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ORIGINAL RESEARCH

Detection and quantification of mRNA in single human polar bodies: a minimally invasive test of gene expression during oogenesis

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ABSTRACT: Proteins and mRNA produced in oogenesis support embryonic development until the zygotic transition, 3 days after fertilization. Since polar bodies can be biopsied with little if any harm to the oocyte, we tested the hypothesis that mRNA originating from expression in the meiotic oocyte is present and detectable in a single polar body prior to insemination. Human oocytes were obtained from patients undergoing controlled ovarian hyperstimulation and intracytoplasmic sperm injection. Immature oocytes were cultured overnight and inspected the following day for maturation. Metaphase II oocytes underwent polar body biopsy followed by reverse transcription without RNA isolation. Sibling oocytes were similarly prepared. Complementary DNA from all samples were pre-amplified over 15 cycles for candidate genes using selective primers. Real-time PCR was performed to detect and quantify relative single-cell gene expression. Polar body mRNA was detected in 11 of 12 candidate genes. Transcripts that were present in greater abundance in the oocyte were more likely to be detected in qPCR replicates from single polar bodies. Pre-amplification of cDNA synthesized without RNA isolation can facilitate the quantitative detection of mRNA in single human polar bodies.

Key words: gene expression / infertility / oocyte quality / oocyte / meiosis

Introduction

The mature oocyte has a unique, cytoplasm-dependent ability to incorporate foreign DNA, regulate gene expression and direct early embryonic differentiation. These functions require specific changes in the mRNA populations that have been documented in experiments using pooled oocytes (Assou *et al.*, 2006; Evsikov and Evsikova, 2009). The clinical importance of healthy oocyte development is evidenced by the impressive pregnancy rates seen with infertile women using assisted reproductive techniques (ART) with oocytes from young, fertile donors. Oocytes from younger women have lower rates of meiotic errors and aneuploidy, which may result from cytoplasm-dependent processes. Although aneuploidy is the most common cause of developmental arrest, screening embryos for aneuploidy does not exclude all embryos of poor prognosis. Despite performing comparative genomic hybridization across 24 chromosomes, many euploid blastocysts, fail to implant and develop into living embryos, suggesting potential problems in either gene expression or endometrial receptivity (Schoolcraft *et al.*, in press).

Oocyte gene expression can be evaluated in messenger RNA, which represents genetic functionality and the downstream effect of epigenetic influences mediating oocyte development. The functional importance of cytoplasmic factors in the oocyte is supported by observations that oocytes can parthenogenically divide without sperm and that enucleated oocytes can support full embryonic development after somatic cell nuclear transfer (Briggs and King, 1952; Rascado *et al.*, 2010). Previous studies of oocyte populations at varying developmental stages have identified rapid changes in mRNA profiles that occur during oocyte maturation and subsequent fertilization, but most data are limited to cohorts of oocytes (Assou *et al.*, 2006; Gasca *et al.*, 2007). Few investigators have evaluated genetic expression in individual oocytes, and no investigators have heretofore evaluated RNA from individual oocytes without destroying the oocyte (Hartshorn *et al.*, 2005; Kocabas *et al.*, 2006; Kurimoto *et al.*, 2007). The developmental potential of individual oocytes varies widely, making it challenging to evaluate successful oogenesis from gene expression in pooled oocytes.

The ability to detect and compare individual differences in oocyte gene expression without harming the oocyte may prove helpful to clinicians caring for patients using ART (Assou *et al.*, 2008). Such a technique would allow embryologists to test for the presence or

abundance of critical mRNA transcripts in the ooplasm. Evaluating this gene expression in the oocyte is important since many of these genes undergo functional control through mRNA turnover and epigenetic processes, which depend on more complex determinants than having an appropriate number of genes (Seli *et al.*, 2005; Biddle *et al.*, 2009; Evsikov and Marín de Evsikova, 2009).

We propose that mRNA can be detected at the level of a single human polar body. Although polar body biopsy has been used for diagnosing maternally derived genetic errors, its clinical utility has been limited because both polar bodies must be sampled and only Mendelian mutations or aneuploidy arising from maternal chromosomes can be detected (Verlinsky *et al.*, 1997, 1999). If mRNA could be detected and assayed in the polar body, assessment of genetic function in the oocyte through analysis of a single polar body and could be performed. Subsequent information could be available within 24 h to assist in selecting embryos for transfer that derived from oocytes with the greatest developmental potential. Alternatively, oocytes could be vitrified following polar body biopsy and prioritized for insemination in situations where the creation of supernumerary embryos is undesirable.

Herein, we attempted to detect and quantify mRNA from a single human polar body. We further test the hypothesis that the relative abundance of mRNA transcripts in a single polar body reflects the relative abundance of that transcript in its sibling oocyte.

Materials and Methods

Human oocyte collection and polar body biopsy

Human oocytes were collected from infertility patients undergoing controlled ovarian hyperstimulation for IVF under a standard clinical protocol. Germinal vesicle (GV) and metaphase I (MI)-staged oocytes that were not mature for a clinically indicated ICSI procedure underwent *in vitro* maturation for 24 h and were used in the study if they extruded a polar body. Written consent was obtained from all patients to use discarded tissue and oocytes for research, and the study was approved by the institutional review board at Women and Infants Hospital. Briefly, 11 patients underwent controlled ovarian hyperstimulation, with gonadotrophin stimulation and pituitary suppression using either a GnRH antagonist or antagonist. Oocytes were aspirated by an ultrasound-guided transvaginal oocyte retrieval 36 h after injection with recombinant HCG. Four hours after retrieval, all oocytes were mechanically stripped of cumulus cells. ICSI was performed in all oocytes with visible polar bodies. After injection of all MII oocytes, any remaining immature oocytes were cultured for 20–24 h in SAGE IVM media (Cooper Surgical, Trumbull, CT, USA). Immature oocytes were examined the next day and oocytes that extruded a polar body were used for our study.

Biopsy and reverse transcription

All biopsies were performed at ×200 magnification after mechanical zona drilling with a polar body biopsy needle (Cook Medical, Bloomington, IN, USA). Polar bodies were aspirated into a glass micropipette with an inner diameter of 20 μm. The polar body was then processed using the Ambion Cells-to-Ct Direct kit (Life Sciences, Carlsbad, CA, USA). Briefly, the polar body was transferred to a lysis buffer containing DNAse I. After a 5-min DNA digestion, DNAse was inactivated with Stop solution. Sibling oocytes were transferred to an identical lysis solution and processed using the same protocol. The lysed specimens were stored on ice for no more than 2 h, whereas other oocytes were biopsied. The samples of DNAse-treated cell lysate were reverse transcribed (RT) without prior RNA isolation, in a final reaction volume of 25 μl. After completing the RT, enzymes were inactivated and cDNA was denatured from RNA by heating to 95°C for 5 min. In order to minimize the sample loss, all processing was performed in the same 700-μl qPCR tube.

The cDNA product from the reverse transcription reaction was then specifically and exponentially amplified for each of the candidate genes over 15 cycles, using the non-biased Ambion PreAMP cDNA amplification kit (Life Sciences). A list of the primers and details of the protocol are shown in Supplementary protocol and Supplementary data, Table SI.

Analyses of gene expression

Gene expression was initially tested in 12 genes across 15 oocyte and polar body pairs (Table I). After pre-amplification, genes were analyzed in triplicate using Real-time TaqMan qPCR with products generated from the same primers that were used in the pre-amplification step (Life Sciences). Two repeat qPCR tests were performed after an initial single-reaction qPCR reaction confirmed the successful reverse transcription and pre-amplification of polar body samples, thus generating a triplicate assay. The gene expression assay is a custom made TaqMan assay that contains both the primers and a gene-specific florescent probe that is dependent on cleavage by DNA polymerase during amplification of the target cDNA sequence. Primers used for qPCR 'gene expression assays' spanned introns in all genes except for *DPPA3* and *18s*. Template RNA had also undergone previous treatment with DNAse I to remove genomic DNA. Location of the primer targets on the gene can be found online (www.allgenes.com).

Table I Candidate genes selected for testing single oocytes and its sibling polar body.

Gene	Function	Relevance
Bcl2L10	Inhibits apoptosis, antioxidant function	Survival factor in oocytes
Ddx4	RNA helicase	Translational regulation during oogenesis and embryogenesis
Dppa3	Regulates transcription	Helps maintain pluripotency during oogenesis
Odc1	Antioxidant and anti-apoptosis	Oocyte survival
Padi6	Post-translational protein modification	Highly expressed in M2 oocytes
Gapdh	Glycolysis	Endogenous control
Dicer	microRNA biogenesis	Critical to mRNA regulation
Ago2	Endonuclease activity in RNA-induced silencing complex (RISC) complex	Necessary for piRNA function
Eif6	Regulates translation of mRNA	Null mutants are lethal at implantation
H1foo	Epigenetic remodeling	Highly expressed in M2 oocytes
Pabp	Binds poly-A tail	Stabilizes oocyte mRNA
Drosha	microRNA processing	Critical to mRNA regulation

Genes were included in the analysis if they generated exponential amplification curves in triplicate for at least one-third of the reactions. Unamplified ribosomal RNA was measured from the same dilute pool of pre-amplified cDNA; however, 18s primers were not included in the pre-amplification master mix. Therefore, 18s RNA only underwent first-strand cDNA synthesis. The levels of two candidate genes were compared with the levels of unamplified 18s RNA. Candidate genes and 18s transcripts were tested in separate wells on the same plate, rather than multiplexed. All reactions used the same 60°C hybridization temperature and qPCR cycle parameters.

Two negative controls were included with every qPCR sample: (i) an oocyte that was processed similarly to the other samples but without addition of reverse transcriptase, and (ii) the template was replaced by nuclease-free water. All qPCR assays were run in triplicate.

Threshold C_t values were set at a level where the slopes of all samples displaying appropriate amplification curves were parallel. Multivariable logistic regression analysis was performed to test if mean oocyte C_t value could predict detection of mRNA in sibling polar bodies. Relative C_t values were compared using two approaches in order to control for differences in polar body size and for global RNA degradation and loss during processing of different samples. First, we compared the relative intracellular abundance of the six different transcripts to each other by ranking the genes in the order of transcript abundance and comparing their median ranks to each other (Fig. 1). Comparison of relative rankings between polar body and sibling oocyte were tested using a sign test with a conservative threshold for statistical significance set using a Bonferroni correction for multiple comparisons. We also measured gene expression in relation to the endogenous level of unamplified 18s ribosomal RNA (Fig. 2).

To test the linearity of the pre-amplification step, a 7-μl aliquot was removed from the same oocyte sample after 0, 3, 6, 9, 12 and 15 cycles of pre-amplification. Aliquots were taken immediately following the extension phase, whereas the thermocycler was held for <30 s. C_t values of each aliquot were then run in triplicate and quantified. This was performed in three single-oocyte samples testing two different candidate genes from each sample. Pre-amplification was tested in six genes. Results were analyzed by linear regression.

Results

The goal of this study was to assay for the presence of mRNA and test the feasibility of quantification; however, some functional consideration was also given to the selection of candidate genes (Table I). Candidate genes were selected if prior studies had suggested they were highly expressed in MII oocytes compared with GV and granulosa cells (Assou *et al.*, 2006). Candidate genes were also selected for their importance in murine and human post-fertilization development. *Ago2*, *Dicer* and *Drosha* were selected for their role in microRNA-mediated mRNA turnover, a process which is likely to be involved in the oocyte to embryo transition following fertilization. *Odc1* and *Bcl2L10* were selected for their antioxidant and anti-apoptotic roles in both oocyte maturation and early embryogenesis (Guillemin *et al.*, 2009; Zhou *et al.*, 2009). *Padi6* and *Eif6* were selected for their role in regulating translation during the oocyte to embryo transition in murine models (Gandin *et al.*, 2008; Yurttas *et al.*, 2008). *Dppa3* and *Ddx4* are highly expressed in MII oocytes and are necessary for transcriptional and translational regulation in oogenesis and early embryogenesis (Assou *et al.*, 2006). *Gapdh* was selected as a housekeeping gene to normalize other transcript levels. *H1Foo* was tested as an additional housekeeping gene for its oocyte specificity; its importance in epigenetic programming; and the high expression of histone transcripts previously identified in starfish oocytes (Klatsky *et al.*, 2010).

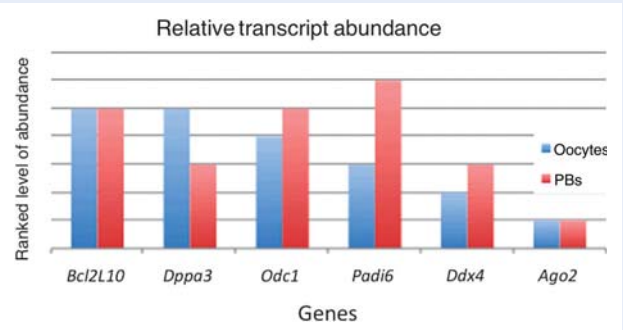

Figure 1 Levels of each transcript were ranked in the order of abundance, relative to each other. Median ranks are shown.

Messenger RNA was detected and quantified for 11 of the 12 candidate genes in both single oocytes and single polar bodies. One transcript, *H1foo*, could not be detected in any of the polar bodies. About 98.9% of replicates from single-oocyte samples yielded an exponential amplification curve for its candidate gene. None of the qPCR reactions amplified product from the negative controls, which included: (i) samples without reverse transcriptase and (ii) qPCR reactions without template.

Pre-amplification of cDNA was necessary to quantify transcript abundance in single polar bodies; therefore, we tested the efficacy of the pre-amplification procedure, to evaluate for bias through uneven amplification of cDNA transcripts, prior to qPCR. Aliquots taken from single-oocyte samples, tested by qPCR before and after every three cycles of 'pre-amplification', demonstrated an exponential increase in cDNA (Supplementary data, Fig. S1). Real-time PCR results showed high correlation ($r = 0.92$) and linear decrease in the C_t value with increasing cycles for a mean efficiency of 95.5% (95% CI: 0.87–1.03). Pre-amplification appeared linear and unbiased over all 15 cycles.

In single polar body samples, half of the candidate genes tested yielded results in at least one-third of the replicates (Table II). Genes that were more reliably detected in polar bodies had consistently more abundant transcripts (lower mean oocyte and polar body C_t values; Table II). Although 12 genes were tested, 6 genes were excluded from the analysis because <33% of polar body qPCR replicates generated quantifiable results (indicated in bold in Table II). These excluded candidate genes, *H1foo*, *Pabp*, *Eif6*, *Drosha*, *Dicer* and *Gapdh* were less abundant in sibling oocytes (Table II). The abundance of mRNA transcripts in the oocyte correlated directly with the odds of successfully detecting and quantifying mRNA abundance in its sibling polar body; for every one unit increase in the oocyte C_t value, the odds of detection in each polar body replicate decreased significantly (OR = 0.73, 95% CI: 0.60–0.88).

We ranked the candidate genes in the order of abundance in each oocyte and polar body sample, in order to compare the relative level expression between genes in the oocyte and polar body. Median ranks are shown in Fig. 1. The similar relative representations of highly expressed transcripts for *BCL2L10* and *Odc1* transcripts, as well as

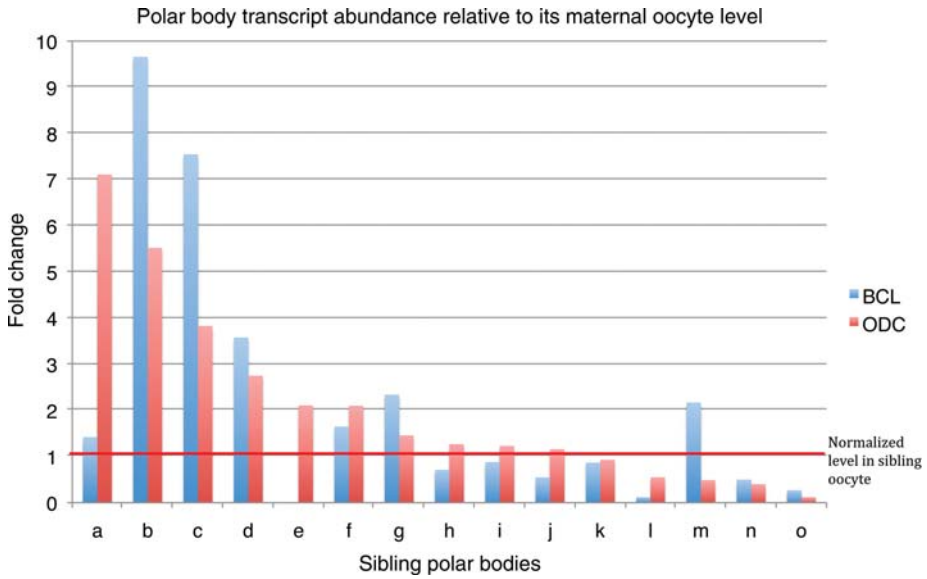

Figure 2 The levels of *Odc1* and *Bcl2L10* transcripts in the polar body were normalized to unamplified 18s ribosomal RNA and compared with levels in their sibling oocyte. Some polar bodies expressed relatively more mRNA transcripts than their sibling oocytes, whereas other polar bodies demonstrated less mRNA.

Table II Transcript levels and detection of mRNA in single polar bodies.

Gene	Mean oocyte C_t value (SD)	Mean PB C_t value (SD)	Probability of detection[a]
Bcl2L10	27.0 (1.9)	35.1 (2.4)	0.74
Odc1	27.9 (1.7)	36.6 (2.2)	0.90
Dppa3	27.9 (2.7)	37.8 (2.6)	0.81
Padi6	28.1 (1.3)	36.5 (1.6)	0.80
Ddx4	28.7 (1.3)	36.6 (1.2)	0.70
Ago2	30.4 (2.4)	37.2 (2.7)	0.39
Drosha	**30.5 (1.7)**	**37.1 (*)**	**0.16**
Gapdh	**32.1 (2.5)**	**37.9 (*)**	**0.21**
EIF6	**31.6 (1.5)**	**38.7 (*)**	**0.24**
PABP	**32.5 (2.5)**	**38.1 (*)**	**0.21**
Dicer	**32.6 (1.5)**	**38.3 (*)**	**0.23**
H1foo	**32.9 (2.5)**	**Undetectable**	**0**

[a]Probability of detection refers to the percentage of qPCR experiments in which transcript was detected. A strong inverse correlation was noted between the mean oocyte C_t value and the probability of detecting that transcript in a sibling polar body (Pearson's correlation coefficient: -0.94, $P < 0.0001$).
*Too few samples to calculate a standard deviation.

the similarly low expression of *Ago2* in both oocytes and polar bodies, can be appreciated. These trends are consistent with the finding that mRNA transcripts were less likely to be detected/quantified in polar bodies if their abundance was lower in its sibling oocyte (Table II).

To test the relative abundance of a single gene between an oocyte and its sibling polar body, we evaluated the normalized expression of two genes, *Bcl2L10* and *Odc1*. These transcripts were highly expressed and reliably detected in most polar body replicates. We initially planned to use pre-amplified GAPDH as a reference; however, this transcript was not reliably detected in polar bodies. Therefore, *BCL2L10* and *Odc1* were normalized using unamplified levels of endogenous 18s ribosomal RNA as a reference. The proportion of each of these two mRNA transcripts varied greatly between the polar body and its sibling oocyte, compared with endogenous rRNA (Fig. 2). In some samples, the $\triangle\triangle C_t$ suggested higher relative levels in the polar body, whereas other samples suggested lower relative levels in the polar body (indicated by fold change between 0 and 1).

Discussion

This is the first report, to our knowledge, documenting the presence of mRNA in a human polar body. This new technique evaluates gene expression in a single oocyte and a single polar body without isolating RNA. Reverse transcription with pre-amplification of cDNA, followed by qPCR allowed detection and quantification mRNA in samples as small as a single polar body. Genes with higher levels of expression in oocytes appear more reliably detectable in the sibling polar body, suggesting that a failure to obtain results in the polar body may relate to oocyte transcript levels that fall below a critical threshold. This finding is consistent with our previous results in starfish oocytes (Klatsky *et al.*, 2010).

In this pilot study, we used unfertilized human oocytes that were cultured for 28–30 h after retrieval. The wide variations seen in the relative proportion of *Bcl2L10* and *Ocd1* transcripts between the polar body and its sibling oocyte may reflect biologic variability resulting from the long duration of oocyte culture without fertilization. Some polar bodies may have been extruded 2 h before biopsy, whereas others may have been extruded by the oocyte 20 h prior. Previous studies have documented the early onset of apoptosis in polar

bodies after oocyte maturation (Fujino *et al*., 1996; Van Blerkom and Davis, 1998). Therefore, variation in polar body transcript profiles relative to the transcript profile in the sibling oocyte may result from some RNA transcripts being less stable in cells undergoing apoptosis, particularly if these transcripts are not freely diffusible and are associated with intracellular complexes or organelles. Similarly, these oocytes may represent a less 'healthy' cohort as they underwent delayed *in vitro* maturation compared with the remainder of their cohort that matured *in vivo* and were inseminated 4 h after retrieval. Alternatively, these findings may reflect epigenetic differences between oocytes in mRNA distribution or turnover. We cannot rule out technical causes to account for this variation, but the results from our linear pre-amplification and the tight standard errors between qPCR replicates for each sample gave confidence in this interpretation.

Interestingly, the differences in the rank order of several genes (Fig. 1) were not as variable as the difference seen between the polar body and its sibling oocyte for *Bcl2L10* and *Odc1* when they were normalized to 18s. The use of ribosomal RNA to normalize levels of mRNA transcripts should be interpreted cautiously and may contribute to the great variation in mRNA levels between the sibling polar body and oocytes depicted in Fig. 2. Use of a highly expressed housekeeping gene may prove more accurate to normalize expression of other mRNA transcripts. Differences in the ranking of relative abundance were statistically significant only for *Padi6* ($P = 0.001$). These differences may reflect either technical variation, statistical chance or differences in the stability of a particular transcript in the polar body when compared with the oocyte. Given the quiescent nature of the MII oocyte, it is unlikely to be attributable to ongoing transcription.

Prior studies have demonstrated that as the primary oocyte develops, it transcribes thousands of genes whose products are necessary for fertilization and early embryonic development. Prior to MI, the GV breaks down and transcriptional factors disengage from chromatin, rendering the cell transcriptionally silent (Sun *et al*., 2007). This is particularly relevant since the human zygotic genome is not activated and cannot transcribe its DNA for 2–3 days following fertilization (Braude *et al*., 1988). Therefore, mRNAs that are needed for fertilization and early embryonic development must be present in the oocyte in sufficient quantity before the first polar body is extruded. These RNA and related proteins guide the majority of intracellular embryonic processes that occur prior to a Day 3 embryo transfer in couples using ART. Detection and analysis of oocyte mRNA may provide insight into developmentally critical processes in the oocyte. We suggest that the extruded polar body may contain a representative transcript profile to that of the oocyte when it completes MI.

No prior investigators have examined RNA in polar bodies, likely due to the technical limitations of biopsy and subsequent single-cell RNA analysis. It may be that gene expression in human oocytes is reflected in mRNA present in the polar body. Images taken by electron microscopy suggest that cytoplasm extruded from the oocyte into the polar body during MI is representative of the ooplasm, containing cortical granules, mitochondria and endoplasmic reticulum (Longo 1997). Similar images of the second polar body revealed absent cortical granules again suggesting a similarity between the ooplasm and polar body cytoplasm.

Polar body biopsy involves careful removal of a polar body through microdissection and can be performed without damaging the sibling oocyte or developing embryo (Verlinsky *et al*., 1990). Interest in polar body biopsy is fueled by the potential to diagnose healthy/unhealthy oocytes prior to fertilization. This ability, coupled with advances in oocyte vitrification would be helpful for patients with ethical objections to fertilizing multiple oocytes and creating supernumerary embryos. One can also imagine using gene expression information from a polar body to prioritize embryos for transfer in an IVF cycle.

As a pilot study, these results should be interpreted conservatively. We demonstrated that advances in molecular genetic techniques now permit detection and quantification of mRNA in individual cells as small as a single polar body. We expect that further refinement of this technique and application to fresh oocyte–polar body pairs will demonstrate intriguing correlations between transcript levels in oocytes and polar bodies. Such findings would be useful not only to clinicians and embryologists, but to researchers studying mRNA dynamics, including mRNA turnover during MI and MII. We appreciate the rapid and dynamic changes in oocyte gene expression and mRNA turnover and suspect that the polar body would reflect the mRNA status of the oocyte at the time of biopsy. Unfortunately, it will not address earlier/later transitions in oocyte transcriptional regulation, but it will give us a snapshot of the oocyte at that critical time in a care decision.

Future studies are needed to evaluate the correlation between mRNA profiles in freshly collected oocytes and polar bodies, as well as to test whether threshold proportions of critical transcripts can predict fertilization and development in sibling oocytes. While the promise of identifying a 'good' oocyte for fertilization remains far off, technological advances and identification of functionally important genes in the oocyte to embryo transition offer new opportunities in the ongoing search for a minimally invasive metric of oocyte developmental potential.

Authors' roles

All experiments and biopsies were carried out by P.C.K. All molecular genetic laboratory procedures were performed under the supervision of G.M.W. and polar body biopsies were performed under the supervision of S.A.C. All authors contributed to study design and preparation of the manuscript.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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