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Gene expression profiles of single human mature oocytes in relation to age

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BACKGROUND: The development competence of human oocytes declines with increasing age. The objective of this study was to investigate the effect of age on gene expression profile in mature human oocytes.

METHODS: mRNA was isolated for whole genome gene expression microarray analysis from metaphase II (MII) oocytes donated by IVF or ICSI patients [10 women aged <36 years (younger) and five women aged 37–39 years (both inclusive) (older)] undergoing controlled ovarian stimulation. The oocytes were donated and prepared immediately after recovery from the follicle. RT–PCR on additional four younger and two older oocytes confirmed the array analysis.

RESULTS: On the basis of 15 independent replicates of single MII oocytes, 7470 genes (10 428 transcripts) were identified as present in the MII oocytes. Of these, 342 genes showed a significantly different expression level between the two age groups; notably, genes annotated to be involved in cell cycle regulation, chromosome alignment (e.g. MAD2L1 binding protein), sister chromatid separation (e.g. separase), oxidative stress and ubiquitination. The top signaling network affected by age was 'cell cycle and organism development' (e.g. SMAD2 and activin B1 receptor).

CONCLUSION: There is a substantial difference between younger and older oocytes in the transcriptional level of genes involved in central biological functions of the oocytes, thus providing information on processes that may be associated with the ageing phenomenon and possibly contributing to decreased fertility.

Key words: gene expression / human oocyte / age / microarray / metaphase II

Introduction

In addition to the gradual decline in the number of ovarian follicles, one of the major causes for the decline in female reproductive capacity with increasing age appears to be an age-dependent decrease in oocyte quality. This relationship has clearly been established in oocyte donation programmes, where women in their forties experience pregnancy potential similar to the age of the donors who often are in their twenties (Navot *et al.*, 1991).

The quality of the fully mature oocyte is the result of an almost 6-month-long maturational process in the developing follicle culminating in ovulation (or atresia along the developmental path). In a very limited time period after ovulation, the oocyte is capable of undertaking a multitude of functions, such as becoming fertilized with a single spermatozoa, de-condensing the sperm head, completing meiosis, creating two pro-nuclei and sustaining the first cleavages until the embryonic genome becomes active. The processes governing oocyte development competence are usually divided into nuclear and cytoplasmic maturation. Nuclear maturation shows a strong age-dependent increase in the aneuploidy rate of both oocytes and embryos created in connection to assisted reproduction treatments (Eichenlaub-Ritter, 1996; Warburton, 2005). Several non-disjunctional mechanisms have been suggested to cause the aneuploidy: failure in meiotic pairing, synapsis, recombination and segregation (Hassold *et al.*, 2007).

The cytoplasmic maturation of the oocytes takes place throughout follicular development and unlike other cells where RNAs and proteins are used within minutes or hours after formations, oocytes store large quantities of mRNA and proteins for days and weeks in an inactivated form (selective cytoplasmic polyadenylation of the mRNAs). Subsequently, the oocyte is capable of recruiting the stored mRNA and protein in a tightly scheduled manner (Gandolfi

and Gandolfi, 2001; Gosden, 2002). In addition, there is an increase in number and spatial organization of cytoplasmic organelles, e.g. the number of mitochondria increases from 10 000 in the primordial oocyte to 100 000 in the fully grown oocyte (Jansen, 2000).

Thus, the complexity and careful regulation of the transcriptional activity of the oocyte dictates its ultimate acquisition of developmental competence (Song and Wessel, 2005; Stitzel and Seydoux, 2007) including (i) the timely translation of stored maternal transcripts to provide the ooplasm with new proteins; (ii) the post-translational modification of stored or newly synthesized protein which sets the exact timing for cellular events and (iii) the processes involved in degradation of proteins and mRNAs which remove molecules that are no longer needed (Evsikov and Marin de, 2009). The transcriptional activities of the oocytes may be influenced by age and may explain the reduced potential of older oocytes when compared with young. However, this hypothesis has not been studied using high throughput techniques in which the entire transcriptional activity has been monitored using microarray technique on single oocytes.

In order to investigate the molecular events, linked to both nuclear and cytoplasmic maturation, which characterize mature oocytes from a group of women with an advanced reproductive age in comparison to a younger patient group, in the present study we performed full-genome microarray analysis on individual oocytes and observed the effect of oocyte age.

Materials and Methods

Patients receiving IVF/ICSI treatment were included. Two age groups were created; the younger group included women less than 36 years of age ($\leq$35), whereas the older age group included women between 37 and 39 years (both included). Only women from whom more than six oocytes were available for their own treatment were asked to consider donation of one oocyte. For the microarray analysis, 10 metaphase II (MII) oocytes from the younger group were analysed. In the older age group, donation occurred less often owing to a reduced number of retrieved oocytes, and a total of five MII oocytes from the older group were analysed.

Treatment and donation

Informed consent was obtained from patients at the Fertility Clinic, University Hospital Copenhagen, Hvidovre Hospital; the study protocol was approved by the Danish Scientific Ethical Committee (Ethical Approval Number: KF 299017).

All patients were treated with short antagonist protocol. Upon confirmation of menstrual bleeding, no ovarian cysts and endometrial thickness $<$5 mm on Day 2–3 of the menstrual cycle, all patients received recombinant FSH (Puregon®, Organon, a division of Schering-Plough, Copenhagen, Denmark) dosed individually for 8–12 days as described (Popovic-Todorovic *et al*., 2004). When the leading follicle reached 12–13 mm in diameter the GnRH antagonist (Orgalutran®, 0.25 mg daily, Organon, a division of Schering-Plough) was administered daily until the day of hCG. When the leading follicles exceeded 16 mm diameter, hCG was administered (Ovitrelle®, 6500 IE, Merck Serono, Copenhagen, Denmark) and oocyte retrieval was performed 36 h later. The donated oocyte was removed from the surrounding cumulus granulosa cells within 30 min after ovum retrieval, initially mechanically with 18G needles followed by enzymatic treatment (Sydney IVF Hyaluronidase, 80 IU/ml, Sydney, Australia).

Each oocyte was checked for the presence of the first polar body and then briefly incubated in Tyrode's solution (MediCult, Jyllinge, Denmark) to remove the zona pellucida, washed twice in phosphate-buffered saline (PBS) and transferred with 2 μl PBS (Mg^{2+} and Ca^{2+} free, 0.1% polyvinyl alcohol, Sigma, Denmark) with RNase inhibitor (Protector RNase Inhibitor, 5 U/μl, Roche Diagnostic, Mannheim, Germany) to a 0.2 ml tube (MicroAmp, Applied Biosystems, Singapore), flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Isolation of RNA from the oocytes

We used the Picopure RNA isolation kit (Arcturus Reagents/Molecular Devices, USA) to isolate total RNA from a single oocyte. The quantity and integrity of the extracted total RNA were determined by Nanodrop (Nanodrop Technologies, USA) and the Bioanalyzer LabChips (Agilent Technologies, USA), respectively.

Microarray analysis

Samples were labelled using the double amplification protocol for small RNA samples according to the manufacturer's guidelines (Affymetrix, Santa Clara, CA, USA). On average 35 ng of total RNA extracted from the oocytes was reverse transcribed into complementary DNA (cDNA) using an oligo-dT primer containing a T7 RNA polymerase promoter. cDNA was used as a template in the *in vitro*-transcription reaction driven by the T7 promoter to synthesize cRNA, which was used as input for the second round of labelling. cRNA was transcribed into cDNA and the T7 promoter was used to drive the second round *in vitro* transcription during which biotin labelled oligo-nucleotides were incorporated into the synthesized cRNA. Fifteen micrograms of labelled sample were hybridized to the HG-U133plus2 GeneChip array (Affymetrix), which test close to 48 000 well-substantiated genes using ~56 000 probe sets. The arrays were washed and stained with phycoerythrin conjugated streptavidin (SAPE) using the Affymetrix Fluidics Station® 450, and the arrays were scanned in the Affymetrix GeneArray® 2500 scanner to generate fluorescent images, as described in the Affymetrix GeneChip® protocol.

Microarray data analysis

The Cel files were imported into the statistical software package R v. 2.7.2 using BioConductor v. 2.8 (Gentleman *et al*., 2004) and gcRMA modelled using quantiles normalization and 'lowess' summarization (Bolstad *et al*., 2003). The modelled log-intensity of 56 400 probe sets was used for high-level analysis of selecting differentially expressed genes. Genes were defined as being differentially expressed in a class comparison, if they were selected in the univariate two-sample *t*-test. A probe set is defined as being differentially expressed, if the *P*-value is below 0.05, the fold change is larger than 1.5 and the difference of means is larger than 50 (real values) between the patients groups ($\leq$35 years; 37–39 years, both inclusive).

Detection calls were derived using the MAS5 algorithm implemented in the Bioconductor package, affy (Gautier *et al*., 2004). This algorithm calculated a Wilcoxon signed rank-based *P*-value for the presence/absence of each particular gene on the array, as implemented in the Affymetrix Microarray Suite version 5. The output is a detection call that can take on the values of present, absent or marginally absent. We filtered for present genes in the two patient groups, $\leq$35 years and between 37 and 39 years (both inclusive), respectively. A gene is defined to be present in a particular age group, if we find expression in 80–100% of the samples. No genes were found to be exclusively detected in one of the two patient groups, using this above filtering criteria.

All samples are MIAMI compliant and are handled according to Standard Operating Procedures in the Microarray Center. The 15 samples were

submitted to ArrayExpress at European Molecular Biology Laboratory using MIAMIexpress. The experiment accession number is E-MEXP-2347.

Gene ontology enrichment analysis

The gene ontology (GO) enrichment analysis was performed to define overrepresentation of functional categories of the selected genes. For the genes defined as being differentially expressed, we applied the gene enrichment analysis implemented in the software package dChip (www.dchip.org), which calculates binomial approximated *P*-values for the hyper geometric distribution to determine enrichment. The test compares the proportion of genes in the sub-categories of the molecular function in the gene list to the proportion of all genes on the array found in that functional category. For a more detailed view of enriched genes, as well as enrichment analysis of the GO groups, biological process and cellular compartment, we used the GO enrichment tool implemented in the data expression software Partek version 6.4 (Partek®, St. Louis, MO, USA). Here, the baseline of the analysis is the genes interrogated by the array. The GO enrichment analysis is performed by applying a χ^2 test comparing the proportion of the selected genes that are part of a functional group, compared with proportion of the genes in the baseline (all genes on the array) that are part of the same functional group. If a functional group has an enrichment score above 1, the functional category is said to be over-expressed or enriched. Pie diagrams of the distribution of the number of genes in each of the enriched GO categories were produced using the pie chart functionality implemented in MicroSoft Excel 2007.

Biological networks

Biological networks of the differentially expressed genes were generated in Ingenuity pathway analysis tools from Ingenuity Systems Inc. (www.ingenuity.com). The list of differentially expressed genes was imported into Ingenuity and each gene identifier was overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were then generated based on their connectivity. A network score was calculated based on the hyper-geometric distribution and calculated with the right-tailed Fisher's exact test. The score is the negative log of this *P*-value. The score takes into account the number of network eligible molecules in the network and its size, as well as the total number of network eligible molecules analyzed and the total number of molecules in the knowledge base that could potentially be included in the network. The score represents the chance of getting a network containing at least the same number of network eligible molecules by chance when randomly picking the number of genes that can be in networks from the knowledge base. The top (highest scoring) networks are presented.

Validation of microarray results by quantitative RT–PCR

Total RNA was extracted from six MII oocytes (four younger and two older) using the PicoPure® RNA Isolation Kit (MDS, Inc., Ontario, Canada). The total RNA was used as input to an RNA amplification step using the WT-Ovation™ Pico RNA Amplification System from NuGEN (NuGEN Technologies, Inc., CA, USA). Ten nanograms of the amplified single-stranded cDNA was used for RT–PCR analysis using the TagMAN platform (Applied Biosystems).

Expression levels of three genes were measured: SMAD2, MRPL43 (mitochondrial ribosomal protein L43) and ANAPC4 (anaphase promoting complex subunit). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for reference. Primers used to amplify GAPDH, SMAD2, MRPL43, ANAPC4 in all real-time PCR reactions were (all 5′ to 3′): gAPDH_forward GGTGCTGAGTATGTCCTGGA, GAPDH_reverse GTGG TTCACACCCATCACAA, SMAD2_forward CTCCAGGTATCCCATCG AAA, SMAD2_reverse GTCGGGGCACTAATACTGGA, MRPL43_forward ACTGGGTCGCTATGTGCAG, MRPL43_revers CACGGACGC GAGTTTACATA, ANAPC4_forward CACCCCCTAACACAGAAGGA, ANAPC4_reverse CTGGCTTTTGCAAACACTGA. PCR conditions: following an initial step at 50°C for 2 min and a denaturation step at 95°C for 2 min, amplification was performed for 40 cycles at 95°C for 10 s and at 60°C for 45 s. All PCR reactions were performed in triplicate in 96-well formats, in a 10 μl final volume with Fast SYBR Green Master Mix (Applied Biosystems) using an ABI 7900HT instrument (Applied Biosystems). Data were analyzed using the Absolute Quantification (Standard Curve).

Results

The baseline characteristics of women in the two age groups were comparable, besides higher basal FSH at Day 2 or 3 in the menstrual cycle and higher total amount of FSH used for the controlled ovarian stimulation in the older compared with the younger women (Table I).

A total of 15 microarrays, representing 10 younger (≤35 years; mean: 31.1 years) and 5 older (between 37 and 39, mean: 37.8 years) individual MII oocytes were performed. The present call ranged from 28.2 to 39.3% with a mean of 32.8 ± 0.9. Using the criteria for present as being expressed in at least 80% of the samples in both age groups, 10 428 probe sets representing 7470 genes were present in the human MII oocyte (Supplementary Data, Table S1). In this list of expressed genes in the MII oocytes, 5213 has a biological process GO term assigned and are included in the pie diagram, presenting the main functional categories of cellular component, biological process and molecular level present in the MII oocytes (younger and older together; Supplementary Data, Fig. S1A–C). It was found that the main functional category of the biological process was 'cellular processes' representing the following sub-processes, with enrichment scores in parentheses: cellular metabolic process (65.7), cellular organization (27.6), cell cycle (24.4), cell division (19.4), microtubule-based processes (6.5) and chromosome segregation (5.3). In 'the

Table I Patient demographics and baseline characteristic in the two age groups for study of gene expression profiles in MII oocytes.

	Younger	Older
Number of donated oocytes	10	5
Age (years)	31.1 ± 2.7 (27–35)	37.8 ± 1.3 (37–39)
FSH CD2–3 (IU/l)	4.5 ± 1.7	8.9 ± 3.3*
Length of infertility (years)	2.1 ± 1.5	1.6 ± 0.9
BMI (kg/m^2)	23.4 ± 2.5	21.0 ± 1.4
Reason for treatment	Male factor: 8 Ovulation defect: 2	Male factor: 2 Unexplained: 2 Ovulation defect: 1
Cycle number	1.2 ± 0.4	1.2 ± 0.4
FSH total (IU)	1505 ± 383	2345 ± 1003*
FSH days	8.9 ± 1.6	8.8 ± 0.8
No. oocytes retrieved	11.9 ± 3.8	11.2 ± 4.6
'Follicle size' (ml)	2.5 ± 0.8	2.5 ± 1.0

Data are mean ± SD (range). Student's *t*-test. *$P < 0.05$ is significant.

metabolic process', the highest enrichment score was in the sub-processes 'macromolecular metabolic process' (29.3) and 'electron transport chain' (10.3), whereas under 'establishment of localization', 'establishment of protein localization' (20.0) and 'establishment of RNA localization' (7.1) were highly enriched.

In the relatively small category 'reproductive process', 100 annotated genes were organized under 'gamete generation' and 14 genes under 'fertilization'.

At the molecular level, 'binding' was the main process representing protein binding (45.5), antigen binding (44.8), nucleotide binding (21.8), nucleic acid binding (20.7), carbohydrate binding (10.4) and chromosome binding (6.8). The following sub-processes under 'catalytic activity' were enriched: 'ligase activity' (15.7), 'isomerase activity' (7.4), 'transferase activity' (6.5), 'RNA splicing activity' (5.5). Under 'translational regulator activity', the 'translation initiation factor activity' (9.3) and 'translation elongation factor activity' (2.8) were enriched.

The comparison of the gene expression profiles in MII oocytes from the younger (27–35 years) and older women (37–39 years) exhibited significant differences (fold change above 1.5) in 351 transcripts and 342 unique genes, of which 102 had fold change above 2.0 (Supplementary Data, Table S2). In comparison to the younger oocytes, 125 genes were up-regulated and 217 genes were down-regulated in the older oocytes (Fig. 1). Thus 4.6% of the genes present in the MII oocytes were influenced by age. The hierarchical cluster diagram demonstrated that expression profiles of younger and older oocytes are distinctly different and showed that older oocytes had greater transcriptome homology than the younger oocytes (Fig. 1). The list of the 342 differentially expressed genes in the MII oocytes was subjected to gene function enrichment analysis to determine which main functional categories were affected by age. Seventy-six GO biological processes were identified at the 0.01 significance level to be under- or over-represented in the older versus the younger MII oocytes. The categorized genes are shown in Supplementary Data, Table S3. These processes were re-categorized into 15 main categories (Table II). Additionally, the 'biological processes' represented in the 342 differentially expressed genes are shown in a pie diagram (Fig. 2).

Ingenuity Software network analysis showed the following networks were highly significantly affected by age (enrichment score): organism development/cell cycle (46), Cell Death/Inflammatory disease/cancer (31), Cell assembly and organization/Molecular transport, protein trafficking (29) and DNA Replication, Recombination and Repair (21).

The top network affected, 'Organism development/cell cycle' is shown in Fig. 3. Several genes in this network as well as other cell cycle annotated genes (Supplementary Data, Table S3) will be discussed.

Result of the quantitative RT–PCR in four younger (mean age: 29.5 years; range: 26–35 years) and two older (mean age: 39 years) MII oocytes revealed excellent agreement with the microarray data (Table III). The FSH doses used in these cycles were comparable in the younger (mean: 1370 IU, range: 1125–1800 IU) and older (mean: 1600 IU, range: 1400–1800 IU) women.

Discussion

This study demonstrates a considerable difference in the gene expression profile of mature MII oocytes between the younger and the older age group. To ensure optimal quality of the MII oocytes, they were prepared immediately after recovery from the follicle. More than 300 genes, many of which appear to be of importance for the development of the fertilized oocyte, are significantly up or down-regulated when the two age groups are compared. One of the most pronounced differences is the number of transcripts annotated to be involved in the cell cycle signalling pathways. Additionally, genes coding for proteins involved in spindle checkpoint regulation, DNA stability and chromosome segregation (e.g. separase) were also influenced by age.

More than 10 000 transcripts were found in the oocyte transcriptome using the present selection criteria, which is an increase of more than 2000 transcripts when compared with previously published studies on individual MII oocytes (Wood *et al*., 2007).

Most frequently, transcriptome analyses are made on samples where oocytes are pooled in groups of 3–10 MII oocytes (Assou *et al*., 2006; Kocabas *et al*., 2006; Jones *et al*., 2008; Wells and Patrizio, 2008; Jaroudi *et al*., 2009), reporting from 4801 to 12 031 transcripts as being present. The variability in number of genes present as reported in these studies may reflect individual and technical variability. Taking advantage of recent refinements in the advanced microarray technology on single cells, we performed the gene expression analysis on individual MII oocytes. Present calls were quite stable with a mean of 32.8 $\pm$ 0.9%. When using the defined criteria for the presence of a gene in the oocytes in both age groups (80–100%, i.e. present at least in 8 of 10 younger and in at least four of five older oocytes), 7470 genes (10 428 transcripts) were identified as present in the MII oocytes.

Comparing our list of present transcripts with the list from Wood (Wood *et al*., 2007) we found an overlap of 6685 transcripts. As the array platform is identical in the two studies, differences in protocols for RNA extraction and handling of the oocytes may explain the difference in number of genes present. In contrast to Wood (Wood *et al*., 2007), we used RNase inhibitor that may have minimized the loss of transcripts during the procedures of extraction, amplification and hybridization. Comparative analyses between animal species have shown a high degree of similarity in genes expressed and signal intensity in oocytes (Vallee *et al*., 2008). In a study analyzing whole gene expression profiles in three sets of 500 MII oocytes from mice, 10 977 transcripts were identified as being present, which is almost identical to the numbers of transcripts in the present study of human individual oocytes.

The majority of published studies on oocyte transcriptome have been conducted on MII oocytes that failed to fertilize after IVF or ICSI (Bermudez *et al*., 2004; Assou *et al*., 2006; Gasca *et al*., 2007; Steuerwald *et al*., 2007; Wells and Patrizio, 2008). These represent suboptimal pools of oocytes, because these oocytes do not possess the capacity to sustain the processes necessary for fertilization and further development, which may also be the case for the *in vitro* matured MII oocytes that were obtained from oocytes found to be immature at ovum retrieval 40 h after hCG injection (Jaroudi *et al*., 2009). In addition, the *in vitro* culture period may have altered the gene expression. In the present study, the oocytes were flash frozen within 30 min after ovum retrieval comparable to the procedure of Wood (Wood *et al*., 2007) and should eliminate the bias described above.

The GO enrichment analysis performed to define under- and over-representation of functional categories at the cellular component, biological process and molecular levels of the 7470 genes present in the

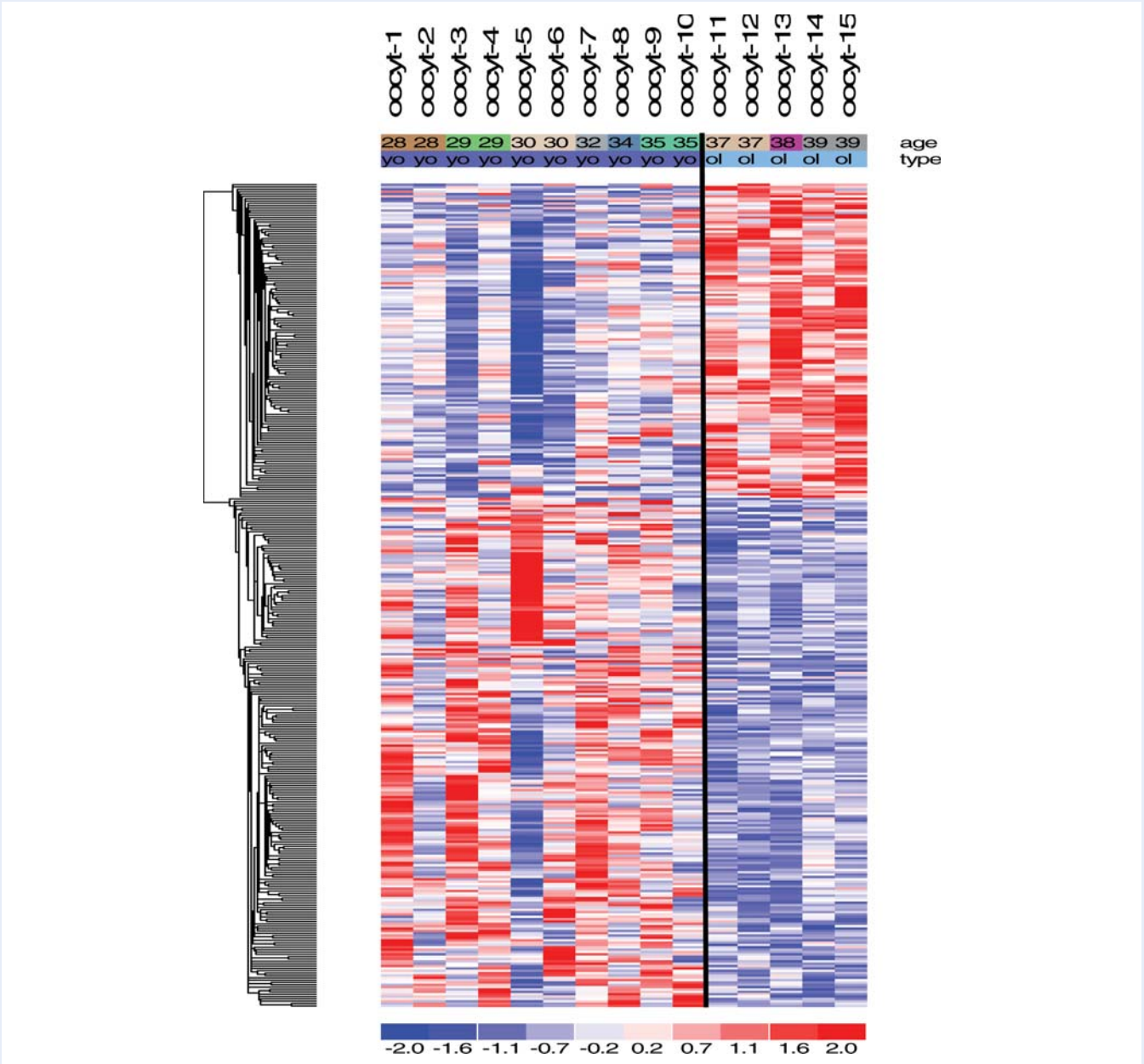

Figure 1 Hierarchical cluster of genes selected as being differentially expressed between the younger (27–35 years; $n = 10$) and older (37–39 years; $n = 5$) MII oocytes.

The expression level for each gene is standardized to have a mean value of 0 and SD of 1. White colour represents the mean value, 0, red colour represents gene expression level above mean and blue colour represents expression below mean. The intensity of the pseudo colour reflects the number of SDs from the mean, as indicated by the lower colour box.

MII oocytes, shows an overview of the functions of the mRNA pooled in the human MII oocytes: a pool that has the competence to direct and fulfill the development of fertilization as well as embryo development to the maternal–embryonic transition and presumably even further (Stitzel and Seydoux, 2007).

As expected the cellular component analysis showed that more than 90% of the transcripts present represent genes involved in intracellular processes. That MII oocytes have few transcripts dealing with extracellular activities has also previously been shown (Assou *et al.*, 2006).

The biological process analysis showed dominance of the following categories: cellular processes (29%), biological regulation (23%), metabolic processes (15%) and developmental processes (9%). In the cellular process category, the main sub-processes of cellular metabolic process, cellular component organization, protein and RNA localization, cell cycle, cell division, microtubule and actin filament processes and chromosome segregation showed the highest enrichment scores. In addition, the following molecular processes were highly overrepresented: protein binding, nucleotide binding, nucleic acid binding, carbohydrate binding and chromosome binding. These findings are

Table II Functional characterization of genes differentially expressed in younger (27–35 years) and older (37–39 years) human MII oocytes.

	Annotational term	Number of differentially expressed genes	*P*-value
Cell compartment	Intracellular	188	0.000074
	Nucleus	75	0.004391
Cell cycle	Cell cycle	27	0.002681
	Mitotic cell cycle	13	0.00148
	Regulation of mitosis	5	0.005004
Spindle/microtubule	Cytoskeleton organization and biogenesis	19	0.00078
	Spindle organization and biogenesis	4	0.000733
Cellular organization	Cellular component organization and biogenesis	61	0.004947
	Regulation of cellular component organization	6	0.002685
	Golgi vesicle transport	9	0.001402
DNA	DNA metabolic process	28	0.000248
	DNA replication	10	0.003983
	Nucleobase, nucleoside, nucleotide metabolic process	87	0.000507
	Hydrolase activity, acting on ester bonds	21	0.004135
DNA repair	DNA repair	10	0.016809
	Response to DNA damage stimulus	13	0.010501
RNA	Regulation of transcription from RNA polymerase II promoter	15	0.008099
	Negative reg of transcript from RNA polymerase II promoter	7	0.008154
Metabolism	Metabolic process	171	0.000035
	Biopolymer metabolic process	122	0.000000
	Catalytic activity	107	0.009242
Post translational	Protein modification process	46	0.000565
protein modification	Post-translational protein modification	43	0.000088
	Protein catabolic process	12	0.000749
Ubiquination	Protein ubiquitination	6	0.00222
	Ubiquitin ligase complex	5	0.00569
Energy	ATP binding	36	0.007735
	Purine ribonucleotide binding	43	0.007701
Apoptosis	Programmed Cell death	20	0.035156
Signalling and	Protein kinase activity	28	0.009097
Response	Transferase activity	45	0.003908
	Transferase activity, transferring phosphorus-containing group	33	0.000216
	Phosphate metabolic process	19	0.002974
	Response to endogenous stimulus	13	0.009925
Miscellaneous	Mesoderm morphogenesis	3	0.006768

The most frequent categories of GO biological processes in the differentially expressed genes were identified using dChip. These biological categories were further re-categorized. Gene overlap exists between the annotated terms. The genes present in the biological categories are shown in Supplementary Data, Table S3.

in agreement with the major processes involved in meiosis and mitosis, implying microtubule attachment to chromosomes and cell cycle progression. The major regulatory mechanism for the processes is the activation of the stored mRNAs, in line with the observed enrichment of translation factor activities. In addition, we noted an over-expression of catalytic activity in the MII oocytes, which confirms previous findings showing that protein and mRNA degradation was important for oocyte maturation including meiosis, fertilization and transition to zygote (Stitzel and Seydoux, 2007). Not surprisingly, the function 'electron transport chain' was highly enriched in the oocytes, which is likely to reflect the high number of mitochondria present in the mature oocyte (Jansen, 2000).

Hierarchical clustering demonstrated that oocyte gene expression was influenced by age. A total of 342 unique genes showed a significant difference in expression in older versus younger oocytes, of which 125 were up-regulated and 217 were down-regulated. Thus, the majority of genes present in the MII oocyte were not influenced by age, while an interesting 4.6% of the genes were found to be affected by age. This figure is similar to findings in mice, where MII oocyte whole transcriptome comparison between young and aged mice showed 4.8% genes were differentially expressed (Hamatani *et al*., 2004). The magnitude of differences between the differential expressed genes in the present study was relatively small (103 genes >2-fold, 239 genes >1.5-fold), which also compares favourably with results in mice (99 transcripts >2.0-fold, 431 transcripts >1.5-fold) (Hamatani *et al*., 2004). Owing to differences in the array platform in the studies, an exact comparison between the lists of differentially expressed human and murine genes is not possible,

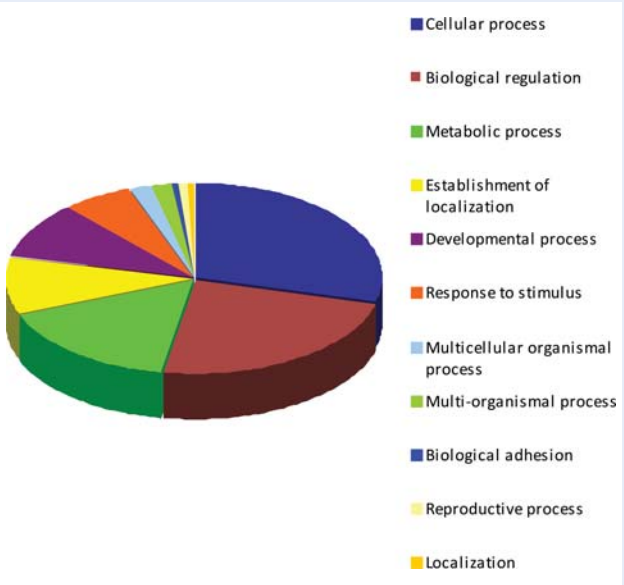

Figure 2 Division of the 342 genes which are differentially expressed in human MII oocytes between younger (27–35 years) and older (37–39 years) oocytes according to GO annotation for 'biological processes'.

although it seems that there are some similarities regarding the functional groups affected by age (Hamatani *et al*., 2004; Pan *et al*., 2008).

The effect of age on gene expression profile in human oocytes has previously been addressed with an array of 8500 human sequences reporting present call at 25% (2125 genes) and 608 transcripts differentially expressed between the age groups: less than 32 years, between 32 and 40 years and above 40 years (based on only 2, 1 and 2 biological replicates, respectively; Steuerwald *et al*., 2007). They also found that up to 28% of transcripts present were affected with age, which is considerably higher than our finding. This discrepancy may reflect that the groups covered different age intervals, and a larger number of genes may be influenced by age when the age of women further exceeds 40 years, which was not studied in the present material. In addition, the use of oocytes failing to fertilize after IVF as well as potential differences in stimulation protocols and ovarian responses between the age groups (Steuerwald *et al*., 2007) may have influenced the gene expression of the oocytes (Bonnet *et al*., 2008). In the present study, all MII oocytes came from women who followed identical stimulation protocols, inducing comparable ovarian responses, though with a significantly higher FSH dosage used in the cycles in the women aged 37–39 years when compared with the cycles in younger women. We interpret our findings to primarily be the result of a difference in age of the women and not primarily to the difference in FSH dosage. This interpretation is supported by the notion that the successful RT–PCR confirmation of the microarray analysis was conducted on oocytes from cycles where the FSH dosages were comparable in the two age groups.

Interestingly, we noted in the hierarchical cluster analysis that the older oocytes had greater transcriptome similarity than the younger oocytes. This may be because the older group has a fairly narrow age range of 3 years (37–39), whereas the younger has a range of 9 years (27–35).

Cell cycle

The GO analysis and the pathway analysis on the genes differentially expressed between the two age groups show that the central functions of the oocyte (e.g. cell cycle and mitosis) are affected by age. This is in agreement with findings in mice oocytes, where 'cell cycle' was the biological function most affected by age (Hamatani *et al*., 2004).

The functional network most significantly affected by age was the network denoted 'organism development and cell cycle' with more than 75% of the pathways affected by age. The network represents several pathways in transforming growth factor-β (TGF-β) signalling, well known to be operative during mammalian preimplantation development and in embryonic stem cells (Sudheer and Adjaye, 2007) and differentially up-regulated in human oocytes compared with somatic cells (Kocabas *et al*., 2006).

SMAD2, which was down-regulated by 2.8-fold with increasing age, is centrally placed in this 'organism development and cell cycle' network and is a member of the so-called SMAD protein family, that is essential for intracellular signalling for members of the TGF-β super-family of peptide growth factors (Nakao *et al*., 1997). SMAD2 was present in all oocytes, confirming findings showing up-regulation of SMAD2 in human MII compared with somatic cells (Kocabas *et al*., 2006), whereas SMAD2 was not reported as present by Wood (Wood *et al*., 2007). Down regulation of SMAD2 in aged oocytes has also been reported in mice (Pan *et al*., 2008). Presence and dosage of SMAD2 have been shown to have an essential role in early embryonic development in studies with mutant mice and *in vitro* culture of mice oocytes with inhibition of SMAD2 signalling (Weinstein *et al*., 1998; Nomura and Li, 1998). The maternally derived SMAD2 has been suggested to be of importance throughout preimplantation development and an intact SMAD2/3 signalling pathway is required for proper maintenance of the inner cell mass as well as pluripotency of human embryonic stem cells (James *et al*., 2005). The action of activin and TGF-β is generally described to be dependent on both SMAD2 and SMAD4 (Sudheer and Adjaye, 2007). SMAD4 ovarian-specific knockout mice are subfertile, suggested to mainly result from cumulus cell defects (Pangas *et al*., 2006). SMAD4 was not expressed in the MII oocytes, indicating that a potential role of SMAD2 in the oocyte and in the zygote until the maternal–embryonic transition is independent of SMAD4. An immunohistochemistry study has shown the presence of SMAD2 throughout the cytoplasm in human MII oocytes, in all cleavage stages as well as in blastocysts (Osterlund and Fried, 2000). One of the activators of SMAD2 is the Activin receptor 1B, which was also highly expressed in MII oocytes, confirming earlier studies (Kocabas *et al*., 2006; Wood *et al*., 2007). We found that the Activin receptor 1B was significantly up-regulated in the older oocytes (1.5-fold). Activin is known to play a central role in folliculogenesis *in vivo* and *in vitro* (Telfer *et al*., 2008) and in the later stages of oocyte maturation (Wang and Ge, 2004).

Cell cycle progression is controlled by activation and subsequent inactivation of a series of cyclin-dependent kinase (Cdk) 1 complexes. Cdk inhibitor 1C (CDKN1C) is a strong inhibitor of several G1

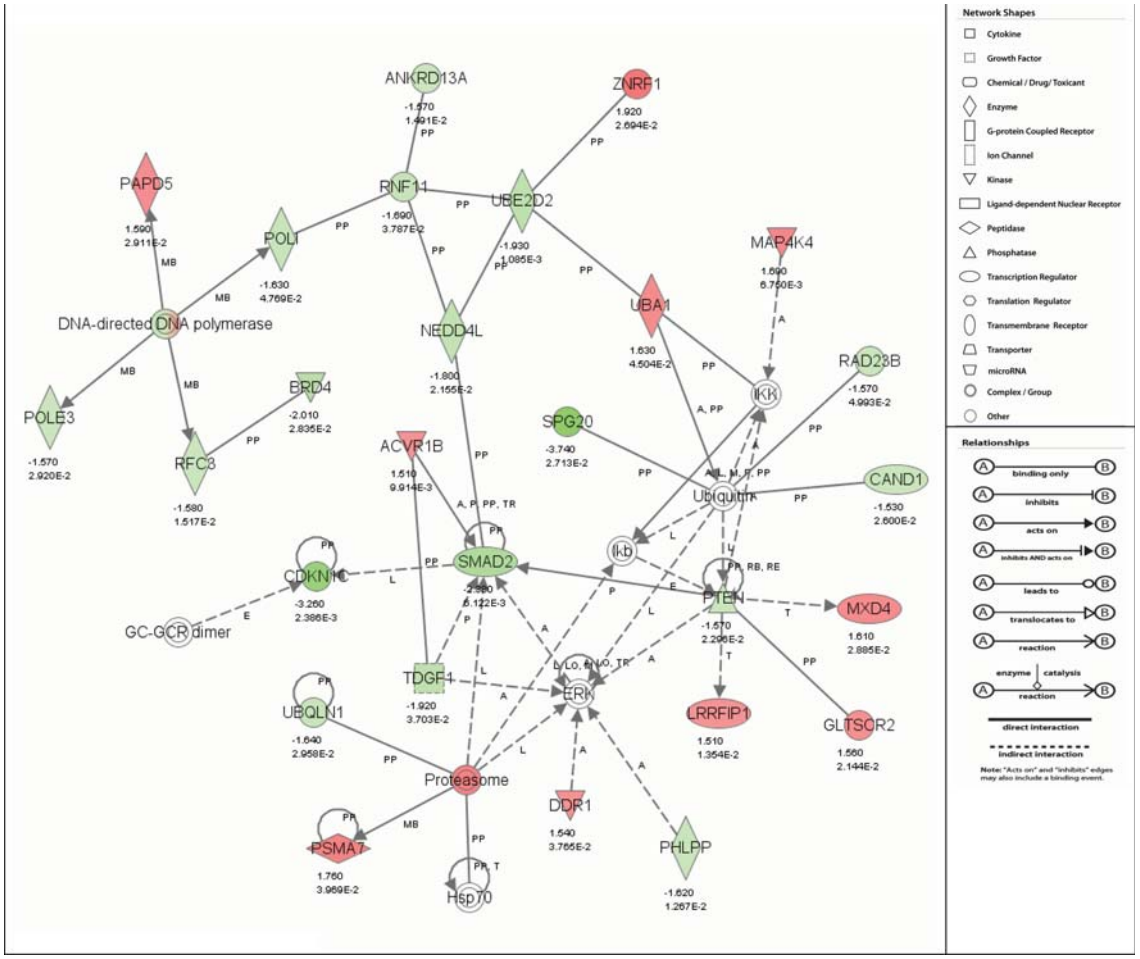

Figure 3 Top network affected by age is the network denoted 'Organism development and cell cycle' generated by Ingenuity Pathways Analysis. The network contains genes such as SMAD2, the Activin 1B receptor, mitogen activated protein (MAP)-kinase 4 and cyclin-dependent kinase inhibitor 1C (CDKN1C). The intensity of colours indicates the degree of up-regulation (red) or down-regulation (green). Node (gene) and line (gene relationships) symbols are described to the right.

Table III Quantitative RT–PCR confirmation of microarray data.

Gene symbol	Ratio by microarray five older oocytes (37–39 years) ten younger oocytes (27–35 years)	Ratio by real-time PCR two older oocytes (39 years) four younger oocytes (26–35 years)
SMAD2 SMAD family member 2	0.42	0.38
MRPL43 mitochondrial ribosomal protein L43	2.50	1.40
ANAPC4 anaphase promoting complex subunit	0.65	0.08

cyclin/Cdk complexes and a negative regulator of cell proliferation (Andrews *et al.*, 2007). The dosage of CDKN1C has been shown to be essential for mouse embryogenesis *in utero* (Zhang *et al.*, 1997), whereas no data are available on the cleavage or blastocyst stages. Our data show that the expression of CDKN1C in oocytes is significantly influenced by age, showing a 3.2-fold down regulation in the older compared with younger oocytes, which could increase the cleavage rate of the embryos with increasing age: the cleavage rate of two pronuclei zygotes has, however, been shown to be unaffected by age (Ziebe *et al.*, 2001), which does not support this finding and interpretation.

Potential marker genes for the ageing process as well as oocyte quality may be found in this signalling network denoted 'organism development and cell cycle' and SMAD2 is suggested to be a candidate for the ageing process, although functional studies are needed to confirm this.

Oxidative stress and protective mechanisms

Chronological oxidative stress increases with reproductive ageing leading to DNA damage (Keefe and Liu, 2009), which is suggested

to explain a part of the lower developmental competence of aged oocytes.

In contrast to the transcriptome study in mice (Hamatani *et al*., 2004), the GO terms 'mitochondrial function' and 'response to stress' were not overrepresented in the genes that were differentially expressed by age in human MII oocytes. Some genes involved in mitochondria function were altered, for example the mitochondrial fission regulator 1 (Mtfr1). We can report that Mtfr1 is highly expressed in human MII oocytes and that the expression level is significantly reduced by age (1.6-fold). In mice testis, Mtfr1 is highly expressed, especially in spermatids and Leydig cells and has been suggested to protect the male gonads against oxidative stress (Monticone *et al*., 2007). Thus, the lower expression level of Mtfr1 in the aged oocytes can be speculated to be leading to a lower capacity to resist oxidative stress. This is in accordance with the observed influence of age on the GO category 'apoptosis/cell death' with, for example, increased transcription of Caspase 9 (1.6-fold) in the older oocytes compared with the younger oocytes, which may reflect increased oxidative stress. An increase in expression level of pro-apoptotic genes in MII oocytes with age has also been shown in mice (Hamatani *et al*., 2004).

The oocytes actively repair damaged DNA including the DNA brought into the zygote (Aitken and De Iuliis, 2007), and studies in mice have shown that the oocyte capacity to repair induced DNA damage is apparently maintained at a high level throughout reproductive life (Guli and Smyth, 1989). The GO analysis of the genes affected by age in the present study showed an over-expression of genes belonging to the terms: 'DNA repair' and 'Response to DNA damage'. We noted that nuclear autoantigenic sperm protein (NASP) was up-regulated (2.8-fold) by age. NASP has previously been shown to be present in MII oocytes (Richardson *et al*., 2006) and suggested to be a key player in the assembly and repair of newly replicated DNA to ensure transition of chromatin from one cell cycle to another (Blake *et al*., 2002). Studies of HeLa cells have shown that loss of NASP causes delay in cell cycle progression and the NASP$-/-$ mutation in mice causes implantation failure (Blake *et al*., 2002). Thus the observed increase in the expression of NASP in our study may be a compensatory mechanism for the oocyte to cope with the increase in DNA damage and sustain the repair capacity in advanced age, as reported in aged mice (Guli and Smyth, 1989).

Another protective mechanism may be hyaluronan (HA) synthesis, as recent studies suggest that oxidative stress induces an increase in HA synthase expression and that the synthesized HA has a protective effect (Campo *et al*., 2004). HA synthase 3 (has3), which is selectively expressed in oocytes (Kimura *et al*., 2002), was highly expressed in the human MII oocytes and significantly higher (1.6-fold) in the older oocytes compared with the younger. Several studies in mice have demonstrated that the addition of HA to culture media effectively prevents fragmentation or segmentation of oocytes and supports the development of *in vitro*-matured, -fertilized and -cultured oocytes to the blastocyst stage (Sato *et al*., 1987; Kano *et al*., 1998). In human IVF, follicular fluid from cycles with successful implantation showed higher levels of than in cycles with failed implantation, while age had no effect on the HA level (Babayan *et al*., 2008). It is not clear what role the potential has3-derived HA from the oocyte takes in this process (Kimura *et al*., 2002), although our finding may reflect a protective response to a higher oxidative stress level with age (Keefe and Liu, 2009).

Meiosis, mitosis and spindle function

The final step in the duplication and distribution of the genome to daughter cells takes place at the metaphase to anaphase transition. At this point, the cohesion complex, connecting aligned sister chromatid pairs, is cleaved by separase allowing the spindle to pull sister chromatids into opposite halves of the cell (Uhlmann, 2001). Cohesin cleavage is essential not only during mitosis, but also in meiosis when it is required for homologue segregation in the first division and probably also for sister centromere separation in the second (Buonomo *et al*., 2000). Over-expression of separase induces premature separation of chromatids, lagging chromosomes and anaphase bridges (Zhang *et al*., 2008), as also confirmed by showing that failure in inhibition of separase activity causes chromosome misalignment during proliferation of the post-migratory primordial germ cells, resulting in mitotic arrest and aneuploidy (Huang *et al*., 2008). Separase was up-regulated (1.6-fold) in the older compared with the younger oocytes.

Just as sister chromatid separation is essential for cell survival, so is precise replication of the genome during the S-phase of the cell cycle and is ensured by cell cycle checkpoints and DNA repair mechanisms. EME1 (essential meiotic endonuclease 1) has been shown to contribute to chromosome stability and euploidy in human cells (Hiyama *et al*., 2006). Small decreases in gene dosage of EME1 promote re-replication and polyploid cells in addition to chromosome aberrations, such as DNA gaps and breaks (Hiyama *et al*., 2006). In the present study EME1 gene expression was down-regulated (1.6-fold) in older oocytes compared with younger, which may increase the rate of cleavage stage embryos with polyploid blastomeres.

A fully functional apparatus for spindle formation is essential for correct chromosome (meiosis) and sister chromatid (mitosis) separation as well as the subsequent cytokinesis. Genes involved in spindle formation and biogenesis were influenced by age in the present study which may partly explain previous data in human MII oocytes, showing compromised chromosome alignment and microtubule matrix in oocytes from the older patient when compared with the younger patient group (Battaglia *et al*., 1996).

Microtubule organization and meiotic spindle formation has been shown to be controlled through the MAPK (mitogen activated protein kinase) pathway. DOCR1 (deleted in oral cancer 1 related) is localized on microtubules in the metaphase I and II spindle and phosphorylated by MAPK. Injecting mice MII oocytes with antisense RNA directed against endogenous DOCR1 mRNA induces disorganized spindles, suggesting a regulatory function on microtubule organization (Terret *et al*., 2003). Here, the aged oocytes displayed 1.6-fold down-regulation of DOCR1 compared with the younger oocytes. Likewise potentially compromising the spindle organization, CSPP (centrosome/spindle pole associated protein) was up-regulated (1.7-fold) in the aged oocytes. Over-expression of CSPP has been shown to impair mitosis and promote multi-polar spindles (Patzke *et al*., 2006), which may be involved in the increase in the degree of fragmentation of cleavage stage embryos observed with increasing age (Ziebe *et al*., 2001).

The spindle checkpoint ensures accurate chromosome segregation by delaying anaphase in response to misaligned sister chromatids during mitosis. Upon checkpoint activation, Mad2 binds directly to Cdc20 and inhibits the anaphase-promoting complex (Xia *et al*.,

2004). A protein, the MAD2L1 binding protein, counteracts the function of Mad2 and is required for the silencing of the spindle checkpoint (Xia *et al*., 2004). In our study this MAD2L1 binding protein was up-regulated (1.6-fold) with age in the MII oocytes. MAD2L1 was expressed in both younger and older oocytes, whereas Gasca *et al.* (2007) reported absence of this protein in MII oocytes but presence in germinal vesicle (GV) oocytes. In the study of Wood *et al.* (2007) MAD2L1 was reported as present. These discrepancies may result from differences in the *in vitro* culture period and quality of the oocyte, since the MII oocytes used by Gasca *et al.* (2007) were oocytes that failed to fertilize after IVF/ICSI. The up-regulation of MAD2L1 may induce suboptimal silencing of the spindle checkpoint adding to the risk of non-disjunction chromosome segregation.

Ubiquitination

The GO analysis showed significant influence of age on the ubiquitination pathway. The most prominent function of ubiquitination is labelling of proteins for proteasomal degradation by the ubiquitin ligases. The proteasomal degradation pathway is essential for many cellular processes, including cell cycle regulation and progression, where cyclins are ubiquitinated, i.e. cyklin A degradated by the ubiquitin ligase, anaphase promoting complex (Havens *et al*., 2006). Additionally, over the last years, evidence has accumulated that several nuclear proteins become ubiquitinated in response to DNA damage (Hofmann, 2009).

The gene for ubiquilin 1, an ubiquitin-like protein, was down-regulated by age (1.6-fold), as were an anaphase promoting complex subunit, ANAPC4 (1.6-fold) and two ubiquitin-conjugating enzyme genes, UBE2D (1.9-fold) and UBE4B (1.8-fold), coding for proteins that catalyze the first step in ubiquitination, although three genes for the ubiquitin-specific peptidases USP2, USP34 and USP42 were up-regulated (2.9, 1.6 and 1.6-fold, respectively). In contrast to this tendency to a reduced ubiquitination by age, the UBE1 gene was up-regulated (1.6-fold) by age and USP9X (1.8-fold) was down-regulated. USP9X is a deubiquitinase, acting as an essential and evolutionarily conserved component in TGF-β and bone morphogenetic protein signalling (Dupont *et al*., 2009) and has been shown to be expressed in oocytes at secondary follicle stage, and highly accumulated in the oocyte cytoplasm in Graafian follicles in adult mice (Noma *et al*., 2002).

These findings showing mainly a reduction in the ubiquitination by age is in accordance with previous findings on murine (Hamatani *et al*., 2004) and human oocytes (Steuerwald *et al*., 2007). A general age-related deterioration of the ubiquitination process, showing less free ubiquitin and more ubiquitin-protein conjugates leading to inefficient recovery and recycling of proteins, has been suggested (Cuervo and Dice, 1998). A recent study comparing the gene expression profile of blood mononuclear cells in women conceiving at 45 years of age with women who had their last delivery before the age of 30, confirms the hypothesis that ubiquitination, ageing and fertility are strongly connected (Gielchinsky *et al*., 2008). Failure in ubiquitination may contribute to accumulation of damaged protein and blockade of the proteasome degradation machinery (Gielchinsky *et al*., 2008). This may lead to alterations in the cell cycle regulation that depends on degradation of cyclins through ubiquitination and proteasome-dependent mechanisms.

The majority of aneuploid conceptions are to the result of errors during maternal meiosis I and II and these errors increase with maternal age (Hassold and Hunt, 2001). Why this increase in aneuploid oocytes and embryos occurs with increasing maternal age is unknown, however, several hypothesis exist. Initial reports have provided evidence that during the oogenesis in fetal life some oocytes are 'set-up' to become atretic or mal-segregate 12–50 years later (Lenzi *et al*., 2005). This supports the 'limited oocyte pool' hypothesis, where the limited number of antral follicles available in older women could lead to suboptimal oocytes for ovulation (Warburton, 2005). Another hypothesis is that over time, although resting in the prophase, changes happen to the oocyte or its milieu inducing damage to the meiotic machinery (Warburton, 2005). The present study does not provide data which add to discussion of the origin of the problem, but instead focuses on the effect of age on the developmental machinery (the transcriptome) present in the oocyte after meiosis has been resumed and maturation has progressed, hence ready for being activated by sperm entry, forming a zygote and cleaving. In relation to the terms nuclear and cytoplasmic maturation, nuclear maturation is used to describe the oocyte maturation stages of the chromosome compartment: GV, MI and MII, although the 'cytoplasmic maturation' covers the organelles, the transcriptome and the proteins stored in the cytoplasm during oogenesis. The transcriptome part of the cytoplasmic maturational process, dictating the acquisition of development competence of the oocyte, is indeed influenced by age.

Taken together our data display substantial differences between the age groups in transcriptional level of genes involved in the central biological functions of the oocytes, from establishment and organization of the second meiotic spindle, through protein metabolism, organelle remodelling and DNA repair to the regulation of meiotic and mitotic divisions. Several of these transcripts that were annotated to be involved in cell cycle regulation, chromosome alignment and sister chromatide separation, showed differential expression in the two age groups. These differences in the transcriptome profile suggest a molecular basis for the age-associated increase in aneuploidy in human oocytes and pre-embryos (Eichenlaub-Ritter, 1996; Warburton, 2005).

Conclusion

We have shown a substantial difference between younger and older oocytes in the transcriptional level of genes involved in central biological functions of the oocytes, thus providing information on processes that may be associated with the ageing phenomenon, and possibly contributing to decreased fertility.

Supplementary Data

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

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