MHR *Basic science of reproductive medicine* **ORIGINAL RESEARCH**

Transcriptomic profiling of human oocytes: association of meiotic aneuploidy and altered oocyte gene expression

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ABSTRACT: The ability to identify oocytes with the greatest potential for producing a viable embryo would be of great benefit to assisted reproductive treatments. One of the most important defects affecting oocytes is aneuploidy. Aneuploidy is also closely related with advancing maternal age, a phenomenon not well understood. This study combined a comprehensive cytogenetic investigation of 21 oocytes with a detailed assessment of their transcriptome. The first polar body was removed from all oocytes and aneuploidy assessed using comparative genomic hybridization. Preliminary mRNA transcript data were produced with the use of microarrays for seven of the corresponding oocytes (three normal and four aneuploid). The results obtained for normal and aneuploid oocytes were compared and 327 genes were found to display statistically ($P < 0.05$) significant differences in transcript levels. Ninety-six of these genes were further assessed in seven aneuploid and seven normal oocytes using real-time PCR. The results indicated that aneuploidy is associated with altered transcript levels affecting a subset of genes. A link between mRNA transcript numbers and age was also observed. The possibility that different transcript levels in the oocyte have an impact on cellular pathways remains to be proven. However, it may be significant that some of the highlighted genes produce proteins involved in spindle assembly and chromosome alignment. Additionally, several genes with altered amounts of transcript produce cell surface or excretory molecules, and could potentially serve as targets for non-invasive oocyte aneuploidy assessment.

Key words: meiosis / gene expression / non-invasive aneuploidy assessment / oocyte / transcriptome

Introduction

In the context of assisted reproductive treatment (ART), an oocyte is considered competent when it is able to complete cytoplasmic and meiotic maturation, undergo fertilization, sustain early embryonic development and ultimately lead to a successful pregnancy (Li *et al.*, 2008). The complex interactions between the oocyte, follicular cells and endocrine systems are crucial for the acquisition of competence, as illustrated by numerous previous studies (Eppig, 1991; Eppig *et al.*, 1997, 2002; Assou *et al.*, 2006; Feuerstein *et al.*, 2007).

However, as demonstrated by recent clinical and research observations, many of the oocytes collected during assisted reproductive procedures are abnormal, cytogenetically or otherwise, and thus unable to produce viable embryos. This may provide a partial explanation for the low live birth rate per oocyte retrieved (about 7%)

and per embryo transferred (about 15%) following ART (Kovalevsky and Patrizio, 2005; Patrizio and Sakkas, 2009). The ability to identify the most competent oocytes would be of great benefit to *in vitro* fertilization (IVF) treatments, allowing the embryos with the highest implantation potential to be prioritized for transfer to the uterus.

Although numerous strategies for evaluating embryos have been proposed, the options for testing oocytes remain extremely limited (Patrizio *et al.*, 2007). Routine oocyte analyses are restricted to a simple assessments of nuclear maturity (i.e. presence of the first polar body is indicative of a mature MII oocyte). Unfortunately, these methods provide only a vague indication of oocyte competence and are incapable of detecting the presence of chromosome abnormalities in the oocyte or other more subtle metabolic or genetic anomalies. Although some studies have attempted to assess oocyte cytoplasm, the surrounding cumulus cells, or the extruded first polar

body, these measures are not widely practiced (Coticchio *et al.*, 2004; Wang and Sun, 2007).

Aneuploidy is extremely common in human oocytes, and is one of the major factors negatively influencing reproductive success. Literature suggests that ~5% of all clinically recognized pregnancies are chromosomally abnormal, carrying a trisomy or a monosomy (reviewed in Hassold *et al.*, 2007). The vast majority of these pregnancies culminate in miscarriage, although a few trisomies (e.g. trisomies 13, 18, 21) are sometimes capable of survival to term, resulting in the birth of babies with congenital defects and/or mental retardation (Hassold and Hunt, 2001).

The vast majority of aneuplodies detected during pregnancy are due to chromosome segregation errors that occurred during oogenesis. Karyotyping studies of human oocytes have described two main mechanisms leading to aneuploidy: the first involves the non-disjunction of entire chromosomes, observed during both meiotic divisions (Zenzes and Casper, 1992), whereas the second involves the premature division (predivision) of a chromosome into its two constituent chromatids, followed by their random segregation, upon completion of the first meiotic division (Angell, 1991).

Data obtained from the cytogenetic analysis of human oocytes have clearly shown a direct relationship between advancing maternal age and increasing aneuploidy rates. Specifically, such studies suggest that the expected aneuploidy rate in the oocytes of women under 25 years of age is ~5%, increasing to 10–25% in the early thirties and typically exceeding 50% in women over 40 (Sandalinas *et al.*, 2002; Kuliev *et al.*, 2003; Pellestor *et al.*, 2003; Hassold *et al.*, 2007; Fragouli *et al.*, 2009a, b).

During IVF cycles, many of the morphologically normal embryos transferred to the uterus, either do not achieve implantation or spontaneously abort during early pregnancy. In many cases, the presence of aneuploidy is likely to be the underlying cause of this failure. It has been suggested that the use of preimplantation genetic screening (PGS) to aid in the identification and preferential transfer of embryos free of chromosome errors could lead to improved IVF success rates (Munne *et al.*, 1993; Gianaroli *et al.*, 1999). Standard PGS strategies employ fluorescent *in situ* hybridization (FISH) and examine up to 12 chromosomes in blastomeres, biopsied from cleavage stage embryos (Colls *et al.*, 2007; Mantzouratou *et al.*, 2007). Various studies have reported a positive outcome after the use of PGS using FISH (Gianaroli *et al.*, 1999; Munne *et al.*, 2005; Schoolcraft *et al.*, 2009). Others, however, failed to see any improvements (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007).

The reason why the preferential transfer of embryos diagnosed euploid using PGS has not always been associated with improved IVF outcome may be related to diagnostic errors due to chromosomal mosaicism (Wells and Delhanty, 2000; Voullaire *et al.*, 2002; Munné, 2006; Hanson *et al.*, 2009; Vanneste *et al.*, 2009a, b). Although most mosaic embryos turn out to have abnormalities in all of their cells, a minority contain a mixture of aneuploid and normal cells and could be incorrectly diagnosed following single blastomere analysis. Problems caused by mosaicism can be avoided by looking at the chromosomes of the oocyte, since any abnormality present in a gamete will be present in every cell of the resulting embryo.

An alternative explanation for the poor PGS results obtained by some laboratories may be excessive damage done to the embryo during biopsy. Biopsy of two cells has been shown to be detrimental to embryo implantation (De Vos *et al.*, 2009) and it is likely that even single cell biopsy has some small effect, and possibly a large impact if poorly performed (Cohen *et al.* 2007). For PGS to increase implantation rates, the improvement in embryo selection it provides must more than compensate for any potential reduction in implantation caused by the biopsy. One possible solution to difficulties related to embryo biopsy is the development of non-invasive methods of detecting aneuploidy.

We sought to gain an improved understanding of the origin of meiotic errors in oogenesis and to identify novel molecular markers of aneuploidy that could be utilized for non-invasive screening. For this purpose, we opted to compare the transcriptomes of aneuploid oocytes with those of chromosomally normal oocytes, searching for genes showing consistent differences in transcript number. Adjustment of polyadenylated mRNA levels represents one of the principal mechanisms utilized by cells in order to regulate cellular pathways and for this reason quantification of gene transcripts from individual genes may help to reveal the processes occurring within a cell at a given moment. In the case of oocytes, abnormal levels of mRNA transcripts may expose the basis of biological problems predisposing to aneuploidy and provide information concerning competency, maturity and general viability (Wells *et al.*, 2005; Wells and Patrizzio, 2008). If the affected genes produce proteins expressed on the cell surface or excreted from the cell, the development of non-invasive assays might also be possible.

Materials and Methods

Patient details

This research was conducted using Institutional Review Board approved protocols and with patient signed informed consent. Ten karyotypically normal women undergoing IVF treatment due to either tubal (seven patients) or male factor (three patients) infertility, agreed to donate mature MII oocytes towards this research project. The average maternal age was 34.5 years (age range: 30–41 years). None of the patients had any evidence of ovarian pathology. Nine of the 10 women were being treated at Tecnobios IVF, Bologna, Italy, while the remaining patient underwent an IVF cycle at Yale Fertility Center, New Haven, CT, USA. Table 1 summarizes their details, along with the number of donated oocytes and the type of mRNA analysis technique with which they were examined. The ovarian stimulation protocol and subsequent oocyte collection was as described in Borini *et al.* (2006) for the nine women who had their IVF treatment in Technobios, whereas the protocol used for the Yale patient was as described in Patrizio and Sakkas (2009).

MII oocytes and corresponding first polar bodies

Twenty-one mature MII oocytes and their corresponding polar bodies (PBs) were processed during the course of this study. All oocytes were morphologically normal and unexposed to sperm. Prior to conducting analysis of gene transcript levels, the PBs underwent comparative genomic hybridization (CGH) analysis to examine their chromosome complement and indirectly that of the corresponding oocytes. All oocytes were processed and analysed individually using either microarray analysis or real-time PCR with TaqMan low density arrays (TLDAs).

Table I Patient details.

Patient no.	Maternal age years	Indication for ART	No. of oocytes examined	Poly-A mRNA analysis
1	30	Tubal factor	7	Array
2	31	Tubal factor	1	TLDAs
3	32	Male factor	1	TLDAs
4	32	Tubal factor	2	TLDAs
5	33	Tubal factor	2	TLDAs
6	35	Tubal factor	3	TLDAs
7	35	Tubal factor	2	TLDAs
8	36	Tubal factor	1	TLDAs
9	40	Male factor	1	TLDAs
10	41	Male factor	1	TLDAs

TLDAs, TaqMan low density arrays.

Oocyte and PB and processing

Micromanipulation of single oocytes and PBs took place in sterile conditions, with solutions and equipment treated to remove/inactivate RNA degrading enzymes. Sample collection was performed rapidly in order to minimize mRNA degradation or any induced changes in gene expression. All oocytes were transferred to a drop of acidified Tyrode's solution (Medicult, USA), allowing the zona pellucida to be removed. First PBs were separated from oocytes and were prepared for CGH analysis, as outlined in Fragouli *et al.* (2006a, b). Each oocyte was briefly washed in calcium and magnesium free phosphate buffered saline (Invitrogen, USA) containing 0.1% w/v polyvinyl alcohol (Sigma, USA) and 0.4 U/ml RNasin Plus RNase inhibitor (Promega, USA). The oocytes were subsequently transferred to microcentrifuge tubes and then immediately frozen and stored at −80°C until RNA extraction took place.

CGH analysis of PBs

The entire CGH procedure took place as described in Fragouli *et al.* (2006a, b). In brief, all PBs were lysed and the DNA subjected to whole genome amplification using degenerate oligonucleotide primed-PCR (DOP-PCR). The amplified DNA was labelled with Spectrum Green-dUTP (Abbott, USA) using a nick translation kit (Abbott, USA). Reference DNA (1 ng), derived from a karyotypically normal female (46,XX), was also amplified with DOP-PCR and labelled with Spectrum Red-dUTP (Abbott, USA). The test and reference DNAs were co-precipitated and hybridized to slides covered with many cells in metaphase. The hybridization time was 72 h.

Microscopy, image analysis and interpretation

After a series of post-hybridization washes (Fragouli *et al.* 2006a, b), metaphase spreads were photographed using an Olympus BX 61 fluorescent microscope equipped with a cooled charge-coupled device system. Ten metaphases were captured per hybridization and the images analysed using Cytovision CGH software (Version 3.9, Applied Imaging, USA), allowing a red–green ratio to be calculated for each chromosome. Equal sequence copy number between the test and reference DNAs was seen as no fluctuation of the ratio profile from 1:1. Test sample under-representation was seen as fluctuation of the ratio profile in favour of the red colouration (ratio below 0.8), whereas test sample over-

representation was seen as fluctuation of the profile towards the green colouration (above 1.2). Such fluctuations were respectively scored as losses or gains in the test sample. Distinction between chromosome and chromatid errors took place as described previously (Fragouli *et al.*, 2006a, b). Previous studies have confirmed the reciprocal nature of chromosomal losses and gains in oocytes and PBs (Gutiérrez-Mateo *et al.* 2004; Fragouli *et al.*, 2006a, b).

Oocyte RNA extraction and amplification

Extraction of RNA from single oocytes was achieved with the use of Stratagene's Absolutely RNA Nano-prep Kit (USA), according to the manufacturer's instructions. A two round *in vitro* transcription followed, providing amplification of the extracted RNA to a level sufficient for subsequent microarray analysis (Wells and Patrizzio, 2008). For this purpose, a combination of two different commercially available kits was employed. The first amplification round utilized the TargetAmp-2Round aRNA Amplification Kit 2.0 (Epicentre, USA), whereas the second round employed the NanoAmp RT-IVT Labeling Kit (Applied Biosystems, USA). Both amplification rounds took place according to the manufacturer's protocols. During the second round of amplification, digoxigenin labelled nucleotides were incorporated into the RNA, permitting subsequent chemiluminescent detection of cRNA after hybridization to a microarray. Amplified RNA integrity and concentration were assessed using a Bioanalyser and a Nanodrop spectrophotometer, respectively, prior to microarray hybridization.

Microarray details

The Applied Biosystems Human Genome Survey Microarray was employed to examine the transcriptional activity of seven different oocytes. This was a highly sensitive microarray consisting of 32 878 60-mer oligonucleotide probes for the interrogation of 29 098 genes. Chemiluminescent detection was accomplished using the Applied Biosystems Chemiluminescent Detection Kit and image capture and processing was achieved with an Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. Microarray hybridization and image acquisition was carried out by the Environmental and Occupational Health Sciences Institute (EOHSI, UMDNJ-Rutgers).

Real-time PCR via TLDAs

Verification of the microarray results involved the use of a customized TLDAs (Applied Biosystems, USA), a platform permitting high-throughput, low reaction volume, real-time PCR. TLDA cards consist of 384 (4 × 96) wells pre-loaded with specified sequence detection (TaqMan) probes. The TLDA platform was used to perform duplicate analysis of 96 genes, including two endogenous controls, in each of 14 oocytes. The housekeeping genes chosen were glyceraldehyde-3-phosphate dehydrogenase (GADPH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1), since previous studies had indicated that these genes are suitable for the purposes of quantification and normalization of transcript numbers in oocytes (Kuijk *et al.*, 2007; Mamo *et al.*, 2007).

As with the microarray analysis, oocyte RNA was extracted with the use of Stratagene's Absolutely RNA Nano-prep Kit (USA), according to the manufacturer's instructions. Following standard cDNA synthesis, each sample was subjected to 15 cycles of amplification using an oligonucleotide pool, comprised of primer pairs specific to each of the genes to be analysed. This occurred in a total volume of 50 μl. After completion of this preamplification step, each product was mixed with 50 μl of 2 × TaqMan Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems). The mixture was then transferred to a TLDA card and 1 μl of reaction mixture was distributed into each of the wells containing gene-specific TaqMan probes by centrifugation. TLDA cards were placed in the Applied Biosystems Prism 7900HT Sequence Detection system in order for the PCR amplification to occur. The PCR amplification conditions were as follows: 2 min at

50°C and 10 min at 94.5°C for 40 cycles of 30 s at 97°C and 1 min at 59.7°C. ΔCT values were determined by normalization to both housekeeping genes using the RQ SDS manager software (Applied Biosystems).

Bioinformatics and statistical analysis

Signals from each of the microarrays were subjected to quantile normalization and filtered for signal to noise ratios >3 (detectability filter). Relative fold changes were calculated for comparison of individual samples. PANTHER, an online tool supported by Applied Biosystems (http://www.pantherdb.org/) was used for further data analysis. PANTHER assigned genes with differences in transcript abundance to different categories based upon either biological or molecular function, and compared the number of genes in each category to a reference list comprising all of the genes in the human genome. A binomial statistics tool was employed to compare multiple clusters of genes to a reference list, statistically determining over- or under-representation of genes in individual PANTHER categories (biological process, molecular function or pathway; Cho and Campbell, 2000). The data analysis statistics also included a Bonferroni correction for multiple testing. By identifying over- and under-represented classes of genes, an indication of processes likely to display altered activity in aneuploid oocytes was obtained. All samples are MIAMI compliant and are handled according to Standard Operating Procedures in the Microarray Center. Data from the 7 oocytes which underwent microarray analysis were submitted to ArrayExpress at European Molecular Biology Laboratory (http://www.ebi.ac.uk/microarray-as/ae/) using MIAMIexpress. The experiment accession number is E-MEXP-2745. Statistical analysis of TLDA real-time PCR data employed RealTime StatMiner™ version 3.0 software (Integromics™ S.L, Spain). Specifically, hierarchical clustering employing complete linkage with Pearson correlation and a two-way ANOVA were used to compare samples.

Results

Comprehensive cytogenetic analysis of PBs

We used CGH to examine the chromosome complement a total of 21 first PBs from 10 different patients. Results were obtained from all investigated PBs and were used to divide the oocytes into two groups, one including all those characterized as haploid normal (23,X) and another including all the abnormal oocytes.

Table II shows the cytogenetic results obtained during the course of this study. All 21 oocytes were assessed via CGH analysis of the first PB and chromosome abnormalities were detected in 11 (52.4% oocyte aneuploidy rate). The abnormalities were observed in oocytes donated by 8 of the 10 women who participated in this investigation (average maternal age 33.8 years, age range 30–41 years). Careful analysis of CGH data confirmed the presence of two distinct mechanisms leading to maternal aneuploidy: (i) whole-chromosome non-disjunction, affecting 7 of the 11 abnormal oocytes and accounting for 11 of the 15 chromosome errors; (ii) unbalanced chromatid pre-division, which affected 4 of 11 abnormal oocytes and was responsible for 4 of 15 chromosome errors. Eleven of the 15 chromosome anomalies involved losses of chromosome material in the first PB (corresponding to a gain in the MII oocyte) and the remaining four involved gains of chromosome material in the first PB (corresponding chromosome loss in the MII oocyte).

Microarray analysis of mRNA transcripts from normal and aneuploid oocytes

mRNA transcripts derived from three normal and four aneuploid oocytes were assessed using microarrays. To avoid possible

Table II Summary of data from the CGH analysis of first PBs and details of the type of gene expression analysis of the corresponding oocytes.

Patient no.	PB no.	PB CGH result	Oocyte karyotype	Oocyte characterization	Poly-A mRNA analysis
1	1-4PB	23,X	23,X	Normal	Array
	1-6PB	23,X	23,X	Normal	Array
	1-22PB	23,X,−Xq25-q28	23,X,+Xq25−q28	Abnormal	Array
	1-25PB	22,−X	24,X,+X	Abnormal	Array
	1-26PB	23,X	23,X	Normal	Array
	1-28PB	23,X,−3,+13	23,X,+3,−13	Abnormal	Array
	1-31PB	22,X,−2	24,X,+2	Abnormal	Array
2	2-2PB	20,X,−14,−15,−22	26,X,+14,+15,+22	Abnormal	TLDAs
3	3-3PB	22,X,−16	24,X,+16	Abnormal	TLDAs
4	4-1PB	23,X,−Xcht	23,X,+Xcht	Abnormal	TLDAs
	4-2PB	23,X	23,X	Normal	TLDAs
5	5-2PB	23,X	23,X	Normal	TLDAs
	5-5PB	22,X,−22	24,X,+22	Abnormal	TLDAs
6	6-1PB	23,X	23,X	Normal	TLDAs
	6-2PB	23,X	23,X	Normal	TLDAs
	6-3PB	23,X,−16cht	24,X,+16cht	Abnormal	TLDAs
7	7-1PB	23,X	23,X	Normal	TLDAs
	7-3PB	23,X	23,X	Normal	TLDAs
8	8-2PB	23,X,+16cht,+22cht	23,X,−16cht,−22cht	Abnormal	TLDAs
9	9-11PB	23,X	23,X	Normal	TLDAs
10	10-1PB	24,X,+17	22,X,−17	Abnormal	TLDAs

differences in gene expression related to variability in the genetic background of patients, rather than aneuploidy, all the oocytes tested by microarray were derived from the same woman (patient 1 in Tables I and II).

Previous studies had demonstrated that amplification of RNA samples produces a range of fragment sizes, varying from less than 50 to over 4000 nucleotides in length (mean ∼500 nucleotides) and that the quantity of RNA generated, ranges from 1 to 31 µg, averaging at ∼12 µg. The seven oocyte samples described above all yielded >5 µg of RNA, sufficient for microarray hybridization.

Signals from each of the seven microarrays used were subjected to quantile normalization and filtered for signal to noise ratios >3 (detectability filter). Relative fold changes were calculated for comparison of individual samples. Out of the 29 098 genes examined, 6226 had transcripts detectable in all of the samples. Additionally, 327 genes displayed statistically different ($P < 0.05$) numbers of transcripts when aneuploid and normal oocytes were compared. One hundred and eighty-one genes displayed reduced amounts of mRNA in the abnormal samples and 146 showed a proportionate increase. Sixty-one of these genes showed highly significant alterations ($P < 0.01$) in relative transcript copy number.

Genes were classified according to their molecular function and the biological processes in which they are involved. Of the 327 showing differences in the number of transcripts, 134 were of unknown molecular function/biological process. The remaining genes could be placed into 27 distinct categories of biological process, ranging from cell cycle regulation to oogenesis and embryogenesis. Additionally, microarray analysis suggested that at least 44 different pathways might be malfunctioning or abnormally activated/repressed in the aneuploid oocytes. Examples included glycolysis and pyruvate metabolism, DNA replication and apoptosis. Supplementary data, Tables SI and SII catalogue the genes with unusually low and high numbers of mRNA transcripts, respectively, and give details about their molecular functions, which biological processes they participate in, and the pathways they regulate. The pi-chart in Fig. 1 illustrates the 27 biological processes which were affected in the aneuploid oocytes.

Among the biological processes displaying the greatest disturbance in transcript levels were spindle assembly, chromosome alignment and segregation. Various genes regulating this process, such as KIF2B and TUBB4Q, displayed an increase in the relative quantities of mRNA transcripts, whereas others such as ASPM showed a decrease. Moreover, altered patterns of mRNA species were seen for other genes involved in the regulation of genomic stability, the G2/M checkpoint, the ubiquitin proteosome pathway, nucleic acid metabolism and chromatin packaging/remodelling, and metabolism of lipids, fatty acids and steroid hormones.

From a diagnostic perspective, it was interesting that several genes displaying highly significant alterations in transcript number ($P < 0.01$)
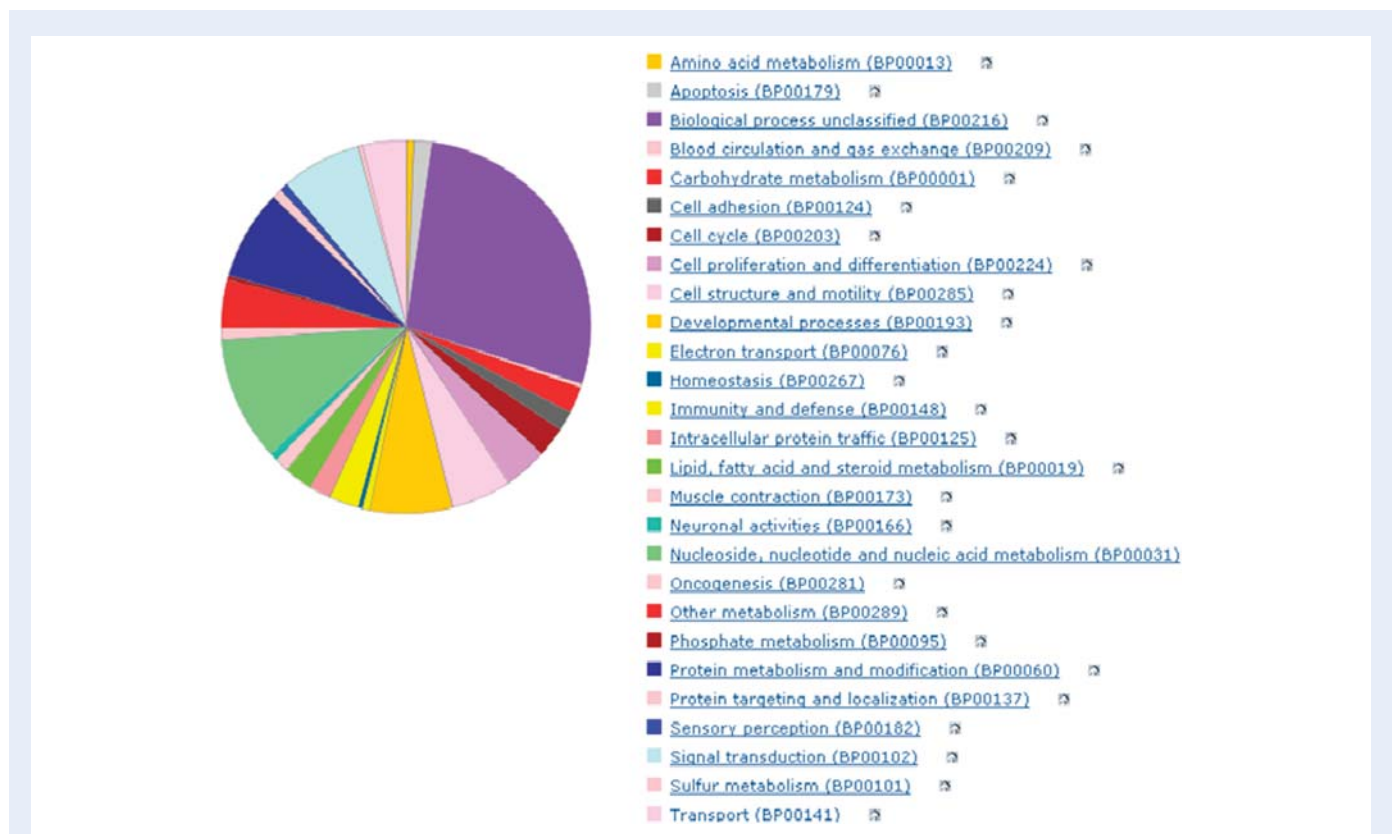
- Amino acid metabolism (BP00013)
- Apoptosis (BP00179)
- Biological process unclassified (BP00216)
- Blood circulation and gas exchange (BP00209)
- Carbohydrate metabolism (BP00001)
- Cell adhesion (BP00124)
- Cell cycle (BP00203)
- Cell proliferation and differentiation (BP00224)
- Cell structure and motility (BP00285)
- Developmental processes (BP00193)
- Electron transport (BP00076)
- Homeostasis (BP00267)
- Immunity and defense (BP00148)
- Intracellular protein traffic (BP00125)
- Lipid, fatty acid and steroid metabolism (BP00019)
- Muscle contraction (BP00173)
- Neuronal activities (BP00166)
- Nucleoside, nucleotide and nucleic acid metabolism (BP00031)
- Oncogenesis (BP00281)
- Other metabolism (BP00289)
- Phosphate metabolism (BP00095)
- Protein metabolism and modification (BP00060)
- Protein targeting and localization (BP00137)
- Sensory perception (BP00182)
- Signal transduction (BP00102)
- Sulfur metabolism (BP00101)
- Transport (BP00141)

Figure 1 The pi-chart shows the 27 biological process which were affected in the aneuploid oocytes. One hundred and thirty-four of the 327 genes with statistically significant differential expression ($P < 0.05$) were considered as 'unclassified' as far as their molecular function and the biological process they participated in were concerned.

coded for cell surface, or excreted proteins, or influenced metabolic pathways generating excretory products. The proteins and other products generated might serve as targets for the development of non-invasive assays for aneuploidy screening.

The alterations in mRNA transcript numbers were not associated with the specific chromosome abnormalities present in individual aneuploid oocytes, but rather represented a generalized feature of aneuploidy, being common to all cytogenetically abnormal oocytes.

Verification of microarray data via real-time PCR analysis

In order to verify the results obtained from the microarray analysis, we employed TLDAs. TLDAs are a relatively new innovation in real-time PCR that enable the simultaneous analysis of a larger numbers of genes, compared with conventional real-time PCR approaches. Hence, we were able to further investigate 94 of the 327 genes that showed altered mRNA levels on microarray analysis. These 94 genes have been identified in Supplementary data, Tables SI and SII. We also included two housekeeping genes, namely GAPDH and HPRT1, which served as endogenous controls.

A total of 14 oocytes, 7 normal and 7 aneuploid, were used during this part of the study. These were donated by nine women (patients 2–10 in Tables I and II), of an average maternal age of 35 years (range 31–41 years). The abnormal oocytes were generated by seven of these nine women (patients 2–6, 8 and 10 in Table II) and the average age for this subgroup of patients was 34.3 years (range 31–41 years). The errors scored affected both whole-chromosomes (four oocytes) and single chromatids (three oocytes). The karyotypes of the aneuploid oocytes are shown in Table II.

The data obtained for the 96 genes in the 14 different oocytes were normalized against the results obtained for the two housekeeping genes (GADPH and HPRT). Statistical analysis took place with the use of the RealTime StatMiner™ version 3.0 software (Integromics™ S.L, Spain). Hierarchical clustering, to identify samples showing similar patterns of mRNA transcripts, was performed employing complete linkage with Pearson correlation. Three different comparisons of samples and data took place: (i) The results from chromosomally normal oocytes were compared with those seen in the oocytes carrying whole-chromosome non-disjunction errors; (ii) The quantities of mRNA transcripts seen in the chromosomally normal oocytes were compared with those seen in the oocytes carrying single chromatid errors; (iii) The pattern of transcripts seen in the oocytes carrying whole-chromosome non-disjunction errors was compared with that seen in the oocytes carrying single chromatid errors.

In most cases, hierarchical clustering placed normal and abnormal oocytes into distinct groups, but the separation was not absolute, a few clusters containing both aneuploid and normal samples. However, hierarchical clustering was able to separate normal and aneuploid oocytes coming from the same patient. This suggests that there are distinct differences between normal and abnormal oocytes from the same patient, but variability between patients is significant and may obscure such differences.

Differences in the number of transcripts were observed more clearly when oocytes carrying chromosome errors were compared with those affected by chromatid errors. In this case hierarchical clustering was able to place all four oocytes which became aneuploid due to whole-chromosome non-disjunction together in one group, whilst the remaining three oocytes which became aneuploid due to unbalanced chromatid predivision were placed together in another group. Figure 2 illustrates the cluster diagram resulting from this third sample comparison.

We were also interested in investigating whether maternal age had any impact on the mRNA levels of genes in normal and aneuploid oocytes. For this reason we analysed the data with the use of a two-way ANOVA. The women who donated oocytes were grouped according to maternal age (35 years or less, 36–40 years, 41 years or more) and four different comparisons took place for each of these three groups: (i) The number of mRNA transcripts seen in the normal oocytes was compared with the quantities seen in the aneuploid oocytes; (ii) The level of transcripts seen in the normal oocytes was compared with those seen in the oocytes carrying whole-chromosome errors; (iii) The number of transcripts seen in the normal oocytes was compared with those seen in the oocytes carrying single chromatid errors; (iv) The number of transcripts seen in the oocytes carrying chromosome errors was compared with the number seen in the oocytes carrying chromatid errors.

This statistical analysis indicated that maternal age and/or aneuploidy significantly affected the abundance of transcripts from 27 of the 96 examined genes. Twelve of these genes had higher levels of transcripts and 15 showed a decline. Specifically, aneuploidy affected the quantity of mRNA transcripts from 18 genes (Table III), whereas maternal age affected six genes (Table IV). There were significant interactions between the effects of maternal age on aneuploidy (and
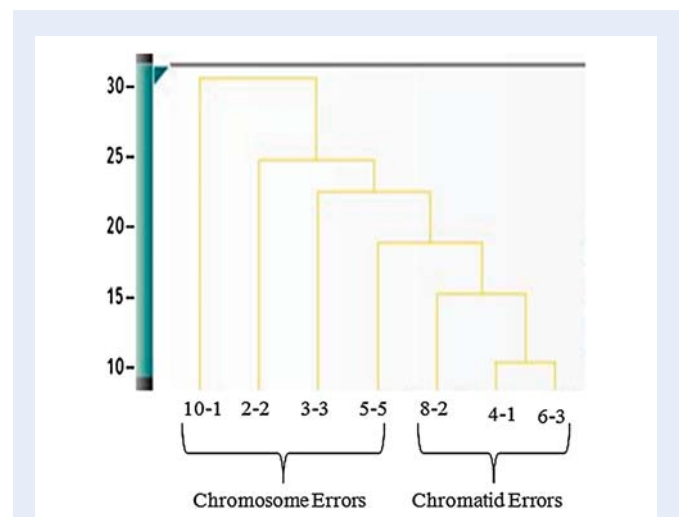
Figure 2 Hierarchical cluster resulting from the data obtained during TLDA analysis of aneuploid oocytes (chromosome and chromatid errors). Hierarchical clustering was able to place the four oocytes which became aneuploid due to whole-chromosome non-disjunction in one group, whereas the remaining three oocytes which became aneuploid due to unbalanced chromatid predivision were placed in another group. This presence of two distinct groups indicates that there are subtle differences between the mechanisms leading to whole-chromosome non-disjunction and to unbalanced chromatid predivision.

Table III TLDA data: chromosome abnormalities and gene expression.

Gene name	Gene symbol	Gene family	Molecular function	Biological process	Pathway	Expression in aneuploid samples
Asp (abnormal spindle)-like, microcephaly-associated (Drosophila)	ASPM	ABNORMAL SPINDLE-LIKE MICROCEPHALY-ASSOCIATED PROTEIN	Cytoskeletal protein	Muscle contraction; Cytokinesis	Inflammation mediated by chemokine and cytokine signalling pathway → Myosin	Under-expressed
Tumor necrosis factor receptor superfamily, member 21	TNFRSF21	TUMOR NECROSIS FACTOR RECEPTOR RELATED	Receptor	NF-kappaB cascade; Induction of apoptosis; cell surface receptor mediated signal transduction; JNK cascade; other intracellular signalling cascade; neurogenesis	Unclassified	Under-expressed
Histone linker H1 domain, spermatid-specific 1	HILS1	HISTONE H1/H5	Histone	Chromatin packaging and remodelling	Unclassified	Under-expressed
Dendritic cell-derived ubiquitin-like protein	DC-UbP	UBIQUITIN DOMAIN CONTAINING 1 PROTEIN-RELATED	Ubiquitin-protein ligase	Unclassified	Unclassified	Under-expressed
Unassigned	Unassigned	DNA MISMATCH REPAIR PROTEIN PMS2	DNA-binding protein	Meiosis	Unclassified	Under-expressed
Pyruvate dehydrogenase (lipoamide) beta	PDHB	DEHYDROGENASE RELATED	Dehydrogenase	Carbohydrate metabolism	Unclassified	Under-expressed
Ligand of numb-protein X 2	LNX2	NUMB-BINDING PROTEIN 2	Cytoskeletal protein	Asymmetric protein localization	Notch signalling pathway → LNXp80	Under-expressed
Transforming growth factor beta regulator 4	TBRG4	CELL CYCLE PROGRESSION 2	Unclassified	Cell cycle	Unclassified	Under-expressed
KIAA0753	KIAA0753	UNCHARACTERIZED	Unclassified	Unclassified	Unclassified	Under-expressed
E74-like factor 1 (ets domain transcription factor)	ELF1	ELF1	Ribosomal protein	mRNA transcription	PDGF signalling pathway → Ets	Under-expressed
Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	MEIS2	HOMEOBOX PROTEIN MEIS	Transcription factor	mRNA transcription regulation; developmental processes; cell proliferation and differentiation; oncogenesis	Unclassified	Under-expressed
Immunity-related GTPase family, M	IRGM	UNCHARACTERIZED	Unclassified	Unclassified	Unclassified	Over-expressed
Unassigned	Unassigned	TROPOMYOSIN	Actin-binding motor protein	Cell motility; cell structure; muscle contraction; muscle development	Unclassified	Over-expressed
Proprotein convertase subtilisin/kexin type 5	PCSK5	SUBTILISIN/KEXIN-RELATED SERINE PROTEASE	Protease	Receptor protein serine/threonine kinase signalling pathway; proteolysis; mesoderm development	Alzheimer disease presenilin pathway → Furin Alzheimer disease-amyloid secretase pathway → Furin	Over-expressed
RCC1 domain containing 1	RCCD1	REGULATOR OF CHROMOSOME CONDENSATION-RELATED	Guanyl-nucleotide exchange factor	Unclassified	Unclassified	Over-expressed

Tubulin, beta polypeptide 4, member Q	TUBB4Q	TUBULIN BETA CHAIN	Tubulin	Chromosome segregation; cell motility; cell structure; intracellular protein traffic	Huntington disease → beta-tubulin	Over-expressed
					Cytoskeletal regulation by Rho GTPase → Tubulin	
					Hedgehog signalling pathway → Microtubule	
Retinoic acid receptor responder (tazarotene induced) 2	RARRES2	UNCHARACTERIZED	Unclassified	Unclassified	Unclassified	Over-expressed
Transmembrane protein 87A	TMEM87A	PTM1	Unclassified	Unclassified	Unclassified	Over-expressed

Table IV TLDA data: maternal age and gene expression.

Gene name	Gene symbol	Gene family	Molecular function	Biological process	Pathway	Expression in aneuploid samples
Phosphatidylserine decarboxylase	PISD	PHOSPHATIDYLSERINE DECARBOXYLASE	Decarboxylase	Phospholipid metabolism	Unclassified	Under-expressed
Resistin like beta	RETNLB	SUBFAMILY NOT NAMED	Unclassified	Unclassified	Unclassified	Under-expressed
Dynein, cytoplasmic, light polypeptide 2B	DNCL2B	DYNEIN LIGHT CHAIN 2	Cytoskeletal protein	Cell motility	Unclassified	Over-expressed
1-acylglycerol-3-phosphate O-acyltransferase 7 (lysophosphatidic acid acyltransferase, eta)	AGPAT7	ACETYLTRANSFERASE-RELATED	Transferase	Other metabolism	Unclassified	Over-expressed
Polycomb group ring finger 3	PCGF3	RING FINGER PROTEIN	Transcription factor	mRNA transcription regulation; developmental processes	Unclassified	Over-expressed
Protocadherin gamma subfamily A, 8	PCDHGA8	PROTOCADHERIN GAMMA	Cadherin	Cell adhesion-mediated signalling; cell adhesion	Wnt signalling pathway → Cadherin	Over-expressed
					Cadherin signalling pathway → Cadherin	

Table V TLDA data: chromosome abnormalities, maternal age and gene expression.

Gene name	Gene symbol	Gene family	Molecular function	Biological process	Pathway	Expression in aneuploid samples
ATG7 autophagy-related 7 homolog (*S. cerevisiae*)	ATG7	UBIQUITIN-ACTIVATING ENZYME E1	Transfer/carrier protein	Intracellular signalling cascade; proteolysis	Ubiquitin proteasome pathway → Ubiquitin-activating enzyme E1	Under-expressed
keratin 80	KRT80	KERATIN, TYPE II, CYTOSKELETAL	Cytoskeletal protein	Ectoderm development; apoptosis; cell structure	Unclassified	Under-expressed
Kinesin family member 2B	KIF2B	KINESIN FAMILY MEMBER 2 (KIF2)	Microtubule-binding motor protein	Meiosis	Unclassified	Over-expressed

vice versa) on three genes (ATG7, KIF2B and KRT80; Table V). These 27 genes with differential transcript numbers, along with details about their function, biological process and pathways, are listed in Tables III, IV and V.

Discussion

The data generated during this study allowed us to compare the transcriptomes of chromosomally normal oocytes with those affected by aneuploidy. The purpose of this analysis was to identify genes displaying consistent differences in activity related to meiotic aneuploidy. A key requirement for an investigation of this type is the correct categorization of oocytes into normal or aneuploid groups. In order to achieve accurate aneuploidy detection, we employed CGH analysis of the first polar body. We have previously validated this technique, confirming that CGH is capable of detecting errors affecting any chromosome or chromatid, and that abnormalities detected in the first polar body are reciprocated in the associated oocyte (Fragouli *et al.*, 2006a, b, 2009a, b).

Analysis of the mRNA content of individual oocytes utilized one of the most comprehensive microarrays available, providing data on more than 30 000 distinct transcripts. In order to reduce the risk that any differences in the number of transcripts detected were simply artefacts, related to the genetic background of patients rather than aneuploidy, we conducted our initial microarray analysis using a set of oocytes all derived from the same woman. However, in order to determine whether the genes detected are of general relevance to aneuploidy, or just specific to the single patient whose oocytes were assessed by microarray, confirmatory real-time PCR analysis was subsequently applied to oocytes from nine different women. For this purpose, we employed TLDAs, which allowed the simultaneous analysis of almost 100 genes in each oocyte, combining the high accuracy of real-time PCR with high throughput. To our knowledge, this is the first application of such technology in single cells.

Although the main purpose of the TLDA analysis was to confirm the relationship between aneuploidy and transcript abundance for a subset of the genes identified by microarray, we were also interested to know whether the number of transcripts was influenced by the age of the donor. This is of particular relevance given the close association between advancing maternal age and the risk of oocyte/fetal aneuploidy. For this reason, we ensured that the 14 oocytes subjected to TLDA analysis were derived from women of various ages (age range 31–41 years, average age 35 years). Statistical analysis of the TLDA data confirmed that not only chromosome abnormality, but also maternal age affected the quantity of mRNA transcripts from some genes in human oocytes.

Comparison of the microarray results from normal and aneuploid oocytes produced a catalogue of 327 genes with different mRNA transcript levels in oocytes containing chromosome errors. One hundred and eighty-one of these genes showed a decrease in the number of transcripts in aneuploid oocytes, with the remaining 146 displaying higher relative abundance. Bioinformatic analysis allowed ~60% (193) of these genes to be classified according to their molecular function. Interestingly, the cellular/molecular roles of the remaining genes are currently unknown, underscoring the unique, highly specialized and poorly understood biology of the oocyte.

Alteration of the number of polyadenylated transcripts from specific genes suggested that multiple biological processes and pathways may have abnormal levels of activity in the aneuploid oocytes. Currently, this possibility remains to be confirmed and will require further studies, assessing protein levels and functional competence of cellular pathways. Confirmatory work is particularly important in the case of transcriptomic analysis of oocytes, since post-transcriptional mechanisms controlling mRNA stability and protein synthesis are used extensively during oocyte maturation and early embryonic development. The data obtained in the current investigation, provides a foundation for future studies, highlighting candidate genes that should be the focus of such analyses.

Processes potentially affected by altered mRNA levels in aneuploid oocytes include spindle assembly and chromosome alignment, chromatin packaging and remodelling, cellular metabolism, DNA replication and apoptosis. Although a direct impact on chromosome segregation and aneuploidy seems plausible for some of these processes, the diversity of the pathways affected seems to indicate that aneuploid oocytes are often abnormal on multiple levels. It may be the case that chromosome malsegregation is just one symptom of a more generalized problem affecting oocytes that become aneuploid.

However, the relatively high frequency of aneuploid pregnancies in humans clearly demonstrates that, such oocytes are usually fertilization-competent and compatible with the establishment of a clinical pregnancy.

Numerous studies using classical and molecular cytogenetic methodologies to examine oocytes and PBs have described abnormalities of both entire chromosomes and single chromatids (Angell, 1991; Sandalinas et al., 2002; Cupisti et al., 2003; Pellestor et al., 2003; Fragouli et al., 2006a, b, 2009b). It has been shown that differences in the recombination patterns between oocyte bivalents predispose towards one of these two types of meiotic error (Hunt et al., 1995; Thomas et al., 2001; Hassold et al., 2007). Interestingly, the application of a hierarchical cluster algorithm to the TLDA data resulted in oocytes carrying chromosome errors being placed in a distinct group, separate from those affected by chromatid abnormalities. The division of the seven aneuploid oocytes into two distinct groups suggests that there may be differences in the factors that predispose to whole-chromosome non-disjunction and unbalanced chromatid predivision.

Among the genes showing altered abundance of transcripts in the aneuploid samples there were four coding for cytoskeletal proteins. Specifically, ASPM (Abnormal spindle-like, microcephaly-associated) and LNX2 (Ligand of numb-protein X 2) had decreased transcript levels, whereas increases were observed for DNCL2B (Dynein, cytoplasmic, light polypeptide 2B), an unassigned gene encoding for an actin-binding protein belonging to the tropomyosin family, the tubulin TUBB4Q (tubulin, beta polypeptide 4, member Q) and KIF2B (Kinesin 2B, a member of the kinesin 13 family of microtubule depolymerases with central motor domain). Voet et al. (2009) determined that ASPM is one of the main regulators of meiotic spindle organization and rotation, and is responsible for correct localization of dynein at the spindle poles. LNX2 encodes for a molecular scaffold protein (Rice et al., 2001), whereas KIF2B is thought to be involved in spindle assembly, chromosome movement and microtubule depolymerization (Manning et al., 2007; Bakhoum et al., 2009). The altered numbers of transcripts from these genes suggests that the corresponding proteins may be produced in either too great or too small amounts. It is conceivable that abnormal concentrations of these proteins could lead to disruption of spindle formation, chromosome alignment and subsequent movement, predisposing to chromosome malsegregation during the first meiotic division.

Differences in the amount of mRNA from genes involved in the regulation of cellular metabolism and energy production was also observed. Specifically, reduced levels of transcripts from the gene PDHB (pyruvate dehydrogenase [lipoamide] beta) were detected in the aneuploid oocytes. PDHB is a component of pyruvate dehydrogenase, a multi-enzyme complex involved in the production of ATP, which includes PDHB, PDHA1 and PDHA2 (Johnson et al., 2007; Johnson and Kaplitt, 2009). The vast majority of processes involved in meiotic maturation are directly dependent on ATP production. Disruption of PDHA1 has been associated with meiotic defects affecting chromatin condensation, microtubule assembly and chromosomal movement (Johnson et al., 2007), suggesting a potential role in the genesis of aneuploidy. Reduction in PDHB levels may lead to decreased quantities of functional pyruvate dehydrogenase. This in turn could further impair mitochondrial metabolism of other ATP-generating substrates that are converted to pyruvate, such as glucose, lactate and various amino acids and result in oocytes at high risk of becoming chromosomally abnormal.

Advancing maternal age influenced the abundance of mRNA transcripts derived from two genes involved in phospholipid metabolism, PISD (phosphatidylserine decarboxylase) which displayed reduced quantities of transcript and AGPAT7 [1-acylglycerol-3-phosphate O-acyltransferase 7 (lysophosphatidic acid acyltransferase, eta)] which showed increased quantities. Both these genes are involved in the production of phosphatidylethanolamine, a key mitochondrion phospholipid (Gohil and Greenberg, 2009). It has been demonstrated that total lack of PISD activity in mice is associated with lethality between Days 8 and 10 of embryonic development (Steenbergen et al., 2005). Examination of these embryos demonstrated that a large number of their mitochondria were aberrantly shaped or fragmented (Steenbergen et al., 2005). AGPAT7, on the other hand, belongs to a family of transferases which are responsible for attaining the correct molecular species of phospholipids, and has been found to be involved with enrichment of phosphatidylethanolamine in the brain (Cao et al., 2008). The lower numbers of PISD transcripts seen with advancing maternal age might lead to the generation of oocytes with defective mitochondria and decreased production of phosphatidylethanolamine and/or ATP. The elevated relative amount of AGPAT7 mRNA may therefore represent a compensatory mechanism, aimed at increasing the production of phosphatidylethanolamine.

Another gene directly associated with phosphatidylethanolamine that displayed altered transcript levels in aneuploid oocytes was ATG7 (autophagy-related 7 homolog). ATG7 is one of many autophagy-related genes and also participates in the ubiquitin proteasome pathway (Geng and Klionsky, 2008). The ubiquitin proteasome pathway labels proteins and other molecules for degradation and removal from the cell, and one means of removal is autophagy. The main function of ATG7 is to mediate the conjugation of ATG8 to phospatidylethanolamine, producing a scaffold protein responsible for the size of the autophagosome, an intracytoplasmic vacuole in which cellular components are enclosed and subjected to enzymatic digestion (Geng and Klionsky, 2008). The lower quantity of ATG7 transcripts detected in aneuploid oocytes, and in oocytes from older women, suggests that production of the corresponding protein may be reduced. This might limit the size of autophagosomes and impair their function, resulting in inefficient removal of cellular debris and damaged organelles. Previous studies have suggested that aneuploidy can be a consequence of compromised autophagy (Mathew et al., 2007).

The presence of chromosome abnormalities in the oocytes was also associated with alterations to the transcripts of several genes involved in the regulation of apoptosis. TLDA analysis confirmed reduced amounts of mRNA from TNFRSF21 (tumour necrosis factor receptor superfamily, member 21), DC-UbP (dendritic cell-derived ubiquitin-like protein) and KRT-80 (keratin 80) in aneuploid oocytes. Additionally, KRT-80 was also affected by maternal age. It is not currently clear whether these genes influence chromosome segregation or whether the altered quantity of mRNA is a consequence of an indirect association with aneuploidy. However, given that apoptotic pathways have roles in the control of the cell cycle and the disposal of cells carrying genetic abnormalities, a direct link with aneuploidy may exist.

Analysis of TLDA results revealed a total of nine genes displaying alterations in the number of transcripts related to advancing maternal age. Differences in oocyte 'gene expression' associated with maternal age have been previously reported, following RT−PCR or microarray analysis (Steuerwald *et al.*, 2001a, b, 2007; Grøndahl *et al.*, 2010). The first two studies indicated that the oocytes of 'older' women have a lower number of transcripts from the MAD2 and BUB1 genes. Both genes are involved in the regulation of the spindle assembly check-point, which is responsible for delaying anaphase initiation if defects in the alignment of chromosomes at the metaphase plate are detected. We did not observe any direct affects of aneuploidy or age on the quantity of MAD2 and BUB1 transcripts, but observed that the total mRNA level was lower in aneuploid and aged oocytes. Although some genes showed evidence of higher relative levels of polyadenylated transcripts in abnormal oocytes, in general, aneuploid oocytes contained less mRNA than those with a normal haploid set of chromosomes. It seems that such oocytes may be poorly resourced in terms of mRNA or that the transcripts have become prematurely degraded. Alternatively, aneuploid oocytes may have reduced polyadenylation of transcripts, making them harder to detect using the methods employed during this study.

Significant gene expression differences were also observed between 'young' and 'old' oocytes in a recent microarray study by Grøndahl *et al.* (2010). The wide variety of cellular processes that were shown to display atypical levels of gene expression associated with age in that study support the notion that deficiencies affecting 'older' oocytes extend beyond a predisposition to errors of chromosome segregation. Interestingly, several of the processes showing altered expression in the Grondahl (oocyte-age) investigation have significant overlap with processes found to be potentially disrupted in the current (oocyte aneuploidy) study, in keeping with the close association between oocyte aneuploidy and advancing maternal age. Affected processes include microtubule dynamics, ubiquitination, signalling and cell cycle control.

Several of the genes that displayed differing numbers of transcripts between normal and aneuploid oocytes produce cell surface or excretory molecules. These include the receptors PTPRM (protein tyrosine phosphatase, receptor type, M), TNFRSF21 (tumour necrosis factor receptor superfamily, member 21) and UNC13B (Unc-13 homolog B), the membrane traffic protein CNIH4 (Cornichon homolog 4) and the transcription factor SDF2 (stromal cell-derived factor 2). Although much work remains to be done, the ready accessibility of these gene-products suggests it may be possible to develop non-invasive assays for the detection of aneuploidy based upon their analysis. This could eliminate the need for oocyte and/or embryo biopsy, transforming PGS and methods of embryo viability assessment.

Another interesting area for future research is the influence of follicular environment and oocyte metabolism on aneuploidy predisposition. During the current study, several genes involved in pathways related to hypoxia and hormonal response displayed altered numbers of transcripts. This suggests that aneuploidy might not be wholly dependent on intrinsic oocyte factors and that the follicular microenvironment may also play a role. The ovarian environment changes significantly with age, particularly in regards to the hormonal milieu. This seems compatible with a potential association between age, meiotic error and microenvironment.

The observed changes in the quantity of mRNA transcripts from metabolism-related genes may be unrelated to aneuploidy, representing another downstream consequence of a more generalized oocyte insufficiency. However, it is also possible that abnormal activity of these pathways predisposes to aneuploidy in some way, creating an intracellular environment in which chromosome malsegregation is more likely to occur. If the latter scenario is proven to be correct, then it may be possible to find ways to support the proper functioning of the affected pathways, and reduce the risk of chromosome malsegregation. In the context of treating infertile couples, this might be achieved via dietary supplementation or with supplementation of medium used for the *in vitro* maturation of oocytes. This remains highly speculative, but represents an exciting area for future research.

To conclude, this is the first study to report comparison of the quantities of polyadylated mRNA transcripts in normal and aneuploid oocytes. This was achieved using a combination of transcriptomic profiling via microarray, comprehensive chromosome screening using CGH, and validatory work employing real-time PCR with TLDAs. The alterations detected are not affected by the specific chromosome involved in the abnormality; rather they appear to be a general, consistent, feature of aneuploid oocytes. The genes affected hint at the cellular pathways that are likely to play a role in the origin of chromosome malsegregation during female meiosis, providing new targets for research into the origin, detection and possibly in the future, prevention of aneuploidy.

Authors' roles

E.F.: carried out experimental work, analysed scientific data, wrote manuscript. V.B.: collected and processed oocytes, carried out experimental work. P.P.: patient consults and recruitment, contributed to manuscript, assisted with grant funding. A.O.: processed oocytes, carried out experimental work. Z.H.: assisted in expression data analysis. A.B.: patient consults and recruitment. J.D.A.D.: provided part of EF's salary during this project. D.W.: carried out experimental work, analysed scientific data, provided part of EF's salary and funding for this project, edited the manuscript.

Supplementary data

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

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