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Age-associated alteration of oocyte-specific gene expression in polar bodies potential markers of oocyte competence

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Abstract

Objective—To confirm that oocyte-specific mRNAs are detectable in the polar body (PB) of MII oocytes and determine the effect of age on oocyte-specific transcript levels.

Design—Prospective study

Setting—Hospital-based academic research laboratory

Animals—CD1 female mice

Intervention(s)—Aged (40–50 weeks) and young (7–9 weeks) mice were administered pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). Oocytes were fertilized *in vitro* to assess fertilization and developmental competence. MII oocytes were obtained and first PBs was removed. mRNA from each PB and its sibling oocyte was reverse transcribed and analyzed by real-time quantitative PCR.

Main Outcome Measure(s)—Fertilization and developmental rates and expression of 6 oocyte-specific genes (*Bmp15*, *Gdf9*, *Hlfoo*, *Nlrp5*, *Tcl1*, and *Zp3*) in PBs and sibling oocytes from young vs. aged mice.

Result(s)—Oocytes from aged mice had lower developmental competence. Four genes (*Hlfoo*, *Nlrp5*, *Tcl1*, and *Zp3*) were differentially expressed in aged vs. young oocytes ($P < 0.05$). All 6 transcripts were present in PBs from aged and young mice at lower levels than in the sibling oocytes; transcript levels were lower in aged PBs compared with young PBs ($P < 0.05$).

Conclusion(s)—There is a significant difference in the transcript levels of oocyte-specific genes in aged vs. young PB that correlates with age-related decreases in oocyte competence. Differences in gene expression in PB may be potential biomarkers of MII oocyte competence.

Keywords

infertility; meiosis; oocyte-specific genes; oocyte quality; age

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Capsule Differences in gene expression in individual polar bodies of MII oocytes may be potential markers for minimally invasive testing of oocyte competence.

INTRODUCTION

Oocyte competence is defined as the potential to undergo maturation, fertilization, and development to the blastocyst stage, ultimately giving rise to live offspring (1). Oocyte competence is the major factor that limits the success of assisted reproduction technologies (ART). Despite the rapid development of ART, the ratio of oocytes harvested to live-born babies is approximately 25:1 for young women (2). This low efficiency is primarily due to poor oocyte quality, which increases as women age and move out of their optimal years of fertility (3, 4). Studies of women in oocyte donation programs have established that reduced oocyte competence is the major cause of age-related decline in fertility (5). The ability to select embryos for transfer that were produced from high-quality MII oocytes would greatly improve the success rate of in vitro fertilization (IVF). Currently, oocyte selection is based on subjective morphological criteria, which have a low predictive power for IVF outcomes (6). Thus, there is an urgent need to identify more objective, predictive, and non-invasive markers of mature oocyte competence (7).

Cytoplasmic factors in the oocyte, including mRNA and proteins, are vital for appropriate growth and maturation of the oocyte as well as for the development of a viable embryo after fertilization. Oocytes acquire these cytoplasmic factors during early oocyte growth and gradually accumulate more during the later stages of follicle growth; however, transcriptional activity essentially ceases at the end of oocyte growth until the resumption of meiosis after fertilization. In mouse, most transcriptional reactivation occurs at the 2-cell stage (8–10). A critical period exists between the time the fertilized oocyte reenters meiosis and the embryonic genome becomes activated, implying that the first days of development depend on protein synthesis from the repository of maternal transcripts from the oocyte. Consequently, the health of early embryos depends on materials and biosynthetic activity already present in the mature egg.

It has been suggested that individual differences in mRNA profiles and transcript abundance within a cohort of oocytes might predict their competence and potential to produce viable embryos and live offspring (11). Oocyte-specific genes have been identified using various transgenic mouse models; these genes influence oocyte growth and development, the integrity of the oocyte-granulosa cell complex, oocyte maturation, fertilization, and early embryonic development (12). As markers of oocyte competence and embryo potential, assessment of oocyte-derived mRNA expression levels may provide a way to more objectively assess and predict the developmental competence of individual oocytes; such an approach may ultimately aid in selection of high-quality oocytes for use in IVF (13). Thus far, there are few quantitative data regarding oocyte transcript levels in individual oocytes, or how oocyte-specific genes correlate to oocyte competence or vary with age (14, 15).

One obvious drawback to assessing individual oocyte competence by quantifying its gene expression profile is the risk of damaging the oocyte during sampling. The polar body (PB) is a cell created by asymmetric division of the oocyte at the time of meiosis and produces a readily accessible test source of genomic material that can be sampled as a proxy for the “sibling” oocyte for diagnostic purposes. Klatsky et al. first reported the presence of mRNA in human PB (16). Reich et al demonstrated that the transcriptome of a human PB accurately reflects its sibling oocyte (17). The ability to quantify mRNA in individual cells—as small as a single PB—opens up the possibility that we can detect and compare individual differences in oocyte-specific gene expression in the PB without harming the oocyte.

Our primary aim was to test the hypothesis that oocyte-specific mRNAs are present and detectable in the first polar body and may be potential markers of oocyte competence. We quantified the mRNA in individual PBs and their sibling MII oocytes from both young and

aged mice. We first compared transcript abundance between PBs and sibling oocytes, and then determined the effect of age on oocyte developmental competence and gene expression levels in PBs and oocytes.

MATERIALS AND METHODS

Animals

We obtained Institutional Review Board (IRB) 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 (12L:12D) 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 (NIH) Guide for the Care and Use of Laboratory Animals.

Oocyte Isolation

MII oocytes were collected from, young (7–9 weeks) and aged (40–50 weeks) female mice after superovulation was induced by intraperitoneal injection of a single dose [5 IU (young mice) or 20 IU (aged mice)] of pregnant mare's serum gonadotropin (PMSG; Calbiochem, La Jolla, CA) followed by the same dose of human chorionic gonadotropin (hCG) (Intervet, Canada) 46–48 hours later. Oocyte-cumulus cell complexes were recovered from ampullae into Leibovitz L15 medium containing 1% fetal bovine serum (FBS) 16 hours after hCG administration. Oocytes were dissociated from the surrounding cumulus cells using 0.3 mg/ml hyaluronidase (Sigma, St. Louis, MO) in order to identify MII oocytes. After the removal of cumulus-corona cells, only those MII oocytes that had extruded a PB were collected. The MII oocytes were then subjected to either *in vitro* fertilization or separation of the PB and sibling oocyte.

In vitro Fertilization and Blastocyst Culture

For *in vitro* fertilization of oocytes, motile sperm were prepared from a sperm suspension collected from the cauda epididymis of proven CD-1 male breeder mice as described previously (18). A total of 15–20 MII oocytes were placed in 50 μ l of IVF medium microdrops containing 1×10^6 sperm/ml and incubated under mineral oil for 7–8 h at 37 °C and 5% CO₂. Oocytes were then washed 3 times in fresh KSOM to remove all bound sperm and transferred into a 20- μ l drop of fresh KSOM drop overnight. Embryos that cleaved to the 2-cell stage were characterized as fertilized. Embryos were washed in KSOM and cultured to the blastocyst stage. Blastocyst formation rate was scored after 5 days of culture. Embryos were cultured in 3 replicate experimental groups of at least 30 zygotes.

PB and Sibling Oocyte Separation

MII oocytes that had extruded an intact PB of normal size were collected; oocytes with fragmented PBs or enlarged PBs were excluded from this study (Fig. 1). Each MII oocyte was briefly treated with acidic Tyrode's solution (Sigma) to remove its zona pellucida and then subjected to gentle pipetting to separate the PB from its coupled “sibling” oocyte (Fig. 1). Single PBs 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, 5nM DTT) (19). Sibling oocytes were transferred to an identical lysis solution and processed using the same protocol. The lysed specimens were stored at –20°C.

Reverse Transcription (RT)

Total mRNA within lysed single PBs and their sibling MII oocytes was reverse transcribed using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA) without prior RNA purification from the lysate. 10⁶ copies of a plasmid-derived RNA transcript (pw109, Perkin-Elmer GeneAmp RNA PCR kit) were added to each sample prior to RT as an exogenous control for efficiency of reverse transcription (20). Samples with less plasmid RNA than the pAW109 control were excluded from further study. RT was performed by adding the following to each sample: 7.7 µl nuclease-free water, 2 µl 10× RT buffer, 0.8 µl dNTP (2.5 mM of each dNTP), and 3 µl random primers (0.1 µg/µl). Reactions were incubated at 65°C for 5 minutes then cooled to room temperature (approximately 5 minutes). Next, 2 µl of 100 mM dithiothreitol, 0.5 µl RNase inhibitor (40 U/µl), and 1 µl reverse transcriptase (50 U/µl) per sample were added; the reactions were incubated at 42°C for 60 minutes and then terminated by heating to 70°C for 15 minutes. The resulting complementary cDNA samples were stored at -80°C.

Real-time Quantitative PCR (qPCR)

Gene expression levels in PBs and sibling oocytes from young and aged mice were determined for 6 candidate genes (Table 1). Real-time qPCR was performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Forest City, CA). For each reaction, 0.5 µl cDNA, 0.5 µl primers, 5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), and 4 µl nuclease-free water were added to a final volume of 10 µl. 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 3 times with a negative control of reaction mixture with no cDNA added.

Statistical Analysis

The calculation of gene expression was as follows, using *Bmp15* in aged oocyte as an example: $Bmp15 = 2^{-\Delta\Delta Ct}$; $\Delta\Delta Ct = [Ct(Bmp15) - Ct(Rpl18)]_{aged} - [Ct(Bmp15) - Ct(Rpl18)]_{young}$. Statistical comparisons for qRT-PCR results were analyzed using *t*-test. Chi square analysis was used to analyze categorical data. Data were reported as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Oocyte Competence and Embryo Development

We first investigated the rates of fertilization and development to the blastocyst stage. A total of 138 and 112 MII oocytes were collected from young and aged mice, respectively. In young mice, 116/138 oocytes were fertilized (84.1%), and 96/116 2-cell embryos were subsequently cultured to the blastocyst stage (82.8%). In aged mice, 78.6% (88/112) of MII oocytes cleaved into 2-cell stage embryos, of which 70.5% (62/88) developed to the blastocyst stage. The normal fertilization rate was lower for aged oocytes compared with young oocytes, but the difference was not significant (78.6% vs 84.1%, respectively). Development rates to the blastocyst stage were significantly lower for aged oocytes compared with young oocytes (70.5% vs. 82.8%, $P < 0.05$). There were no morphologic differences in the mature oocytes and developing embryos between the 2 groups, however (Fig. 2).

Relative mRNA Expression Levels in Oocytes and PBs from Young vs. Aged Mice

The expression of 6 oocyte-specific transcripts (Table 1) was analyzed in 30 individual PBs and sibling MII oocytes from young (n=20) and aged (n=10) mice using real-time qPCR. Compared with young oocytes, there was a significantly lower level of *H1foo*, *Nlrp5*, *Tcl1*,

and *Zp3* transcripts in aged oocytes ($P < 0.05$; Fig. 3A). Although *Gdf9* and *Bmp15* transcript levels were also lower in aged oocytes compared with young oocytes, the difference was not significant.

Transcripts of the 6 selected oocyte-specific candidate genes were detected by real-time qPCR in individual PBs from both aged and young MII oocytes. PBs from aged mice had significantly lower levels of all 6 transcripts compared with PBs from young mice ($P < 0.05$; Fig. 3B).

Relative Abundance of Selected mRNA Transcripts in PBs vs. Sibling Oocytes

The relative levels of all 6 oocyte-specific mRNA transcripts were lower in individual PBs compared with their sibling MII oocytes from both aged and young animals. To determine the effect of age 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 change in transcript abundance in PBs. The change in transcript level was between 0.12-fold (*Bmp15*) and 0.44-fold (*Zp3*) in aged mice and between 0.21-fold (*Bmp15*) and 0.82 fold (*Zp3*) in young mice (Fig. 3C). For all 6 genes, the fold decrease in relative transcript abundance in PBs compared with sibling oocytes was statistically significantly greater in aged mice vs. young mice ($P < 0.05$); in other words, the PBs from young mice had relative transcript levels that were closer to those of their sibling oocytes than did the PBs from aged mice compared to their sibling oocytes (dashed line, Fig. 3C).

DISCUSSION

Decreasing oocyte competence with maternal aging is a common feature in females of most mammalian species. Age-dependent oocyte deficiencies include both meiotic abnormalities and cytoplasmic deficiencies. Functional changes associated with oocyte aging include decreased fertilization rates, polyspermy, and either arrested embryonic development or spontaneous abortion (21). In the present study, we demonstrated that the oocytes from aged mice exhibited a reduced rate of development to blastocysts than oocytes from young mice. Our data confirm those of others that demonstrated lower competence of aged oocytes than young oocytes for *in vitro* development (22). How age affects the inherent features of the oocyte that determine its developmental potential is still poorly understood. Most research has emphasized the importance of chromosomal abnormalities because of the well established increase in aneuploidy with increasing maternal age. Yet many euploid blastocysts also fail to implant and develop into living embryos, supporting the idea that age-related abnormalities in the ooplasm may also reduce oocyte competence and negatively impact embryo development (23, 24). In human oocytes, cytoplasmic aging has been associated with a decline in mitochondrial function, a reduction in transcripts from genes participating in the spindle assembly checkpoint, and an increase in the frequency of apoptosis (25–28). Similar age-related changes have been described in the mouse model, where low oocyte developmental competence has been associated with various oocyte abnormalities, including chromosome scattering, chromosome decondensation, alteration of gene expression patterns, and high rates of DNA fragmentation (29–31).

As we were most interested in finding genes associated with mouse oocyte developmental competence, six oocyte-specific genes were chosen because of the essential roles that they play in mouse oocyte development, maturation, and fertilization, and early embryo development. The role of *Gdf9* and *Bmp15* in folliculogenesis and oocyte maturation has been demonstrated in mice (32–34). *Zp3* plays an important role in forming the mouse zona pellucida, which is critical for fertilization (35, 36). *H1foo* is indispensable for meiotic maturation of the mouse oocyte (37). *Nlrp5* and *Tc11* also play vital roles during the first stages of embryo development, and the absence of their protein products in the mouse

oocyte has a direct impact on the cleavage potential and on the progression of the embryo beyond the blastocyst stage (38–40). Therefore, to investigate age-dependent molecular changes in the oocyte that may impact oocyte competence, we compared these oocyte-specific gene expression levels in young and aged mice.

In this study, we found that *H1foo*, *Nlrp5*, *Tcl1* and *Zp3* transcript levels were relatively lower in aged oocytes compared with young oocytes. Hamatani et al (30) also found a number of genes involved in reproduction and oocyte-specific genes had lower expression with aging. These results may suggest that there is a lower capacity of aged oocytes to produce and store proteins necessary for oocyte function in the time between fertilization and embryonic genome activation. These analyses are a first step to explore the molecular factors associated with variations in reproductive competence of individual oocytes, and suggest that it may be possible to detect and quantify specific oocyte-derived mRNAs to predict the developmental competence of individual oocytes. This approach may lead to more objective criteria for the selection of MII oocytes for IVF, which is currently limited to subjective, morphologic assessment (13).

In female meiosis, the two meiotic divisions are unequal resulting in the extrusion from the oocyte of two small polar bodies. The first PB is extruded as the oocyte matures and resumes meiosis I prior to ovulation. The second PB is extruded following fertilization and resumption of meiosis II. To conserve nutrients, the majority of the cytoplasm is segregated into the ovum to prepare for subsequent embryonic development if fertilization occurs. The PB contains relatively little cytoplasm, is extremely small compared with the ovum, and eventually degenerates. Others have demonstrated that the chromosomes within the first PB are identical to those in the oocyte, and have the same genetic and reproductive potential (42). Previously published studies have also examined the association between first PB morphology and oocyte quality, subsequent rates of implantation, and rates of pregnancy. Ebner et al found embryos with an intact first PB had increased rates of blastocyst formation and implantation compared with embryos that had a fragmented first PB (43). Navarro et al found that an enlarged PB is related to poorer rates of fertilization, cleavage, and embryo quality (44). However, these data are considered to be controversial and need to be confirmed (45).

Polar body biopsy involves careful removal of a polar body through microdissection, which has been proven to be a safe procedure that can be used with high precision and without negative impact on *in vitro* embryo development in mice and humans (46–49). Reich et al (17) analyzed over 12,700 unique mRNAs and miRNAs from oocyte samples and compared them with 5,431 mRNAs recovered from the sibling PBs. Their results demonstrated that detection and quantification of mRNA in PBs is possible and that the PB mRNA reflects the transcript profile of the MII oocyte. This work led to our proposal that biopsying the PB would allow testing for gene expression in the oocyte without damaging the sibling oocyte or developing embryo. The detection and analysis of PB mRNA may provide insight into oocyte quality in the context of IVF.

This is the first report, to our knowledge, that documents the ability to detect oocyte-specific mRNAs in the first PB of mouse MII oocytes and assess the effect of age on gene expression levels in the first PB. We were able to detect and quantify all 6 candidate oocyte-specific mRNA transcripts in individual PBs from the MII oocytes of aged and young mice. We found that the abundance of transcripts was lower but detectable in PBs versus their sibling oocytes. The first PB degenerates rather quickly and frequently begins to degenerate before ovulation; approximately 70% of first PBs degenerate within 6 hours after maximal nuclear maturation (50). DNA fragmentation has been observed in the first PB, suggesting that the degeneration of PB is likely to be an apoptotic process (51, 52). Therefore, the lower relative

abundance of PB transcripts relative to that of the sibling oocyte may be due to instability of the transcripts in PB that are undergoing apoptosis. However, it remains unclear which factors determine individual oocyte and species-specific differences in PB degeneration rates, and how the timing of PB degeneration affects oocyte-specific gene expression in PB (50).

In this study, we also found that the levels of oocyte transcripts in aged mice were lower than in young mice, and that the patterns of expression in the MII oocytes were mirrored in their PB. Our results also showed that the abundance of transcripts in PB relative to the sibling oocyte is lower in aged animals compared with young animals. An increased susceptibility to apoptosis during reproductive aging has been previously described in both human and mouse oocytes (28, 31), and Fujino et al reported that DNA fragmentation was observed more frequently in freshly recovered oocytes from aged mice compared with young mice (31).

Our work characterizing age-related molecular changes in MII oocytes also has clinical implications. Lim et al (53) found that oocytes with signs of slight degeneration have no morphologic differences from healthy oocytes, and may be deemed suitable for fertilization and embryo transfer. However, these embryos often do not implant or implant transiently. This may explain why, despite a comparable number of good quality embryos transferred, older women achieve a much lower pregnancy rate. This also suggests that the current morphological markers used to select oocytes for fertilization may be inadequate. Alternatively, assessing the presence or abundance of critical mRNA transcripts in the oocyte—or, as a proxy, the PB—may offer a more objective, predictive approach to determining oocyte competence. Using polar body gene expression information to identify the most competent oocytes would be of great benefit in IVF treatment, allowing the embryos with the highest implantation potential to be prioritized for transfer to the uterus.

In conclusion, this is first study to report a comparison of the quantities of oocyte-specific mRNA transcripts in PB from young and aged mouse MII oocytes. This study demonstrates that it is possible to accurately quantify several genes in a single PB using real-time qPCR, and these genes may serve as biomarkers of oocyte competence. Future studies are needed to further evaluate the correlation between the timing of PB degeneration and dynamic changes in mRNA turnover in PB, as well as to identify the functionally important genes in the PB that may reflect biological differences between oocytes. The availability of technical platforms and whole genome microarrays will accelerate our understanding of the molecular processes within the PB and oocyte (54), with the ultimate goal of identifying predictive biomarkers of oocyte competence and developing minimally invasive assays for clinical use.

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FIGURE1.

Morphology of the first PB (A-C) and the separation of the first PB from its associated MII oocyte (D, E). A: intact PB of normal size; B: fragmented PB; C: enlarged PB; D: Remove ZP; E: Separation of first PB from its sibling oocyte. Bar=50µm.

FIGURE2. Morphology of MII and fertilized oocytes from young (A–C) and aged (D–E) mice. (A,D) MII oocyte; (B,E) 2-cell embryo; (C,F) blastocyst. Bar=50μm

FIGURE 3.

A: Oocyte-specific genes expression in MII oocytes from young and aged mice. Results are normalized to each transcript in MII oocyte from young mice as 1 (the calibrator). Compared with young oocytes, there was a significantly lower level of *H1foo*, *Nlrp5*, *Tcl1*, and *Zp3* transcripts in aged oocytes. Error bars represent SD. * significance relative to young mice ($P < 0.05$)

B: Oocyte-specific genes expression in PB from young and aged mice. Results are normalized to each transcript in PB from young mice as 1 (the calibrator). PBs from aged mice had significantly lower levels of all 6 transcripts compared with PBs from young mice. Error bars represent SD. * significance relative to young mice ($P < 0.05$)

C: Relative abundance of each single gene in PBs compared with its sibling oocyte. If the normalized mRNA level of each transcript in sibling oocytes is set at 1, the abundance of the transcript in PBs was consistently lower than that in the sibling oocytes. In addition, transcript levels were significantly lower in aged animals compared with young animals. Error bars represent SD. Horizontal line indicate the normalized level in sibling oocyte. * significance relative to its sibling oocyte ($P < 0.05$)

TABLE 1

Oocyte-specific genes selected for testing 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; required for ovarian folliculogenesis	NM 008110.2
<i>H1foo</i>	Epigenetic remodeling, essential for meiotic maturation	NM 138311.2
<i>Nlrp5</i>	Required for normal early embryogenesis	NM 011860.2
<i>Tc11</i>	Participates in early embryonic development	NM 009337.3
<i>Zp3</i>	Necessary for fertilization	NM 011776.1