Vitrification at the pre-antral stage transiently alters inner mitochondrial membrane potential but proteome of in vitro grown and matured mouse oocytes appears unaffected

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Background: Vitrification is a fast and effective method to cryopreserve ovarian tissue, but it might influence mitochondrial activity and affect gene expression to cause persistent alterations in the proteome of oocytes that grow and mature following cryopreservation.

Methods: In part one of the study, the inner mitochondrial membrane potential ($\Delta$Ψm) of JC-1 stained oocytes from control and CryoTop vitrified pre-antral follicles was analyzed by confocal microscopy at Day 0, or after culture of follicles for 1 or 12 days. In part two, proteins of in vivo grown germinal vesicle (GV) oocytes were subjected to proteome analysis by SDS polyacrylamide gel electrophoresis, tryptic in-gel digestion of gel slices, and one-dimensional-nano-liquid chromatography of peptides on a multi-dimensional-nano-liquid chromatography system followed by mass spectrometry (LC-MS/MS) and Uniprot Gene Ontology (GO) analysis. In part three, samples containing the protein amount of 40 GV and metaphase II (MII) oocytes, respectively, from control and vitrified pre-antral follicles cultured for 12 or 13 days were subjected to 2D DIGE saturation labeling and separated by isoelectric focusing and SDS gel electrophoresis (2D DIGE), followed by DeCyder™ analysis of spot patterns in three independent biological replicates. Statistical and hierarchical cluster analysis was employed to compare control and vitrified groups.

Results: (i) Mitochondrial inner membrane potential differs significantly between control and vitrified GV oocytes at Day 0 and Day 1, but is similar at Day 12 of culture. (ii) LC-MS/MS analysis of SDS gel fractionated protein lysates of 988 mouse GV oocytes revealed identification of 1123 different proteins with a false discovery rate of <1%. GO analysis assigned 811 proteins to the ‘biological process’ subset. Thirty-five percent of the proteins corresponded to metabolic processes, about 15% to mitochondrion and transport, each, and close to 8% to oxidation-reduction processes. (iii) From the 2D-saturation DIGE analysis 1891 matched spots for GV-stage and 1718 for MII oocyte proteins were detected and the related protein abundances in vitrified and control oocytes were quantified. None of the spots was significantly altered in intensity, and hierarchical cluster analysis as well as histograms of p and q values suggest that vitrification at the pre-antral stage does not significantly alter the proteome of GV or MII oocytes compared with controls.

Conclusions: Vitrification appears to be associated with a significant transient increase in $\Delta$Ψm in oocyte mitochondria, which disappears when oocyte/cumulus cell apposition is restored upon development to the antral stage. The nano-LC-MS/MS analysis of low numbers of oocytes is useful to obtain information on relevant biological signaling pathways based on protein identifications. For quantitative comparisons, saturation 2D DIGE analysis is superior to LC-MS/MS due to its high sensitivity in cases where the biological material is very limited. Genetic background, age of the female, and/or stimulation protocol appear to influence the proteome pattern. However, the quantitative 2D DIGE approach provides evidence that vitrification does not affect the oocyte proteome after recovery from transient loss of cell–cell interactions, in vitro growth and in vitro maturation under tested conditions. Therefore, transient changes in mitochondrial activity by vitrification do not appear causal to persistent alterations in the mitochondrial or overall oocyte proteome.

Key words: oocyte quality / mitochondria / follicle development / gene expression


Introduction

Cryopreservation of female germ cells is an option to preserve reproduction capacity in aggressive cancer treatment that threatens the health of somatic and germ cells in the ovary (Jadoul et al., 2010; Noyes et al., 2011; Smitz et al., 2010). By retransplantation of cryopreserved ovarian material, followed by natural conception, or by in vitro fertilization of cryopreserved oocytes and subsequent embryo transfer, female fertility could be successfully restored (reviewed by Donnez et al., 2011). Recent studies analyzing preservation at ultrastructural, functional and developmental level indicates that vitrification is a fast and safe protocol for cryopreservation of ovarian materials (Isachenko et al., 2009; Smith et al., 2010; Ubaldi et al., 2010; Cobo and Diaz, 2011; Martinez-Burgos et al., 2011; Sheikh et al., 2011; Trokoudes et al., 2011). Nevertheless, several studies showed different subcellular alterations or disturbances in the intrinsic cellular homeostasis of oocytes after vitrification or slow rate freezing compared with untreated controls, which may contribute to chromosomal misalignment at metaphase II (MII), modification in reactive oxygen species (ROS) dependent redox status and gene expression, or in a higher level of DNA fragmentation (Larman et al., 2007; Huang et al., 2008; Coticchio et al., 2009; Tatone et al., 2010). Overall mRNA abundance may become decreased by vitrification in human oocytes (Chamayou et al., 2011). The exposure to high concentration of potentially genotoxic cryoprotective agents (CPA) like ethylene glycol, dimethyl sulfoxide or propylene glycol during vitrification might induce alterations in proteins and cellular integrity (Fahy, 2008; Smitz et al., 2009; Tatone et al., 2010a), which could affect gene expression in the oocyte itself or in the somatic cells of the follicle, ultimately affecting the oocyte proteome, health and developmental potential irreversibly (Aye et al., 2010; Smitz et al., 2010).

To avoid hyperstimulation and exposures to potentially harmful hormones in some cancer patients, and prevent reintroduction of malignant cells, cryopreservation of immature follicles, and in vivo or in vitro growth and maturation using two- or three-dimensional culture systems to obtain MI oocytes avoids risks by malignant cell contamination and can also become an attractive approach to optimize yield of fertilizable oocytes for fertility preservation (Xu et al., 2009; Smitz et al., 2010). We recently showed that vitrification of pre-antral follicles from the ovary of prepubertal mice by the CryoTop method (Kuwayama et al., 2005) resulted in high survival rates, although it significantly delayed folliculogenesis in the subsequent 12 days of culture compared with fresh controls, possibly as a result of a transient loss of contacts between oocyte and granulosa cells by transzonal projections after vitrification (Trapphoff et al., 2010a). Although the in vitro growth pattern of oocytes was similar between control and vitrified groups, and in vitro ovulated MI oocytes from culture of vitrified pre-antral follicles possessed a normal spindle, well-aligned chromosomes and euploid constitution (Trapphoff et al., 2010a), it cannot be excluded that vitrification induces osmotic stress, and damage to mitochondria (Isachenko et al., 2003; Nottola et al., 2009; Hashimoto et al., 2010; Turuthum et al., 2010; Zhou et al., 2010). The transient loss of transzonal projections might influence homeostasis and gene expression in the oocyte within the follicle irreversibly.

Studies in mouse follicles and oocytes suggest that granulosa cell derived nitric oxide suppresses high mitochondrial membrane potential in subcortical mitochondria of oocytes during growth and maturation and thereby may protect from oxidative stress. The transient loss of communication between the oocyte and the surrounding granulosa cells after vitrification (Trapphoff et al., 2010a) could disturb processes that control inner mitochondrial membrane potential of subcortical mitochondria (Van Blerkom et al., 2008), gene expression and/or paracrine signaling by growth factors (e.g. Hutt and Albertini, 2007; Gilchrist, 2008; Su et al., 2009) or by follicular fluid activating substance (Grändahl, 2008) and could thereby induce persistent changes in expression patterns and the proteome of the oocyte.

In the first part of the study, we isolated oocytes from control and CryoTop vitrified pre-antral follicles and assessed mitochondrial inner membrane potential (ΔΨm) in subcortical and perinuclear cytoplasm of mitochondria right on Day 0 (vitrification and release from cryoprotectant), at Day 1 when many oocytes in vitrified follicles have transiently lost their transzonal projections, and after 12 days of culture when full apposition between oocyte and granulosa cells is restored (Trapphoff et al., 2010a), in order to obtain information on transient or persistent influences of vitrification on mitochondrial distribution and activity pattern.

In the second part of the study we initially isolated in vivo grown germinal vesicle (GV) oocytes from large antral follicles of unprimed young adult mice at the late diestrus stage for a qualitative analysis by a nano-LC-MS/MS approach to find out whether mitochondrial proteins are detectable from a limited number of oocytes, and which developmentally relevant proteins can be identified from 988 GV oocytes of this mouse strain. In total, 1123 proteins were identified with a high confidence level [false discovery rate (FDR) < 1%] by our approach. Comparisons to data from a recent study using basically the same methodology (Wang et al., 2010) revealed reproducibility of the approach but also identified some interesting differences between the proteome of oocytes from unstimulated and stimulated cycles.

In the last part of the study, we quantitatively assessed the influence of vitrification on the oocyte proteome of in vitro grown GV as well as MI oocytes. Due to the limited sample amounts available from in vivo grown oocytes, the ultrasensitive 2D saturation DIGE technique was used, which had been previously shown to facilitate spot quantification in 2D gels containing only 500 ng total protein (Berendt et al., 2009). More than 1700 spots were quantified from each gel of GV and MI stage oocytes. The comprehensive quantitative characterization of differences in protein abundance in this sensitive approach indicates that no significant quantitative changes in these proteins are induced by vitrification at the pre-antral stage, confirming safety of CryoTop vitrification within limits of resolution and experimental conditions.

Materials and Methods

Animals and oocyte isolation and culture

CS7/B6j X CBA/Ca female F1 hybrids were housed in a temperature controlled environment with a 12 h light/dark cycle and fed with R/M pellets (Ssniff, Soest, Germany) and water ad libitum. Experiments were done with the permission of the University of Bielefeld authorities and in accordance with German law.

Ovaries were isolated from 2 to 4 months old mice at late diestrus of the natural cycle (as determined by vaginal smears) and were transferred to isolation media (37°C) (M2 medium; Sigma, Munich, Germany, supplemented with 10 mg/ml bovine serum albumin, PAA, Coelbe, Germany).

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Fully grown GV oocytes were obtained from large antral follicles by puncture with hypodermic needles (Terumo, Eschborn, Germany) and removal of cumulus cells by gentle pipetting. Pools of 60–100 denuded GV oocytes were briefly washed four times in phosphate-buffered saline (PBS; Oxoid, Wesel, Germany) at 37°C and collected into vials within a minimal volume of PBS (<1 μl). Samples from 12 mice with a total of 988 GV oocytes were frozen and stored at −80°C.

**Isolation of pre-antral follicles, in follicle culture, and vitro growth and maturation of oocytes**

Pre-antral follicles (diameter 110–130 μm; 3b follicles; Pedersen and Peters, 1968) were isolated from ovaries of mice aged 12 to 14 days of age (C57/Bl6 X CBA/Ca mice) and cultured individually in 96-well microtiter plates (Coming, Amsterdam, Netherlands) in an atmosphere of 5% CO2 at 37°C and saturated air humidity (Cortvriendt and Smitz, 2002; Van Wemmel et al., 2005; Trapphoff et al., 2010a) in 50 μl culture medium (α-MEM glutamax, Invitrogen) supplemented with 5% fetal calf serum, 5 mg/ml insulin and transferrin, 5 ng/ml sodium selenite (Sigma-Aldrich, Munich, Germany) containing 10 μl/ml recombinant follicle stimulating hormone (GnRH) covered with 30 μl mineral oil (Invitrogen), as described previously (Trapphoff et al., 2010a). For further details, see Supplementary Materials 1. Survival rates and follicle development were analyzed (Trapphoff et al., 2010a). Oocytes were harvested from vitrified and control follicles at GV stage or MII at Day 12 or 13, respectively, for quantitative proteome analysis. For studies on mitochondrial inner membrane potential GV oocytes were isolated from follicles after isolation with and without vitrification (Day 0), or after 1 day or 12 days of culture.

**CryoTop vitrification of pre-antral follicles**

CryoTop vitrification was performed with slight modifications as described for human oocytes by Kuwayama et al. (2005). More detailed information can be found in Supplementary Materials 1. Before transfer to 96-wells and start of culture, follicles were washed twice in Leibovitz medium for 5 min at 37°C.

Rates of follicle survival, development to the antral stage and maturation to MII (first polar body extrusion) were calculated for control and vitrified group as previously described (Trapphoff et al., 2010a).

**Analysis of relative abundance of mitochondria with higher or lower inner membrane potential in control and vitrified group**

GV oocytes were freed from granulosa cells by gentle digestion with 2 mg/ml collagenase type IV (Sigma, Munich, Germany) in Leibovitz L-15 media containing 1 μM colistamide for 20 min at 37°C followed by pipetting and release of the oocyte. Day 12 GV oocytes were freed from cumulus cells without collagenase by pipetting with thin glass capillaries. Consecutive staining of DNA by Hoechst 33342 (0.5 μg/ml; Sigma) and JC-1 (1.5 μM; Molecular Probes, Gottingen, Germany) was done in Leibovitz L-15 media supplemented with 1 μM colistamide at 37°C each followed by immediate live imaging by confocal microscopy (Van Blerkom et al., 2002). Details of quantitative analysis are found in Supplementary Materials 1. The quantitative fluorescence ratio was determined with LCS software for three different areas: subcortical area (in outer 10% surface area of the oocyte; Fig. 1A), perinucleus region (area within the inner subcortical zone) and overall ratio for the whole cross-section area (Fig. 1A).

**Lysis of GV oocytes for 1D SDS gel electrophoresis**

Lysis was carried out by adding 30 μl Lämmli buffer to 988 oocytes, 5 min 95°C heating (Thermomixer 5436, Eppendorf, Hamburg, Germany) and 5 min sonication (Sonorex RK 100, Bandelin, Berlin, Germany). The procedure of heating and sonication was repeated three times. After 5 min centrifugation at 14,000 g (GS 15R, Beckman, Krefeld, Germany) the supernatant was stored at −20°C.

**1D polyacrylamide SDS gel electrophoresis**

SDS-electrophoresis (overall gel size 7 cm (l) × 8.5 cm (W) × 0.75 mm) was performed as detailed in Supplementary Materials 1.

**Gel slicing and tryptic in-gel digestion**

The gel lane was cut into 28 slices (Fig. 1A) using a scalpel and submitted to tryptic in-gel digestion (details in Supplementary Materials 1). Prior to LC-MS/MS analysis the peptides were solved in 40 μl 0.1% formic acid.

**LC MS/MS analysis**

The 1D-nano-LC was performed on a multi-dimensional-nano-liquid chromatography system (Ettan MDSLC, GE Healthcare, Freiburg, Germany). For further details see Supplementary Materials 1. Mass spectrometry was performed on an LTQ Orbitrap XL instrument (Thermo Scientific) on-line coupled to the nano-LC system. For electrospray ionization a distal coated SilicaTip (FS-360-20-10-D-20, New Objective, Woburn, USA) and a needle voltage of 1.4 kV was used. The MS method consisted of cycles of one full MS scan (Mass range: 300–2000 m/z) and five data dependent MS/MS scans (35% collision energy). The dynamic exclusion was set to 180 s.

**Database search and data analysis**

The MS/MS data were searched with Mascot V 2.1.03 (Matrix Science, Boston, USA) using the following parameters: (i) Enzyme: Trypsin, (ii) Fixed Modification: Carbamidomethyl (C), (iii) Variable modifications: Oxidation (M); (iv) Peptide Tol. 25 ppm, (v) MS/MS TOL. 0.8 Da, (vi) Peptide charge 1+, 2+ and 3+, (vii) Instrument ESI-TRAP and (viii) Allow up to 1 missed cleavages. Details of statistical evaluation are in Supplementary Materials 1.

**Gene ontology analyzes**

Gene Ontology (GO) Analyses was performed by the GO tool provided by the Uniprot community (http://www.uniprot.org/). It clusters proteins according to terms provided by the GO project (Ashburner et al., 2000).

**Comparison of identified proteins with the dataset of Wang et al.**

The comparison of our list of gene names with the list provided by Wang et al. was performed using the Access software (Microsoft).

**Lysis and labeling of oocytes for 2D saturation DIGE analysis**

Lysis of fully grown GV stage and MII mouse oocytes from pre-antral follicle culture (vitrified and controls; culture period 12 or 13 days, respectively) was carried out as described (Berendt et al., 2009) and detailed in Supplementary Materials 1. The internal pooled standard (IPS) was prepared from 5 μg of in vivo grown GV oocytes from large antral follicles and labeled with Cy3 saturation dye using corresponding TCEP and Cye.
Dye concentrations as for the in vitro grown oocytes. Labeled samples were stored at \(220^\circ\text{C}\).

### 2D gel electrophoresis

Prior to isoelectric focusing (IEF), 0.25 \(\mu\)g of the Cy5-labeled sample was mixed with 0.25 \(\mu\)g Cy3-labeled IPS. For IEF Immobiline™ DryStrips pH 4-7, 24 cm (GE Healthcare Freiburg; Germany) were rehydrated overnight in 450 \(\mu\)l rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v Pharmalyte pH 3–10, 13 mM DTE) using a reswelling tray (GE Healthcare). Dry strips were overlaid with DryStip™ Cover Fluid (GE Healthcare) to prevent evaporation. IEF was performed using an Ettan IEPphor (GE Healthcare). Samples were applied to the first dimension gel using anodic cup loading. Focusing was performed for a total of 38.25 kVh. Prior to SDS–PAGE, IEF strips were equilibrated for 10 min in 15 ml equilibration buffer (100 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.5% w/v DTE) containing 200 \(\mu\)l saturated bromophenol blue solution. SDS–PAGE was performed using an Ettan Dalt 6 system (GE Healthcare). IPG strips were loaded onto the second dimension gels (0.375 M Tris–HCl pH 8.8, 12.5% acrylamide/bisacrylamide, 0.1% w/v SDS, 0.1% w/v APS, 0.014% v/v TEMED) and overlaid with 0.5% w/v agarose in SDS running buffer (25 mM Tris, 192 mM glycin, 0.2% w/v SDS). Gels were run in groups of six with 10 mA per gel for 1 h and 40 mA per gel for 6 h in 1 \(\times\) SDS running buffer for the anodic chamber and 2 \(\times\) SDS running buffer for the cathodic chamber.

### Imaging and quantitative analysis of 2D DIGE

Gels were scanned using a Typhoon 9400 fluorescence scanner (GE Healthcare) with a photomultiplier voltage of 670 V. Remaining parameters were set according to the manufacturer’s protocol. The images were analyzed using DeCyder™ Differential Analysis Software version 6.5 (GE Healthcare). The spot detection parameter ‘estimated number of spots’ was set to 10 000 and spots with a volume lower than 30 000 were excluded.
Statistical analysis
Student’s t-test was used for follicle developmental scores. Follicle survival, progression to MII and arbitrary units [a.u.] of ratios of red to green fluorescence intensities were compared between control and vitrified groups using the χ²-test with Yates correction. P < 0.01 was considered significant.

Protein quantification experiments were evaluated by the DeCyder™ 6.5 software (Student’s t-test). The p and q value histograms were generated by the R software (open source).

Results
Part one: Analysis of $\Psi_{\text{mit}}$ in GV stage oocytes of control and vitrified group
J-C-1 staining showed that mitochondria are enriched in the peripheral ooplasm, close to the oolemma (blue arrows, Fig. 1A) and in the perinuclear region of control and vitrified GV stage oocytes (yellow arrows, Fig. 1A).

Comparing the relative ratio of red to green fluorescence in optical sections in line scans through the center of J-C-1 stained oocytes by confocal microscope showed that the relative ratio of mitochondria with high versus low $\Psi_{\text{mit}}$ is similar in the subcortical domain between the control and the vitrified group right after vitrification and thawing at Day 0 (Fig. 1B, left panel, lightest blue and green columns) while there is a small but significant difference between the two groups in the overall and perinuclear ratio ($P < 0.001$; n = 123 and 138 for control and vitrified group, respectively).

In contrast, the average ratio of mitochondria with high to low $\Psi_{\text{mit}}$ becomes significantly increased compared with Day 0 in both experimental groups ($P < 0.001$; Fig. 1B left and middle panels) at Day 1 of culture. There were also significant differences in the relative proportion of mitochondria with high or low $\Psi_{\text{mit}}$ between oocytes from the control (n = 123) and the vitrified groups (n = 89) at Day 1 (Fig. 1A, B, middle green and blue columns; $P < 0.001$). The overall ratio of mitochondria with high $\Psi_{\text{mit}}$ is increased about three times in the vitrified group from Day 0 to Day 1, and was significantly higher than the control in the subcortical ($P < 0.001$; blue arrow) as well as the perinuclear domain. While the relative ratio/arbitrary units is similar in all mitochondrial domains of the control oocytes, it differs from each other in the vitrified group, with a significantly higher ratio in the subcortical region compared with the perinuclear domain ($P < 0.01$).

In GV oocytes that grew in vitro during 12 days of pre-antral follicle culture up to the antral stage (n = 114), the overall ratio of red to green fluorescence is similar to the Day 1 ratio and slightly but significantly lower in the perinuclear/cytoplasmic domain of the control compared with Day 1 ($P < 0.001$). It is significantly increased in the subcortical and cytoplasmic domain of the control Day 12 compared with the Day 1 oocytes ($P < 0.001$). The ratio drops significantly from Day 1 to Day 12 in all domains in the vitrified group (n = 82) (Fig. 1B, right green columns, $P < 0.001$). As a consequence, at Day 12 mitochondrial $\Psi_{\text{mit}}$ is not significantly different between control and vitrified groups in all domains. Thus, vitrification significantly affects mitochondrial activity with respect to mitochondrial inner membrane potential when apposition between oocyte and granulosa cells has been lost at Day 0 and especially at Day 1, but there is a recovery during culture and with the restoration of the transzonal projections the $\Psi_{\text{mit}}$ also recovers to a state that is comparable to control after 12 days of culture.

Part two: Identification of proteins in GV stage mouse oocytes by LC-MS/MS
In order to obtain more information on the proteome of maturation and developmental competent GV-stage mouse oocytes, and to assess sensitivity of proteome analysis by LC-MS/MS with respect to oocyte numbers, we collected large numbers of GV stage oocytes from antral follicles of unprimed C57/B16 x CBA/CA female mice at late diestrous of the natural estrous cycle.

To reduce sample complexity prior to qualitative mass spectrometry, the protein lysate of 988 mouse oocytes, corresponding to 25 μg total protein, was prefractionated on a 12% SDS polyacrylamide gel (Fig. 2A). The Coomassie stained gel was sliced into 28 pieces (Fig. 2A), each of which was individually subjected to in-gel trypsinization followed by nano LC-MS/MS analysis using an Orbitrap XL mass spectrometer (Fig. 2B shows examples of a Base Peak Ion chromatogram and MS/MS spectra).

A total of 250 664 MS/MS spectra were acquired and subjected to Mascot data processing. Mascot data were statistically analyzed using the Scaffold software tool. Applying a FDR of <1% on the protein level and a minimum number of two different peptides per protein, 23 983 spectra could be assigned to 6660 different peptides corresponding to 1123 different proteins (Supplementary data, Table S1). The decoy database search led to only 1 hit, demonstrating an FDR far below 1%.

The entire set of proteins was subjected to GO analyses. Eight hundred and eleven proteins were assigned to the ‘biological process’ subset (Fig. 2C, left panel). Within this subset 479 proteins could be assigned to the cluster ‘metabolic processes’, 302 to the cluster ‘biological regulation’, 96 to ‘signaling’, 40 to ‘reproduction’ and 36 proteins to the cluster ‘death’. A total of 155 identified proteins could be matched to the cluster ‘developmental processes’ (Fig. 2C, right panel). Within ‘developmental processes’ 121 and 53 proteins, respectively, could be assigned to the subclusters ‘anatomical structure development’ and ‘anatomical structure morphogenesis’.

Eight proteins are associated with chromosome segregation, five of which are listed in Table I.

GO analysis using the same categories as described by Yurttas et al. (2010) (Fig. 2D) provides evidence that the relative numbers of proteins in ‘metabolic process’ is higher in the GV stage when compared with MII (35% in our sample versus 24% in the MII oocytes analyzed by Yurttas et al., 2010, respectively) while the relative proportion of proteins assigned to ‘mitochondrion’ was 3.8-fold higher in our sample compared with the analysis of MII oocytes (15 versus 4%; Yurttas et al., 2010). This may be a consequence of an increased overall number of protein identifications in our more sensitive approach (1123 versus 150 proteins identified by Yurttas) yielding to an enhanced fraction of proteins with medium abundance in our dataset. If vitrification causes significant abundance differences in these mitochondrial proteins, these should at least in part be captured by our 2D DIGE analysis, addressing >1700 spots. Our study confirmed that a large number of maternal effect proteins and P-body proteins or proteins of RNA metabolism (Table II) that are involved in processes at maturation and after fertilization are already present.
at the GV stage. We also could demonstrate that several proteins recently identified in the MII stage of mouse oocytes and suggested to be involved in reprogramming (Pfeiffer et al., 2011) are already present at the GV stage (Table II, second part). These comprise proteins involved in histone modification like histone acetyl transferase type B (Hat1; Ejlassi-Lassallette et al., 2011) and proteins involved in remodeling chromatin conformation and in DNA repair/recombination like a protein correlated with reprogramming in bovine oocytes (Tpt1; Yarm et al., 2002) as well as the ATP-dependent DNA helicases (Ruvbl1, Ruvbl2; Izumi et al., 2010; for further references, see Table II).

Comparison of our dataset with a much larger dataset obtained from 7000 mouse GV oocytes from a different mouse strain using a similar prefractionation and LC-MS/MS method (Wang et al., 2010) demonstrated the reproducibility of the protein identification by LC-MS/MS. The comparison of the proteome from the GV stage oocytes from adult females in our set with that of stimulated cycles containing prepubertal females showed that the large majority (966 of the 1123 proteins in our sample) could be confirmed to be present in mouse GV stage oocytes of both strains and ages. Of the 157 proteins in our dataset that were not contained in the GV oocyte dataset from Wang et al. (2010), 38 proteins were contained in the dataset derived from MII oocytes or zygotes by the Wang group, and accordingly appear to be expressed in our sample before resumption of maturation or in higher abundance as those in

Figure 2 LC-MS/MS proteome and GO analysis. (A) 1D 12% SDS gel for prefractionation of proteins from 988 GV mouse oocytes. Left lane: Sigma Wide Range Marker. Right lane: protein lysate of 988 mouse GV oocytes. Brackets indicate the 28 fractions subjected to LC-MS/MS analysis. (B) Examples of LC-MS/MS from gel slice 21. Upper panel: Base Peak Ion Chromatogram; X-axis: time, Y-axis: relative signal intensity. Lower Panel: example of MS/MS spectrum; X-axis: m/z values, Y-axis: relative signal intensity. (C) Left panel: Uniprot GO analysis of all identified proteins using the ‘biological processes’ subset of the GO database from the GO project, last updated on 9 December 2008. Right panel: Uniprot GO subcluster analysis of 155 proteins of the cluster ‘developmental process’ of the GO database from the GO project, last updated on 9 December 2008. (D) Uniprot GO analysis of all identified proteins using categories as published by Yurttas et al. (2010).
Table I  Some proteins of GV oocytes identified by GO analysis in the category: ‘Chromosome segregation’.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Serine/threonine-protein phosphatase 2A regulatory subunit A alpha (PP2A subunit A isofrom PR-alpha)</td>
<td>Shugoshin Sgo2 expressed in oocytes is responsible for the centromeric localization of PP2A and the protection against phosphorylation of cohesin Rec8, which prevents precocious chromatid segregation in meiosis I; inhibition of PP2A is required for mitotic entry in Xenopus egg extracts</td>
</tr>
<tr>
<td>ADP-ribosylation factor-like protein 8B (ADP-ribosylation factor-like protein C; hArl8b)</td>
<td>Protein enriched in growth cone of neuronal cells; diet can alter expression of the mRNA in brain and affect neurite formation; human hArl8b’s N-terminal acetylation is involved in p53 regulation and cell viability; novel small G protein indispensable for equal chromosome segregation I in yeast</td>
</tr>
<tr>
<td>Spindle and kinetochore-associated protein 2 (SKA2/Protein FAM33A)</td>
<td>Spindle and kinetochore complex required for normal onset of anaphase in mitosis and likely promoting stable spindle microtubule attachment of chromosomes</td>
</tr>
<tr>
<td>Smc-4 (Structural maintenance of chromosome protein 4)</td>
<td>Component of the condensin I and II complexes; condensin complexes mediate chromosome condensation at M-phase, are essential for chromosome segregation and involved in gene regulation, DNA repair and recombination</td>
</tr>
<tr>
<td>Budding uninhibited by benzimidazole 3-homolog (Bub3)</td>
<td>Spindle assembly protein in oocytes essential for normal chromosome separation; among transcripts significantly different in level of between in vivo derived (IVD) and in vitro produced (IVP) bovine embryos in late 8-cell stage; recruited by poly (ADP-ribose) polymerase (Parp-1) to centromeres in mouse oocytes and implicated in MII arrest</td>
</tr>
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*Proteins identified uniquely in GV stage by present proteome analysis and not found in dataset by Wang et al. (2010).

*Proteins identified in MII or embryo but not GV oocytes by proteome analysis by Wang et al. (2010).

the Wang study. Another 119 proteins have not been identified from any developmental stage analyzed by Wang et al. (2010) (Supplementary data, Table SII). The proteins identified only from mouse GV oocytes by our approach contain a fairly high proportion (7%) of cell cycle associated proteins, in comparison, only 1% of all proteins were belonging to this GO category in the data from GV oocytes by Wang et al. (2010). Several of the proteins are involved in checkpoint control (e.g. the protein SKA2/FAM33A as spindle and kinetochore-associated protein; Table I) and in the status of chromosome configuration/condensation (e.g. protein of the condensin complex of proteins, SMC4; references in Table I). Interestingly, SKA-2 was recently shown to contain an intron with micro-RNA regulating its own expression via the ERG/CPEB pathway (Cao et al., 2010), suggesting that there might be differences in this regulatory loop between the mouse strains or in dependence of age and stimulation protocol. Proteins identified in our dataset by LC-MS/MS analysis in the GV stage and later in the MII stage in the Wang dataset also include a protein recognized initially in budding yeast and presumably involved in spindle checkpoint control, budding uninhibited by benzimidazole 3 homolog (BUB3) (Li et al., 2009). Other regulatory factors like Aurora kinase A, B or C and mitotic centromere associated kinesin were not identified, presumably due to their low abundance. However, the growth factor GDF9 which when affected by vitrification may severely interfere with normal oocyte development and acquisition of competence was recognized in the sample (Erickson and Shimasaki, 2003; Yeo et al., 2009; Sugjura et al., 2010; Wei et al., 2011). These data clearly revealed that a number of proteins required for maturation, support of early embryogenesis, and chromatin reprogramming after fertilization are already present in the fully grown GV stage oocyte. However, especially in mouse with comparatively small oocytes (70 nm diameter), thousands of oocytes are obviously necessary to obtain a more detailed information on a larger fraction of the oocyte proteome. For the detection of quantitative changes between the proteome of control and vitrified groups of in vitro grown oocytes, we therefore employed a 2D DIGE saturation approach. Though this approach does not permit spot identification from analytical gels, it requires only around 10 mouse oocytes per 2D gel for a reliable quantification.

Part three: Effect of vitrification on the quantitative proteome profile of GV oocytes
In the control and vitrified groups, 1142 and 801 pre-antral follicles were isolated, respectively, for the differential quantitative analysis of mouse oocyte proteomes by saturation DIGE analysis. Survival of follicles from Day 0 to Day 4 was 96.4 and 84.5% for control and vitrified group, significantly less in the vitrified compared with the control group (χ²-test, P < 0.001). Follicle development was slightly but significantly delayed in the vitrified group compared with the control (average score 3.65 versus 3.28 at Day 8 and 4.61 versus 4.48 at
Day 12, for control and vitrified follicles, respectively; t-test, \( P < 0.001 \) at Day 8 and 12 of culture), comparable to previous observations (Trapphoff et al., 2010a). From a total of 426 and 213 follicles grown to the antral stage and subsequently stimulated by rEGF and rhCG at Day 12 and in vitro ovulated at Day 13, 323 (75.8%) and 153 (71.8%) oocytes of the control and vitrified group emitted a first polar body, not significantly different from each other, and similar to a recent study (Trapphoff et al., 2010a).

Three independent biological replicates—each consisting of 41–70 oocytes—of in vitro grown GV and MII oocytes from vitrified and control pre-antral follicles cultured for 12 or 13 days, respectively, were lysed under conditions compatible with 2D DIGE saturation

<table>
<thead>
<tr>
<th>Table II</th>
<th>Identified MEG proteins (maternal effect proteins), P-body proteins and proteins in reprogramming that are detected by LC-MS/MS at GV stage, and the effects of knockout or the potential function in oocytes/embryos or other cell types.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knock-out phenotype and/or activity in gene expression or reprogramming</td>
<td>Reference</td>
</tr>
<tr>
<td>MEG and P-body like associated proteins</td>
<td></td>
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<tr>
<td>Dnmt1</td>
<td>Maintains genomic imprints in embryos; deficiency causes early embryo death</td>
</tr>
<tr>
<td>Oep (FLOPED)</td>
<td>Component of cytoplasmic lattices; deficiency causes embryo arrest at 2–4-cell stage</td>
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<tr>
<td>Nlrp5 (MATER)</td>
<td>Present in mitochondria and nucleus; component of cytoplasmic lattices; deficiency causes embryo arrest at 2-cell stage</td>
</tr>
<tr>
<td>Nlrp14</td>
<td>Present in human oocyte and embryo; specific function unknown</td>
</tr>
<tr>
<td>Nucleoplasmin 2/NPM2</td>
<td>Component of nucleolus-like bodies in fully grown GV oocytes; involved in sperm chromatin decondensation; deficiency causes embryo arrest primarily at 1-cell stage</td>
</tr>
<tr>
<td>Padi6</td>
<td>Ribosomal storage; component of cytoplasmic lattices; influences organelle distribution and cytoplasmic microtubules in oocytes; deficiency causes arrest at 2-cell stage</td>
</tr>
<tr>
<td>Dpapa5a</td>
<td>Regulated by Oct4 transcription factor; Reduction in fertility</td>
</tr>
<tr>
<td>Zygote arrest 1/Zar 1</td>
<td>Together with ZAR1-like associated with P-body components in 2-cell stage and possibly involved in RNA processing; deficiency causes embryo arrest at one cell stage</td>
</tr>
<tr>
<td>Ddx6</td>
<td>DEAD-box protein RNA helicase; subcortical association in fully grown oocytes; probable ATP-dependent RNA helicase involved in RNA processing and P-body-like component of mature oocytes</td>
</tr>
<tr>
<td>Hat1</td>
<td>Histone acetyl transferase type B in oocyte; implied in regulation of gene expression and possibly influenced by oocyte ageing and culture conditions prior to zygotic gene activation; proper pattern of acetylation on the H4 tail domain in association with Hat1 appears required for nuclear import and chromatin assembly at S-phase</td>
</tr>
<tr>
<td>Ruvbl1 and Ruvbl2</td>
<td>RuvB-like 1 and 2; ATP-dependent DNA helicases oligomerizing associated with regulation of transcription, RNA modification, DNA repair, and telomere maintenance, and possibly involved in protein complex formation and RNA decay</td>
</tr>
<tr>
<td>Tpt1</td>
<td>Activates pluripotency gene Oct4; protein TCPT (translationally controlled tumor protein) identified in proteome screen as degraded by proteasome at meiotic exit; correlated with reprogramming in bovine oocytes; phosphorylated by polo-like kinase 1 and involved in cytoskeleton and spindle dynamics at anaphase</td>
</tr>
</tbody>
</table>
labeling. Aliquots corresponding to 40 oocytes (1.0 μg total protein) were labeled with Cy5, and an internal standard for all gels was prepared from 200 in vivo grown GV oocytes (5 μg total protein) and labeled with Cy3. From each of the three replicates of GV and MII oocytes, two gels were analyzed, each containing one sample (250 ng total protein) and an aliquot of the internal standard (250 ng). In Fig. 3A and B representative 2D-Gel images of Cy5 labeled GV (Fig. 3A) and MII (Fig. 3B) oocytes are shown, demonstrating the high quality of the gels and the reproducibility of the spot patterns. Gels were matched by the algorithm of DeCyder 6.5, leading to a minimum of 1891 matched spots from GV oocytes and 1718 matched spots from MII oocytes. For quantitative evaluation, a student’s t-test was applied in DeCyder, and the software’s FDR correction feature was applied.

None of the 1891 spots met the criteria set for differential abundance (FDR correction feature applied, P-value < 0.05); among all quantified spots the lowest P-value was 0.49 in the GV oocytes dataset and 0.23 in the MII oocytes dataset. To further check if P-value distribution is biased, e.g. by any experimental or biological effect, histograms of the student’s t-test’s P-values (Supplementary data, Fig. S1) and of corresponding q-values (Supplementary data, Fig. S2) were generated. The P-values histograms showed an almost equal distribution, demonstrating that even an increase of replicates would not lead to the detection of statistically significant abundance alterations between vitrified and non-vitrified samples. In accordance to this finding, the histograms of q-values demonstrate that no significant differences between the spot pattern exists (Supplementary data, Fig. S2). Finally, hierarchical clustering of the spot patterns (Fig. 3C) showed a complete random clustering in the GV and MII stages, again demonstrating that no significant reproducible differences between the spot patterns of vitrified and non-vitrified oocytes can be detected in the proteome represented by the 1891 (GV oocytes) or 1718 (MII oocytes) spots.

Discussion

Mitochondria have diverse activities in the regulation of cellular homeostasis, including ATP production, control of calcium homeostasis, and signaling pathways involved in cell proliferation and apoptosis. In the context of oocyte vitrification, understanding the changes in mitochondrial function and protein expression is crucial for optimizing the success rates of assisted reproduction techniques. The study presented in this paper provides valuable insights into the proteomic changes associated with the vitrification process, which can be leveraged for improving the quality of embryos and the overall success rates of treatments. Further research is needed to elucidate the specific protein targets and pathways affected by vitrification, which could pave the way for developing more effective and efficient protocols for oocyte cryopreservation.

Figure 3  Quantitative differential proteome analysis by saturation 2D DIGE. (A) Images of representative 2D gels (pH 4–7) from Cy5 labeled GV oocytes. Left panel: unvitrified oocytes (control); right panel: vitrified oocytes. (B) Images of representative 2D gels (pH 4–7) from Cy5 labeled MII oocytes. Left panel: unvitrified oocytes (control); right panel: vitrified oocytes. (C) Zoomed view of heat maps from hierarchical cluster analysis of protein spots in GV (upper panel) and MII oocytes (lower panel) with a color gradient for spot intensity ranks (heat map interval −0.5 green to +0.5 red). Columns represent individual samples, rows represent individual spots. Column superscripts indicate sample origin: V, vitrified; C, control. Numbers in column superscripts represent individual biological replicates.
homeostasis, lipid metabolism, apoptosis and redox regulation (Dumollard et al., 2009; Ramalho-Santos et al., 2009; Eichenlaub-Ritter et al., 2010a; Van Blerkom, 2010). Oocytes do not express glycolytic enzymes and rely on activities of granulosa cells to supply them with metabolites such as lactate and pyruvate for the tricarboxylic acid cycle and ATP production by mitochondria (Su et al., 2009; Sutton-McDowell et al., 2010). Mitochondrial oxidative metabolism of pyruvate in the oocyte appears essential at maturation after gap junctional communication ceases since the knockdown of pdh1 (pyruvate dehydrogenase complex) causes meiotic and developmental arrest and spindle aberrations, and interferes with chromosome segregation in the mouse oocyte but does not interfere with oocyte growth when oocytes are still tightly connected to cumulus (Johnson et al., 2007). In vivo, the functional junctional communication appears to contribute to the activity of mitochondria and levels of ROS generation within the oocyte at a low level during growth and meiotic arrest, possibly to minimize risks by oxidative damage. Moreover, there is evidence that nitric oxide produced by granulosa cells is involved in modulating mitochondrial activity/inner membrane potential ($\Delta\Psi^{\text{mit}}$). Thus, the intramitochondrial membrane potential is kept low in subcortical mitochondria of mouse oocytes resting at the GV stage within the intact follicle (Van Blerkom et al., 2008). However, mitochondria in the subcortical domain of the human and mouse oocyte become activated and exhibit increased $\Delta\Psi^{\text{mit}}$ upon cumulus expansion and loss of gap junctional communication at in vivo ovulation, or after mechanical removal of cumulus cells from the zona at the GV stage (Van Blerkom et al., 2008). Similarly, the present study shows that transient loss of contact between the immature GV oocyte and the granulosa cells in the vitrified pre-antral follicle results in significantly increased $\Delta\Psi^{\text{mit}}$, especially at Day 1 after vitrification and in the subcortical domain, concomitant with the loss of tight oocyte–granulosa cell apposition. According to expectation, this implies that the oocyte transiently activates mitochondria after partial or total loss of gap junctional communication and transzonal projections. Since only few oocytes and follicles do not survive vitrification (Trapphoff et al., 2010a), the transient loss of contacts and mitochondrial activation does not lead to cell death or induction of apoptosis, possibly because the oocyte can still obtain metabolites like lactate from cumulus via transporters, and the transiently increased mitochondrial membrane potential/metabolism is tolerated until the rapid restoration of close oocyte/granulosa cell apposition and signaling (Trapphoff et al., 2010a). Differences in mitochondrial activities and redox regulation upon cryopreservation have also been shown by other studies in oocytes or ovarian tissues. For instance, vitrification of human MII oocytes caused a drop in ATP levels, presumably by effects of vitrification on mitochondrial activity, which increased again during recovery after warming (Manipalviratn et al., 2011). About 50% of human vitrified MII oocytes contained a typical, small and slender mitochondria-smooth endoplasmic reticulum aggregate after vitrification by cryoleaf or cryoloop (Nottola et al., 2009). The distribution and mitochondrial membrane potential were altered by vitrification at the 2-pronuclear stage in mouse (Zhao et al., 2009). Exposure of mouse zygotes to vitrification solution per se as well as vitrification procedure including cooling/warming resulted in down-regulation of Bax, Bcl2 and p53 mRNA in blastocysts (Dhali et al., 2007), whereas slow freezing but not vitrification caused profound alterations in the proteome in an initial report using mass spectroscopy for proteome analysis (Larman et al., 2007). Therefore, it was of interest to study the mitochondrial inner membrane potential as one indicator of mitochondrial functionality and the immediate and persistent effects of vitrification with the CryoTop method at the pre-antral stage in the present study. Mitochondria and ultrastructure of oocytes were well preserved in human ovarian slices rapidly vitrified and warmed in cryotubes and then cultured for 24 h (Sheikhi et al., 2011). This is similar to what can be seen after restoration of oocyte/granulosa cell apposition and oocyte growth in vitro to the antral stage in the present study. The vitrification per se did not result in a dramatic immediate change in mitochondrial inner membrane potential at Day 0, suggesting that exposure to cryoprotectant had only a mild influence on mitochondrial integrity and function under the chosen conditions with the CryoTop vitrification protocol. In contrast, the transient loss of transzonal projections profoundly influenced mitochondrial activity at Day 1 in a reversible fashion. Mitochondria were not irreversibly damaged by vitrification as oocyte growth and acquisition of maturational competence was not visibly changed and mitochondrial inner membrane potential was no more significantly different between Day 12 control and vitrified GV oocytes.

Proteasomal degradation of mitochondrial proteins is vital for mitochondrial homeostasis, and transient or permanent increases in ROS may negatively impact such processes, e.g. there are correlations between decreased proteasomal degradation, ROS and increased oxidative stress that are postulated to contribute to senescence (Torres and Perez, 2008). Therefore, it was of interest to study the proteome of oocytes grown from vitrified pre-antral follicles to assess possible irreversible influences of vitrification on gene expression in general, and particularly on the mitochondrial proteome. Mitochondria constitute a major cytoplasmic component of oocytes such that changes in abundance of proteins by vitrification are expected to be visible by proteome analysis.

The qualitative nano-LC-MS/MS approach to study the oocyte proteome led to the identification of 1123 proteins from 988 mouse GV oocytes. For Uniprot GO analysis, the same categories as described by Yurttas et al. were used. Corresponding to the high content of mitochondria in GV oocytes, a considerable proportion of proteins could be assigned to the subcluster ‘mitochondrial’, much higher compared with oocytes in the MII stage as reported by Yurttas et al. (2010). This is consistent with selective degradation of transcripts in oxidative phosphorylation and energy production during maturation (Su et al., 2007), and reduction in abundance of mitochondrial gene transcripts from the GV to the MII stage in the bovine oocyte (8.4% down-regulated at transcript level; Mamo et al., 2011). Nevertheless, it cannot be excluded that our enhanced fraction of proteins in the subcluster ‘mitochondrial’ is simply a consequence of the increased sensitivity of our 1D gel-LC-MS/MS approach (1123 identified proteins versus 150 in Yurttas et al., 2010), facilitating the identification of more proteins expressed at only intermediate levels such as mitochondrial proteins. Overall, this method to assess protein homeostasis appears effective to perform systems biology analysis of protein expression in oocyte development, quality and maturation, although—under high confidence conditions—the number of proteins/peptides addressable is still limited.

One of the first published approaches using LC-MS/MS analysis for mouse oocytes identified 625 proteins from 2700 mature mouse oocytes (Zhang et al., 2009). A recent study of the mouse oocyte...
proteome (Wang et al., 2010) used essentially the same methodology as reported here, but subjected the proteins obtained from 7000 GV-stage mouse oocytes to the analysis and identified 2781 proteins. Compared with the study by Zhang, Wang identified roughly 4.5 times more proteins from three times more oocytes. This might be due to a slightly higher sensitivity of the Wang approach, however, the biological material differed in several aspects: oocytes were obtained from a different strain of mice and from younger adult females (aged 4–12 weeks compared with 8–16 weeks in the present study) in stimulated cycles, 48 h after pregnant mare serum, whereas exclusively adult females (8–16 weeks) and spontaneous cycles were used by the study of Zhang et al. (2009).

Compared with the study presented here, Wang et al. (2010) identified roughly 2.5-fold more proteins (2781 versus 1123 protein IDs) from roughly seven times more mouse oocytes (7000 versus 988). This supports the notion that increases in numbers of oocytes and protein in a sample do not necessarily cause a proportional increase in identified protein/peptides (Arnold and Frohlich, 2011). Nevertheless, improved instrumentation will further enhance the proteomic outcome from a given amount of a sample. This is emphasized by a recent study of a mouse oocyte proteome which identified, with latest MS instrumentation (Orbitrap Velos, Thermo), 3699 proteins from 1884 MII oocytes from primed young B6C3F1 (C57Bl/6) × C3H/HeN) female mice, the largest number so far in this species (Pfeiffer et al., 2011).

While transcriptome analysis using amplification and chip technologies has considerably gained insights into gene expression and signaling pathways in oocytes and the somatic compartment of the follicle, it has its pitfalls and shortages (Su et al., 2007; Salisbury et al., 2009; Potireddy et al., 2010; Seli et al., 2010), mainly because the preparation of RNA greatly influences outcomes (Scantland et al., 2011). Transcription profiling in oocytes to assess dynamics of gene expression in maturing oocytes is also limited by the fact that oocytes become transcriptionally quiescent at the end of the growth phase before resumption of maturation. At this point, cellular homeostasis is mainly controlled by sequential spatiotemporal recruitment as well as by degradation or storage of mRNA, some of which have long half-lives (Bachvarova et al., 1980; Brower et al., 1981; De Leon et al., 1983). Messenger RNA processing causes presence of a variety of isoforms (Salisbury et al., 2009). Differential translation is regulated by presence on polysomes and regulation by conserved sequences in the 5' untranslated region of mRNAs. Repression or expression can be regulated by various binding proteins and small noncoding RNAs. In addition, the cell cycle and stage-specific post translational modification, activity and degradation of proteins influence the transcriptome and actual gene expression patterns on an additional level (for discussion, see Seli et al., 2010). To attain information on the status of the oocyte proteome it is therefore indispensable to analyze the proteins and their isoforms to examine the dynamic processes before and after maturation (Arnold and Frohlich, 2011). Current LC-MS/MS analyzes have substantially improved the knowledge of the proteome and allow the comparison of protein to mRNA expression levels and the dissection of the relative contribution of mRNAs and proteins to oocyte maturation and embryogenesis (Pfeiffer et al., 2011). However, it remains challenging to compare proteome data generated by different experimental groups qualitatively and quantitatively in repeat experiments with mouse oocytes to obtain in depth information on minor changes in abundance of any individual protein or protein isoform according to stage, treatment or environment since each group and experiment still requires isolation of hundreds of oocytes for replicate experiments. For instance, Powell et al. (2010) used kit technology, in which proteins are labeled by conjugation to the thiol group of the cysteine residues or to Lys-residues through an adapter linker that converts the ε-amino group of lysine into a thiol group to assess the relative abundance of labeled proteins after LC-MS/MS of high- and low-quality bovine oocytes. One hundred bovine oocytes corresponding to roughly 9 µg proteins were required to obtain meaningful information on major differences between the two groups. This corresponds roughly to the protein amount of about 400–500 mouse oocytes for any experimental group. This suggests that a replicate experiment would require isolation of at least 1000–1500 mouse oocytes from vitrified and control group each to identify major differences in the proteome.

Instead of following this approach, the data of the LC-MS/MS analysis in this study were used to compare our dataset with that of Wang et al. (2010), revealing that the large majority of the 1123 proteins in our sample, 923 proteins, could be confirmed to be present in mouse GV stage oocytes showing the high reliability and reproducibility of the method. Of the 157 proteins in our dataset that were not contained in the GV oocyte dataset from Wang and co-workers, 38 proteins were also contained in the dataset derived from MII oocytes or zygotes by Wang et al. (2010). The latter may therefore differ slightly in relative abundance in the two studies, e.g. as a result of differences in the genetic background, or due to differences in female age, the follicular status and hormonal stimulation between the GV oocytes collected for analysis. In the past, we have shown that the mouse oocytes from unstimulated females obtained by late diestrus of the natural cycle from large antral follicles are fully grown and of high quality. Depending on culture conditions, most have apparently in vivo acquired a large quantity of proteins that are needed for maturation to MII and faithful chromosome segregation (Hu et al., 2001; Cukurcan et al., 2003). This is supported by the present observations in which many maternal proteins that are essential and contribute to cell cycle regulation, chromosome separation and embryogenesis and proteins presumably involved in reprogramming are already present in the GV oocytes. Stimulation of folliculogenesis in young mice as used by Wang et al. (2010) might recruit follicles of slightly different quality and permit development to the antral stage and growth of oocytes that might have suboptimal quantities of proteins. Further studies have to confirm this in view of increasing evidence that hyperstimulation may affect the quality of oocytes in the mouse (Edgar et al., 1987; Van Blerkom and Davis, 2001; Combelles and Albertini, 2003; Wang et al., 2006). In contrast to the 38 proteins which are present in dataset of both studies but at different stages, another 119 proteins have not been identified from any developmental stage analyzed by Wang et al. (2010).

Within the proteins only identified from the C57Bl7 × CBA/Ca females, GV oocytes by our approach an overproportional representation of proteins in the subcluster ‘cell cycle’ (7%) is revealed (compared with 4% in the entire dataset and 1% in the study by Wang et al., 2010). Moreover, three out of eight proteins clustering into ‘chromosome segregation’ are also not contained in the Wang dataset. Considering these results and the possible permissive cell cycle control in aged CBA/Ca mice (reviewed by Vogt et al., 2008;
Eichenlaub-Ritter et al., 2010b; Nagaoka et al., 2011) as well as the prediction of precarious loss of molecules in chromosome cohesion in aged oocytes (Hunt and Hassold, 2010; Jessberger, 2010) makes it interesting to assess the abundance of such uniquely identified oocyte proteins in the proteome of GV oocytes of aged females as this appears now feasible and might provide information on age-related changes in predisposition to chromosome non-disjunction.

However, it has to be kept in mind that the analysis so far still covers only a fraction of the thousands of proteins expected to be present in oocytes according to transcriptome data. In view of the immense expenditure to collect thousands of oocytes for prefractionation and LC-MS/MS, it appeared reasonable to use an initial 2D DIGE saturation labeling approach instead of a nano LC-MS/MS based one to assess the long-lasting influence of vitrification on mouse oocytes in the present study, which requires much smaller sample amounts and lower numbers of oocytes (Berendt et al., 2009), although it is not possible to assess the identity of proteins in spots without a master gel of several thousand oocytes for spot analysis by mass spectrometry. The observations reveal that highly reproducible spot patterns from proteins and protein isoforms can be obtained by this method from a low number of only 10 oocytes per 2D gel. Moreover, posttranslational modifications (e.g. phosphorylation) represent a potential target of alteration by the vitrification process, and 2D gel-based approaches facilitate the separation and quantification of protein isoforms differing in their isoelectric point. Our 2D DIGE analysis using the dedicated DeCyder software package provided clear evidence for equal expression levels of all 1700 addressed proteins in vitrified and non-vitrified samples from both GV oocytes and MII oocytes. This result was further supported by a hierarchical cluster analysis and histograms of p- and q-values (Supplementary data, Figs S1 and S2).

Moreover, in the large number of 1891 (GV oocytes) or 1718 (MII oocytes) spots that could be reproducibly recognized and quantified, one can expect the representation of at least a part of high and medium abundance proteins like mitochondrial proteins or components of the proteome with essential functions in maturation or early development (we have demonstrated the containment of those in our LC-MS/MS dataset of 1123 proteins). For instance, the RNA-binding factor YBX2 (Y box protein 2, also termed MSY2) represents 2% of all proteins in mouse oocytes and is a critical factor in mRNA storage, stability and turnover (Yu et al., 2001; Medvedev et al., 2008, 2011). YBX2 knockout interferes with transcriptional silencing in the growing GV oocyte, and induces gross alterations in expression patterns, spindle aberrations and arrest of embryogenesis (Yu et al., 2004; Medvedev et al., 2011). This protein was identified in the GV oocyte proteome in our study, that from Wang et al. (2010) and in the MII oocyte proteome (Pfeiffer et al., 2011). Since we did not detect any differences in the 1891 spots of separated proteins in 2D DIGE, this represents evidence that vitrification does not have a significant influence on the presence or abundance of this important factor in the control of gene expression at maturation and early development.

The depth of any currently available proteome analysis is still very limited, and therefore it remains open whether low abundance factors, e.g. transcription factors or regulatory enzymes that are synthesized, transiently activated by posttranslational modifications or degraded rapidly at any stage of oogenesis, become affected by vitrification at the pre-antral stage. It should also be mentioned that protein complexes are inevitably destroyed by the chaotropic reagents used in a proteomic analysis and therefore, no knowledge can be gained from our experiments about alterations or functional impairments of protein complexes by the vitrification procedure. However, the observations in the current study provide strong evidence that there are no major persistent changes in the proteome and only transient changes in mitochondrial activity by exposure of follicles to high concentrations of cryoprotectant.

This is in agreement with a previous study failing to detect long-lasting adverse effects of vitrification on growth, maturation, spindle, chromosome segregation and methylation imprinting in in vitro grown and matured mouse oocytes (Trapphoff et al., 2010a). However, further studies are required to assess sensitivity of oocytes to vitrification at different stages and safety of the technique, particularly since there are indications that vitrification at the GV stage followed by in vitro maturation does not alter genomic imprint patterns (Al-Khtib et al., 2011) but the methylation of promoter regions of developmental genes (Milroy et al., 2011). Whether this impacts gene expression and the proteome of the oocyte and embryo warrants further studies. Wang et al. (2011) noted successful growth and maturation of mouse oocytes but reduced development to MII and to the blastocyst after IVF of oocytes derived from pre-antral follicle culture isolated from vitrified compared with fresh ovarian slices. Preliminary analysis of development to blastocyst of in vitro fertilized oocytes from control and vitrified pre-antral follicles using the current protocol (Trapphoff et al., 2010b) did not detect significant differences in blastocyst formation between the groups. This emphasizes that size of the sample, possibly cooling rate and influences from the surrounding tissue can impact follicle and oocyte quality and protocols used in vitrification have to be optimized individually to achieve optimal results. The present report hopefully contributes to present perspectives for most effective ways of proteome analysis of oocyte/embryo quality to assess safety of vitrification and cryopreservation and improve treatment in human fertility preservation.

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

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Authors’ roles
All authors substantially contributed to conception and design of the study, and interpretation of results, and approved the final draft of the manuscript. M.D. and T.T. were involved in data collection (proteome analysis and oocyte/follicle culture and mitochondrial analysis, respectively) and manuscript writing; T.F. contributed substantially to data mining and critical discussion of the manuscript; G.A. and U.E. contributed to manuscript drafting, critical discussion and coordination of the study.


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