proc Natl Acad Sci U S A*. 2015;112(17):5527-5532.
8. Christenson LK, Gunewardena S, Hong X, Spitschak M, Baufeld A, Vanselow J. Research resource: preovulatory LH surge effects on follicular theca and granulosa transcriptomes. *Mol Endocrinol*. 2013;27(7):1153-1171.
9. Norris RP, Freudzon M, Mehlmann LM, *et al*. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development*. 2008;135(19):3229-3238.
10. McKey J, Cameron LA, Lewis D, Batchvarov IS, Capel B. Combined iDISCO and CUBIC tissue clearing and lightsheet microscopy for in toto analysis of the adult mouse ovary. *Biol Reprod*. 2020;102(5):1080-1089.
11. Irving-Rodgers HF, Hummitzsch K, Murdiyarso LS, *et al*. Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. *Cell Tissue Res*. 2010;339(3):613-624.
12. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA. Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction*. 2010;140(5):655-662.
13. Vigone G, Shuhaibar LC, Egbert JR, Uliasz TF, Movsesian MA, Jaffe LA. Multiple cAMP phosphodiesterases act together to prevent premature oocyte meiosis and ovulation. *Endocrinology*. 2018;159(5):2142-2152.
14. Egbert JR, Fahey PG, Reimer J, *et al*. Follicle-stimulating hormone and luteinizing hormone increase $Ca^{2+}$ in the granulosa cells of mouse ovarian follicles. *Biol Reprod*. 2019;101(2):433-444.
15. Eppig JJ. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev Biol*. 1982;89(1):268-272.
16. Eppig JJ. Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte-granulosa cell complexes developed in vitro from preantral follicles. *Biol Reprod*. 1991;45(6):824-830.
17. Owen CM, Jaffe LA. Luteinizing hormone stimulates ingression of granulosa cells within the mouse preovulatory follicle. *Biol Reprod*. Published online October 17, 2023. Doi:10.1093/biolre/ioad142

18. Eppig JJ, Wigglesworth K, Pendola F, Hirao Y. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod*. 1997;56(4):976-984.
19. Sugiura K, Su Y-Q, Diaz FJ, *et al*. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development*. 2007;134(14):2593-2603.
20. Clift D, Schuh M. A three-step MTOC fragmentation mechanism facilitates bipolar spindle assembly in mouse oocytes. *Nat Comm* 2015;6(1):7217.
21. Clarke HJ. Transzonal projections: essential structures mediating intercellular communication in the mammalian ovarian follicle. *Mol Reprod Dev*. 2022;89(11):509-525.
22. Bianco S, Bellefleur A-M, Beaulieu É, *et al*. The ovulatory signal precipitates LRH-1 transcriptional switching mediated by differential chromatin accessibility. *Cell Rep*. 2019;28(9): 2443-2454.e4.
23. Jonas K, Fanelli F, Huhtaniemi IT, Hanyaloglu AC. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *J Biol Chem*. 2015;290(7):3875-3892.
24. Jean-Alphonse F, Bowersox S, Chen S, Beard G, Puthenveedu MA, Hanyaloglu AC. Spatially restricted G protein-coupled receptor activity via divergent endocytic compartments. *J Biol Chem*. 2014;289(7):3960-3977.