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Follicle microenvironment-associated alterations in gene expression in the mouse oocyte and its polar body

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Abstract

Objective—To determine whether the follicle environment modulates oocyte-specific gene transcript levels in cultured oocytes and polar bodies (PBs).

Design—Animal study.

Setting—Large academic research center.

Animal(s)—CD1 mice.

Intervention(s)—In vitro growth of secondary mouse follicles in 0.25% or 1.5% alginate (ALG) in a three-dimensional culture system.

Main Outcome Measure(s)—Relative transcript levels of *Gdf9*, *Bmp15*, *Nlrp5*, *Tcl1*, and *Zp3* were measured by real-time quantitative reverse transcriptase–polymerase chain reaction in oocytes during in vitro follicle development and oocyte maturation and in their first PBs after removal from metaphase II (MII) eggs.

Result(s)—All transcripts decreased earlier in oocytes cultured in 1.5% ALG compared with 0.25% ALG. Transcript levels were lower in MII eggs cultured in 1.5% ALG compared with in 0.25% ALG. All genes were expressed in PBs, and transcript levels were lower in PBs cultured in 1.5% ALG compared with in 0.25% ALG. Abundance of all transcripts was lower in PBs than in their sibling oocytes.

Conclusion(s)—Local follicle environment modulates oocyte-specific gene expression in the oocyte and first PB. There is a significant difference in the transcript levels of oocyte-specific genes in PBs of 1.5% versus 0.25% ALG that correlates with ovarian environment-related decreases in oocyte competence.

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Discuss: You can discuss this article with its authors and with other ASRM members at <http://fertstertforum.com/jiaoz-follicle-culture-oocyte-quality/>

Z.-X.J. has nothing to disclose. T.K.W. has nothing to disclose.

Keywords

Infertility; meiosis; oocyte-specific genes; oocyte quality; follicle culture

Oocyte competence (or oocyte quality) is defined as an oocyte's ability to mature, be fertilized, and give rise to normal offspring (1). The ovarian follicular microenvironment and maternal signals, mediated primarily through the granulosa and cumulus cells, are responsible for supporting oocyte growth, development, and the gradual acquisition of oocyte competence. The bidirectional communication between oocytes and somatic cells in the follicle are complex, occurring via multiple coordinated pathways, mutual signaling events, and regulatory loops that together move an oocyte towards competence. This level of complexity has made it difficult to characterize the critical features of an oocyte that are required to achieve competence for fertilization and embryo development—the features of a “good egg” (2).

In vitro ovarian follicle growth (IVFG) and in vitro maturation (IVM) provide a valuable tool with which we can study the critical and complex interactions regulating follicle and oocyte development. We use alginate (ALG) as a three-dimensional follicle culture matrix for the encapsulation and maturation of ovarian follicles to produce high-quality oocytes that can be fertilized and give rise to offspring (3). Our prior work using the ALG follicle culture system demonstrated that the physical properties of the follicle microenvironment alter the developmental fate of the encapsulated follicle, leading to differences in oocyte competence (4–6). This finding is consistent with what we know about the ovary, as healthy, growing follicles gradually move from the fibrous, more rigid outer cortex to a more permissive growth environment in the core (7). What remains unclear is how oocyte gene expression varies in response to the movement of growing follicles from one ovarian microenvironment to another.

During the growth period, the oocyte stores RNA and proteins that will be necessary for resuming and completing maturation, supporting fertilization, and initiating embryo development. Knockout experiments have identified oocyte-specific genes that influence oocyte growth and development, the integrity of the oocyte-granulosa cell complex, oocyte maturation, fertilization, and early embryonic development (8). *Gdf9* and *Bmp15* can regulate granulosa cell functions such as proliferation, differentiation, cumulus expansion, and oocyte maturation (9–11). *Zp3* plays an important role in forming the mouse zona pellucida (ZP), which is critical for fertilization (12, 13). *Nlrp5* and *Tcl1* also play vital roles during the early stages of embryo development, and the absence of their protein products in the mouse oocyte has a direct impact on cleavage potential and the progression of the embryo beyond the blastocyst stage (14–16). Understanding oocyte gene expression patterns and how they change in relation to their environment may help untangle the molecular and cellular processes that determine oocyte competence.

One challenge in fertility medicine today is how to identify “good eggs,” that is, which oocytes are most likely to be fertilized, develop into embryos, and result in the birth of healthy offspring. Currently, oocyte selection is based on subjective morphological criteria, which have a low predictive power for IVF outcomes (17). Thus, there is an urgent need to

identify more objective, predictive, and noninvasive markers of mature oocyte competence (18). One hypothesis is that the quality of oocyte is based on the presence of the appropriate set of mRNA and proteins stored during folliculogenesis (19). It has been suggested that differences in mRNA profiles and transcript abundance within individual oocytes might predict their competence and potential to produce viable embryos and live offspring (20). However, one obvious drawback to this approach is the risk of damaging the oocyte during sampling. Others have demonstrated the presence of mRNA in the human polar body (PB), a cell created by the asymmetric division of the oocyte at the time of meiosis, and found that the transcriptome accurately reflects that of its sibling oocyte (21, 22). The ability to quantify mRNA in the PB opens up the possibility of detecting and comparing differences in oocyte-specific gene expression in individual PBs to predict the quality of their sibling oocytes.

In this study, we used our in vitro follicle culture system to determine the changes in gene expression profiles of oocyte-specific genes in oocytes from different follicle stages and in different follicle microenvironments. We also quantified and compared mRNA transcripts in individual PBs and their sibling MII oocytes after IVFG and IVM.

MATERIALS AND METHODS

Animals

We obtained Institutional Review Board permission to perform the animal experiments in this study. CD1 mice were housed and bred in a controlled barrier facility within Northwestern University's Center for Comparative Medicine (Chicago, IL) in a temperature- and light-controlled environment (12 hours light:12 hours dark) and were provided with food and water ad libitum. All mice were maintained in accordance with the policies of Northwestern University's Animal Care and Use Committee and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

ALG Hydrogel Preparation, Follicle Isolation, Encapsulation, and Culture

Sodium ALG preparation, follicle isolation, encapsulation, and culture were all done as described elsewhere (3). Aliquots of charcoal-stripped and sterilized sodium ALG were reconstituted with sterile 1× PBS to concentrations of 1.5% and 0.25% (w/v) before use. Multilayered secondary follicles (130–150 μm) were mechanically isolated from 15-day-old female mice. Individual follicles were encapsulated into ALG beads prepared at various concentrations (1.5% and 0.25%; w/v) and then were plated one follicle per well in 96-well plates in 100 μL of growth media. Encapsulated follicles were cultured at 37°C in 5% CO₂ for 8 days. Follicles were considered dead if the oocyte was no longer surrounded by a granulosa cell layer or if the granulosa cells had become dark and fragmented and the follicle had decreased in size.

IVM of Oocytes

After 8 days of culture, follicles were retrieved from the ALG bead and transferred to maturation media for 16 hours, and then oocytes were denuded as described elsewhere (4). The oocytes were considered to have undergone germinal vesicle breakdown if a germinal

vesicle was not visible. If a PB was present in the perivitelline space, the oocytes were classified as metaphase II (MII). Fragmented or shrunken oocytes were classified as degenerated.

Oocyte and PB Collection

After IVM, only those oocytes that had extruded an intact PB were collected as described elsewhere (23). Single oocytes or PB in 0.5 μ L PBS were transferred to the bottom of 0.5-mL thin-wall Eppendorf tubes containing 1.5 μ L lysis buffer (0.8% Igepal, 1 U RNasin/ μ L, 5 nM DTT) (24). The specimens were stored at -80°C .

Follicle and Oocyte Measurement

For each follicle and oocyte, photographs were taken and measurements were made using a calibrated micrometer (Fisher micromaster, 1 mm with 0.01-mm division) under an inverted microscope. All photographs were imported into ImageJ 1.33U (National Institutes of Health) for measurement (4). The diameter of follicles containing oocyte was measured in duplicate from the outer layer of theca cells. The diameters of oocytes were measured without the ZP.

Characterization of Gene Expression

As previously described, total mRNA within a lysed single cell was reverse transcribed using the AccuScript High Fidelity First Strand cDNA Synthesis Kit (Stratagene) without prior RNA purification (23). To normalize for variations in mRNA content among individual PBs and oocyte lysates, 10^6 copies of a plasmid-derived RNA transcript (pw109, Perkin-Elmer GeneAmp RNA PCR kit) were added to each sample before reverse transcription (RT) as an exogenous control for efficiency of RT (25). Samples with less pw109 RNA than controls were excluded from further study. RT was performed using random hexamers according to the manufacturer's protocol (23). The resulting cDNA samples were stored at -80°C .

Gene expression levels in oocytes or PBs were determined for five candidate genes (Table 1). Real-time quantitative polymerase chain reaction (qPCR) was performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems). For each reaction, 0.5 μ L cDNA, 0.5 μ L primers, 5 μ L TaqMan Universal PCR Master Mix (Applied Biosystems), and 4 μ L nuclease-free water were added to a final volume of 10 μ L. PCR cycling conditions were 95°C for 10 minutes, followed by 50 amplification cycles of 95°C for 15 seconds and 60°C for 1 minute. To maximize accuracy, each sample was run in triplicate with a negative control of reaction mixture with no cDNA added.

Statistical Analysis

Statistical comparisons for RT-qPCR results were analyzed using the *t* test. The χ^2 analysis was used to analyze categorical data. Data were reported as mean $\pm$ SD. $P < .05$ was considered statistically significant. All statistical calculations were performed using the software GraphPad Prism version 4.0.

RESULTS

Effect of ALG Rigidity on Folliculogenesis

We first confirmed the effect of the microenvironment on in vitro–cultured follicle growth. Follicles were cultured in either 0.25% or 1.5% ALG for 8 days. Follicle survival rates were 83.3% (50/60) in 0.25% ALG and 78.3% (47/60) in 1.5% ALG, indicating that follicle survival was not significantly affected by the ALG condition. Follicles also increased in size in both ALG conditions (Fig. 1). Follicles in 0.25% ALG reached a mean diameter of $368.6 \pm 7.3 \mu\text{m}$, significantly greater ($P < .05$) than follicles cultured in 1.5% ALG, which reached a final diameter of $299.3 \pm 8.1 \mu\text{m}$.

Effect of ALG Rigidity on Oogenesis

Diameters of oocytes ranged from $66 \mu\text{m}$ (day 2) to $74 \mu\text{m}$ (day 8; median values). Oocytes in day 2 follicles were significantly smaller than those in day 4, 6, and 8 follicles ($P < .05$). From day 6, the diameters of 1.5% ALG oocytes were smaller than those of 0.25% ALG oocytes, but this difference was not statistically significant (Supplemental Fig. 1).

Effect of ALG Rigidity on Oocyte-Specific Gene Expression

The five selected genes were expressed in oocytes at all stages of in vitro follicle development ($n = 7\text{--}10$ in each stage), with high variability in transcript levels per oocyte. In the 0.25% ALG group, the expression of four of the oocyte transcripts (*Gdf9*, *Bmp15*, *Tcl1*, and *Zp3*) was highest in oocytes of large preantral follicles on day 6 of culture. All five transcripts then decreased in oocytes on day 8; however, the decrease occurred earlier in the 1.5% ALG group compared with in the 0.25% ALG group. In the 1.5% ALG group, the transcript levels of *Gdf9*, *Bmp15*, and *Tcl1* increased slightly on day 4, while *Nlrp5* and *Zp3* were highest on day 2 of culture. The levels of all five oocyte transcripts in the 1.5% ALG group were significantly lower on day 8 compared with on day 2 ($P < .05$; Fig. 2).

Effect of ALG Rigidity on Oocyte Maturation

After 8 days of culture, follicles were removed from the ALG beads and stimulated with hCG for 16 hours to induce oocyte maturation. There were no morphological differences in the mature oocytes between the ALG concentration groups (Fig. 1). There were also no significant differences in the mean diameter of oocytes between the two groups ($72.6 \pm 1.9 \mu\text{m}$ in 0.25% ALG vs. $70.9 \pm 2.1 \mu\text{m}$ in 1.5% ALG); however, the percentage of oocytes cultured in 0.25% ALG that extruded the first PB (MII%) was 86% (43/50) and 63.8% (30/47) in 1.5% ALG. There were statistically significant differences between the two ALG culture groups ($P < .05$).

Effect of ALG Rigidity on Oocyte-Specific Gene Expression in MII Oocytes

Expression of the five oocyte-specific transcripts was analyzed in 20 individual oocytes from follicles that were cultured in either 0.25% ($n = 10$) or 1.5% ($n = 10$) ALG. To determine the effect of ALG concentration on relative transcript levels in the MII oocyte, we set the normalized level of each transcript in the MII oocytes from follicles cultured in 0.25% ALG at 1 and then determined the fold change in transcript abundance in oocytes

from follicles cultured in 1.5% ALG. The levels of transcripts in MII oocytes in the 1.5% ALG group were lower (between 0.36-fold for *Nlrp5* and 0.59-fold for *Gdf9*) compared with the levels in the 0.25% ALG group ($P<.05$; Fig. 3A).

Effect of ALG Rigidity on Oocyte-Specific Gene Expression in the First PB

The expression levels of the same five oocyte-specific transcripts were analyzed in 20 individual sibling PBs removed from MII oocytes in the 0.25% and 1.5% ALG groups ($n = 10$ in each group). Transcripts of all five genes were detected in individual PBs from both groups. Consistent with our findings in the sibling oocytes (Fig. 3A), PBs from the 1.5% ALG group had significantly lower levels of all five transcripts compared with PBs from the 0.25% ALG group ($P<.05$; Fig. 3B). The pattern of transcript abundance was very similar between oocytes and sibling PBs for the five genes.

Relative Abundance of Selected mRNA Transcripts in PBs Versus Sibling Oocytes

The relative levels of all five oocyte-specific mRNA transcripts were lower in individual PBs compared with their sibling MII oocytes from follicles cultured in either 0.25% or 1.5% ALG (Fig. 3C). To determine the effect of follicle environment on relative transcript levels in the PB and sibling oocyte, we set the normalized level of each transcript in the sibling oocyte as 1 and then determined the fold difference in transcript abundance in PBs. The difference in transcript level was between 0.54-fold (*Bmp15*) and 0.83-fold (*Zp3*) in the 0.25% ALG group and between 0.46-fold (*Bmp 15*) and 0.77-fold (*Zp3*) in the 1.5% ALG group. For all five genes, the lower relative transcript abundance in PBs compared with their sibling oocytes was not statistically significantly different between the two ALG culture groups (Fig. 3C).

DISCUSSION

In this study, we used two different concentrations of ALG (1.5% and 0.25%) to investigate the physiological effects of the microenvironment on follicle and oocyte development. There were no significant differences in the follicle survival rates, which were comparable to those previously reported (4). However, follicles reached a greater diameter in 0.25% ALG compared with 1.5% ALG, confirming our previous observation that a less rigid microenvironment was permissive to growth. The more rapid increase in follicle size in the 0.25% ALG group could be attributed to more rapid theca and granulosa cell proliferation. Xu et al. (4) found that ALG concentration not only affected the proliferation and differentiation of the adjacent theca cells but also regulated granulosa cell proliferation and differentiation within the follicle, suggesting that mechanical forces exerted on the theca cells are translated to the internal granulosa cell population.

It has been shown that the interaction between the mammalian oocyte and its surrounding somatic cells is important for the earliest stages and contributes to normal oocyte development and cumulus cell differentiation (26, 27). During folliculogenesis, oocytes synthesize and accumulate RNAs the most in the earliest phases of development, which coincides with active proliferation of follicular cells; however, by the end of growth (antral stages), silencing of transcriptional activity and degradation of some mRNA will be the

predominant processes (28–30). Pan et al. analyzed global gene expression during in vivo oocyte growth and found that a number of genes were down-regulated at the time of antrum formation, which coincides with the acquisition of oocyte meiotic competence (31). Consistent with what occurs in vivo, we found that for the five genes analyzed in oocytes in 0.25% ALG group, the highest levels of mRNA expression were detected in oocytes in antral stage follicles (day 6 follicles) and then decreased in oocytes from day 8 follicles. Our observations with the three-dimensional ALG culture system agree with those of Sanchez et al., who studied six oocyte-specific genes in oocytes from two-dimensional in vitro follicle culture (32).

By contrast, the five gene transcripts decreased earlier (day 4 follicles) in oocytes cultured in 1.5% ALG, and significantly decreased in oocytes on day 8 follicles. The transcript levels were lower than those of oocytes cultured in 0.25% ALG. These results suggest that a more rigid follicle microenvironment limits the storage of gene products in the oocyte during folliculogenesis, although it remains unclear how mechanical signals on the follicle affect downstream synthesis and accumulation/storage of transcripts during oocyte growth. One group has reported that granulosa cells mediate oocyte global transcriptional silencing in preovulatory mouse oocytes (33).

As described by other investigators, a defined oocyte-specific gene expression pattern arising during folliculogenesis is crucial for the acquisition of oocyte developmental competence; conversely, deficiencies in gene expression or dynamics that occur during follicle development may be linked to impaired oocyte competence (30, 34). In this study, although the final size of oocytes was not significantly different between the two groups, oocytes from follicles in the 1.5% ALG group produced fewer mature oocytes. We previously found that the normal fertilization rate (5.6% vs. 41.5%) and development rate to the blastocyst stage (0.0% vs. 29.4%) were significantly lower for oocytes from follicles cultured in 1.5% ALG compared with those cultured in 0.25% ALG (4, 5). These results indicate that deficiencies in storage of products in the oocyte during folliculogenesis may in turn negatively affect MII oocyte developmental competence (35) and support the idea that the physical properties of the ovarian environment regulate oocyte developmental competence (36). This concept may provide a unique perspective on disorders in which follicles are unable to advance beyond an immature stage and the associated oocytes are of poor quality, such as in polycystic ovary syndrome (PCOS). Wei et al. investigated the expression patterns of *Gdf9* and *Bmp15* during oocyte maturation in women with PCOS and in a control group. They found that after ovarian stimulation, the expression of *Gdf9* and *Bmp15* mRNA displayed dynamic changes during oocyte maturation in the control group but their expression in oocytes from PCOS patients was lower and showed no dynamic change (37).

PB biopsy involves careful removal of a PB through microdissection, which has been proven to be a safe procedure that can be used in mice and humans. We and others have suggested that the PB provides a readily accessible “test” source of genomic material that can be sampled for diagnostic purposes as a proxy for the sibling oocyte (38, 39). In the current study, we were able to detect and quantify all five of the candidate oocyte-specific mRNA transcripts in individual PBs. As expected based on the unequal distribution of

cytoplasmic content into the oocyte and PB during meiosis, the abundance of transcripts for the five genes was lower but detectable in PBs compared with their sibling oocytes. Furthermore, given that the oocyte and its sibling PB share ooplasm before division, we were not surprised to find that the pattern of differences in gene expression in the 0.25% and 1.5% groups were the same in both oocytes and PBs. Thus, we have shown that the relative abundance of mRNA transcripts in a single PB faithfully reflects the relative abundance of that transcript in its sibling oocyte. Reich et al. (22) reported similar results for human oocytes and their sibling PBs. They analyzed over 12,700 unique mRNAs and miRNAs from oocyte samples and compared them with 5,431 mRNAs recovered from the sibling PBs. They demonstrated that detection and quantification of mRNA in human PBs are possible and that the human PB mRNA transcriptome reflects that of its sibling MII oocyte. This work and our own led us to propose that the detection and quantification of oocyte-derived mRNAs in PBs could be used to predict the developmental competence of individual oocytes and establish a more objective set of criteria than morphology alone for the selection of oocytes for use in IVF (18, 40). Future studies will need to clarify what changes in specific genes are associated with oocyte competence.

In conclusion, this study demonstrated that oocyte-specific gene expression levels in the oocyte are affected by the microenvironment of the follicle or the rigidity of the ALG matrix in in vitro culture. We also found that it is possible to accurately quantify several oocyte-specific genes in a single PB and that transcript levels mirror those in the sibling oocyte. Further experiments will prospectively evaluate the relationship between oocyte-specific gene expression levels in PBs and the fertilization and embryonic developmental competence of the sibling oocyte to determine the predictive value of gene expression as a biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Representative photographs of multilayered secondary follicles encapsulated in 0.25% (A–F) or 1.5% (G–L) ALG and cultured for 8 days. Oocytes were isolated at the end of culture and matured. A multilayered secondary follicle encapsulated in ALG (A, G) increased diameter after 4 days (B, H) and 6 days (C, I) culture and finally developed into antral follicles (D, J). After hCG induction of oocytes on day 8, the cumulus-oocyte-complex (*arrow*) dispersed (E, K) and the oocyte resumed meiosis and extruded the first PB (F, L). Bar = 100 μ m (A–D, G–J) and 50 μ m (F, L).

Jiao. Oocyte transcripts in polar bodies. *Fertil Steril* 2013.

FIGURE 2.

Oocyte-specific gene expression in oocytes from follicles cultured in 0.25% or 1.5% ALG.

Results are normalized to each transcript on day 2 of culture in each group (set to 1 as the calibrator). Error bars represent SD.

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FIGURE 3.

Oocyte-specific gene expression in MII oocytes and their first PBs from follicles cultured in 0.25% vs. 1.5% ALG. (A) Oocyte-specific gene expression in MII oocytes. Results are normalized to each transcript in MII oocytes from follicles culutred in 0.25% ALG (set to 1 as the calibrator). Compared with oocytes from the 0.25% ALG group, there was a significantly lower level of all transcripts in the 1.5% ALG group. *Error bars* represent SD. *Significance relative to the 0.25% ALG group ($P<.05$). (B) Oocyte-specific genes expressed in the first PB of MII oocytes. Results are normalized to each transcript in PBs from the 0.25% ALG group (set to 1 as the calibrator). Compared with PBs from the 0.25% ALG group, there was a significantly lower level of all transcripts in the 1.5% group. *Error bars* represent SD. *Significance relative to 0.25% ALG group ($P<.05$). (C) The relative abundance of each of the five oocyte-specific genes in PBs compared with their sibling oocytes. If the normalized mRNA level of each transcript in the sibling oocyte is set at 1, the abundance of the transcripts in the PBs was consistently lower than in the sibling oocytes. For all five genes, the fold decrease in relative transcript abundance in PBs compared with sibling oocytes was not statistically significantly different between the two culture groups. *Error bars* represent SD. The *horizontal line* indicates the normalized transcript level in sibling oocytes from each culture group.

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TABLE 1

Oocyte-specific genes selected for RT-qPCR in individual PBs and sibling oocytes.

Gene	Function	Accession no.
<i>Bmp15</i>	Involved in oocyte maturation and follicular development	NM 009757.4
<i>Gdf9</i>	Regulates oocyte growth and function; regulates granulosa cell growth, differentiation, and cumulus expansion	NM 008110.2
<i>Nlrp5</i>	Required for normal early embryogenesis; depletion of NLRP5 blocks early embryogenesis	NM 011860.2
<i>Tcl1</i>	Participates in early embryonic development	NM 009337.3
<i>Zp3</i>	Necessary for ZP formation and fertilization	NM 011776.1

Note: Accession no. = NCBI reference sequence accession number.

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