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Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes

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BACKGROUND: Cryopreservation is now considered as an efficient way to store human oocytes to preserve fertility. However, little is known about the effects of this technology on oocyte gene expression. The aim of this study was to examine the effect of the two cryopreservation procedures, slow freezing and vitrification, on the gene expression profile of human metaphase II (MII) oocytes.

METHODS: Unfertilized MII oocytes following ICSI failure were cryopreserved either by slow freezing or by the Cryotip method for vitrification. After thawing, total RNA was extracted and analyzed using Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays. The gene expression profiles and associated biological pathways in slowly frozen/thawed and vitrified MII oocytes were determined and compared with those of non-cryopreserved MII oocytes used as controls.

RESULTS: Both cryopreservation procedures negatively affected the gene expression profile of human MII oocytes in comparison with controls. However, slowly frozen and vitrified MI oocytes displayed specific gene expression signatures. Slow freezing was associated with down-regulation of genes involved in chromosomal structure maintenance (*KIF2C* and *KIF3A*) and cell cycle regulation (*CHEK2* and *CDKN1B*) that may lead to a reduction in the oocyte developmental competence. In vitrified oocytes, many genes of the ubiquitination pathway were down-regulated, including members of the ubiquitin-specific peptidase family and subunits of the 26S proteasome. Such inhibition of the degradation machinery might stabilize the maternal protein content that is necessary for oocyte developmental competence.

CONCLUSIONS: The low pregnancy rates commonly observed when using human MII oocytes after slow freezing–thawing may be explained by the alterations of the oocyte gene expression profile.

Key words: human MII oocyte / cryopreservation / slow freezing / vitrification / microarray

Introduction

From an ethical and clinical point of view, there is an urgent need to efficiently store human oocytes in order to preserve fertility. Two cryopreservation methods are currently available: slow freezing and vitrification (Gardner *et al.*, 2007). Experimental data suggest that vitrification affects oocyte physiology to a lower degree than slow freezing (Kuleshova and Lopata, 2002; Kuwayama *et al.*, 2005; Valojerdi and Salehnia, 2005; Chamayou *et al.*, 2006; Borini *et al.*, 2008; Cobo *et al.*, 2008; Loutradi *et al.*, 2008; Cao and Chian, 2009; Dessolle *et al.*, 2009; Fasano *et al.*, 2010; Gualtieri *et al.*, 2011). Specifically, survival and fertilization as well as embryo cleavage and implantation rates were higher when vitrified rather than slowly frozen human mature oocytes were used, leading to 1.8 births and 1.2 births per 100 thawed oocytes, respectively (Dessolle *et al.*, 2009). Although the implantation potential and resulting pregnancy rates are indicators of the

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Table I Studies investigating the impact of cryopreservation procedures on the gene expression profile of oocytes.

Studies	Species	Samples	Approaches	Number of targeted genes	Cryopreservation procedures	Outcome
Liu *et al.* (2003)	Mouse	Pre-antral follicles isolated from fresh and frozen-thawed ovarian tissue followed by IVM	Microarray	588	SF	Weakly affected
Succu *et al.* (2008)	Ovine	*In vitro* matured (MII) oocytes	Quantitative real-time RT–PCR	8	V	Affected
Isachenko *et al.* (2009)	Human	Ovarian tissue cryopreservation followed by *in vitro* culture	Quantitative real-time PCR and RT–PCR	1	SF, V	Affected by vitrification
Di Pietro *et al.* (2010)	Human	MII oocytes after IVF cycle	real-time RT–PCR	8	V	Not affected
Turathum *et al.* (2010)	Canine	Cryopreserved immature oocytes (GV) followed by IVM	RT–PCR	5	V	Weakly affected
Habibi *et al.* (2010)	Mouse	*In vitro* matured (MII) oocytes	Nested quantitative PCR	3	V	Affected
Anchamparuthy *et al.* (2010)	Bovine	Primary follicles (GV) in the ovarian tissue followed by IVM and cryopreserved MII oocytes	Quantitative real-time RT–PCR	4	V	Affected
Lee *et al.* (2008)	Mouse	Germinal vesicles collected after ovarian tissue cryopreservation	Quantitative real-time RT–PCR	2	SF	Affected
Chamayou *et al.* (2011)	Human	MII oocytes after IVF cycle	RT–PCR	18	SF, V	Affected
Current study	Human	MII oocytes after IVF cycle	Microarray	27 585	SF, V	Affected

IVM, *in vitro* maturation; SF, slow freezing; V, vitrification.

efficacy and safety of the used cryopreservation protocol (Cobo and Diaz, 2011), these data provide limited information on the impact of these methods on oocyte physiology. Moreover, although the first child conceived using cryopreserved MII oocytes was born more than 25 years ago (Chen, 1986), only around 1000 children have been born after oocyte cryopreservation (Yang *et al.*, 2007; Chian *et al.*, 2008; Wennerholm *et al.*, 2009), thus limiting the available data.

A few studies have provided molecular evidence on the impact of cryopreservation on the oocyte gene expression profile (Liu *et al.*, 2003; Lee *et al.*, 2008; Succu *et al.*, 2008; Isachenko *et al.*, 2009; Anchamparuthy *et al.*, 2010; Di Pietro *et al.*, 2010; Habibi *et al.*, 2010; Turathum *et al.*, 2010; Chamayou *et al.*, 2011). Most of these works concerned mainly other mammalian species and reported that, in metaphase II (MII) oocytes, cryopreservation mainly affected the expression of genes related to oxidative stress [members of the heat shock protein family, superoxide dismutase 1), apoptosis (members of the BCL2 family, death receptors) and cell cycle (cyclin B, members of the histone family, polymerases; Liu *et al.*, 2003; Lee *et al.*, 2008; Succu *et al.*, 2008; Anchamparuthy *et al.*, 2010; Habibi *et al.*, 2010; Turathum *et al.*, 2010]. The conclusions of these studies are somewhat contradictory (Table I), although proteomic analyses of mouse MII oocytes confirmed the significant negative impact of slow freezing on oocyte physiology compared with vitrification (Larman *et al.*, 2007; Katz-Jaffe *et al.*, 2008).

Therefore, in order to describe in detail the effect of slow freezing and vitrification on the abundance of specific transcripts in oocytes, we compared the gene expression profiles of cryopreserved and non-cryopreserved human unfertilized MII oocytes by using a DNA microarray approach.

Materials and Methods

Patients' characteristics

The study population included 48 normal responder patients (aged <36 years), referred for ICSI due to male infertility, with one or two previous failed cycles. Patients did not have gynecological disorders, such as polycystic ovary syndrome (PCOS). Unfertilized MII oocytes were donated after written informed consent. The research project was approved by the ethics committee of our institution.

Metaphase II oocyte retrieval and oocyte culture

After controlled ovarian stimulation, cumulus–oocyte complexes (COCs) were collected by vaginal puncture under ultrasound echo-guidance 36 h after administration of 5000 IU human chorionic gonadotrophin. COCs were immediately placed in G-MOPS^{TM} PLUS (Vitrolife Fertility Products) at 37°C for few minutes and then transferred to GIVF PLUS culture medium (Vitrolife Fertility Products). Oocytes were denuded of cumulus cells by enzymatic treatment with 80 UI/ml hyaluronidase solution (SynVitro^{R}Hyadase, MediCult) to assess nuclear maturity. Mature MII oocytes were used for ICSI and then individually cultured in micro-drops of fertilization medium under paraffin mineral oil (Vitrolife Fertility Products). Fertilization was evaluated 18–20 h after ICSI and unfertilized MII oocytes were collected 24, 48 and 72 h post-microinjection for the present study. The proportion of oocytes collected at 24, 48 and 72 h was 75, 20 and 5%, respectively, and was similar between the study groups. Degenerated, unfertilized MII oocytes were excluded. Unfertilized MII oocytes from a given patient were either directly placed in RLT RNA extraction buffer (RNeasy Micro Kit, Qiagen) (control group: non-cryopreserved MII oocytes), or cryopreserved. Three pools of

non-cryopreserved MII oocytes were prepared [a total of 54 oocytes from 11 patients in pools of 15 ± 3.2 oocytes), placed in RLT RNA extraction buffer and frozen at −80°C until use.

Slow oocyte freezing and thawing

A modified version of the slow freezing protocol by Fabbri *et al.* (1998) was used. Briefly, MII oocytes were incubated in two successive cryoprotectant solutions (1,2-propanediol and sucrose) to obtain a progressive and complete dehydration. The base medium for all freezing solutions was Cryo-PBS (Freeze-Kit I™, Vitrolife). Oocytes were first incubated in Cryo-PBS containing 1.5 M 1,2-propanediol for 10 min, followed by Cryo-PBS with 1.5 M 1,2-propanediol and 0.1 M sucrose. Individual oocytes were then placed in plastic straws and transferred into an automated freezing machine (Cryopreservation Minicool 40PC, Air liquide) at 23°C. The temperature was progressively reduced to −8°C at a rate of −2°C/min and seeding was induced manually in proximity of liquid nitrogen. Straws were cooled to −30°C at a rate of −0.3°C/min and then to −150°C at a rate of −50°C/min. Straws were then transferred into a liquid nitrogen tank for long-term storage.

For thawing, straws were first warmed at room temperature for few seconds and then immersed in the following cryoprotectants solutions to rehydrate oocytes (Thaw-Kit I™, Vitrolife): 1.0 M 1,2-propanediol + 0.2 M sucrose (5 min), 0.5 M 1,2-propanediol + 0.2 M sucrose (5 min) and 0.2 M sucrose (10 min). The survival rate of unfertilized oocytes was 50%.

Three pools of surviving MII oocytes were prepared (a total of 59 oocytes from 16 patients in pools of 18 ± 1.4 oocytes), placed in RLT RNA extraction buffer (RNeasy Micro Kit, Qiagen) and frozen at −80°C until use.

Oocyte vitrification

Oocytes were vitrified using the following: medium 199 (M199)-based solutions (Vitrification Freeze Kit, Irvine Scientific): 7.5% dimethyl sulfoxide (DMSO) + 7.5% ethylene glycol (EG) + 20% dextran serum substitute (DSS; equilibration solution) and 15% DMSO + 15% EG + 20% DSS + 0.5 M sucrose (vitrification solution).

Oocytes were first placed in the equilibration solution at room temperature for 7–8 min and then in the vitrification solution for 30 s. Individual oocytes were then placed in cryotips and transferred to liquid nitrogen tanks for long-term storage.

Cryotips were first warmed by immersion in a 37°C water bath for 3 s and then placed in the M199-based thawing solution (1 M sucrose + 20% DSS). Cryoprotectants were progressively removed using the following M199-based dilution and washing solutions: 0.5 M sucrose + 20% DSS (dilution solution) and 20% DSS (washing solution). The survival rate of unfertilized oocytes was >90%.

Four pools of surviving MII oocytes were prepared (a total of 86 oocytes from 21 patients in pools of 21.5 ± 4.6 oocytes), placed in RLT RNA extraction buffer (RNeasy Micro Kit, Qiagen) and frozen at −80°C until use.

Complementary RNA preparation and microarray hybridization

RNA was extracted from the MII oocyte pools using the micro RNeasy Micro Kit (Qiagen). The amount and integrity of the total RNA samples were assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Complementary RNA (cRNA) was prepared by two rounds of amplification according to the manufacturer's 'double amplification' protocol (two-Cycle cDNA Synthesis Kit, Invitrogen) starting from total RNA. Labeled fragmented cRNA (12 μg) was hybridized to oligonucleotide probes on Affymetrix HG-U133 Plus 2.0 arrays. Each pool of MII oocytes was put on a microarray chip.

Data processing

Scanned GeneChip images were processed using the AGCC (Affymetrix GeneChip Command Console) software. Microarray data were analyzed using the Affymetrix Expression Console software and normalization was performed with the RMA (Robust Multiarray Averaging) algorithm to obtain an intensity value signal for each probe set.

Microarray data analysis

To compare the gene expression profiles of the three groups (non-cryopreserved, slowly frozen/thawed and vitrified MII oocytes), we performed an unsupervised classification with hierarchical clustering using a coefficient of variation (CV) ≥100% between samples (de Hoon *et al.*, 2004).

To identify genes that were differentially expressed in non-cryopreserved and slowly frozen/thawed MII oocytes and in non-cryopreserved and vitrified MII oocytes, we first performed a selection based on a fold ratio >5 or <0.2 of the mean signal intensity between groups. Then, the significance analysis of microarrays (SAM, Stanford University, USA, Tusher *et al.*, 2001) technique was used. SAM provides the mean or median fold change (FC) values and a false discovery rate (FDR) confidence percentage based on data permutation. Selected genes (FC >2 and FDR <5%) were then analyzed using Ingenuity (http://www.ingenuity.com) to identify the specific biological pathways/functions.

Quantitative RT–PCR analyses

Amplified RNA (0.5 μg), used for DNA microarray chips, from non-cryopreserved (one pool containing 17 oocytes), slowly frozen/thawed (two pools with 17 and 21 oocytes, respectively) and vitrified (two pools with 15 and 21 oocytes, respectively) MII oocyte pools was used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) according to the manufacturer's recommendations (Applied Biosytems). For qPCR, 2 μl (of a 1:4 dilution) first-strand DNA were added to a 10 μl reaction mixture containing 2.5 μM of each primer and 5 μl of 2× LightCycler 480 SYBR Green I Master mix (Roche). DNA was amplified over 50 cycles with the annealing temperature at 63°C using the Light Cycler 480 detection system (Roche) and values were normalized to *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) expression for each sample using the following formula: $E_{\text{testedprimer}}^{\Delta ct} / E_{\text{HPRT}}^{\Delta ct}$ ($E = 10^{-1/\text{slope}}$), $\Delta Ct = Ct_{\text{control}} - Ct_{\text{unknown}}$, where E corresponds to the efficiency of the PCR reaction. The E value is obtained by a standard curve that varies in function with the primers used. The non-cryopreserved MII oocyte sample was used as control. Each sample was analyzed in duplicate and multiple water blanks were included.

Results

Gene expression profiles of non-cryopreserved and cryopreserved human MII oocytes

To perform unsupervised clustering, a first selection of the raw microarray data was carried out based on the CV (≥100%) of the signal intensity of all samples and 158 probe sets were selected. Unsupervised clustering (Fig. 1) showed that the four pools of vitrified MII oocytes branched around the three pools of non-cryopreserved MII oocytes.

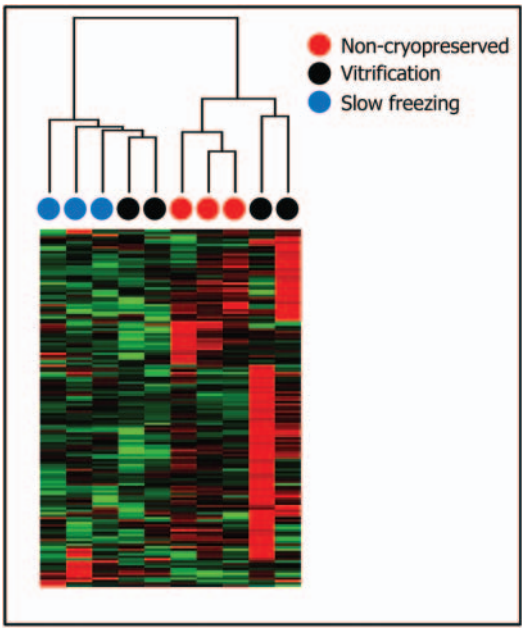

Figure 1 Unsupervised hierarchical clustering of non-cryopreserved, vitrified and slowly frozen human MII oocytes. Red, up-regulated; green, down-regulated.

A

	Number of genes	
	Up	Down
Non-cryopreserved *versus* slow freezing	7	381
Non-cryopreserved *versus* vitrified	99	509

B

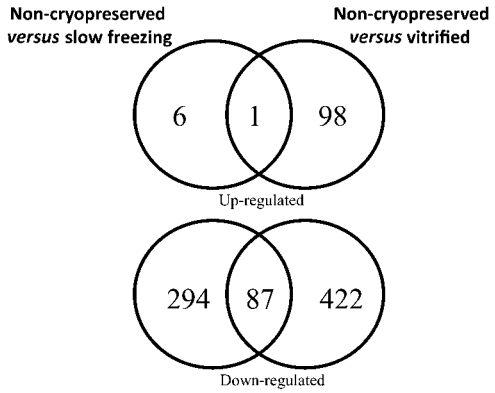

Figure 2 (**A**) Number of genes significantly modulated between non-cryopreserved MII oocytes and slowly frozen or vitrified MII oocytes. (**B**) The venn diagram of transcripts up- or down-regulated between the slowly frozen or vitrified MII oocyte group in comparison with the non-cryopreserved control group.

SAM analyses of the gene expression profiles identified 388 genes (381 down- and 7 up-regulated) that were differentially expressed in the non-cryopreserved and slowly frozen MII oocyte pools and 608 genes (509 down- and 99 up-regulated) that were differentially expressed in the non-cryopreserved and vitrified MII oocyte pools (Fig. 2A). Most of these differentially expressed genes were down-regulated in cryopreserved MI oocytes in comparison with controls (non-cryopreserved MII oocytes).

An intersection of the lists of genes that were down-regulated in frozen and vitrified MII oocytes indicated that 87 of these genes were shared (Fig. 2B) and supervised clustering of these 87 genes confirmed these results (Fig. 3B). Figure 3A shows the 10 genes that were most down-regulated in both cryopreserved groups. Genes that were down-regulated in both vitrified and slow frozen MII oocytes belonged mainly to the 'nitric oxide signaling' pathway (*CAV1*, *PDGFC* and *SLC7A1*), the 'ovarian cancer signaling' pathway (*GJA1* and *LEF1*), the 'role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis' (*F2RL1*, *IL6ST* and *PLCL1*) and the 'clathrin-mediated endocytosis signaling' cascade (*ITGB1*, *PIK3C2A*, *PP3CA* and *TFRC*).

Quantitative RT–PCR was used to validate the down-regulation of *SLC38A2*, *TXNRD1* and *GJA1* in both vitrified and slow frozen MII oocytes (Fig. 3C).

Differential gene expression in slow frozen and vitrified MII oocytes

Besides the small common molecular signature (87 genes), slowly frozen and vitrified MII oocytes displayed specific and exclusive gene expression signatures that were characterized by a very high percentage of down-regulated genes (98% in the slow frozen and 81% in the vitrified MII oocyte pools) in comparison with non-cryopreserved MII oocytes. More specifically, among the 381 down-regulated genes of the slow frozen/thawed group, 294 were exclusive to this cryopreservation procedure. Among the 509 down-regulated genes specific to the vitrified group, 422 were exclusive to the vitrification procedure (Fig. 2). The 10 most down-regulated genes in each group are reported in Table II.

Gene ontology analyses have been performed with the lists of down-regulated genes exclusive to each cryopreservation procedure.

In the vitrified MII oocyte group, the top two canonical pathways amongst the 422 down-regulated genes were the protein ubiquitination pathway [*DNAJB4* (−5.1), *DNAJB14* (−6.7), *DNAJC15* (−5.1), *PSMD7* (−5.4), *PSMD14* (−6.1), *SMURF2* (−18.9), *UBC* (−6.3), *UBE2B* (−6.7), *USO1* (−5.1), *USP2* (−8.2), *USP10* (−5.1), *USP37* (−5.9), *USP44* (−13.4) and *USP9X* (−10.7), *P*-value = 1.08E−03] (Fig.) and growth hormone signaling pathways [*IGF1R* (−8.4), *IRS1* (−7.3), *JAK2* (−6.2), *PIK3CA* (−6.5), *PIK3R1* (−7.7), *PRKCA* (−7.6) and *RPS6KA5* (−5.8), *P*-value = 1.21E−03]. The three other top canonical pathways affected by down-regulation in vitrified MII oocytes are included in the Supplementary data, Table S1. Moreover, the most affected molecular and cellular function was the cell cycle which included mRNA polyadenylation genes specifically down-regulated in the vitrified MII oocyte pools (Table III).

In the slowly frozen MII oocyte group, the top two canonical pathways amongst the 294 down-regulated genes were those involved in the role of BRCA1 in DNA damage responses [*ATF1* (−5.6), *BARD1* (−5.0), *CHEK2* (−5.7), *FANCL* (−8.1) and *GADD45A*, *P*-value = 2.63E−03] and the aldosterone signaling pathway [*SGK1* (−6.1), *HSPD1* (−5.9), *HSPA14* (−5.3), *DUSP1* (−6.4), *DNAJC9*

A

Symbol	Name	Non-cryopreserved *versus* slow freezing Fold change	Non-cryopreserved *versus* vitrified Fold change
SLC38A2	Solute carrier family 38, member 2	−26.62	−11.96
TXNRD1	Thioredoxin reductase 1	−21.19	−7.67
HIPK2	Homeodomain interacting protein kinase 2	−17.22	−6.26
GJA1	Gap junction protein, alpha 1	−16.83	−20.71
SLC38A1	Solute carrier family 38, member 1	−16.58	−12.23
IL6ST	interleukin 6 signal transducer	−15.78	−9.59
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	−15.23	−5.18
NBN	Nibrin	−15.05	−5.48
CAV1	Caveolin 1	−15.03	−5.16
CRIM1	Cysteine rich transmembrane BMP regulator 1	−8.18	−21.44

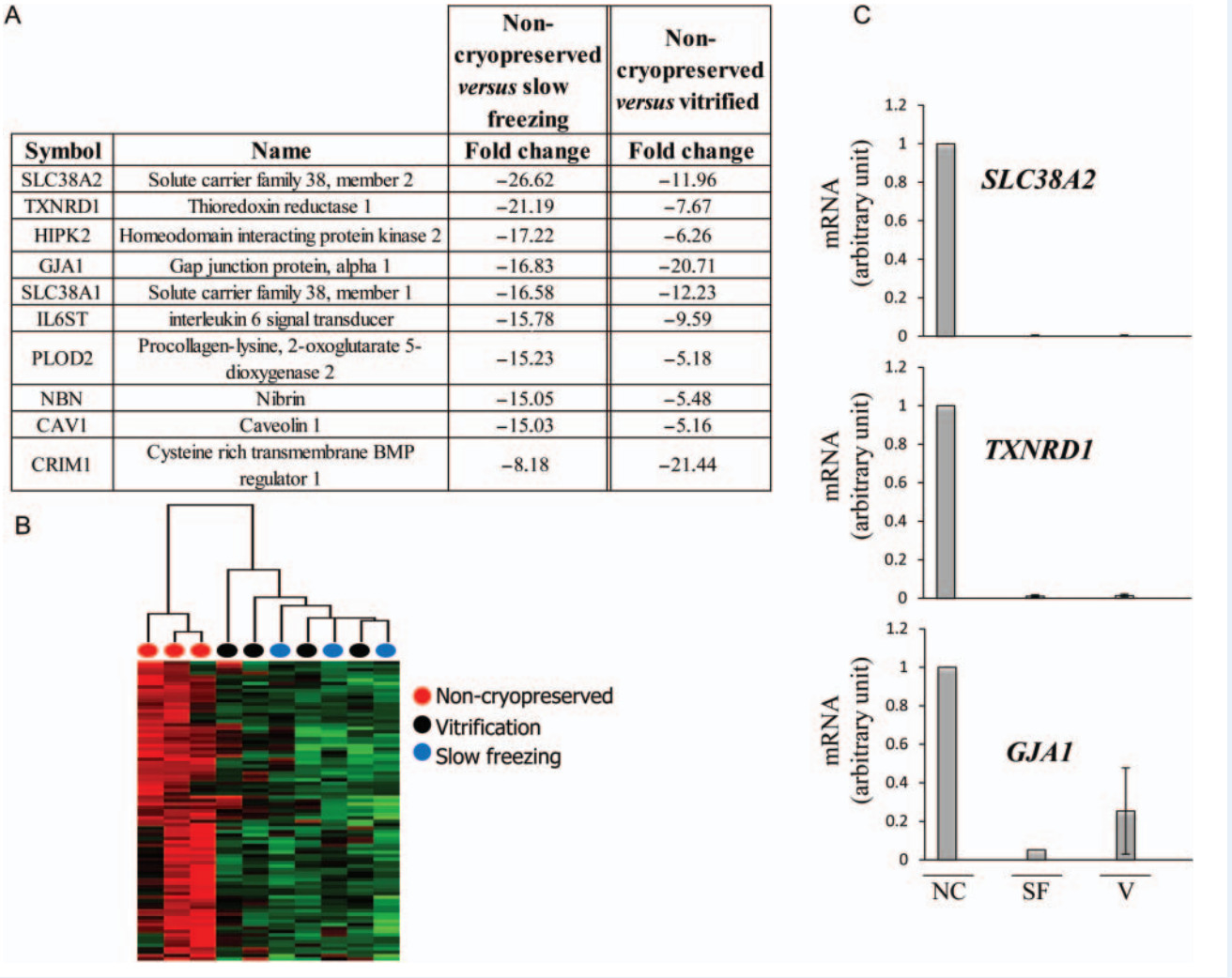

Figure 3 Supervised hierarchical clustering of non-cryopreserved, vitrified and slowly frozen human MII oocytes. (**A**) The 10 most down-regulated genes in both the cryopreserved MII oocyte groups compared with fresh oocytes. (**B**) Supervised cluster analysis using the 87 genes that were down-regulated in both the vitrified and slowly frozen human MII oocytes in comparison with the non-cryopreserved MII oocytes. (**C**) Validation by qRT–PCR of the down-regulation of three of these shared genes. Bars represent the mean ± SEM. NC, non-cryopreserved; SF, slow freezing; V, vitrification.

(−6.2), *DNAJC6* (−7.5) and *DNAJA1* (−7.2), *P*-value = 1.6E−02]. The three other top canonical pathways affected by down-regulation in slowly frozen MII oocytes are included in the Supplementary data, Table S1. In addition, the most affected molecular and cellular function was the cell cycle and this included many genes involved in chromosome structure maintenance [*KIF2C* (−5.5), *KIF3A* (−5.3), *KIF11* (−5.4), *KIF14* (−8.5) and *FAM33A* (−9.6)] and cell cycle regulation (*GADD45A*, *CHEK2* and *CDKN1B*; Table III) that were specifically down-regulated in the slowly frozen MII oocyte group compared with non-cryopreserved MII oocytes.

Discussion

Here we report that slowly frozen/thawed and vitrified unfertilized MII oocytes are characterized by differential down-regulation of specific transcripts in comparison with non-cryopreserved MII oocytes, suggesting that both cryopreservation procedures lead to loss of the mRNA content. Oocyte developmental competence depends on the accumulation of maternal proteins and mRNAs during oogenesis, and reduced developmental competence is considered to be one of the main reasons of IVF failure.

The control of gene expression and translation during oocyte maturation and the early stages of embryogenesis depend on the polyadenylation levels of maternal mRNAs. Polyadenylation levels are regulated by the poly(A) polymerase PAPOLA, a key enzyme responsible for the addition of poly(A) at the 3′ end of pre-mRNA, and by the cleavage and polyadenylation specific factor (CPSF) that facilitates both RNA cleavage and poly(A) synthesis (Laishram and Anderson, 2010). Both *PAPOLA* and *CPSF2* were down-regulated in vitrified MII oocytes in comparison with non-cryopreserved and slowly frozen oocyte groups, suggesting that vitrification is associated with a general alteration of the mRNA content. This hypothesis is reinforced

Table II List of the genes that were most significantly down-regulated exclusively in vitrified MII oocytes or exclusively in slowly frozen MII oocytes in comparison with the control group (non-cryopreserved MII oocytes).

Symbol	Name	FC	
		Non-cryopreserved versus slow freezing	Non-cryopreserved versus vitrified
PGM2	Phosphoglucomutase 2	−31.50	
AASDHPPT	Aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	−17.76	
CYR61	Cysteine-rich, angiogenic inducer, 61	−16.87	
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	−15.73	
GBE1	Glucan, branching enzyme 1	−15.29	
TXNDC	Thioredoxin-related transmembrane protein 1	−15.07	
ATP6V1A	ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A	−14.21	
TMEM45A	Transmembrane protein 45A	−13.88	
PREPL	Prolyl endopeptidase like	−13.82	
LIN7C	lin-7 homolog C	−13.39	
THAP6	THAP domain containing 6		−19.32
TSC22D2	TSC22 domain family, member 2		−18.75
KLHL20	Kelch-like 20		−16.40
EIF2S1	Eukaryotic translation initiation factor 2, subunit 1 alpha		−16.31
PRPF4	PRP4 pre-mRNA processing factor 4 homolog		−15.03
AKAP1	A kinase anchor protein 1		−14.93
OSBPL8	Oxysterol binding protein-like 8		−14.70
EIF1AX	Eukaryotic translation initiation factor 1A, X-linked		−14.27
THEMIS	Thymocyte selection associated		−14.26
PCYOX1	Prenylcysteine oxidase 1		−14.22

by the observation that *H2AFZ*, a member of the H2A histone family, was specifically up-regulated in vitrified MII oocytes, because its accumulation has been related to reduced transcription in specific DNA regions (Hardy *et al.*, 2009). In addition, studies in mice have shown that this histone variant is required for embryonic development as the lack of functional *H2AFZ* leads to embryonic lethality (Faast *et al.*, 2001). *PAPOLA* and *H2AFZ* were previously reported to be both down-regulated after vitrification of *in vitro* matured ovine oocytes (Succu *et al.*, 2008). This discrepancy could be explained by species-specific differences.

On the other hand, in the slowly frozen MII oocyte group, the specific down-regulation of genes involved in chromosome structure maintenance and cell cycle regulation may lead to cellular development arrest. Among these genes, only *GADD45A* was previously reported to be affected by the slow freezing procedure; in mouse preantral follicles, it was up-regulated in contrast to the present results (Liu *et al.*, 2003).

Down-regulation of transcripts that are known to play a role in the acquisition of developmental competence was previously reported in mature ovine and human oocytes after cryopreservation (Succu *et al.*, 2008; Chamayou *et al.*, 2011). Based on clinical results, it seems that the biological functions affected by the two cryopreservation techniques are different with a more deleterious impact of the slow freezing procedure (Gook and Edgar, 2007). This idea is supported by our findings. Indeed, although many transcripts were down-regulated in both slowly frozen and vitrified oocytes compared with non-cryopreserved oocytes, very few of these genes were shared by the two groups of cryopreserved oocytes, suggesting that each cryopreservation procedure negatively and differently affect the expression profile of human MII oocytes. Specifically, following vitrification many genes involved in the ubiquitination pathway were down-regulated, including several members of the ubiquitin-specific peptidase family of cysteine protease and subunits of the 26S proteasome (Fig. 4). The inhibition of the degradation machinery could possibly stabilize the maternal protein content that is necessary for oocyte developmental competence. This hypothesis is reinforced by the observation that embryo development to blastocyst stage was significantly delayed following IVF of vitrified/warmed ovine MII oocytes, suggesting that minimal biological functions are maintained after vitrification (Succu *et al.*, 2008).

Conversely, the down-regulation in slowly frozen MII oocytes of genes associated with DNA repair, transcriptional regulation in response to DNA damage, cell cycle regulation and maintenance of chromosomal stability (Yoshida and Miki, 2004) may be associated with more severe molecular damages than in vitrified oocytes, thus compromising the biological functions and affecting oocyte developmental competence.

The way this study was carried out (and, as a consequence, the reported results) needs to be further discussed. In particular, unfertilized aging oocytes, which were collected mainly 24 or 48 h

Table III Genes related to the cell cycle that were differentially expressed either in slowly frozen or in vitrified MII oocytes in comparison with non-cryopreserved MII oocytes.

Exclusive to the slowly frozen/thawed oocyte group				Exclusive to the vitrified oocyte group			
Gene ID	**Symbol**	**Name**	**FC**	**Gene ID**	**Symbol**	**Name**	**FC**
227013_at	LATS2	Large tumor suppressor	−8.9	204822_at	TTK	Monopolar spindle 1-like 1	−7.5
225684_at	FAM33A	Spindle and kinetochore-associated complex subunit 2	−9.6	205899_at	CCNA1	Cyclin A1	6.4
204634_at	NEK4	Never in mitosis gene a-related kinase 4	−7.5	204645_at	CCNT2	Cyclin T2	−5.6
201458_s_at	BUB3	Mitotic checkpoint component	−12.2	227299_at	CCNI	Cyclin I	−6.4
204444_at	KIF11	Kinesin family member 11	−5.4	222962_s_at	MCM10	Minichromosome maintenance complex component 10	−7.2
203725_at	GADD45A	Growth arrest and DNA-damage-inducible, alpha	−9.1	207318_s_at	CDC2L5	CDC2-related protein kinase 5	−6.1
237241_at	ECT2	Epithelial cell transforming sequence 2 oncogene	5.2	202717_s_at	CDC16	Cell division cycle 16 homolog	−6.1
210002_at	GATA6	GATA binding protein 6	−7.4	226400_at	CDC42	Cell division cycle 42	−6.5
209408_at	KIF2C	Mitotic centromere-associated kinesin	−5.5	212720_at	PAPOLA	Poly(A) polymerase alpha	−12.2
210416_s_at	CHEK2	CHK2 checkpoint homolog	−5.7	204460_s_at	RAD1	DNA repair exonuclease REC1	−5.4
209112_at	CDKN1B	Cyclin-dependent kinase inhibitor 1B	−5.1	232238_at	ASPM	Abnormal spindle homolog	−6.7
201200_at	CREG1	Cellular repressor of E1A-stimulated genes 1	−6.3	225994_at	CPSF2	Cleavage and polyadenylation specific factor 2	−5.6
218397_at	FANCL	Fanconi anemia, complementation group L	−8.1	200853_at	H2AFZ	H2A histone family, member Z	7.9
226660_at	RPS6KB1	Ribosomal protein S6 kinase	−5.4	207828_s_at	CENPF	Centromere protein F	7.6

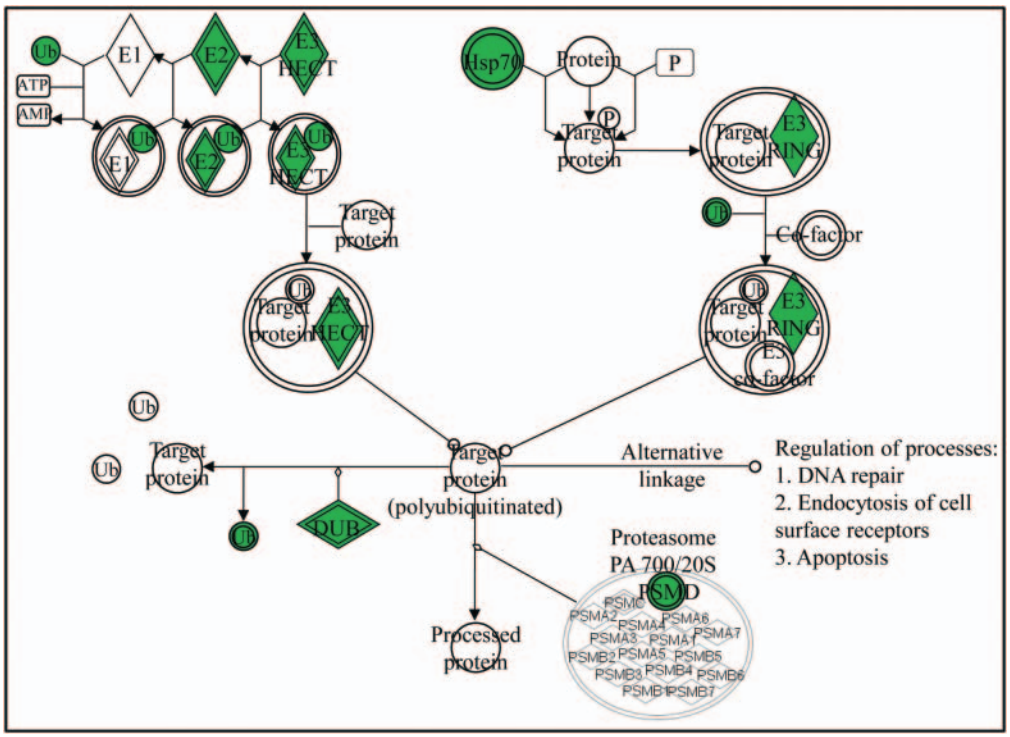

Figure 4 The protein ubiquitination pathway is the most strongly affected functional network in vitrified MII oocytes. The Ingenuity Pathway software was used to identify the functional pathways affected by the two cryopreservation procedures. The figure shows the genes belonging to the protein ubiquitination pathway that were down-regulated (green color) following vitrification. Uncolored genes were not identified as differentially expressed in our study, but were integrated into the network for computational grounds. In this network, edge types are indicatives: a plain line indicates direct interaction, a dashed line indicates indirect interaction, a line without arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates that one acts on the other.

post-microinjection, were used because the French bioethical laws forbid research using fresh oocytes. The use of aging oocytes requires caution with the microarray data, as the kit used for cRNA preparation amplifies poly-A RNA, and there is a correlation between adenylation and the time course of degradation of maternal RNAs. However, since all samples were amplified using the same technology, any bias should affect all samples equally. In theory, this should not prevent the identification of expression differences related to treatment. Furthermore, the Cryotip closed system was employed for oocyte vitrification to comply with the French bioethical laws. Recent studies reported that the type of device used for vitrification plays an important role in the developmental capability of oocytes (Kuwayama *et al*., 2005; Kuwayama, 2007; Rienzi *et al*., 2010). Specifically, although the closed system has been successfully used for vitrification of human blastocysts, the developmental potential of such oocytes is reduced in comparison with that of oocytes that have been vitrified using open systems, such as Cryotop or Cryoloop (Kuwayama, 2007). The beneficial effect of the open methods on oocyte developmental competence is possibly linked to the use of a minimal volume of the droplets of solution in which oocytes are vitrified, thus increasing the cooling and the warming rates which may contribute to the improved survival. In addition to the already reported clinical impact of the closed vitrification procedures on the pregnancy outcome, our study shows that oocytes stored using this Cryotip system are characterized by a high number of down-regulated genes.

Conclusions

Both slow freezing and vitrification using the Cryotip system differentially affect the gene expression profile of human unfertilized MII oocytes by reducing overall transcript abundance compared with non-cryopreserved MII oocytes. Functional annotations of the down-regulated genes suggest that slow freezing has more deleterious consequences on the oocyte developmental competence than the vitrification procedure. The lower implantation and pregnancy rates of human oocytes after slow freezing may be explained by the specific alterations to their gene expression profiles. The use of slowly frozen human oocytes should be reconsidered in the light of these results, although more investigations are required to confirm these findings.

Supplementary data

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

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Authors' roles

M.C. performed the microarray and qRT–PCR experiments, analysed and interpreted the data, and wrote the paper; H.D. performed the microarray and qRT–PCR experiments, analysed and interpreted the data and wrote the paper; R.K. performed cryopreservation procedures; A.S. contributed to data interpretation and participated in the paper revision; D.H. contributed to data interpretation and participated in the paper revision; S.H. conceived the experiments, participated in the paper revision and gave final approval.

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Conflict of interest

None declared.

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