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Detection and quantification of maternal-effect gene transcripts in mouse second polar bodies: potential markers of embryo development competence

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Abstract

Objective—To test the hypothesis that quantification of mRNAs originating the second polar body (PB₂) provides a non-invasive tool for assessing embryo quality.

Design—Prospective study

Setting—Hospital-based academic research laboratory

Animals—CD1 female mice

Intervention(s)—MII oocytes obtained from 7- to 8-week-old mice after pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) priming. After *in vitro* fertilization, the PB₂ was biopsied from zygotes followed by reverse transcription. Real-time PCR was performed to quantify gene expression levels in single PB₂. The sibling zygotes were continuously cultured to blastocyst stage.

Main Outcome Measure(s)—Embryo developmental competence and six maternal-effect gene (*Dnmt1*, *Mater*, *Nobox*, *Npm2*, *Tcl1* and *Zar1*) transcripts in the PB₂

Results—PB₂ mRNA was detected in all candidate genes. Transcripts that were present in greater abundance in the zygote were more likely to be detected in qPCR replicates from single PB₂. 4 candidate genes (*Dnmt1*, *Nobox*, *Npm2* and *Tcl1*) expression levels in PB₂ between two groups (2-cell embryo vs. blastocysts) approached statistical significance.

Conclusion—PB₂ may contain a representative transcript profile to that of the zygote after fertilization. Differences in gene expression in PB₂ may be potential biomarkers of embryo quality.

Keywords

infertility; meiosis; maternal-effect genes; embryo quality

Despite remarkable progress in both the clinical and embryological aspects of assisted reproductive technologies (ART), the live birth rate is still disappointingly low. The ratio of oocytes harvested to live-born babies is approximately 25:1 for young women (1). 85% of

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embryos produced *in vitro* and transferred in the uterus fail to develop into an infant (2). The utilization of the most competent embryo during *in vitro* fertilization (IVF) is crucial to ensure a successful pregnancy. Currently, the morphological criteria and the standard cytogenetic methods used to select and classify embryo are not sufficient for predicting the IVF outcomes. The lack of reliable predictors of oocyte/embryo developmental competence hampers the effectiveness of assisted reproductive technology (ART) (3, 4). Thus, there is an urgent need to identify more objective, predictive, and non-invasive markers to choose the embryos with the highest implantation potential to be prioritized for transfer to the uterus (5).

Development of a mammalian embryo starts with fertilization, the fusion of sperm and egg and formation of a totipotent zygote. After fertilization, the mouse sperm is incorporated into the cytoplasm of the egg and provides DNA for the male pronucleus, which is essential for egg activation. However, earlier studies have demonstrated that the sperm play no major role in cleavage-stage embryogenesis, the maternal genome controls virtually all aspects of early animal development (6, 7). The most conclusive evidence that stored maternal-effect determinants are required for embryonic developmental competence has come from loss-of-function studies in the mouse. Loss of maternal *Mater*(8), *Zar1* (9) and *Npm2* (10) transcripts causes the arrest of development at embryonic genome activation stage. Embryos derived from *Tc1*-null females have delayed cleavage-stage progression with decreased fecundity (11). *Nobox* plays an important role of regulation embryonic genome activation, pluripotency gene expression, and blastocyst cell allocation (12). As markers of embryo quality, assessment of maternal-effect mRNA expression levels may provide a way to more objectively assess and predict the developmental competence of embryo; such an approach may ultimately aid in improving implantation rate in IVF (13). However, due to the drawback that assessing maternal-gene expression profile has the risk of damaging the oocyte during sampling, it is still not known whether maternal-effect molecular markers that could be used to predict the developmental competence of embryo.

A polar body (PB) is the byproduct of an oocyte meiotic division. The first PB (PB₁) is extruded as the oocyte matures and resumes meiosis I prior to ovulation. The second PB (PB₂) is extruded following fertilization and resumption of meiosis II. There is no clear evidence of the fate of the PB in any mammal including the mouse, which is the commonly used research model. However, the PB is generally considered as waste material, and therefore not essential to embryo development. In recent years the PB has gained prominence as they have been used as a DNA source representative of the oocyte for genetic testing (14). PB₁ mRNA also is being considered as a proxy for the oocyte in tests for oocyte competence and embryonic viability (15–17). The ability to quantify mRNA in a single PB—opens up the possibility that we can detect and compare individual differences in gene expression in the PB without harming the oocyte (18).

The PB₂ is produced by asymmetric cytokinesis ~2h after sperm-egg fusion, ~10h before major zygotic transcription in mouse (19). PB₂ contains about 4.5% of the zygote volume and one maternal chromosome set and a hemi-spindle. PB₂ can be present in the perivitelline space of the developing embryo for several days and usually completely decays before the embryo reaches the blastocyst stage (20, 21). Isolation of the PB₂ after fertilization can be regarded as a non-invasive proxy for cytoplasmic sampling of the oocyte from which the zygote was formed (22).

In this study, we have set out to examine the expression of six maternal effect genes in the PB₂. The candidate genes were chosen based on their important roles in early embryo development in mouse (Table 1). We first compared transcript abundance between PBs and

their sibling zygotes, and then we asked whether that relative abundance of mRNA transcripts in PB₂ is a marker of embryo developmental competence.

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.

Collection and culture of MII oocytes

MII oocytes were collected from 7- to 8-week-old female mice after superovulation was induced by intraperitoneal injection of a single dose (5 IU) 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. Cumulus oophorous complexes (COC) were recovered from ampullae into Leibovitz L15 medium containing 1% fetal bovine serum (FBS) 14 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. All oocyte and embryo culture was in humidified CO₂ (5% (v/v) in air) at 37°C.

Collection of Zygotes Following *In vitro* Fertilization (IVF)

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. A total of ~30 MII oocytes were placed in 50 µl of IVF medium microdrops containing 1×10⁶ sperm/ml and incubated under mineral oil for 4 h. Zygotes were chosen as those having a clear pronuclei, but do not have an extant PB₁ to assist with identification of PB₂ extrusion and reduce contamination with mRNA from earlier stages. Following incubation, each zygote was then washed 3 times in fresh KSOM to remove all bound sperm.

Polar Body Biopsy and Blastocyst Culture

For laser microdissection, a zygote was aspirated with a holding capillary tube. After positioning, a single hole in the ZP was drilled with the XYClone laser system (Hamilton Thorne Biosciences). The PB₂ was gently aspirated into a 19 µm biopsy pipette and put separately from the zygote. Subsequently single PBs were aspirated into a fine glass needle and transferred to the bottom of 0.5-ml thin-wall Eppendorf tubes containing 1.5 µl lysis buffer (0.8% Igepal, 1U RNAsin/µl, 5nM DTT) (23). The Sibling zygotes were either transferred to an identical lysis solution or washed in KSOM and then transferred into a 15-µl drop fresh KSOM cultured to the blastocyst stage individually. Culture media was changed every 48 hours. The lysed specimens were flash-frozen in liquid nitrogen and stored at –80°C in preparation for cDNA synthesis.

Reverse Transcription (RT)

Total mRNA within lysed single PBs and their sibling zygotes was reverse transcribed using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA) without prior RNA purification from the lysate. To normalize for variations in mRNA

content among individual PB and oocyte lysates, 10^6 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 RNA recovery and efficiency of reverse transcription (24). Samples with less pw109 RNA than controls were excluded from further study. RT was performed by adding the following to each sample: 7.7 μ l nuclease-free water, 2 μ l $10\times$ RT buffer, 0.8 μ l dNTP (2.5 mM of each dNTP), and 3 μ l random primers (0.1 μ g/ μ l). Reactions were incubated at 65°C for 5 minutes then cooled to room temperature (approximately 5 minutes). Next, 2 μ l of 100 mM dithiothreitol, 0.5 μ l RNase inhibitor (40 U/ μ l), and 1 μ l reverse transcriptase (50 U/ μ l) 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.

Real-time Quantitative PCR (qPCR)

Gene expression levels in PB₂ and sibling zygotes 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 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 3 times with a negative control of reaction mixture with no cDNA added.

Statistical Analysis

Statistical comparisons for qRT-PCR results were analyzed using *t*-test and one-way ANOVA. Linear regression analysis was performed to test if mean oocyte Ct value could predict detection of mRNA in sibling polar bodies. Stepwise multiple regression analysis was constructed to determine the predictive value for embryo development. The calculation of gene expression was as fold change ($2^{-\Delta\Delta Ct}$) and relative mRNA level [$\Delta Ct = Ct_{(Target)} - Ct_{(18s)}$. The lower ΔCt means the higher mRNA expression level in this study]. Data were reported as mean $\pm$ SD. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the software GraphPad Prism version 4.0.

RESULTS

Analysis of Relative mRNA Expression Levels in Zygote and PB₂

We first investigated the presence of mRNA to test the feasibility of our quantification methods. Candidate maternal-effect genes were selected for their importance in murine post-fertilization development (Table 1). The expression of 6 maternal-effect transcripts was analyzed in individual PB₂ and sibling zygote (n=10 in each group) using real-time qPCR. mRNA was detected and quantified for all of the 6 candidate genes in both zygote and single PB₂. None of the qPCR reactions amplified product from the negative controls. About 99.4% of replicates from single zygote samples yielded an exponential amplification curve for its candidate gene. In single PB₂ samples, transcripts of all the 6 selected candidate genes were detected by real-time qPCR. However, the amplification rate was lower in the PB compared with its sibling zygote (78.9% vs. 99.4%). Genes that were more reliably detected in polar bodies had consistently more abundant transcripts (lower mean oocyte and polar body Ct values; Table II).

Relative Abundance of Selected mRNA Transcripts in PB₂ vs. Sibling zygote

The relative levels of all 6 maternal-effect mRNA transcripts were lower in individual PB₂ compared with their sibling zygote. We set the normalized level of each transcript in the

sibling zygote at 1, and then determined the fold change in transcript abundance in PB₂. The change in transcript levels was between 0.52-fold (*Tc1l*) and 0.93-fold (*Nobox*) (Fig. 1).

Genes in PB₂ Reflect the Embryo Developmental Competence

After 5 days culture, we categorized embryos into four groups according to morphological changes during preimplantation development (Each group was evaluated in a minimum of 10 embryos): 2-cell embryo (maternal-zygotic transition, n=11), cleavage embryo (increase in cell number to 4–8 cells, n=12), morulae (polarization and cellular flattening, n=10), and blastocyst (formation of blastocoels, n=12).

In the first analysis, the relative mRNA transcripts per PB₂ resulting in different developmental stage embryos were compared. We found *Dnmt1*, *Nobox* and *Npm2* expression were significantly higher in PB₂ that yielded blastocysts vs. 2-cell embryo. These differences were also found in PB₂ *Npm2* expression yielding cleavage embryo vs. 2-cell embryo. Moreover, *Tc1l* was significantly up-regulated in the PB₂ of sibling zygote that produced embryos with high developmental potential (Fig. 2, $P < 0.05$).

After statistically significant correlations of gene expression to embryo quality were found, a stepwise multiple regression analysis was performed to characterize further the candidate genes as predictors of embryo developmental competence. Results showed that there was a statistically significant relationship at the 99% confidence level between embryo quality and *Dnmt1* expression and at the 95% confidence level for *Tc1l* expression. The predictive model was $Y = 9.726 - 0.248X_{Dnmt1} - 0.351X_{Tc1l}$ ($R^2 = 0.61$).

DISCUSSION

This is the first report, to our knowledge, documenting the quantification of maternal-effect transcripts in mouse PB₂, and providing an insight into the relationship between gene expression in PB₂ and embryo quality.

We first analyzed the transcripts expressed in individual zygotes alongside their polar body siblings, and show that after the PB₂ is extruded, the transcripts of both single zygotes and single polar bodies can be assessed quantitatively. Global measurements have indicated that during oogenesis, mRNA is actively transcribed and accumulated in growing oocytes. Fully-grown oocytes contain about 20 to 45% of all mouse genes (25). Oocyte become transcriptionally quiescent during meiotic maturation in the ~12 hours prior to ovulation and a large fraction of the maternally supplied mRNAs is degraded by the two-cell stage (26, 27). Although the mechanisms responsible for these RNAs degradation are incompletely understood, this degradation must have some selectivity (28). Gene expression profiling experiments have provided evidence that some maternal transcripts are degraded rapidly after fertilization if they encode proteins that play a role in specific events during meiosis and are detrimental for development after fertilization; others show a later decrease that coincides with the major onset of transcription at the two-cell stage if the maternal factors are required to bridge the interregnum between the maternal and embryonic control of development. However, how mouse oocytes store and degrade maternal mRNAs is not well understood (28–31). Our results also show the wide variations in the probability of detection of the 6 maternal-effect genes in PB₂ and transcripts that were present in greater abundance in the zygote were more likely to be detected in qPCR replicates from single polar bodies. The understanding of which factors determine maternal-effect genes degeneration in individual zygote and species-specific differences in degeneration rates could be used to find links between specific molecular features and the embryo's acquisition of development competence.

When we compared the transcripts between PB₂ and the sibling zygote, the results showed that the abundance of transcripts for the 6 genes would be lower but varied in PBs compared to their sibling oocytes. VerMilyea et al. compared transcriptome profiles of paired single cell and sub-cellular structures obtained micro surgically from mouse oocytes to totipotent embryos and demonstrated there is transcriptome asymmetric within mouse zygotes but not between early embryonic sister blastomeres (22). It is well documented that the targeted localization of cytoplasmic mRNA is a key mechanism by which asymmetric patterning is achieved in metazoan oocytes and early totipotent embryos that establish stem cell lineages (32). These works and our own led us to propose that the PB₂ signature transcriptome might serve to inform strategies for predicting embryo viability (22, 33, 34). Microarray approach is a very useful tool for the discovery of the genomic profiling of zygotes and PBs. We expect that further study will demonstrate intriguing correlations between transcript levels in zygotes and PBs. Such findings would be useful not only to characterize normal development, but also to understand the effects of exposure to abnormal culture environments on preimplantation development.

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 (35, 36). For successful transition into a clinical application using this approach, we biopsied the PB₂ and continuously culture the sibling zygotes in order to evaluate the relationship between maternal-effect gene expression levels in PB₂ and the embryonic developmental competence of its sibling zygotes to determine the predictive value of gene expression as biomarkers. We found the transcript levels of *Dnmt1*, *Nobox*, *Npm2* and *Tcl1* in PB₂ resulting in blastocysts were significantly up-regulated compared with those resulting in 2-cell stage embryos. Successful preimplantation embryo development depends on the oocyte be sufficiently equipped with stored maternal products. Up to 10% of maternal mRNA persist until the blastocyst stage of development and thus could have a critical roles that extends beyond ensuring embryonic gene activation (EGA) (37). Wells et al revealed that embryos with patterns of gene expression appropriate for their developmental stage have superior viability to those displaying atypical gene activities (38). Our prior work quantified 6 maternal-effect mRNA transcripts in PB₁ from young and aged mouse MII oocytes and demonstrated that 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 (17). This study and our previous data led us to propose that a PB biopsy to measure maternal-effect mRNA abundance may serve as biomarkers of embryo competence (39). Future studies are needed to evaluate dynamic changes in mRNA turnover in PB₂, as well as to identify a cohort of transcripts that are reliably detected in PB₂, whose expression levels are predictive of the developmental competence of a given zygote and its resulting embryo.

Early embryonic development is regulated both genetically and epigenetically. During the development process, the epigenetic signature changes as the cell enters fertilization and/or differentiation. DNA methylation is an important epigenetic event, regulating gene expression and chromatin structure in any developmental processes. To maintain methylated DNA, DNA methyltransferases (DNMTs) are necessary. *Dnmt1* enzyme is the main methyltransferase by far. Genetic studies have suggestion *Dnmt1* may play a role in the methylation of repeat element in growing oocytes and embryogenesis (40–42). Assou et al. found that *Dnmt1* were over expressed in mature MII oocytes and decreased progressively in embryos on Day3. In our study, *Dnmt1* were highly expressed in PB₂, which was significantly up-regulated in PB₂ associated with embryo development potential. Indeed, Li et al. demonstrated that targeted mutation of DNA methyltransferase gene results in embryonic lethality. A high level of *Dnmt1* is necessary and sufficient to ensure maintain DNA imprints and therefore be a biomarker for optimal embryonic development. The

further study of the methylome and the epigenome is important because minor defects can lead to serious human disease (43).

The availability of technical platforms and whole genome microarrays will accelerate our understanding the molecular programme controlling the preimplantation embryo development and identifying specific gene function during preimplantation development. The PB samples indirectly offer the possibilities to gain deep insights into the intricate mechanisms underlying the development of embryo competence. The unraveling of this information will ultimately lead to improved efficiency and health outcomes (i.e. reduced prematurity/perinatal mortality rate and maternal and pediatric complications) of ART. With the availability of a reliable approach capable of identifying the best quality oocytes/embryos, multiple embryo transfer may no longer be necessary in the future.

In conclusion, this is first study to report a differential gene expression between mouse PB₂ from zygote resulting in different embryo development potential. This analysis is a novel concept, and demonstrates that it is possible to accurately quantify several genes in a single PB using real-time qPCR, and these genes may serve as new biomarker candidates to assess the embryo quality. Future studies are needed to correlate gene function and regulation to specific events in early embryonic development, which can facilitate the identification of new biomarkers. These tools may ultimately lead to non-invasive tests for oocyte or embryo quality. Once fully validated, these new approaches are expected to improve oocyte and embryo selection, leading to increased implantation rates (44).

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FIGURE 1.

The relative abundance of each of the 6 maternal-effect genes in PB₂ compared with their sibling zygote. If the normalized mRNA level of each transcript in the sibling zygotes is set at 1, the abundance of the transcripts in PB₂ was consistently lower than in the sibling zygote. Fold change: $2^{-\Delta\Delta C_t}$; $\Delta\Delta C_t = [C_{t(\text{Target})} - C_{t(18s)}]_{\text{PB2}} - [C_{t(\text{Target})} - C_{t(18s)}]_{\text{zygote}}$. Error bars represent SD.

FIGURE 2.

PB₂ gene expression in relate to the different stages of preimplantation embryo development. *Dnmt1*, *Nobox* and *Npm2* expression were significantly higher in PB₂ that yielded blastocysts vs. 2-cell embryo. These differences were also found in PB₂ *Npm2* expression that yielded cleavage embryo vs. 2-cell embryo. Moreover, *Tcl1* was significantly up-regulated in the PB₂ of sibling zygote that produced embryos with high developmental potential. Relative mRNA level $\Delta Ct = Ct_{(Target)} - Ct_{(18S)}$. Significance differences was observed between groups with same letters ($P < 0.05$).

Table 1

Maternal-effect genes selected for testing in individual PBs and sibling zygotes
 Oocyte-specific genes selected for testing in individual PBs and sibling oocytes

Gene	Function	Accession No.
<i>Dnmt1</i>	Necessary for embryogenesis	NM_001199431.1
<i>Mater</i>	Required for normal early embryogenesis	NM_011860.2
<i>Nobox</i>	Regulation of embryonic genome activation, pluripotency gene expression, and blastocyst cell allocation	NM_130869.3
<i>Npm2</i>	Control the activation of sepcific maternal mRNAs necessary for early embryogenesis	NM_181345.3
<i>Tc11</i>	Participates in early embryonic development	NM_009337.3
<i>Zar1</i>	Play essential roles during the oocyte-to-embryo transition	NM_174877.3

Table 2

Transcript levels and detection of mRNA in single polar bodies

Gene	Mean zygote Ct value (SD)	Mean PB Ct value (SD)	Probability of detection ^a
<i>Dnmt1</i>	28.29 (1.54)	35.50 (1.94)	0.92
<i>Mater</i>	30.80 (1.83)	37.17 (0.62)	0.65
<i>Nobox</i>	29.05 (1.64)	35.39 (1.15)	0.85
<i>Npm2</i>	30.86 (2.66)	36.42 (2.73)	0.75
<i>Tcl1</i>	27.94 (2.11)	35.27 (2.08)	0.88
<i>Zar1</i>	28.45 (1.66)	34.70 (1.48)	0.84

^aProbability of detection refers to the percentage of qPCR experiments in which transcript was detected. A strong inverse correlation was noted between the mean zygote C_t value and the probability of detection that transcript in a sibling polar body (Pearson's correlation coefficient: -0.88, *P*<0.05)