REPRODUCTION

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Maternal age effect on mouse oocytes: new biological insight from proteomic analysis

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Abstract

The long-standing view of 'immortal germline vs mortal soma' poses a fundamental question in biology concerning how oocytes age in molecular terms. A mainstream hypothesis is that maternal ageing of oocytes has its roots in gene transcription. Investigating the proteins resulting from mRNA translation would reveal how far the levels of functionally available proteins correlate with mRNAs and would offer novel insights into the changes oocytes undergo during maternal ageing. Gene ontology (GO) semantic analysis revealed a high similarity of the detected proteome (2324 proteins) to the transcriptome (22 334 mRNAs), although not all proteins had a cognate mRNA. Concerning their dynamics, fourfold changes of abundance were more frequent in the proteome (3%) than the transcriptome (0.05%), with no correlation. Whereas proteins associated with the nucleus (e.g. structural maintenance of chromosomes and spindle-assembly checkpoints) were largely represented among those that change in oocytes during maternal ageing; proteins associated with oxidative stress/damage (e.g. superoxide dismutase) were infrequent. These quantitative alterations are either impoverishing or enriching. Using GO analysis, these alterations do not relate in any simple way to the classic signature of ageing known from somatic tissues. Given the lack of correlation, we conclude that proteome analysis of mouse oocytes may not be surrogated with transcriptome analysis. Furthermore, we conclude that the classic features of ageing may not be transposed from somatic tissues to oocytes in a one-to-one fashion. Overall, there is more to the maternal ageing of oocytes than mere cellular deterioration exemplified by the notorious increase of meiotic aneuploidy.

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Introduction

The 'maternal age effect' in reproduction, characterized by a negative relationship between maternal age and reproductive success, is a poorly understood phenomenon. Several hypotheses have been proposed. While the receptivity of the uterus and the ovarian reserve of follicles can explain the 'maternal age effect' in part, it is known that age-related decline of a female's fertility is also rooted in the quality and developmental potential of her oocytes. Unlike the male germline, the bulk of oocytes do not have a gonial stem cell population. Oocytes spend most of their time quiescent in primordial follicles, mature over days or weeks during follicular growth and then become quiescent again near the time of ovulation, when gene transcription is silenced (Bouniol-Baly *et al.* 1999). Thus, ageing of oocytes may be viewed as a life-long maintenance of cellular homeostasis in the same cell, unlike ageing of the male germline. Some molecular factor(s) within oocytes might deteriorate as the potential mother ages, compromising, for example, the function of the chromosomal apparatus or the ability to scavenge reactive oxygen species resulting from mitochondrial reactions (Wilding *et al.* 2005, Miao *et al.* 2009).

The synthesis of the aforementioned molecular factor(s) relies on gene transcription that oocytes largely perform during follicular growth prior to ovulation, and the transcriptional activities of oocytes may be influenced by maternal age. Microarray and RT-PCR methods have revealed that maternal ageing is accompanied

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by changes in the levels of oocytic mRNAs involved in mitochondrial function, apoptosis, oxidative stress, cell cycle regulation, chromosome stability and epigenetic modification, in both mouse and human oocytes (Hamatani *et al*. 2004, Tarin *et al*. 2004, Tatone *et al*. 2006, Steuerwald *et al*. 2007, Ma & Schultz 2008, Pan *et al*. 2008, Grondahl *et al*. 2010, Esteves *et al*. 2011). However, searching these studies for culprits elicits a list of candidate genes that is quite short, featuring *Bcl2* and *Bax* (mitochondrial function and apoptosis); *Txndc9* (*Apacd*), *Sod1* and *Txn1* (oxidative stress); *Mad2l1* (*Mad2*) and *Bub1* (spindle assembly checkpoint (SAC)); *Atrx*, *Brca1*, *Numa1* and *Smc1β* (spindle assembly and chromosome integrity/stability); and *Dmap1*, *Dnmt1*, *Dnmt3A* and *Hdac1/2* (epigenetic modification). Furthermore, the fold-change of these mRNAs in oocytes during maternal ageing is low (sometimes as low as 1.4- to 1.5-fold).

The hypothesis that maternal ageing of oocytes has its roots in aberrant gene transcription, as opposed to other steps of the gene expression cascade, is largely unproven and is difficult to reconcile with the transcriptional silencing that occurs in oocytes near the time of ovulation. Advances in 'omics' research show that intermediate steps of the gene expression cascade, as well as post-translational protein modification and degradation, can affect levels of functionally available proteins independent of transcription (Vogel & Marcotte 2012). Thus, we consider that the protein level may offer novel insights into the changes in gene expression that oocytes undergo during maternal ageing. The proteome, an accessible 'missing link' between transcriptome and phenotype, can be analysed using high-resolution mass spectrometry (MS)-based proteomics, which allows for the accurate identification of thousands of proteins in somatic tissues during *in vivo* ageing (Walther & Mann 2011).

Previous MS studies on mice analysed the proteome of mature oocytes (Ma *et al*. 2008, Zhang *et al*. 2009) and processes of oocyte maturation (Vitale *et al*. 2007, Wang *et al*. 2010, Cao *et al*. 2012, Monti *et al*., 2013) in young donors, but not oocyte quality and composition during maternal ageing. As MS is not inherently quantitative, Wang *et al*. (2010) relied on the simultaneous comparison of signal intensities between replica samples from young donors, in a so-called label-free approach that requires high numbers of oocytes. Scarce samples, such as oocytes from aged donors, are hardly amenable to the replica collection of hundreds if not thousands of oocytes. These scarce specimens can be quantified using a defined amount of isotopically labelled reference, which is added ('spike-in') to the non-labelled oocyte lysate prior to processing for MS. This method is called SILAC (stable isotope labelling of amino acids in cell culture; Geiger *et al*. 2011). F9 embryonal carcinoma (EC) cells are appropriate as a labelled reference for oocytes as they can easily be cultured feeder-free, have stem cell properties and should harbour the majority of all oocyte proteins (although with different relative abundances). Practically, F9 cells allow for the efficient metabolic labelling of the SILAC reference *in vitro*, overcoming the difficulty of directly labelling oocytes *in vivo*.

We analysed maternal ageing of mouse oocytes on the protein level, using SILAC technology and high-resolution MS, to define its signature at a level closer to phenotype than mRNA. These oocytes had aged up to 1 year inside the ovaries and were analysed immediately after ovulation so as to appreciate the effect of maternal age on the oocyte. This should not be confused with the post-ovulatory ageing of oocytes in the oviduct or in a culture medium (Lord & Aitken 2013). We compared and confronted the quantitative protein data with the predictions of mRNA ageing studies of oocytes and somatic cells. The analysis of two maternal age transitions (puberty to mature age, first; mature age to climacterium, second) allowed us to disregard proteins that keep steady throughout life and to focus on those proteins whose abundance is changing, i.e. age regulated in the second age transition. Overall, correlation between quantitative changes in the proteome and in the transcriptome was nearly nil. Gene expression and gene ontology (GO) analyses revealed a distinction in the cellular components and biological processes (BPs) affected by ageing in oocytes compared to somatic cells. Proteins associated with the nucleus were featured predominantly among those that changed (declined) in oocytes during maternal ageing, thereby fulfilling a classic expectation of oocyte ageing, i.e. progressive loss of precision in chromosome maintenance and segregation. By contrast, the number of changing proteins associated with oxidative stress/damage was very small. Our first conclusion is that proteome analysis of mouse oocytes may not be surrogated with transcriptome analysis: the two molecular portraits do not correlate. Our second conclusion is that the classic features of ageing may not be transposed from somatic tissues to oocytes in a one-to-one manner. Overall, there is more to the maternal ageing of oocytes than a mere cellular deterioration exemplified by the notorious increase in meiotic aneuploidy. This is in accord with our functional observations of the increased ability of old oocytes to support blastocyst formation, although reduced post-implantation development is predicted as a result of maternal aneuploidy.

Materials and methods

Gene, mRNA and protein nomenclature

We followed the rules of the Jackson Laboratory published as of January 2014 (http://www.informatics.jax.org/mgihome/nomen/gene.shtml#gene_sym). Briefly, gene names are written in italic format (e.g. *Hprt*), mRNA names are written in italic format and also specified to be mRNA (e.g. *Hprt* mRNA),

protein names are not written in italic format and only uppercase letters were used (e.g. HPRT).

Ethics statement

This mouse ageing study was performed in accordance with the recommendations of the Federation of Laboratory Animal Science Associations (FELASA) and with the ethical permit issued by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of the state of North Rhine-Westphalia, Germany (permit number: 87-51.04.2010.A160). Every effort was made to preserve animal welfare during the prolonged period of time necessary to complete the study.

Oocyte collection

Germinal vesicle (GV) B6C3F1 oocytes were collected from the ovarian follicles 48 h after injection of pregnant mare serum gonadotrophin (Intergonan, Intervet, Unterschleißheim, Germany). Metaphase II (MII) oocytes were collected from the oviductal ampullae 14 h after the injection of human chorionic gonadotrophin (Ovogest, Intervet), as described (Esteves *et al.* 2011). Oocytes used were ovulated from 3 week (pubertal), 8 ± 1 week (mature) and 58 ± 10 week (climacteric) old B6C3F1 mice.

Karyotype analysis

MII oocytes were processed for chromosome counting by air-drying and Hoechst dye staining, as described (Zuccotti *et al.* 1998). As chromosome spreads with fewer than 20 chromosomes may be the result of chromosome loss during the spreading procedure, only supernumerary chromosomes are considered reliable. Therefore, the degree of aneuploidy is measured by the ratio of chromosome spreads with >20 chromosomes over chromosome spreads with ≥ 20 chromosomes.

Embryo production

Embryos were produced by intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT) or parthenogenesis (PA) of MII B6C3F1 oocytes and cultured in α-MEM medium, as described (Esteves *et al.* 2011). Nucleus donors were C57Bl/6J for ICSI and OG2 (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J) for SCNT.

Confocal microscopy immunofluorescence of oocytes

The immunofluorescence and imaging protocol was described previously (Schwarzer *et al.* 2012). The primary antibodies purchased from Santa Cruz Biotechnology (Heidelberg, Germany) were anti-STAG1 (sc-54466), anti-OCT4 (sc-8628), anti-ZAR1 (sc-55994) and anti-BUB1 (sc-18286). Appropriate Alexa Fluor-tagged secondary antibodies (Invitrogen, Life Technologies, Darmstadt, Germany) were matched to the primaries (Alexa Fluor 488 Donkey Anti-Mouse, A-21202; Alexa Fluor 568 Donkey Anti-Goat, A-11057; Alexa Fluor 647 Donkey Anti-Rabbit, A-31573). The fluorescence signal intensity was quantified using Image J (U.S. National Institutes of Health, Bethesda, MD, USA).

Transcriptomics

For each age group, 20 oocytes were collected in biological triplicates. Microarray data of the MII oocytes were obtained using the Agilent platform, as described (Pfeiffer *et al.* 2011) and deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ttgzzeoaqyuwuvs&acc=GSE42959. Total RNA was extracted using the ZR RNA Microprep Kit (Zymo Research Corporation, Irvine, CA, USA). A two-round linear amplification protocol employing a linear two-step TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre, Madison, Wisconsin) was used to generate biotin-labelled cRNA. Similar to other studies, cDNA was synthesized using mRNA-specific poly(dT) primers binding the poly(A) tail of mRNA (Hamatani *et al.* 2004, Kocabas *et al.* 2006). It is possible that amplification with poly(dT) primers distorts the representation of the original mRNA population by amplifying preferentially those mRNAs that have a longer poly(A) tail instead of those mRNAs with short or no poly(A) tail (Oliveri *et al.* 2007). However, the use of poly(dT) primers ensures that only those transcripts are amplified that are amenable to translation at the time of sampling.

Isotopic labelling

F9 EC cells were grown for several passages in RPMI 1640 medium (PAA, Cölbe, Germany), supplemented with 10% dialysed FCS (Sigma), heavy amino acids $^{13}C_6^{15}N_2$-L-lysine (K8) and $^{13}C_6^{15}N_4$-L-arginine (R10; Silantes, Martinsried, Germany) and glutamine and the antibiotics penicillin and streptomycin (Gibco, Life Technologies, Darmstadt, Germany). Labelling efficiency was examined by small-scale in-solution digests of the heavy labelled F9 cells and analysed by LC-MS/MS (see below). Labelled EC cells were used as a spike-in standard when the 100 most intense proteins showed a labelling efficiency $>97.8\%$ (see Supplementary Fig. 1A, see section on supplementary data given at the end of this article). In order to quantify the labelling efficiency, light F9 cells were grown in the same medium except for the heavy amino acids, which were replaced by the conventional amino acids $^{12}C_6^{14}N_2$-L-lysine (K0) and $^{12}C_6^{14}N_4$-L-arginine (R0; Sigma), labelled as 'light' to discriminate them from their 'heavy' counterparts.

Protein isolation, fractionation and MS

Zona-free oocytes and F9 cells were lysed in SDS lysis buffer (4% SDS, 100 mM Tris/HCl, pH 7.5, 0.1 M dithiothreitol, total volume 70 µl), as described (Pfeiffer *et al.* 2011). We used the spike-in SILAC technology combined with high accuracy MS (Geiger *et al.* 2011) to quantify age-related proteome changes in mouse oocytes.

The spike-in method employs an independently prepared internal or spike-in standard (in our case the F9 EC cells, heavy) against which the test proteome (the oocyte, light) is measured after mixing them in a 1:1 ratio. The mixtures were processed by the FASP–SAX procedure (Wisniewski *et al.* 2009). The peptide fractions obtained from the FASP–SAX procedure were analysed by LC-MS/MS on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA),

equipped with an Easy nano-LC system and a nano electrospray source (both from Proxeon, Odense, Denmark). Briefly, peptides were separated by reversed-phase chromatography on fused silica capillary chromatography columns (15 cm length, ID 75 µm; New Objectives, Inc., Woburn, MA, USA) that were packed in-house with Reprosil pure C18 material (3 µm; Dr Maisch, Ammerbuch, Germany). We used gradients from 2 to 27% of buffer B (80% acetonitrile and 0.5% acetic acid) for the SAX flow through fractions, for the pH 11 washing step and for the first elution at pH 8; from 5 to 35% B for SAX elution steps at pH 6 and 5; and from 8 to 40% B for the SAX elution steps at pH 4 and 3. Each gradient lasted 190 min and was followed by a gradient over 10 min to 90% B and further elution at 90% B for 5 min before the column was equilibrated again with starting buffer A (0.5% acetic acid). The mass spectrometer was operated in data-dependent mode (positive ion mode, source voltage 2.1 kV), automatically switching between a survey scan (mass range $m/z=350$–1650, target value$=1\times10^6$; resolution $R=60$ K; lock mass set to background ion 445.120025) and MS/MS acquisition of the 15 most intense peaks by collision-induced dissociation in the ion trap (isolation width $m/z=2.0$; normalized collision energy 35%; dynamic exclusion enabled with repeat count 1, repeat duration 30.0, exclusion list size 500 and exclusion duration set to 90 s; double charge and higher charges were allowed).

Protein identification and quantification

Raw data were processed by MaxQuant Software (v 1.2.2.5, Martinsried, Bavaria, Germany) involving the built-in Andromeda search engine. The search was performed against the International Protein Index database (mouse IPI version 3.73; Kersey *et al*. 2004) concatenated with reversed sequence versions of all entries and supplemented with common contaminants. Parameters defined for the search were trypsin as the digesting enzyme, allowing two missed cleavages; a minimum length of six amino acids; carbamidomethylation at cysteine residues as fixed modification, oxidation at methionine and protein N-terminal acetylation as variable modifications. The maximum allowed mass deviation was 20 ppm for the MS and 0.5 Da for the MS/MS scans.

Protein groups were identified with a false discovery rate set to 1% for all peptide and protein identifications separately, when there were at least two matching peptides, at least one of which was unique to the protein group. Primary quantification was performed using the heavy F9 lysate mix as an internal standard, and ratios between corresponding heavy and light peptide versions were normalized to this mix and expressed as H/L (i.e. heavy/light SILAC internal standard/sample). All these primary protein ratios are the means of at least two (heavy and light) peptide ratios. Direct comparison of oocyte samples was achieved by a 'ratio-of-ratios' calculation, which is possible because the internal standard was the same for all samples (Geiger *et al*. 2011, Monetti *et al*. 2011).

The MS proteomics data have been deposited into the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino *et al*. 2013) with the dataset identifier PXD000512.

Accuracy and reproducibility of our SILAC approach

We applied two controls to validate our spike-in SILAC approach. These controls are based on the 'ratio-of-ratios' method (Geiger *et al*. 2011). In the first control, we examined the heavy:light ratio distribution of F9 cells and the heavy:light ratio distributions of F9 cell/oocyte mixtures. The latter were markedly broader than the former (judged by the number of standard deviations, s.d., into which 90% of the values fall; see below), confirming the expected larger dissimilarity of the F9/oocyte mixture over the F9/F9 mixture (Supplementary Fig. 1B). In the second control, we examined the 'ratio-of-ratios' distributions of the samples of pubertal and climacteric oocytes relative to mature age oocytes and of mature age replicates against each other. To this end, we harvested one pool of 1400 oocytes from mature mice (collected from 47 mice all on the same day; note that the number of climacteric mice needed for an equivalent experiment would be around six times larger) and split it into two mature age replicates. The protein data associated with the mature age replicates are available from the PRIDE archive. We provide the interval (number of s.d from the zero centre) into which 90% of the ratio values fall as a measure of how broad or narrow the distributions are. These are 9.957 s.d. for the pubertal vs mature oocytes, 10.189 s.d. for the climacteric vs mature oocytes, but 4.286 s.d. for the mature age replicates (Supplementary Fig. 1C, D and E). These results indicate that diversity between the different age groups is larger than within the same age group – a prerequisite to analyse the oocytes of different maternal ages comparatively. Unless otherwise stated, the mature age group was used as a reference to analyse the variation and concordance of protein and transcript levels during maternal ageing.

Bioinformatics and statistical data analysis

The microarray raw data were imported into the R environment (R Core Team 2012). We quantile-normalized the data and filtered out probe sets of a low signal level using the Agi4x44Preprocess package (Bioconductor; Gentleman *et al*. 2004) with default parameters. Expression values of probe sets mapping to the same gene were averaged, yielding 22 334 unique entities. The correlation of mRNA and protein abundance values was calculated by Kendall rank (non-parametric) τ correlation coefficients.

In the proteome data provided by the MaxQuant Mass Spectrometer Data Analysis Software, proteins are placed in one group if the identified peptide set of one protein is equal to or contained in a second protein's peptide set. Then, the proteins with the highest peptide count in a group are retained. The IPI (updated to release 3.87) identifiers of these proteins were mapped to ENTREZ identifiers with an existing MGI symbol. We obtained a single ENTREZ identifier for 96.22% of the protein groups to which we assigned the logarithm (to base 2) of the heavy:light ratio calculated by MaxQuant. Ties that may occur due to isoforms (splice variants) or close paralogs that are not distinguishable based on the peptides identified are handled by downweighting the logarithmic heavy:light ratio, defining the base b of the logarithm as $b=2+(T-1)/T$, where T is the number of ties. Thus, proteins with ties have lower

influence in subsequent analyses. In the case of proteins mapping to the same ENTREZ identifier, we average the logarithmic heavy:light ratios. We subjected the proteins identified to further analysis using a 'ratio-of-ratios' (Geiger *et al*. 2011) describing the 'age-transition-based expression change' from pubertal to mature and from mature to climacteric. Specifically, we subtracted the log2 F9:oocyte ratio of the mature age group from the log2 F9:oocyte ratio of the pubertal group, and we subtracted the log2 F9:oocyte ratio of the climacteric group from the log2 F9:oocyte ratio of the mature age group.

Correlation analysis of transcriptome and proteome

We asked whether maternal ageing within the proteome–transcriptome intersection causes regulated changes in the proteome of oocytes compared to their transcriptome. We considered that the analytical methods of LC-MS/MS and microarray may have different dynamic ranges; we, therefore, calculated their correlation using a statistical method that is independent of the absolute amount of change. The Kendall tau (τ) coefficient of correlation measures the similarity of the proteome (X) and transcriptome (Y) protein/gene orderings when ranked by their respective changes of abundance, regardless of any threshold. In other words, it measures the similarity of the orderings of the data (X and Y) when ranked by each of the quantities. If the agreement between the two rankings is perfect (i.e. the two rankings are the same), the coefficient has a value of 1. If the disagreement between the two rankings is perfect (i.e. one ranking is the reverse of the other), the coefficient has a value of -1. If X and Y are independent, then the coefficient is zero.

GO BP overrepresentation analysis

In order to address the concern of a sampling bias of the proteome with respect to the transcriptome, we sampled 1000 random sets from the transcriptome, of the same size as the proteome, and calculated the GO semantic similarity score between the proteome and the transcriptome and between the random sets and the transcriptome using the GOSemSim package (Yu *et al*. 2010).

Considering both proteome age transitions, two gene lists ranked by the expression values were subjected to GO BP overrepresentation analysis. Following Ackermann & Strimmer (2009), we applied a cut-off-free protocol to each gene list, following rank square transformation, treating positive and negative expression changes in the same way. We then calculated the Mann–Whitney *U* test statistics for all gene sets that correspond to GO BP terms, investigating whether genes in these gene sets rank higher in the transformed ranking than the genes not in the gene set. Then, we drew 1000 random sets of the same size from all genes in the detected proteome, for each GO BP term. Again using the transformed ranking, we calculated the Mann–Whitney *U* test statistics for the random sets, thus obtaining *P* values describing the significance of the GO term enrichment. We also applied an algorithm in processing the ontology terms that returns more specific GO BP terms by considering the dependencies between them (Alexa *et al*. 2006); we refer to this algorithm as the 'elimination algorithm'. Finally, GO term heat maps were generated with a *P* value cut-off of 0.01, and a gray gradient corresponding to the *P* value from light gray (slight overrepresentation, high *P* value) to dark gray (marked overrepresentation, low *P* value), using white as default (no overrepresentation).

Results

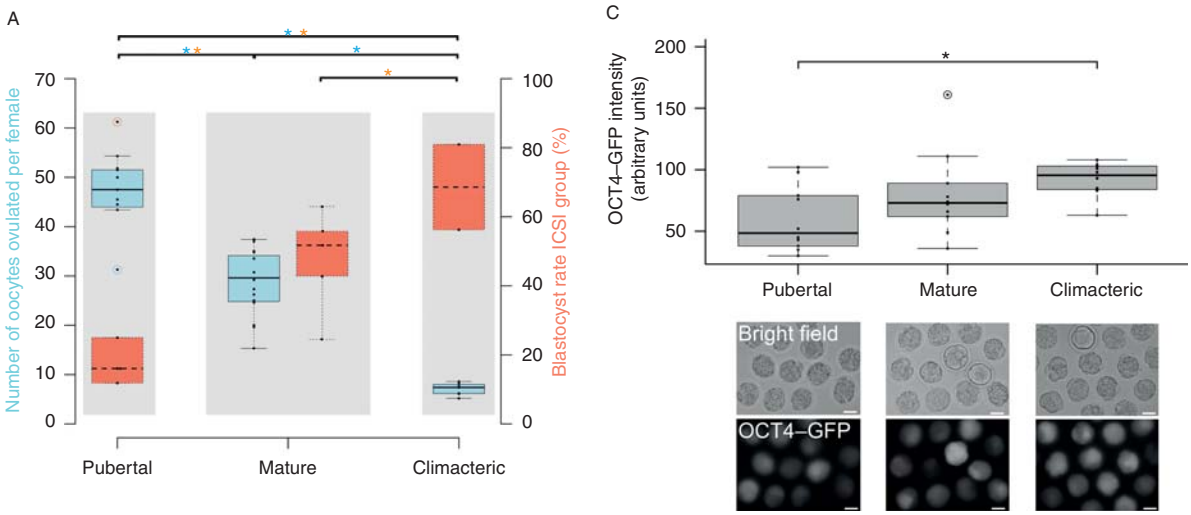
Maternal ageing of oocytes features both deterioration and improvement of functional properties

In order to give our proteomic analysis a phenotypic foundation, we firstly obtained a functional portrait of the MII oocytes ovulated by B6C3F1 mice aged 3 weeks (pubertal), 8 ± 1 weeks (mature) and 58 ± 10 weeks (climacteric). As expected, the number of oocytes ovulated decreased (Fig. 1A) and the frequency of hyperhaploid karyotypes increased during maternal ageing: 7% (1/13), 0% (0/39) and 30% (9/30) respectively. In spite of these negative changes, the proportion of oocytes competent for blastocyst formation increased with maternal age, as measured by blastocyst rates at 96 h after ICSI (Fig. 1A).

Higher cell numbers accompanied the increase in blastocyst rates. The blastocysts' total cell numbers in the ICSI group increased significantly with maternal age (pubertal: 36.7 ± 13.6 cells, $n=32$; mature: 45.8 ± 15.7 cells, $n=33$; climacteric 55.8 ± 14.2 cells, $n=16$; ANOVA, $P=0.0001$; multiple comparisons using *t*-test, $P\leq0.027$).

In addition to fertilization (ICSI), developmental competence was also tested by parthenogenesis (PA) and by SCNT from cumulus cells (Fig. 1B). PA lacks contribution from sperm; SCNT eliminates a possible effect of meiotic aneuploidy on development. In both PA and SCNT, maternal age correlated positively with an increase in blastocyst rate at 96 h (Fisher's exact test, $P<0.01$). Cumulus cells from a transgenic donor harbouring green fluorescent protein (GFP) under the control of the regulatory element of Pou5f1 i.e. Oct4 (*Oct4–GFP*) were used for SCNT, which allows the visualization of pluripotent gene expression. Higher OCT4–GFP intensity, which is a predictor of developmental competence (Cavaleri *et al*. 2008), was observed in derivative cloned morulae obtained from the oocytes of older mothers (ANOVA, $P=0.0364$; multiple comparisons using *t*-test, $P=0.011$ for climacteric vs pubertal age; Fig. 1C).

Although it is well established that deterioration of genome stability (e.g. meiotic aneuploidy) is a hallmark of oocyte ageing, our data attest to the presence of other features that clearly do not deteriorate with maternal ageing, as revealed by improved blastocyst formation, blastocyst cell numbers and *Oct4* transgene expression. Our aim here is to shed light on how oocytes age in molecular terms. The variable rates and qualities of blastocyst formation document that the nature of the



Developmental rates to blastocyst obtained from pubertal, mature age and climacteric oocytes after SCNT, ICSI and PA.

Oocyte donor (weeks)	SCNT			ICSI			PA		
	One-cell	Blastocysts (%)	*n*	One-cell	Blastocysts (%)	*n*	One-cell	Blastocysts (%)	*n*
Pubertal (3)	306	28 (9.2±3.4)†*	4	330	63 (19.1±32.3)†*	5	476	156 (32.8±31.1)†*	5
Mature (8±1)	415	160 (38.6±4.2)†*	5	296	132 (44.6±14.8)†*	5	239	166 (69.5±7.4)†*	4
Climacteric (58±10)	258	126 (48.8±5.4)†*	3	132	95 (72.0±17.5)†*	2	270	210 (77.8±3.3)†*	2

†Fisher's exact test, pairwise comparison related to the one-cell stage and within groups (e.g. SCNT), *P<0.01.
n, number of replicates.

Figure 1 Increased developmental potential of oocytes ovulated from B6C3F1 mice of advanced maternal age. (A) Numbers of oocytes ovulated in association with blastocyst rates after ICSI in pubertal, mature and climacteric mice. Significance was tested by ANOVA, pairwise comparison was made with a multiple comparison *t*-test (*$P \leq 0.05$). (B) Blastocyst rates observed after fertilization (ICSI), parthenogenesis (PA) and cloning (SCNT) given as both total (sum over replicates) and mean ± s.d. Significant differences in blastocyst rates between age groups were compared using pairwise Fisher's exact test. (C) Bright field and fluorescent images of morulae after SCNT from OG2F1 cumulus cells (transgenic for *Oct4-GFP*); size bar, 50 μm. Significance was tested by ANOVA (*$P \leq 0.05$).

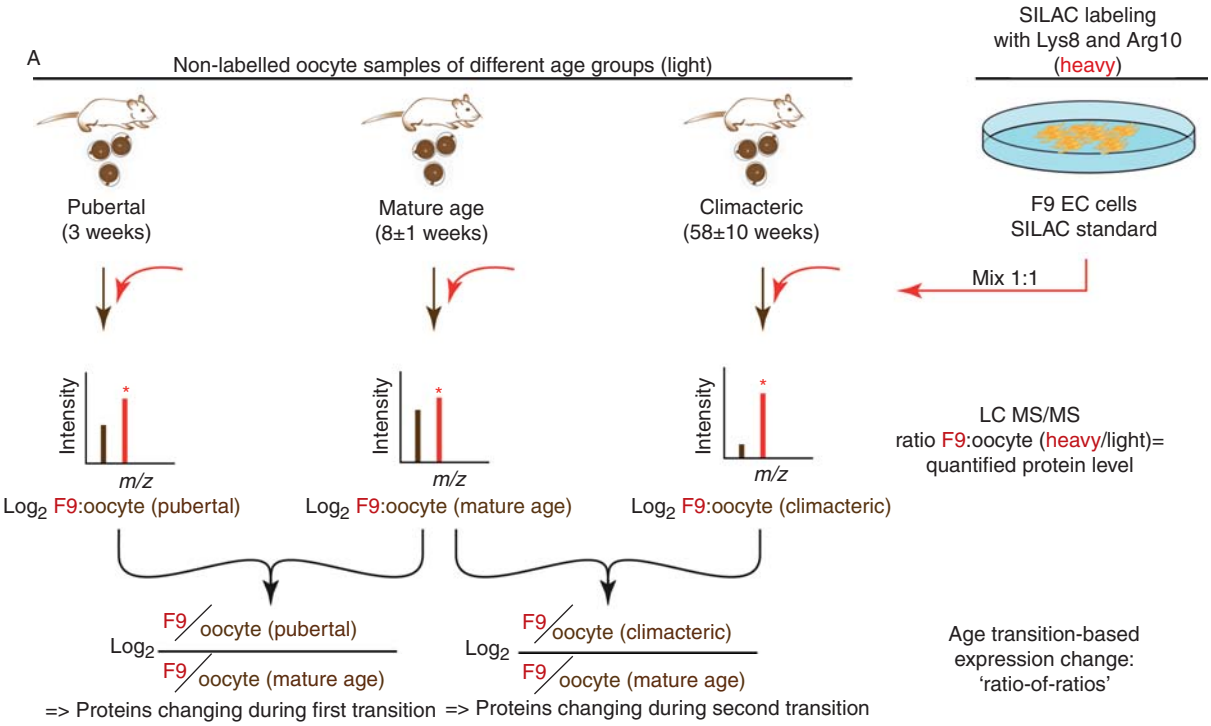
starting material – the oocytes – was not constant during maternal ageing, beyond the notorious phenotype of meiotic aneuploidy. The features of maternal ageing may be rooted in the nucleus or in the cytoplasm of the oocyte. While these cellular components are physically mixed in MII oocytes (absence of nuclear envelope), they can be disentangled *in silico* by performing GO analysis of gene expression data in the domain 'cellular component'. We considered that since proteins relate more closely to cell phenotypes than mRNAs, protein analysis may reveal aspects of the maternal age effect that could not be grasped so far by transcript studies.

A SILAC screen of the proteome of MII mouse oocytes of different maternal age

Seven hundred zona-denuded MII oocytes were used for each of the three age groups (3 weeks, pubertal; 8±1 weeks, mature age and 58±10 weeks, climacteric; see Fig. 2A for a graphical visualization of the SILAC experimental set-up). While 15 and 24 female mice were sufficient in the pubertal and mature age groups respectively 140 female mice were required in the climacteric group to obtain 700 oocytes. Given the scarce amount of material, especially in the climacteric group, we adopted a spike-in (SILAC) quantitative method for proteome analysis, coupled to high-resolution liquid chromatography combined with MS (LC-MS/MS). We used F9 cells labelled isotopically with heavy lysine (Lys8) and heavy arginine (Arg10) as material for the spike-in. Oocyte lysates were mixed 1:1 to lysates of F9 cells that had been labelled efficiently (97.8%; Supplementary Fig. 1A). Thus, the proteins detected in both oocytes and F9 cells are quantifiable relative to each other in this study. Using a linear ion-trap orbitrap hybrid mass spectrometer (LTQ Orbitrap) and the MaxQuant Proteomic Software, we were able to identify a total of 3268 different protein groups, of which 2654, 2639 and 2617 groups were found in pubertal, mature and climacteric MII oocytes respectively (pubertal ∩ mature, 2450 groups and mature ∩ climacteric, 2451 groups). Here, each protein group identified is defined by an F9:oocyte ratio (heavy:light isotopic ratio). Accuracy and reproducibility of our SILAC

approach were validated (see Supplementary Fig. 1B and C and 'Materials and methods' section).

The protein groups identified were mapped to ENTREZ identifiers with an existing MGI symbol. Owing to ties that may occur because of isoforms/splice variants (see 'Materials and methods' section for details), mapping resulted in 2773 gene identities for the pubertal age group, 2757 for the mature and 2732 for the climacteric



B

Amount \ Age group	Pubertal (3 weeks)	Mature age (8±1 weeks)	Climacteric (58±10 weeks)	Simultaneously detected in all age groups
No. of transcripts (microarray)				22 334
No. of detected protein groups	2654	2639	2617	
No. of corresponding gene identities (proteins)	2773	2757	2732	2324
No. of proteins – without probe on microarray:				140
– with corresponding transcripts below detection limit:				118
No. of gene identities in transcriptome–proteome intersection				2066
No. of gene identities (proteins) detected in age transitions (independent of transcript detection)	2450 (first age transition)		2451 (second age transition)	
No. of gene identities (proteins) changing more than four fold in age transitions (independent of transcript detection)	55 (first age transition)		49 (second age transition)	25 (in both age transitions)

Figure 2 Schematic overview of the SILAC workflow and summary. (A) Oocyte lysates were each mixed 1:1 with labelled F9 cell lysate (SILAC reference standard, 'spike-in'). Mixtures were fractionated and analysed by LC-MS/MS. Primary quantification was performed using the heavy F9 cells as SILAC internal standard (*): ratios between corresponding heavy (F9 cells) and light (oocyte) peptide versions were used to determine protein expression levels (expressed as H/L, heavy/light, i.e. SILAC internal standard/sample). Secondary quantification (age transition-based expression change) was performed by dividing the individual H:L peptide ratios of the age groups to be compared (a 'ratio-of-ratios' calculation). (B) Summary of the amounts of transcripts/proteins detected, identified and analysed under the specified criteria.

age group. A summary of all proteins identified and all peptides detected with their mass accuracies is provided in Supplementary Tables 1, 2 and 3, see section on supplementary data given at the end of this article. Representative protein spectra are shown in Supplementary Fig. 2. A total of 2324 of these mapped gene identities were common to the three age groups (Fig. 2B and Supplementary Table 4). The raw protein data associated with the three age groups are available at the ProteomeXchange Consortium (see link in 'Materials and methods' section).

We performed conventional microarray (Agilent, Santa Clara, California) analysis of these oocytes in parallel with the proteome analysis. The transcriptomes were generated from 20 MII oocytes collected in biological triplicates for each age group (pubertal, mature and climacteric). A total of 22 334 gene identities were common to the transcriptomes of the three age groups (Fig. 2B). The raw microarray data associated with this manuscript are available at the Gene Expression Omnibus (see link in 'Materials and methods' section).

A 140 of the 2324 gene identities of the proteome had no corresponding probe on the microarray (Supplementary Table 5, see section on supplementary data given at the end of this article); cognate mRNA was below detection level for another 118 gene identities, despite the presence of the probe on the microarray (Supplementary Table 6). Hence, 2066 gene identities were simultaneously detected in the transcriptome and proteome in all age groups (transcriptome–proteome intersection, Supplementary Table 7). A summary of the numbers of proteins detected is provided in Fig. 2B.

Bioinformatics analysis based on GO terms revealed that the two datasets for the proteome and the transcriptome feature similar BPs. The GO semantic similarity (GOSemSim) score is 0.899, which is within the interval defined by the lower and upper quartiles (0.894 and 0.901 respectively) of the distribution of the similarity scores between the random sets and the transcriptome (see 'Materials and methods' section for details on how the GOSemSim was calculated). Although the number of proteins identified is smaller than the number of transcripts, the proteins we detected can thus be considered representative of the complete proteome.

Quantitative change in the oocyte proteome is not predictable from cognate mRNAs

The protein analysis conducted so far has been qualitative (presence/absence). In order to appreciate the quantitative impact of maternal ageing on the oocyte proteome, we analysed protein abundances, as defined by the 'ratio-of-ratios' (Geiger *et al*. 2011; see Fig. 2A and 'Materials and methods' section). This reflects age-transition-based expression changes, which describe the transition from pubertal to mature (first age transition) and from mature to climacteric (second age transition), using mature as the point of reference. We adopted a fourfold threshold to set significance, because it is in a large excess of the variation (max. 0.41-fold) shown by housekeeping gene products HPRT1, H2AFZ and PPIA (Mamo *et al*. 2007; Fig. 3A) and because twofold fluctuations in proteomics can also be seen in technical replicates (Walther & Mann 2011). Of the 2066 proteins shared by all age groups and with detected mRNA, 48 and 42 proteins varied greater than or equal to fourfold in the first and in the second age transition respectively. The mathematical union ($\cup$) of these two groups of proteins yields 69 proteins (3% of 2066). In contrast to proteins, the abundance of only one mRNA, namely *Cenp-e*, changed fourfold during the first age transition and none in the second. CENP-E is a kinesin-like protein associated with kinetochores. Its mRNA was identified as age-regulated in mouse oocytes (Pan *et al*. 2008).

Overall, the quantitative change in the oocyte proteome (69/2066 proteins; 3%) exceeded the quantitative change in the protein–matched transcriptome (1/2066 genes 0.05%) (χ^2 test, $P=2.46\times10^{-16}$), and the concordance of the two is essentially nil. The overall lack of correlation between proteome and transcriptome, calculated on all 2066 genes/proteins, is summarized by a Kendall τ rank correlation coefficient that is not significantly different from zero: 0.0262 in the first age transition ($P=0.0743$) and 0.0053 in the second age transition ($P=0.7184$, see also 'Materials and methods' section).

The most differently expressed proteins of the ageing oocyte proteome are predominantly localized in the nucleus

Because quantitative change (greater than or equal to fourfold) in the oocyte transcriptome is not correlated with that in the proteome, we decided to consider all proteins detected in subsequent analyses, irrespective of whether they have cognate mRNAs or not. This decision resulted in a slight increase in the number of changing (greater than or equal to fourfold) proteins, from 48 to 55 in the first age transition and from 42 to 49 in the second age transition. These proteins are listed in Table 1 along with the direction (↗, increase and ↘, decrease) of quantitative change and the GO domain 'cellular component' with which they are annotated. The two age transitions (first, puberty to mature age and second, mature age to climacterium) will be contrasted with each other in order to identify those protein changes that are specific to the old age.

The relationship of the 55 and 49 age-regulated proteins (Table 1) to classic features of ageing is described hereafter. Roles in spindle assembly, maintenance of centrosome integrity and chromosome segregation are featured by HAUS7, ACTR1B, BUB1 and TNT. Another classic feature of ageing, namely oxidative

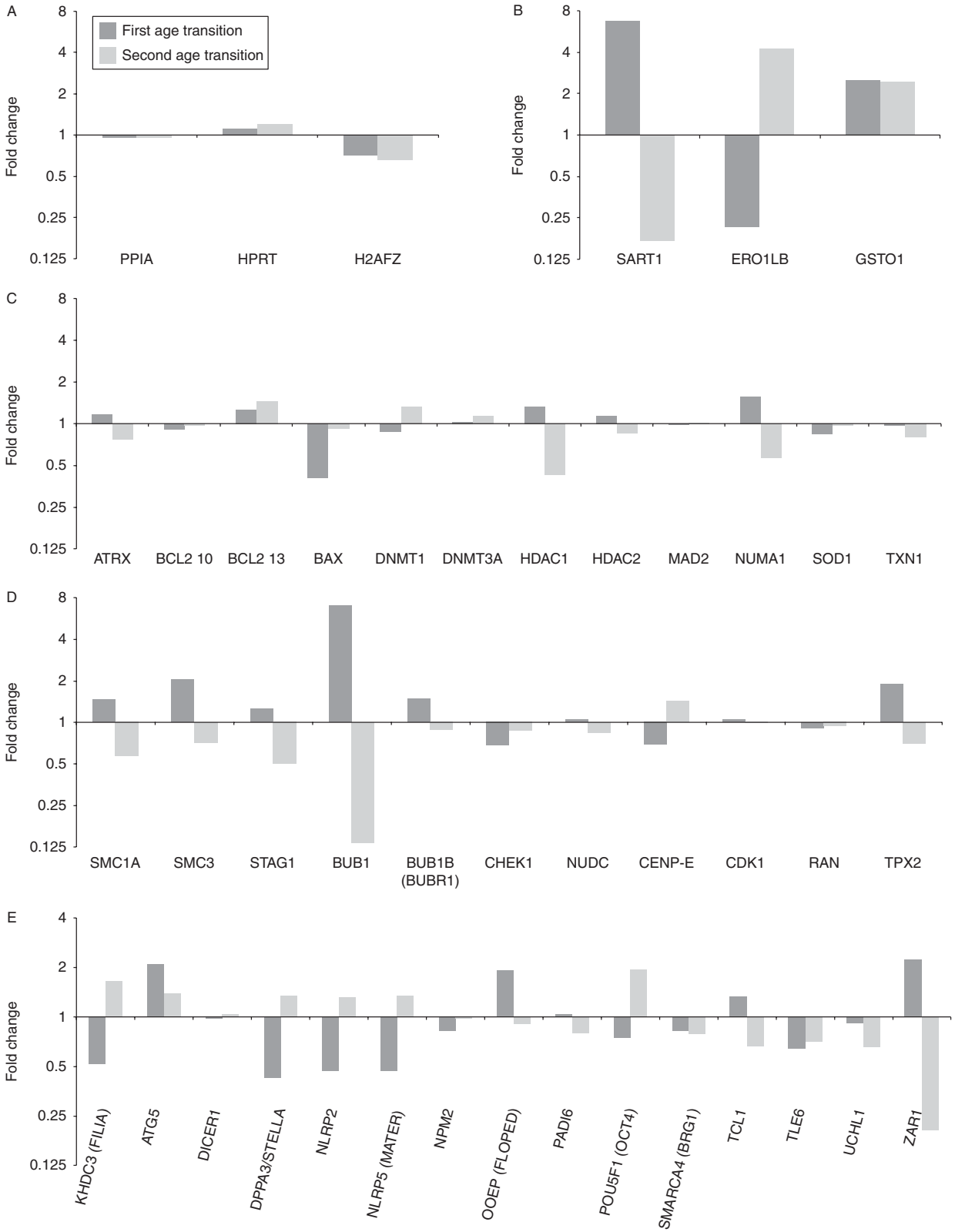


Figure 3 Expression profiles of selected proteins in B6C3F1 oocytes during maternal ageing. Change in protein abundances of (A) housekeepers, (B) factors related to oxidative stress, (C) culprits of ageing revealed by transcriptome studies, (D) structural maintenance of chromosomes (SMC) and spindle assembly checkpoints (SACs) and (E) maternal-effect factors. Values are expressed as fold-change during first and second age transition.

Table 1 Proteins undergoing change in expression during the first (puberty to mature age) and second age transition (mature age to climacteric) in excess of fourfold (in alphabetical order).

First age transition			Second age transition		
Factor	Expression trend	Cellular component	Factor	Expression trend	Cellular component
4931406C07RIK	↘	N	ACTR1B[a]	↘	C
ABCC4	↘	C	ADI1	↗	C+N
ACTR1B[a]	↗	C	AP1G1	↗	C
ARFGAP2	↗	C+N	ARG2	↗	C
ASAH1	↘	C	ARHGAP1	↗	C
BUB1[a]	↗	N	BUB1[a]	↘	N
C87499	↗	NA	CPOX[a]	↘	C
CASP3	↘	C+N	DCAF13	↘	N
CCDC69	↗	NA	DENR[a]	↘	NA
CHMP3	↗	C	DNAJB4	↗	C+N
CPOX[a]	↗	C	DPYSL3	↘	C
CRNKL1	↗	N	DSP	↗	C
D6ERTD527E	↗	NA	DUSP9	↘	C
DENR[a]	↗	C	EML4[a]	↘	C
EML4[a]	↗	C	ERLIN2	↗	C
ERO1LB[a]	↘	C	ERO1LB[a]	↗	C
FAM50A	↗	N	FBXW28	↗	NA
GOLGB1	↗	C	HAUS7[a] (UCHL5IP)	↘	C+N
HAUS7[a] (UCHL5IP)	↗	C	HIST1H1C	↘	N
HMOX1	↘	C+N	HSD17B11	↗	C
KBTBD7	↘	NA	JUP	↗	C+N
LPIN2	↗	C+N	MDN1[a]	↘	N
MDN1[a]	↗	N	MPHOSPH10[a]	↘	N
MPHOSPH10[a]	↗	N	MYL1[a]	↗	C
MYL1[a]	↘	C	PAPOLA	↘	C+N
OOSP1	↘	E	PPM1H[a]	↘	NA
PANK2	↘	C	PTK2[a]	↘	C+N
PDCD11	↘	C+N	RBM10[a]	↘	N
PEX19	↘	C+N	RDH11[a]	↗	C
PLAT	↘	E+C	REEP2[a]	↘	C
PLEC	↗	C	RNF214	↗	NA
PPM1H[a]	↗	NA	RNPEP	↘	C
PTK2[a]	↗	C+N	RPP30	↘	N
PTK7	↘	C	SART1[a]	↘	N
RBM10[a]	↗	N	SEC22A	↗	C
RDH11[a]	↘	C	SENP3[a]	↘	C+N
REEP2[a]	↗	C	SORBS1[a]	↗	C+N
SART1[a]	↗	N	TCL1B1[a]	↗	NA
SENP3[a]	↗	C+N	TCL1B3	↘	NA
SKI	↘	C+N	TDRKH[a]	↘	C
SORBS1[a]	↘	C+N	THOC2[a]	↘	N
SURF6	↗	N	TRIM71	↘	C
TAGLN2	↘	NA	TTN[a]	↘	C+N
TAX1BP1	↘	NA	TUBA3A	↗	C
TCL1B1[a]	↘	NA	URB1	↘	N
TDRKH[a]	↗	C	WDR36[a]	↘	NA
THOC2[a]	↗	N	ZAR1	↘	C
TMSB4X	↘	C+N	ZC3H11A[a]	↘	NA
TTN[a]	↗	C+N	ZFP598	↘	NA
TUBA3A	↘	C			
UTF1	↘	N			
VWA5A	↘	N			
WDR36[a]	↗	NA			
ZC3H11A[a]	↗	NA			
ZP1	↘	N			

C, cytoplasmic; N, nuclear; NA, not annotated.
[a]Proteins changing in excess of fourfold in both age transitions.

stress, is represented in SART1 and ERO1LB. These two proteins are very loosely related to oxidative stress according to the database of the European Bioinformatics Institute (http://www.ebi.ac.uk), and they are not known to play a role in mouse oocytes. Other age-regulated proteins have a relationship to oocyte quality and include ZAR1 (maternal-effect factor), PAPOLA (mRNA maturation) and TCL1B1 (developmental potential). PAPOLA is the poly(A) polymerase α, which adds adenosine residues to create

the 3′-poly(A) tail of mRNAs (Rapti *et al*. 2010). TCL1B1 is associated with developmental potential in early embryos (Sharov *et al*. 2003). Other proteins that changed in abundance include members related to cytoskeleton assembly/organization (e.g. TMS4BX, ACTR1B, TUBA3A, MYL1, PTK7, EML4, PLEC, PTK2 and JUP) and to protein modification (e.g. CRNKL1, MDN1, MPHOSPH10, DENR and TRIM71).

Inspection of the GO domain 'cellular component' reveals that the proteins in the second age transition that increased with ageing are mainly cytoplasmic, while those that decreased are mainly nuclear (Table 1; χ^2 test, $P=0.017$). This allocation is not significantly different from 1:1 in the first age transition (χ^2 test, $P=0.375$). Among the 49 proteins changing in the second age transition, 24 also change in the first transition, while 25 changed only in the second age transition. Reflecting findings already described above, these 25 proteins have a prevalence of cytoplasmic terms among the increasing proteins and of nuclear terms among the decreasing proteins (χ^2 test, $P=0.054$). PAPOLA and ZAR1 are among the latter (Table 1).

Proteins of known candidate genes in the ageing oocyte proteome

We were surprised in our threshold-based analysis (Table 1) that, except for BUB1, the culprits of oocyte ageing were missing, such as BCL2 and BAX (mitochondrial function and apoptosis; Tatone *et al*. 2006), APACD, SOD1, TXN1 (oxidative stress; Hamatani *et al*. 2004, Steuerwald *et al*. 2007), MAD2L1 (SAC; Steuerwald *et al*. 2007, Grondahl *et al*. 2010), ATRX, BRCA1, NUMA1, SMC1B (spindle assembly and chromosome integrity/stability; Hodges *et al*. 2005, Hall *et al*. 2007, Pan *et al*. 2008), and DMAP1, DNMT1, DNMT3A, HDAC1/2 (epigenetic modification; Ma & Schultz 2008, Pan *et al*. 2008). Therefore, we searched for them directly, disregarding the fourfold threshold. APACD, BRCA1, DMAP1 and SMC1B were not detected among the candidates, while the ones that were detected varied less than twofold, except BAX, HDAC1 (Fig. 3C) and BUB1 (Fig. 3D). In contrast to these known culprits of oocyte ageing, three as yet uncharacterized proteins loosely related to oxidative stress were found to change in excess of twofold, namely ERO1LB, SART1 and glutathione *S*-transferase omega 1 (GSTO1; Fig. 3B).

We continued our analysis focusing on the proteins responsible for genome stability (e.g. ploidy) and embryonic genome activation (e.g. maternal-effect factors). While SMC1B was not detected in our study, two other members (SMC1A and SMC3) of the structural maintenance of chromosomes (SMC) complex, which holds the sister chromatids together, were detected in oocytes of all three age groups, but their abundance varied less than twofold (Fig. 3D). By contrast, the SMC cofactor STAG1 decreases in excess of twofold in climacteric oocytes. We have tested and confirmed the STAG1 profile *in situ* – directly in MII oocytes of the three age groups – using confocal immunofluorescence microscopy and compared the measured intensities. The antibody results matched the LC-MS/MS results (Fig. 4A and A′; pubertal vs mature age, $P<0.0001$; climacteric vs mature age, $P<0.0001$; Dunnett's test).

While the abundance of MAD2L1 and other proteins of spindle assembly and chromosome segregation varied less than twofold, another component of the spindle checkpoint, BUB1 (McGuinness *et al*. 2009), varied more than sevenfold (Fig. 3D). As with STAG1, BUB1 abundance was tested by immunofluorescence analysis (Fig. 4B and B′), but it could not be confirmed. This discrepancy between SILAC and immunofluorescence data is discussed below.

A maternal-effect factor, ZAR1, is among the most differently expressed proteins of the ageing oocyte proteome (Table 1). We also examined the other maternal-effect factors. Of the 27 maternal-effect proteins known (Li *et al*. 2010), 17 were detected in oocytes of all three age groups (Fig. 3E). MATER (NLRP5), the maternal antigen that embryos require, is paradigmatic of the maternal-effect factors (Tong *et al*. 2000) and was detected together with the three other members of the subcortical maternal complex (SCMC), namely OOEP (FLOPED), KHDC3 (FILIA) and TLE6 (Li *et al*. 2010). Abundance of these factors was stable during maternal ageing, as was the abundance of other prominent maternal-effect factors, e.g. STELLA (DPPA3) and OCT4, but not ZAR1. ZAR1 abundance declined in oocytes of climacteric females, as confirmed *in situ* using confocal immunofluorescence and comparison of the measured intensities (Fig. 4C and C′; Dunnett's test for ZAR1 pubertal vs mature age, $P=0.6108$ and climacteric vs mature age, $P=0.0043$). OCT4 abundance increased slightly in oocytes of climacteric females, but immunofluorescence analysis revealed a small yet significant decline in OCT4 levels (Dunnett's test, $P\leq0.0218$; Fig. 4D and D′). These discrepancies between SILAC and immunofluorescence data are discussed below.

Molecular signature of oocytes during maternal ageing

The most differently expressed proteins of the ageing oocyte proteome were, only in part, those we had expected based on mRNA studies published. In previous transcriptome studies, only a small number of GO BP were affected in old oocytes, based on thresholds that were sometimes as low as 1.4- to 1.5-fold (Hamatani *et al*. 2004, Pan *et al*. 2008, Esteves *et al*. 2011). Thus, we set out to perform GO analysis to discover the signature of the oocyte proteome during maternal ageing.

We performed GO overrepresentation analysis using ranks instead of age-transition-based expression changes so that the conclusions of the GO analysis are valid

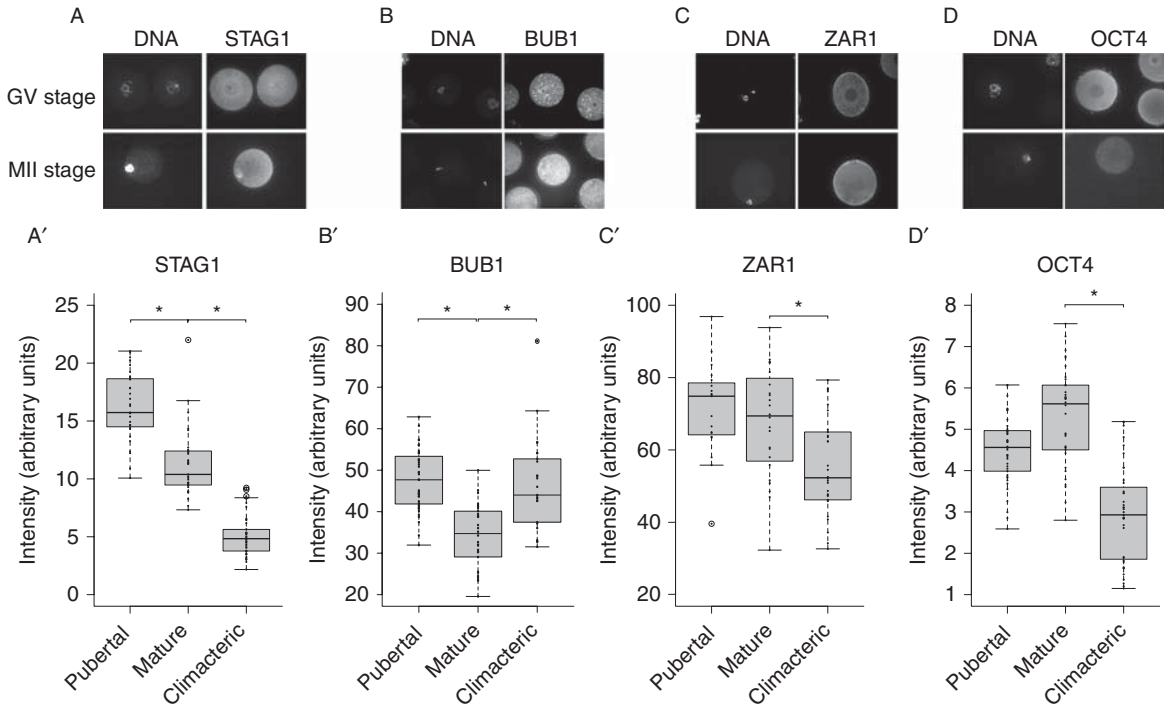


Figure 4 SILAC abundance of selected oocyte proteins validated *in situ* by immunofluorescence. (A, B, C and D) Representative pictures of immunofluorescence staining of STAG1, BUB1, ZAR1 and OCT4 on GV and MII stage oocytes, with DNA counterstaining (YOPRO-1, Life Technologies, Darmstadt, Germany). (A′, B′, C′ and D′) Results of the quantification of the immunofluorescence signal in MII oocytes, performed with Image-J Software. Significance was tested by ANOVA, pairwise comparison was made with the Dunnett's test, taking the mature age as reference (*$P \leq 0.05$).

irrespective of any threshold that we may set, such as fourfold (see 'Bioinformatics and statistical data analysis' section for details). We further applied the 'elimination algorithm' (Alexa *et al*. 2006) to account for redundant GO terms. We considered all the proteins of each age transition (2450 and 2451 respectively), independent of any threshold and of the detection of the protein itself in the other age transition (Fig. 2B and Supplementary Tables 8 and 9, see section on supplementary data given at the end of this article). Our overrepresentation analysis in GO BP terms shows that the two age transitions feature different BPs (Fig. 5, the larger the enrichment, the more the colour shift to dark gray). During the first age transition, processes related to 'regulation of blood pressure', 'stem cell maintenance' and 'cellular response to light stimulus' are overrepresented. During the second age transition, however, the analysis shows overrepresentation of BPs associated with 'RNA processing', 'mRNA splicing, via spliceosome', 'positive regulation of nucleocytoplasmic transport' and 'heart morphogenesis' (Fig. 5), among others. In line with the most differently expressed proteins, these are not the BPs one would expect based on the GO analyses of transcriptome data, which feature terms related to, for example, oxidative damage and stress response (Hamatani *et al*. 2004, Pan *et al*. 2008, Esteves *et al*. 2011) and inflammation (Sharov *et al*. 2008). This discrepancy is also discussed below.

Discussion

Although the oocytes ovulated by older mice complied with the expected reduction in number and increase of meiotic aneuploidy, other commonly expected changes could not be verified. Oocytes of older mice, for example, were superior to younger counterparts at accumulating cells during cleavage, forming blastocysts and expressing Oct4–GFP, irrespective of the developmental stimulus (ICSI, PA and SCNT). These observations confirm and extend our previous study (Esteves *et al*. 2011). Thus, while the well-known fact of maternal age-dependent deterioration of oocyte ploidy was confirmed, similar deterioration is not applicable to all the properties of oocytes, some of which are preserved or even improved. The blastocyst phenotypes are evidence that the nature of the starting material – the oocytes – is not constant during maternal ageing and that there is more to oocyte ageing *in vivo* than the notorious increase of aneuploidy. Using the innovative proteomic tool to appreciate this complexity, we are going to discuss that i) proteome analysis of mouse oocytes may not be surrogated with transcriptome analysis and ii) the classic features of ageing may not be transposed from somatic tissues to oocytes in a one-to-one fashion.

So far, the molecular bases of oocyte ageing, e.g. meiotic aneuploidy, have been searched for in the mRNA world. Expectations from mRNAs are now confronted with unprecedented information gained

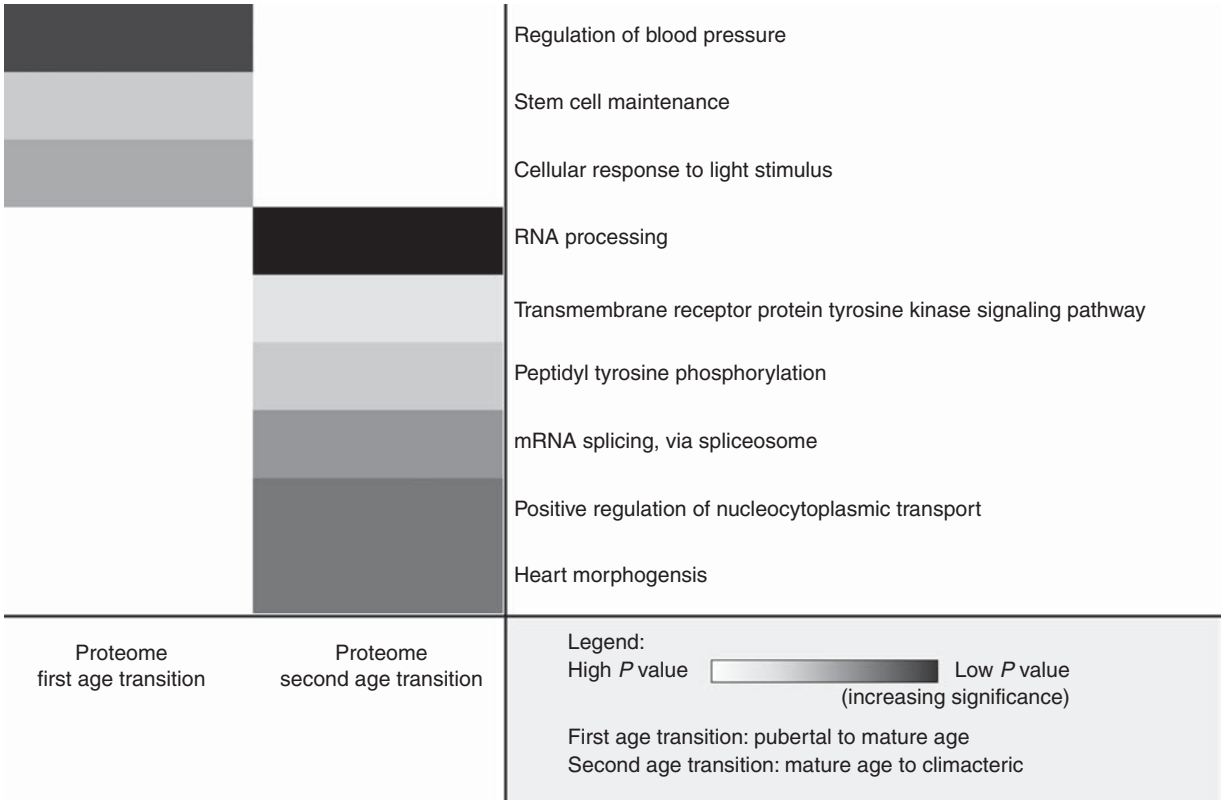


Figure 5 Gene ontology (GO) overrepresentation analysis reveals proteomic signature of oocyte ageing. Overrepresentation heatmap of GO BP terms of 2450 and 2451 proteins detected in the first and second age transition respectively. For the heatmap, the two protein lists were ranked by expression values. Rank square transformation was applied for threshold-free and direction of change (up and down)-independent ranking. Random testing using Mann–Whitney *U* statistics was applied to obtain *P* values (cut-off 0.01). Colour code: gray gradient corresponding to the *P* value, from light gray (light overrepresentation, high *P* value) to dark gray (marked overrepresentation, low *P* value), using white as default (no overrepresentation).

from proteomic analysis, providing us with unexpected results. Our study revealed the highly dynamic character of the oocyte proteome during maternal ageing *in vivo*, whereas proteome and transcriptome were qualitatively similar in GO semantic composition. Overall, there was a higher prevalence of proteins changing in excess of fourfold compared to transcripts (69/2066 vs 1/2066) and minimal concordance between changes of the 2066 pairs of protein and transcript values (Kendall τ close to zero). It should be noted that poly(dT)-primers have been used for the required pre-amplification step when pursuing oocyte microarray analyses. Therefore, only mRNAs with a poly(A) tail that could effectively be translated at the time of sampling, i.e. the MII stage oocyte, have been analysed. This may explain why certain proteins have been detected for which no corresponding mRNA could be identified. It is reasonable to assume that these RNAs had existed during oocyte maturation but lost their poly(A) tail at the time of ovulation to mark them for degradation. This fact may also have influence on the mRNA expression levels detected in MII oocytes in general and may partially explain the missing correlation between transcriptome and proteome in oocytes. However, the transcriptome to proteome correlation also has been described to be poor when analysing stably growing cell lines that are in a steady-state and we deem it, therefore, very unlikely that our correlation analyses have been hampered by technical matters (Akan *et al*. 2012). Further, it is not surprising that protein fluctuations are inherently larger than mRNA fluctuations. Schwanhausser *et al*. (2011) observed that distributions of protein copy number per cell have two to three times (in log10 scale) higher median and higher variation compared with mRNA. Whether these larger fluctuations shed light on the maternal age effect in oocytes is crucial.

Climacteric oocytes being hard to come by (140 mice needed for 700 oocytes), we relinquished the replicates and the conventional approach of setting twofold thresholds combined with *P* values in order to make the call of significance. Instead, we combined a spike-in method (SILAC internal reference) with a higher threshold of fourfold change. This threshold is higher than the difference observed by Walther & Mann (2011) in their comparison of ageing in different somatic tissues and substantially higher (about ten times) than the variation observed in housekeeping proteins (0.4-fold variation), which are overall stable. Searching the oocyte

proteome for a group of changes (e.g. GO terms) that would stand out from the background, our SILAC study revealed several proteins that account for the malfunction of the chromosomal apparatus, but few that reflect oxidative stress or damage. As this study is the first of its kind, one has to be careful not to over-interpret data. Yet, we want to discuss some individual genes that specifically caught our attention. Our SILAC results revealed change in the abundance of the SMC cofactor STAG1, as well as in that of the SAC protein BUB1 and the histone deacetylase protein HDAC1. This latter protein modulates the ability of chromosomes to interact with spindle microtubules and is depleted in climacteric oocytes, thereby jeopardizing proper chromosome segregation (Ma & Schultz 2008). Unlike STAG1 and BUB1, our SILAC results reveal no marked change in the abundance of the core cohesin factors SMC1A and SMC3. It has been proposed that the efficiency of oxidative phosphorylation in the ageing oocyte is degraded by free radical attack (Wilding *et al*. 2005); however, neither metabolism- nor oxidative stress-associated genes featured substantial change of abundance in our quantitative proteome data. A change of proteins very loosely associated with response to oxidative stress, e.g. SART1 and ERO1LB, was detected in the first and in the second age transition; however, a role of these two proteins has not been described in oocytes as of yet.

How reliable are fold-differences of protein abundance in the SILAC measurement? We found a similar abundance trend between our SILAC data and immunofluorescence analysis for STAG1, but not for BUB1. We also examined 'maternal effect factors', i.e. ZAR1 and OCT4 (Li *et al*. 2010), and we confirmed ZAR1, but not OCT4. On the one hand, while antibodies are limited in their ability to detect partially degraded proteins due to the lack of proper 3D structure, proteomics is only dependent on small stretches of peptide sequence and, therefore, a degraded protein might still be detected by LC-MS/MS and MaxQuant. In this respect, while proteomics offers more technical accuracy, immunofluorescence offers more biological relevance. On the other hand, there are many antibodies that detect distinct protein isoforms (e.g. OCT4A only), whereas LC-MS/MS can, in principle, detect all isoforms (contingent on the quality of the database). It is important to note that isoforms were not considered during the SILAC analysis in this study. The prominent maternal factor OCT4, whose A isoform is dispensable for embryo development (Wu *et al*. 2013), varied less than twofold during the age transitions. Although OCT4 abundance correlates positively with developmental potential, it is probably not a precondition, rather it is an effect (Cavaleri *et al*. 2008, Pfeiffer *et al*. 2010).

On the functional level, we observed an increase in developmental rates with ageing in an *in vitro* culture environment outside of the aged female, with the blastocyst stage as the developmental endpoint. This confirms and extends our previous study (Esteves *et al*. 2011). Although we should not aim to explain the developmental performances on the basis of the proteome detected (which is still incomplete, despite the best analytical technology used), we note an interesting age-dependent change in the abundance of two factors that may facilitate, or impede, development. The abundance of GSTO1 increased in both the first and second age transition. Although there is no study of GSTO1 in mammalian oocytes, impaired synthesis of glutathione is indicted as the main cause for compromised developmental potential of pubertal mouse oocytes in mice (Jiao *et al*. 2013); hence, the increased abundance of GSTO1 in old oocytes may facilitate development. Abundance of the cell death inducer BAX was high in pubertal oocytes and decreased in the first age transition. Induction of *Bax* gene expression by *in vitro* culture conditions correlates with reduced developmental rates of mouse embryos (Chandrakanthan *et al*. 2006); hence, the higher abundance of BAX in pubertal oocytes may explain, at least in part, their lower developmental competence compared with mature-age and climacteric oocytes. Certainly, the possibilities are not exhausted here, and there may be additional factors whose accumulation (developmental agonists) or depletion (developmental antagonists) in oocytes could explain the increase in developmental potential observed during maternal ageing.

Although the proportion of blastocyst formation-competent oocytes increased with maternal age, aneuploidy would impair subsequent development. Follow-up of the embryos into post-implantation development is beyond the scope of this study and would not add to what is known already (Lopes *et al*. 2009). The higher blastocyst potential of older oocytes, however, does suggest that other features of ageing oocytes (including non-nuclear processes) may be less affected by maternal ageing than chromosome stability. The majority of proteins that underwent quantitative change in the second age transition are classified as cytoplasmic in the GO domain 'cellular compartment'. Among the nuclear proteins not directly associated with SMC, our data revealed a reduced abundance of the poly(A) polymerase α (PAPOLA) and HDAC1, and an increased abundance of TCL1B1 in old oocytes. As the recruitment of many mRNAs for translation depends on the length of their poly(A) tail (Kosubek *et al*. 2010), the reduction in the abundance of PAPOLA may affect the translation efficiency of mRNAs and could further exacerbate the already poor correlation between transcriptome and proteome. We speculate that, at least for certain cellular functions, oocytes can react to maternal ageing by operating a shift in how gene transcripts are used, leading to the protein profiles not matching the mRNA profiles. PAPOLA is also one of the 118 proteins with no detected cognate mRNA, together with HDAC1/2

(member of the 'reprogrammome'; Supplementary Fig. 3, see section on supplementary data given at the end of this article; Pfeiffer *et al*. 2011), suggesting that the amount of enzyme for these two factors may not be replenished without *de novo* transcription. We speculate that this might be a fatal hurdle for SCNT embryos because *de novo* transcription of the *Papola* gene is dependent on prior nuclear reprogramming, which is known to be inefficient. TCL1B1, which showed an increase in abundance in old oocytes, is a member of the T cell leukaemia/lymphoma 1 (TCL) family and is specifically expressed in oocytes and stem cells. Importantly, Tcl1 is a known downstream target of *Pou5f1* (Hu *et al*. 2008), which is one of the four factors that reprogramme differentiated somatic cells to become pluripotent cells (Takahashi & Yamanaka 2006). TCL1B1 is implicated in the developmental potential of mouse embryos (Sharov *et al*. 2003). The abundance of TCL1B1 is increased almost tenfold in old oocytes, suggesting an explanation for their increased blastocyst rate.

When we examined the molecular signature of the ageing oocyte proteome, we could not confirm the BPs indicted by previous transcriptome studies as also being altered in the ageing proteome, even though the proteome is representative of the transcriptome as judged by GO semantic similarity. Transcriptome studies pointed at genes involved in mitochondrial function, oxidative damage and stress responses (Hamatani *et al*. 2004), genes associated with protein folding/response to unfolded proteins, protein metabolism/metabolism, intracellular transport and cell cycle (Pan *et al*. 2008), as well as stress response and response to oxidative damage (Esteves *et al*. 2011). Although these three studies also found other families of genes responsive to maternal ageing, the number of genes was small and their fold-change was low (sometimes as low as 1.4- to 1.5-fold). The new technology of SILAC MS can now inform the study of oocyte ageing. The most significant terms of BP in the oocyte proteome include 'stem cell maintenance' in the first age transition and 'RNA processing' in the second age transition. It is tempting to speculate that these terms are consistent with a 'memory' of the oogonium (stem cell)-to-oocyte transition (a recent event in ovaries of 3-week-old mice) and with a change in the mode of transcript usage at old age respectively. As the MII oocyte is a transcriptionally silent, terminally differentiated non-dividing cell, it cannot use the window of opportunity of S-phase to impose new epigenetic marks that alter transcription, such as cycling somatic cells; thus, it changes its phenotype through post-transcriptional actions. This may partly explain why the GO terms commonly found enriched in ageing somatic tissues (e.g. inflammation) did not characterize the ageing oocyte proteome. This observation is in line with the conclusion of Sharov *et al*. (2008), who compared the ageing ovary and the ageing testis with ageing somatic tissues of mice, finding that ageing of germ cells generally shows a different pattern of gene expression changes than ageing of somatic organs.

There is one final issue raised by our study. We have characterized the maternal age-effect on oocytes, but what is the underlying cause? Perhaps, the answer to this question has to be searched in the somatic niche of the ovary. The role of the niche has been investigated in the testis. Spermatogonial stem cells (SSCs) do not age or age very slowly, as shown by the successful consecutive transplantation of SSC populations from the testes of old mice to the testes of young recipients. These transplanted SSCs are preserved long past the normal life span of the old donor, for more than 3 years (Ryu *et al*. 2006). These observations suggest that paternal infertility results from deterioration of the somatic niche, not from deterioration of the germ cells. Ideally, one would like to perform a similar investigation also on the ovary, to test whether the quality of oocytes is preserved beyond the life span of the female by transplanting her oocytes to a younger niche. In principle, this experiment is feasible (Eppig & Wigglesworth 2000), but very challenging. Until it is performed, we think that the available literature already hints to an effect of the niche on oocyte ageing. Selesniemi *et al*. (2011) showed that adult mice maintained under 40% caloric restriction (CR) did not exhibit maternal age-related increases in oocyte aneuploidy, chromosomal misalignment on the metaphase plate, meiotic spindle abnormalities or mitochondrial dysfunction, all of which occurred in oocytes of age-matched controls that were fed *ad libitum*. As the bulk of oocytes have already formed at the time of CR treatment, and as CR suppresses ovulation (Nelson *et al*. 1985), we think that the effect of CR is unlikely to depend on the oocytes themselves, rather on their niche. Could the above considerations be extrapolated to humans? Caution should always be exercised in extrapolating, due to species-specific differences in life span, oogenesis, duration of the ovarian cycle and onset of menopause (Velde & Pearson 2002). The average life expectancy of B6C3F1 females is 128 weeks (Myers 1978). The climacteric mice used in our study were aged up to 68 weeks, which would correspond to women 44 years old, assuming a linear relationship and a human life expectancy of 84 years for females. Thus, the cells of our study could have been even older than 68 weeks, which is not a problem with liver, kidney and brain (Walther & Mann 2011), but it is a problem with the ovary as it is depleted of oocytes long before the animal dies. Human oogonia divide more times than mouse oogonia until they enter meiosis (Wallace & Chalkia 2013), and these divisions may introduce DNA mutations. Perhaps more importantly, if we follow up on the effect of CR in mice, then we should consider that the basal metabolic rate per gram of body weight is seven times greater in mice than in humans (Demetrius 2005). Therefore, the human niche may have a different, i.e. slower rate of ageing than the mouse niche. The net outcome of a slower rate of ageing over a longer period of time (decades) is difficult to predict.

Taken together, our data indicate that ageing of oocytes on the protein level generally shows a different pattern of gene expression changes than ageing on the mRNA level, and the former does not resemble somatic ageing. The possibilities of modern proteomics are far from exhausted (e.g. extended protein coverage, detection of post-translational modifications) and future work will contribute to a sharper definition of the maternal age effect in oocytes, including, but not limited to, the correspondence between protein immunodetection and LC-MS/MS results.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0126.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C Schwarzer performed isolation and purification of RNA from oocytes, immunoconfocal embryo imaging and participated in data analysis; B Wang helped with the immunoconfocal embryo imaging; M Siatkowski and G Fuellen performed bioinformatics data analyses; H C A Drexler designed and performed proteomics measurements; N Baeumer performed RNA processing and microarray measurements; M J Pfeiffer helped with the mouse work; M Boiani performed oocyte collection for proteomic analysis, karyotype analysis, micromanipulations, embryo production and participated in data analysis; and C Schwarzer, M Siatkowski, M J Pfeiffer, G Fuellen and M Boiani wrote the paper. All authors read and approved the paper.

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