Front. Med. 2021, 15(1): 144–154
https://doi.org/10.1007/s11684-020-0792-7

RESEARCH ARTICLE

Effects of vitrification and cryostorage duration on single-cell RNA-Seq profiling of vitrified-thawed human metaphase II oocytes

Ying Huo[1,2,3,4,*], Peng Yuan[1,3,4,*], Qingyuan Qin[1,3,4,*], Zhiqiang Yan[1,3,4,5], Liying Yan[1,3,4,6], Ping Liu[1,3,4], Rong Li[1,3,4,6], Jie Yan (✉)[1,3,4], Jie Qiao[1,3,4,5,6]

[1]*Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China;* [2]*Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing 100191, China;* [3]*Beijing Key Laboratory of Reproductive Endocrinology and Assisted Reproduction, Beijing 100191, China;* [4]*Key Laboratory of Assisted Reproduction, Ministry of Education, Beijing 100191, China;* [5]*Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China;* [6]*National Clinical Research Center of Obstetrics and Gynecology, Beijing 100191, China*

Abstract **Oocyte cryopreservation is widely used for clinical and social reasons. Previous studies have demonstrated that conventional slow-freezing cryopreservation procedures, but not storage time, can alter the gene expression profiles of frozen oocytes. Whether vitrification procedures and the related frozen storage durations have any effects on the transcriptomes of human metaphase II oocytes remain unknown. Four women (30–32 years old) who had undergone IVF treatment were recruited for this study. RNA-Seq profiles of 3 fresh oocytes and 13 surviving vitrified-thawed oocytes (3, 3, 4, and 3 oocytes were cryostored for 1, 2, 3, and 12 months) were analyzed at a single-cell resolution. A total of 1987 genes were differentially expressed in the 13 vitrified-thawed oocytes. However, no differentially expressed genes were found between any two groups among the 1-, 2-, 3-, and 12-month storage groups. Further analysis revealed that the aberrant genes in the vitrified oocytes were closely related to oogenesis and development. Our findings indicated that the effects of vitrification on the transcriptomes of mature human oocytes are induced by the procedure itself, suggesting that long-term cryostorage of human oocytes is safe.**

Keywords human metaphase II oocyte; vitrification; cryostorage duration; single-cell RNA-Seq; lncRNA

Introduction

Oocyte cryopreservation has become a clinically viable option for women who are facing age-related or iatrogenic decreases in both the quality and quantity of their oocytes because of declining oocyte quality, invasive cancer treatment, premature ovarian failure, and polycystic ovary syndrome [1]. Oocyte cryopreservation is a breakthrough technique for women who elect to delay childbearing, require gonadotoxic therapy, or perform egg donation [2–4].

The first human oocyte reported to be cryopreserved was preserved via the slow-freezing technique, in which the rate of cooling can be programmed in low concentrations of cryoprotectant agents to minimize intracellular ice crystal formation. Over the last decade, vitrification has replaced slow-freezing as the technique of choice for cryopreserving oocytes. This method is performed with an ultrarapid cooling time and a very high cryoprotectant concentration to minimize ice crystal formation, thereby resulting in high rates of cell survival, fertilization, embryo development, and pregnancy [5–8]. At present, approximately 4000 live births have been obtained via oocyte vitrification since the first birth of a healthy baby following oocyte vitrification in 1999 [9,10].

As the largest human cells, oocytes have relatively lower surface area-to-volume ratios and are more likely to retain water during the freezing process than other cell types.

Received January 2, 2020; accepted April 17, 2020
Correspondence: Jie Yan, yanjiebjmu@bjmu.edu.cn
*These authors contributed equally to this work.

Therefore, oocytes are more vulnerable to damage caused by intracellular ice crystal formation during cryopreservation procedures than other cells. Moreover, dramatic osmotic changes in cryoprotective agents may further seriously damage oocytes [11]. Cryopreservation-induced oocyte damage includes zona pellucida thickening, premature cortical granule exocytosis, meiotic spindle disruption, and aneuploidy [12–16]. Additionally, studies on ovine [17], bovine [18], canine [19], murine [20], and human [21,22] oocyte cryopreservation using the vitrification method and human studies using slow freezing [8,22,23] have revealed that cryopreservation deregulates a subset of genes that involved in oxidative stress, apoptosis, cell cycle, chromosomal organization, RNA splicing and processing, DNA repair, cellular response to DNA damage and to stress, calcium ion binding, malate dehydrogenase activity, and mitochondrial activity. Furthermore, Stigliani *et al*. [8] demonstrated that the length of storage time does not alter the gene expression profiles of human metaphase II (MII) oocytes following slow freezing. Hence, we can infer from available evidence that the potential damage resulting from oocyte cryopreservation might be attributed to the cryopreservation process per se rather than storage time.

Aside from inducing dysregulated gene expression, the slow-freezing cryopreservation technique reportedly affects cellular proteomics. Studies on mice have shown that exposure of mouse oocytes to 1,2-propanediol can alter oocyte proteins and thus affect fertilization and early embryo development [24,25]. Following the slow-freezing technique, free-radical production due to disruption of the oocyte plasma membrane may induce protein efflux, leading to downregulation of proteins. By contrast, the exposure of oocytes to cytotoxic cryoprotectant agents may induce upregulation of several stress proteins.

Among the indicators of the efficacy and safety of cryopreservation techniques, implantation ability and subsequent pregnancy rates are the most critical factors [7,26–30]. However, at present, only limited information about the long-term effects of cryopreservation after thawing on human oocytes and embryos is available. Recently, Stigliani *et al*. [8] demonstrated that storage time does not alter the gene expression profiles of human MII oocytes following the slow-freezing method. Nevertheless, no study has been designed to evaluate whether storage time can affect the gene expression of vitrified-thawed human oocytes. Therefore, this study aimed to investigate the effects of different storage durations after open vitrification on the gene expression patterns in human MII oocytes at the single-cell level. Herein, we compared the transcriptomes of vitrified-thawed human MII oocytes after 1, 2, 3, and 12 months of storage in liquid nitrogen with those of fresh ones using single-cell RNA sequencing analysis.

Materials and methods

Ethical approval and informed consent

This study was reviewed and approved by the Reproductive Study Ethics Committee of Peking University Third Hospital (Research License 2013SZ017). All oocytes were obtained from patients undergoing *in vitro* fertilization (IVF) who decided to donate their surplus oocytes. Written informed consent was obtained from all participants.

Patient selection and oocyte collection

All MII oocytes used in the current study were donated by patients undergoing IVF at the Center for Reproductive Medicine of Peking University Third Hospital following standard clinical protocols. The data for a control group consisting of three fresh human MII oocytes were downloaded from our previously published research [31]. Four patients aged 30–32 years who were diagnosed with tubal factor infertility without any ovarian pathology were enrolled in this prospective study. A total of 13 oocytes from these four patients were randomly divided into four groups according to storage time: 1-, 2-, 3-, and 12-month groups.

Oocyte vitrification and thawing

MII oocytes were vitrified and thawed as previously described [10]. In brief, the oocytes were first equilibrated in 7.5% (v/v) ethylene glycol (EG, Sigma, cat. 293237, USA) + 7.5% (v/v) dimethyl sulfoxide (DMSO, Sigma, cat. D2650, USA) solution for 5 min at room temperature. These oocytes were then transferred into vitrification solutions composed of 15% (v/v) EG + 15% (v/v) DMSO + 0.5 mol/L sucrose (Sigma, cat. V900116, USA) for less than 1 min at room temperature. Finally, these oocytes were immediately loaded onto sterile iVitri straws (Reprobiotech Corp., cat. RBC-S-008, USA) and directly transferred into liquid nitrogen for storage lasting 1, 2, 3, or 12 months.

The frozen oocytes were thawed step by step by using different concentrations of sucrose solution. First, the oocytes were expelled from the straws into 1.0 mol/L sucrose and incubated for 3 min at 37 °C. These oocytes were then successively transferred into 0.5, 0.25, and 0 mol/L sucrose medium for 3 min at room temperature. Finally, the thawed oocytes were cultured in human tubal fluid (LifeGlobal, cat. LGGF-100, Belgium) medium at 37 °C under 5% CO_2 in humidified air for 2 h.

Single-cell transcriptome library preparation

Individual oocytes were first isolated and added to lysate

buffer by mouth pipetting. These single-cell samples were then directly amplified following the Smart-Seq2 method by Genome.cn. The DNA concentration of each library was measured using a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). The fragment distribution of the amplified products was detected using an Agilent 2100 high-sensitivity DNA assay kit (Agilent Technologies, CA, USA).

RNA-Seq library preparation and sequencing

Amplified cDNA products were used as input for library construction of the single-cell transcriptomes. First, the sample cDNA was sheared into 300 bp fragments by using a Bioruptor® sonication system (Diagenode Inc.). Second, terminal repair, addition of A-tailing, and ligation of sequencing adapters to the fragmented cDNA were performed. The sequencing adapter products were amplified using PCR, and the samples were distinguished from each other by adding different index tags. Finally, electrophoresis was performed with the PCR amplification products in 2% agarose gels, the 350–450 bp DNA bands were extracted from the gels with a CWBIO gel extraction kit, and the DNA was dissolved to create the final libraries.

After library construction, the insertion size was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA), and the accurate insertion size was quantified with a TaqMan fluorescent probe by using an AB StepOnePlus real-time PCR system (valid library concentration $>$ 10 nmol/L).

In addition, the index-coded samples were clustered using a HiSeq PE cluster kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. Finally, the sequences of the libraries were analyzed on an Illumina HiSeq platform with 150 bp paired-end sequencing.

Single-cell RNA-Seq data analysis

The single-cell RNA-Seq data was analyzed as previously described [32,33]. In brief, the raw sequencing data were processed to remove the library adapter sequences, amplification primers, and 3′ polyA sequences. Then, the clean data were aligned to the human genome 19 by using TopHat2. Afterward, Cufflinks was used to calculate the gene expression levels in the form of fragments per kilobase of transcript per million fragments mapped reads (FPKM) values. Only genes with FPKM values $>$ 1 in at least one sample were analyzed for their expression levels to ensure accuracy and remove the auxiliary data. The data were transformed by $\log_2(x+1)$ for the subsequent analyses.

The expression patterns of different oocytes were then analyzed via principal component analysis (PCA). Transcript integrity number (TIN) was applied to measure RNA degradation in the oocytes. Differentially expressed genes for different groups were evaluated by multiple *t*-tests. False discovery rate (FDR)-corrected *P* values were calculated using the Benjamini–Hochberg method. The differentially expressed genes were defined on the basis of multiple *t*-tests with criteria of $P < 0.05$, FDR < 0.05, and a fold change ($\log_2$-converted FPKM) > 2 or < 0.5.

Functional annotation analyses of the differentially expressed genes were performed via Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The dynamic expression patterns of known long noncoding RNAs (lncRNAs) were analyzed using the GENCODE lncRNA database.

Results

In the current study, we used 16 donated human MII oocytes to compare the transcriptomes of fresh and vitrified-thawed ones with different storage times at the single-cell level. Overall, 110 Gb of raw data were generated with an average of 28.1 million paired reads per oocyte, and the read length was 150 bp.

Transcriptional profiles of different MII oocytes

First, we evaluated the sequencing data quality. The average mapping rate was 91.33% ± 1.67%. On average, we detected the expression of 12 280 out of 26 364 RefSeq genes, indicating that almost 47% of known human genes were covered by our data.

Then, we analyzed all the data via PCA. Results showed that most of the cryopreserved oocytes clustered together, showing a pattern distinct from that of the fresh oocytes (Fig. 1A).

We applied TIN to measure RNA degradation during the process of oocyte cryopreservation. The TIN metric is a reliable measurement of RNA integrity at both the transcriptome and transcript levels. TIN scores can be calculated after the RNA-Seq data are produced. The relative median TIN of cryopreserved oocytes was approximately 40% that of fresh oocytes (Fig. 1B).

Storage time did not affect the gene expression profiles of mature human oocytes

Pairwise comparisons were performed to analyze the gene expression patterns among the 1-, 2-, 3-, and 12-month groups. However, no differentially expressed genes were found between any two cryopreserved groups (Fig. 1C).

Vitrification-thawing procedures altered the gene expression profiles of mature human oocytes

A total of 1987 differentially expressed genes were found in the 13 vitrified-thawed oocytes compared with the fresh MII oocytes. Among the differentially expressed genes,

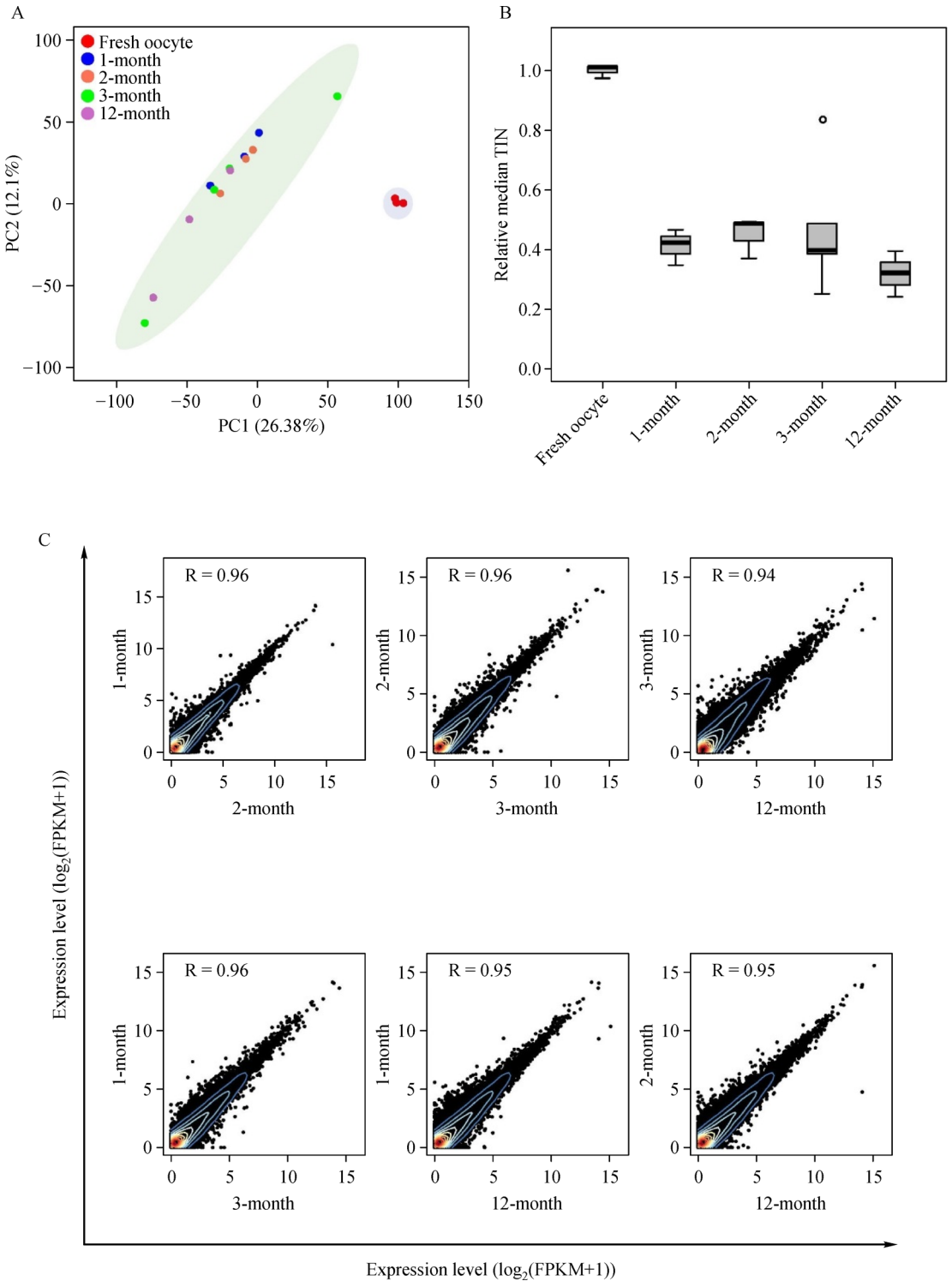

Fig. 1 Quality control of the RNA-Seq data and pairwise comparisons between cryopreserved groups. (A) PCA of the transcriptomes of single human oocytes. Oocytes with the same cryostorage duration are indicated with symbols of the same shape. PC1 and PC2 represent the top two dimensions of the genes showing differential expression among these oocytes. (B) Relative median TIN of single human oocytes. The median TIN of each single cell was divided by the average median TIN of fresh MII oocytes. (C) Pairwise comparisons between two groups among the 1-, 2-, 3-, and 12-month cryostorage groups.

1646 genes were downregulated, whereas 341 were upregulated (Fig. 2A).

To obtain insights into their potential functional roles, we further analyzed all 1987 differentially expressed genes via GO and KEGG analyses.

Among the downregulated genes, the main enriched GO terms included 65 different biological process terms. The upregulated genes were related to 29 unique biological processes (Table S1). The important biological processes of the downregulated and upregulated genes are presented in Fig. 2B and 2C, respectively. In particular, cell cycle and meiosis are the two important biological processes for the functions of oocytes. Notably, two meiosis-related genes, namely, *TUBGCP5* and *NCAPD2*, were considerably downregulated (Supplementary Fig. S1A). In addition, cell cycle-related genes, including *NCAPD2* and *TUBB4B*, were significantly downregulated in cryopreserved MII oocytes, whereas *RANGAP1* and *ANP32E* were upregulated (Supplementary Fig. S1B). We also found that two genes related to cellular response to heat, namely, *HSPA1A* and *HSPA1B*, were significantly downregulated in cryopreserved MII oocytes (Supplementary Fig. S1C). Other important differentially expressed genes that were closely related to oocyte growth and development, such as microtubule-based process, methylation, ubiquinone biosynthetic process, the tricarboxylic acid cycle, and the MAPK cascade, were graphically displayed (Supplementary Fig. S1D–1H).

Moreover, we performed KEGG analysis to explore the related signaling pathways. We found that some important pathways, including carbon metabolism; metabolic pathways; gap junction; axon guidance; pathogenic *Escherichia coli* infection; the citrate cycle; pyrimidine metabolism; 2-oxocarboxylic acid metabolism; biosynthesis of antibiotics; alanine, aspartate, and glutamate metabolism; the GnRH signaling pathway; biosynthesis of amino acids; and RNA transport were enriched for the fresh oocytes (Fig. 2D, Supplementary Table S1). Interestingly, oxidative phosphorylation, lysosome pathways, Huntington's disease pathways, regulation of lipolysis in

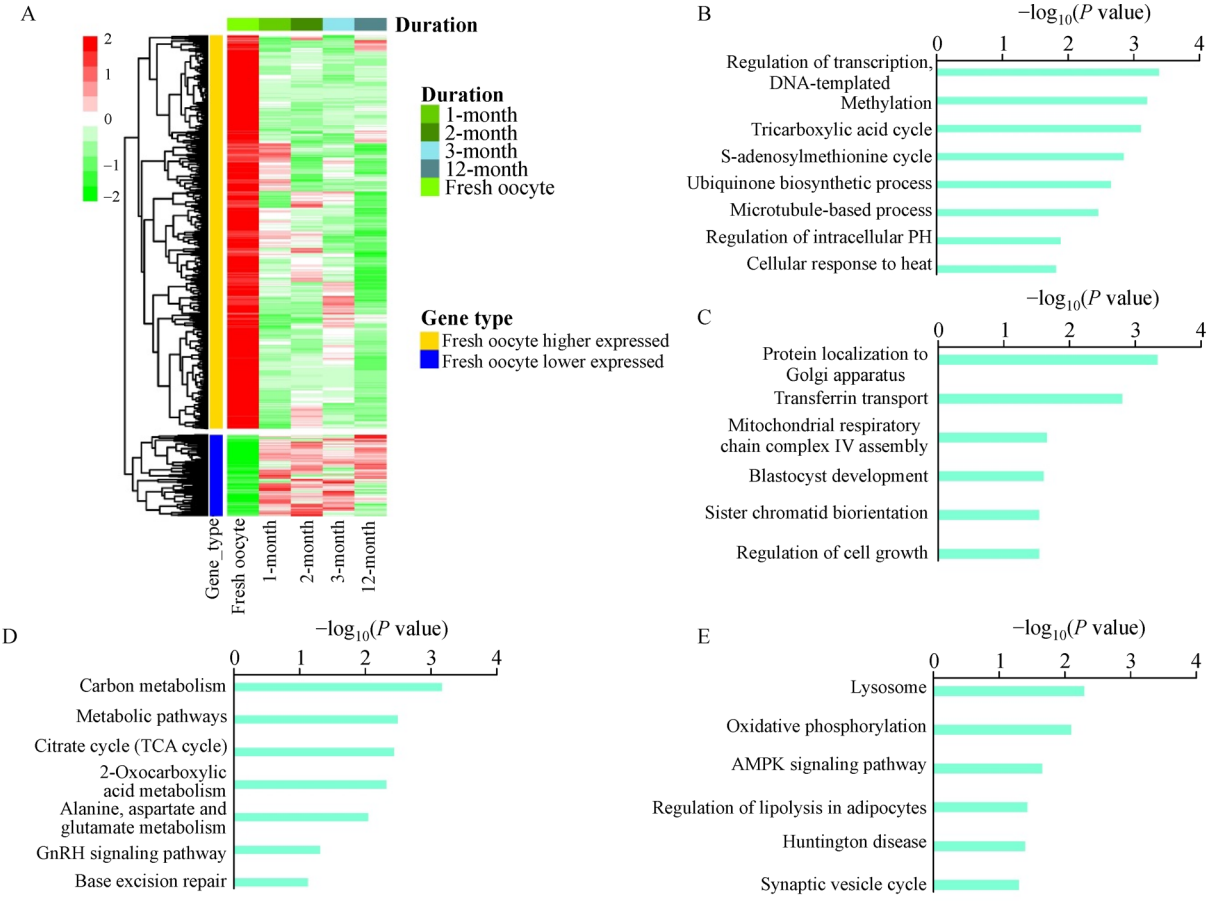

Fig. 2 Differentially expressed genes between fresh and cryopreserved human MII oocytes. (A) Heatmap of the differentially expressed genes between fresh and cryopreserved oocytes. The expression level of each gene was normalized using the *Z*-score. (B) Enriched GO terms of the downregulated genes in cryopreserved oocytes. (C) Enriched GO terms of the upregulated genes in cryopreserved oocytes. (D) Enriched KEGG pathways of the downregulated genes in cryopreserved oocytes. (E) Enriched KEGG pathways of the upregulated genes in cryopreserved oocytes.

adipocytes, the synaptic vesicle cycle, and the AMPK signaling pathway were upregulated in the cryopreserved oocytes (Fig. 2E, Table S1).

To further investigate the effects of storage time on gene expression in cryopreserved oocytes, we compared the gene expression profiles of oocytes in different storage time groups with those of fresh ones. Results showed 520 downregulated and 93 upregulated genes in the 1-month group, 499 downregulated and 95 upregulated genes in the 2-month group, 194 downregulated and 28 upregulated genes in the 3-month group, and 575 downregulated and 40 upregulated genes in the 12-month group (Fig. 3A, Tables S2–S5). Possible overlap among these aberrant genes was assessed using Venn diagrams. Results showed 52 overlapping downregulated genes. GO analysis revealed that these 52 genes were enriched for GO terms mainly related to cellular heat acclimation, regulation of protein ubiquitination, and ATP metabolic processes (Fig. 3B and 3D, Table S6). The aforementioned GO terms were mainly associated with *HSPA1A* and *HSPA1B*, which were described earlier. As shown in Fig. 3C, only four genes, namely, *SCAND1*, *HERC2P7*, *PET100* and

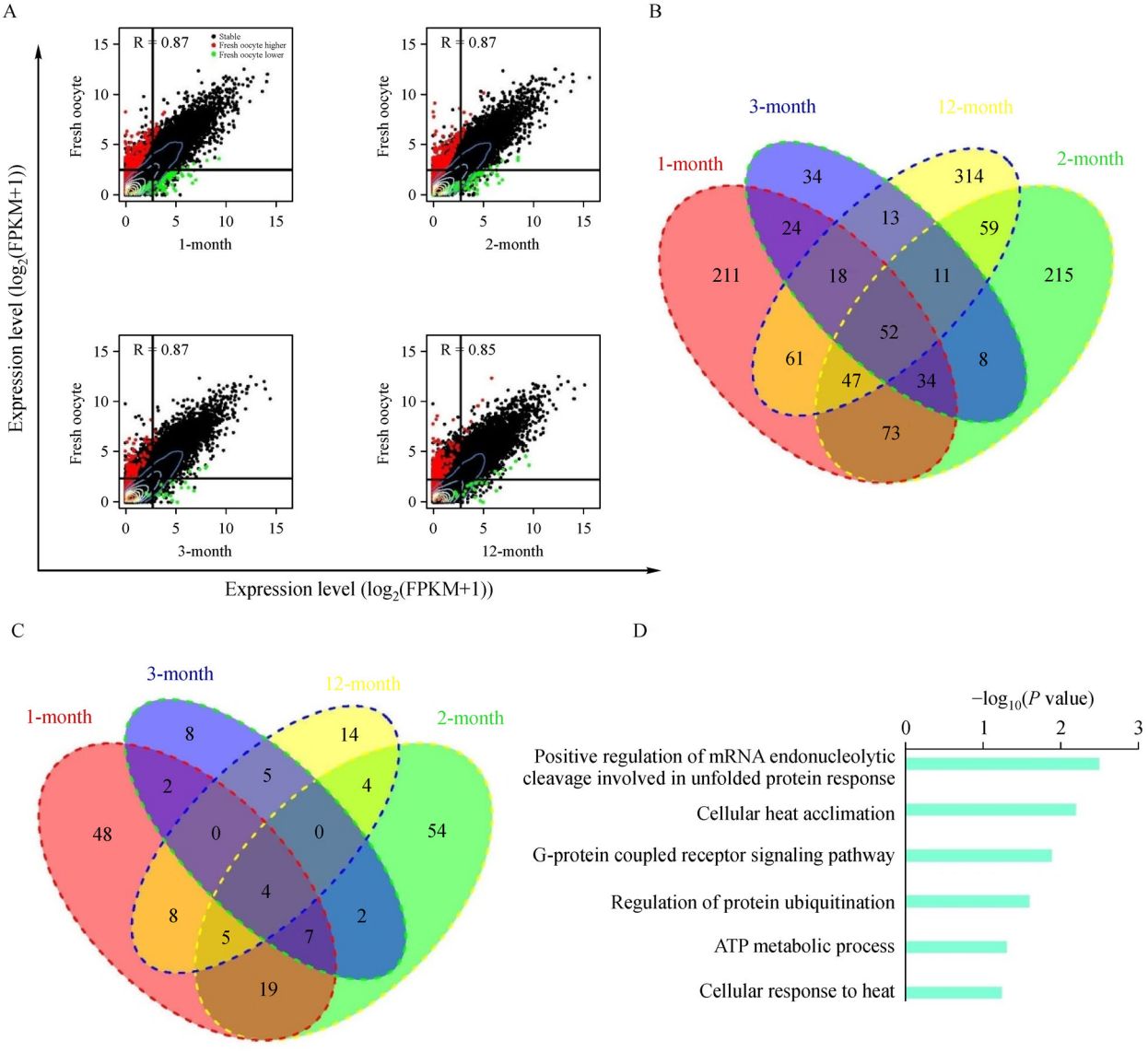

Fig. 3 Differentially expressed genes between fresh oocytes and cryopreserved oocytes in four groups. (A) Scatter plot of the average gene expression between fresh and cryopreserved oocytes. Downregulated genes in cryopreserved oocytes are shown in red, upregulated genes are depicted in green, and stably expressed genes are presented in black. (B) Venn diagram of the common downregulated genes in the four cryopreserved groups in comparison with the fresh group. (C) Venn diagram of the common upregulated genes in the four cryopreserved groups in comparison with the fresh group. (D) Enriched GO terms of the 52 common downregulated genes among the four cryopreserved groups.

TRAPPC1, were located at the intersection of the upregulated gene sets. However, the upregulation of these genes might have been a stochastic event, as the genes were not enriched in any pathway.

Dynamic expression of lncRNAs

The expression patterns of lncRNAs were analogous to those of the aforementioned RefSeq genes, as unsupervised hierarchical clustering analysis indicated that the lncRNAs of most cryopreserved oocytes were clustered together, with two exceptions (one belonging to the 3-month group and one belonging to the 12-month group) that presented a distinct cluster pattern (Fig. 4A). A total of 440 lncRNAs were differentially expressed in the 13 vitrified-thawed oocytes compared with the fresh MII oocytes, of which 395 lncRNAs were downregulated and 45 were upregulated (Fig. 4B, Table S7).

A total of 62, 51, 75, and 75 differentially expressed lncRNAs were found in the 1- (58 downregulated and 4 upregulated), 2- (51 downregulated and 0 upregulated), 3- (67 downregulated and 8 upregulated), and 12-month groups (72 downregulated and 3 upregulated), respectively, compared with the control group (Fig. 4C, Tables S8–S11). Specifically, among the significantly downregulated lncRNAs in the vitrified-thawed oocytes, five lncRNAs, namely, *CTB-180A7.6*, *AP000320.7*, *OOEP-AS1*, *RP11-59H7.3*, and *RP4-785G19.5*, overlapped,

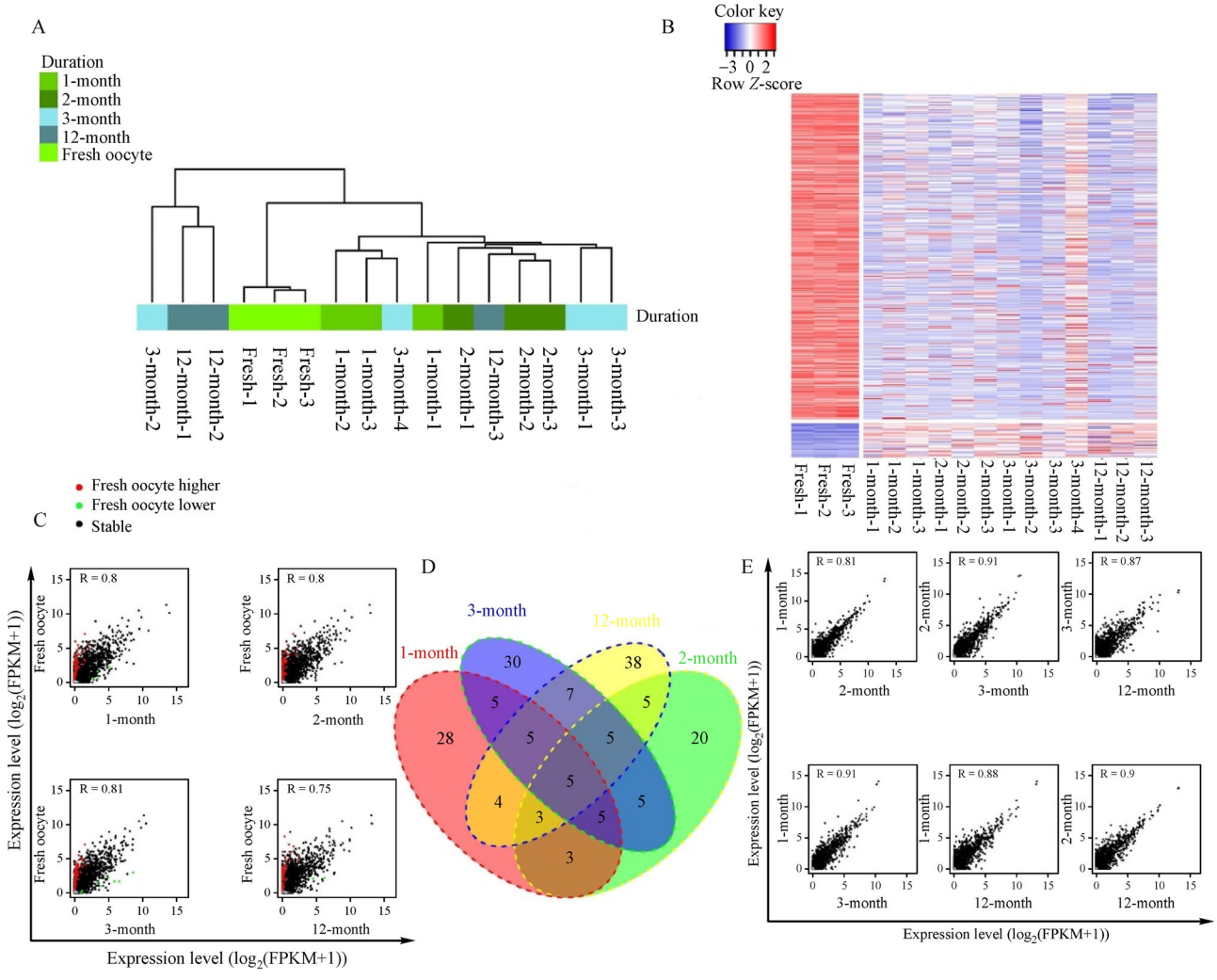

Fig. 4 Expression patterns of lncRNAs among fresh and cryopreserved human MII oocytes. (A) Unsupervised hierarchical clustering of the lncRNA expression profiles of 16 samples. (B) Heatmap of the differentially expressed lncRNAs between fresh and cryopreserved oocytes. *Z*-scores with colors from blue to red indicate the expression levels from low to high. (C) Scatter plot of lncRNA expression between the fresh oocyte group and the 1-, 2-, 3-, and 12-month cryopreserved groups. (D) Venn diagram of the commonly downregulated lncRNAs in the four cryopreserved groups in comparison with the control group. (E) Scatter plot of the average gene expression between two groups among the 1-, 2-, 3-, and 12-month groups.

whereas no upregulated lncRNAs were found to overlap among the four cryopreserved groups (Fig. 4D). No differentially expressed lncRNAs were found between any of the two cryopreserved groups, consistent with the results for the differentially expressed genes (Fig. 4E).

Discussion

As the largest cells in the human body, mature MII oocytes have unique biochemical and physical characteristics. Given that they contain a high proportion of water in the cytoplasm, MII oocytes are vulnerable to damage caused by intracellular ice crystal formation during cryopreservation. Currently, slow freezing and vitrification are the two most commonly used methods in freezing oocytes. Recently, with the introduction and advancement of vitrification techniques, notable improvements have been achieved in ensuring the efficacy and safety of oocyte preservation, as demonstrated by enhanced oocyte survival, fertilization, and pregnancy rates [4,6,34–36].

Prior animal studies have demonstrated that long-term storage of cryopreserved oocytes in liquid nitrogen does not have detrimental effects on the survival, fertilization, embryonic development potential, embryo quality, and gene expression profiles of thawed oocytes [8,37,38]. In the present study, we found that different storage times of vitrified oocytes in liquid nitrogen did not alter the gene expression profiles of the subsequent surviving thawed oocytes. This result was consistent with that of a previous study that reported that the storage time of slow-frozen oocytes does not affect the gene expression profiles of thawed oocytes [8]. These findings suggest the efficacy and safety of long-term storage of frozen oocytes. Collectively, our study and previous studies indicate that the potential damage resulting from oocyte cryopreservation might be due to the cryopreservation procedure per se rather than the storage time. In this regard, these studies have commercial and clinical applications for long-term oocyte banking that can be potentially used for gamete donation programs and for women suffering from fertility disorders.

In the current study, we found that vitrification-thawing procedures may alter the expression of several genes. Overall, fewer mRNAs were extracted from cryopreserved oocytes than from fresh oocytes, revealing that RNA degradation occurred during cryopreservation. The aberrant genes in vitrified oocytes were closely related to oogenesis and development processes, including meiosis, cell cycle, cellular response to heat, microtubule-based processes, methylation, ubiquinone biosynthetic processes, the tricarboxylic acid cycle, the MAPK cascade, sister chromatid biorientation, DNA repair, oxidative phosphorylation, and ATP metabolic processes. Given that high-quality oocytes rely on the precise expression and production of maternal RNAs and proteins, the negative effects of vitrification-thawing procedures on gene expression may affect oocyte quality, leading to poor embryonic development. These results are in agreement with those of previous reports, indicating that cryopreservation procedures may have detrimental effects on oocyte quality [8,17–20,22,23].

HSPA1A and *HSPA1B* were the two most representatively downregulated genes in vitrified-thawed oocytes compared with fresh oocytes. *HSPA1A* and *HSPA1B* are members of the heat shock protein family A (Hsp70). In combination with other heat shock proteins, HSPA1A and HSPA1B proteins are involved in diverse cellular functions, including the regulation of mitosis, the ubiquitin-proteasome pathway, RNA binding, mRNA stabilization, autoimmunity, and inflammatory diseases [39–44]. Intriguingly, the expression levels of *HSPA1A* and *HSPA1B* are correlated with the capacity of embryos to exhibit the heat-shock response, which plays an important role in regulating the successive steps of early embryonic development [45,46]. Another finding that merits our attention was the upregulation of *SCAND1* and *TRAPPC1* in all four groups of cryopreserved oocytes. Although such upregulation seemed to be a stochastic event as these genes were not enriched in any pathway, *SCAND1* and *TRAPPC1* have been shown to have regulatory roles in numerous biological processes. Notably, *SCAND1*, a SCAN family protein that lacks a zinc finger region, is involved in the regulatory mechanism of transcription [47–49]. The gene product of *TRAPPC1*, a component of the multisubunit transport protein particle complex, may play a role in regulating Golgi membrane trafficking [50]. Therefore, alterations in these genes may affect the synthesis of several biological process-related proteins, leading to low oocyte developmental competence. In the present study, we obtained average values for cell-to-cell variability that pave the way for characterizing biological samples at the single-cell level.

In recent years, lncRNAs have attracted the attention of many researchers. In addition to demonstrating that gene expression profiles were affected by vitrification-thawing procedures, this study revealed that *CTB-180A7.6*, *AP000320.7*, *OOEP-AS1*, *RP11-59H7.3*, and *RP4-785G19.5* were downregulated in all four cryopreserved groups compared with the control group. Although the functions of lncRNAs are still uncategorized, studies on humans [31,51–53], human-induced pluripotent stem cells [54], pigs [55], mouse embryonic stem (ES) cells [56], and other subjects [57] have revealed that certain lncRNAs may play important roles in various biological processes, such as chromatin-modifying complex functions, cellular phenotypes, transcriptional regulation, pre-implantation embryonic development, epigenetic regulation, and pluripotency maintenance. Thus, although functional studies are lacking, we speculate that the downregulated lncRNAs

such as *CTB-180A7.6* and *RP4-785G19.5* might be molecular substrates that are involved in the harmful effects of vitrification procedures on cryopreserved oocytes.

However, we have to acknowledge several limitations of the present study. The number of analyzed oocytes was relatively low, and the longest cryostorage duration was only 12 months. Future studies with larger sample sizes and in which the oocytes are kept frozen for longer periods are warranted. Since Chen [58] reported the first live birth of a child developed from cryopreserved oocytes more than 30 years ago, the clinical success rate of oocyte cryopreservation has lagged behind that of embryo cryopreservation [16]. Optimization of oocyte cryopreservation is far from being finished.

In conclusion, our results indicated that the effects of vitrification on the transcriptomes of mature human oocytes are induced by the procedure itself rather than by the storage time. Furthermore, we found, for the first time, that several dysregulated genes and lncRNAs could be involved in the detrimental effects of the vitrification-thawing procedure on cryopreserved oocytes at the single-cell level. These findings provide insights into the molecular mechanisms by which freezing methods and the related storage times affect vitrified-thawed oocytes.

Acknowledgements

We are grateful to the staff of the IVF laboratory of the Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital for their help in oocyte collection. This work was supported by grants from the National Key Research and Development Program (Nos. 2017YFC1002000, 2018YFC-1004001, and 2016YFC1000600), the National Natural Science Foundation of China (Nos. 81571386 and 31429004), and the Capital Health Development Research Project (No. 2018-2-4095).

Compliance with ethics guidelines

Ying Huo, Peng Yuan, Qingyuan Qin, Zhiqiang Yan, Liying Yan, Ping Liu, Rong Li, Jie Yan, and Jie Qiao declare that they have no conflicts of interest. All procedures followed were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for inclusion in the study.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-020-0792-7 and is accessible for authorized users.

References

1. Zhang L, Yan LY, Zhi X, Yan J, Qiao J. Female fertility: is it safe to "freeze?" Chin Med J (Engl) 2015; 128(3): 390–397
2. Harwood K. Egg freezing: a breakthrough for reproductive autonomy? Bioethics 2009; 23(1): 39–46
3. Homburg R, van der Veen F, Silber SJ. Oocyte vitrification—women's emancipation set in stone. Fertil Steril 2009; 91(4 Suppl): 1319–1320
4. Argyle CE, Harper JC, Davies MC. Oocyte cryopreservation: where are we now? Hum Reprod Update 2016; 22(4): 440–449
5. Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, Kato O. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. Reprod Biomed Online 2005; 11(1): 53–57
6. Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P, Alegretti JR, Motta EL. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertil Steril 2010; 94(6): 2088–2095
7. Levi Setti PE, Porcu E, Patrizio P, Vigiliano V, de Luca R, d'Aloja P, Spoletini R, Scaravelli G. Human oocyte cryopreservation with slow freezing versus vitrification. Results from the National Italian Registry data, 2007–2011. Fertil Steril 2014; 102(1): 90–95.e2
8. Stigliani S, Moretti S, Anserini P, Casciano I, Venturini PL, Scaruffi P. Storage time does not modify the gene expression profile of cryopreserved human metaphase II oocytes. Hum Reprod 2015; 30 (11): 2519–2526
9. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: case report. Hum Reprod 1999; 14(12): 3077–3079
10. Zhang L, Xue X, Yan J, Yan LY, Jin XH, Zhu XH, He ZZ, Liu J, Li R, Qiao J. L-proline: a highly effective cryoprotectant for mouse oocyte vitrification. Sci Rep 2016; 6(1): 26326
11. Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. Theriogenology 2007; 67(1): 64–72
12. Gook DA, Osborn SM, Johnston WI. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. Hum Reprod 1993; 8(7): 1101–1109
13. Zenzes MT, Bielecki R, Casper RF, Leibo SP. Effects of chilling to 0 °C on the morphology of meiotic spindles in human metaphase II oocytes. Fertil Steril 2001; 75(4): 769–777
14. Rienzi L, Martinez F, Ubaldi F, Minasi MG, Iacobelli M, Tesarik J, Greco E. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. Hum Reprod 2004; 19(3): 655–659
15. Ghetler Y, Skutelsky E, Ben Nun I, Ben Dor L, Amihai D, Shalgi R. Human oocyte cryopreservation and the fate of cortical granules. Fertil Steril 2006; 86(1): 210–216
16. Bianchi V, Macchiarelli G, Borini A, Lappi M, Cecconi S, Miglietta S, Familiari G, Nottola SA. Fine morphological assessment of quality of human mature oocytes after slow freezing or vitrification with a closed device: a comparative analysis. Reprod Biol Endocrinol 2014; 12(1): 110
17. Succu S, Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Berlinguer F, Naitana S, Ledda S. Vitrification of *in vitro* matured ovine oocytes affects *in vitro* pre-implantation development and mRNA abundance. Mol Reprod Dev 2008; 75(3): 538–546
18. Anchamparuthy VM, Pearson RE, Gwazdauskas FC. Expression pattern of apoptotic genes in vitrified-thawed bovine oocytes. Reprod Domest Anim 2010; 45(5): e83–e90

19. Turathum B, Saikhun K, Sangsuwan P, Kitiyanant Y. Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrinol 2010; 8(1): 70
20. Habibi A, Farrokhi N, Moreira da Silva F, Bettencourt BF, Bruges-Armas J, Amidi F, Hosseini A. The effects of vitrification on gene expression in mature mouse oocytes by nested quantitative PCR. J Assist Reprod Genet 2010; 27(11): 599–604
21. Di Pietro C, Vento M, Guglielmino MR, Borzì P, Santonocito M, Ragusa M, Barbagallo D, Duro LR, Majorana A, De Palma A, Garofalo MR, Minutolo E, Scollo P, Purrello M. Molecular profiling of human oocytes after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes. Fertil Steril 2010; 94(7): 2804–2807
22. Monzo C, Haouzi D, Roman K, Assou S, Dechaud H, Hamamah S. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. Hum Reprod 2012; 27(7): 2160–2168
23. Chamayou S, Bonaventura G, Alecci C, Tibullo D, Di Raimondo F, Guglielmino A, Barcellona ML. Consequences of metaphase II oocyte cryopreservation on mRNA content. Cryobiology 2011; 62 (2): 130–134
24. Larman MG, Katz-Jaffe MG, Sheehan CB, Gardner DK. 1,2-propanediol and the type of cryopreservation procedure adversely affect mouse oocyte physiology. Hum Reprod 2007; 22(1): 250–259
25. Katz-Jaffe MG, Larman MG, Sheehan CB, Gardner DK. Exposure of mouse oocytes to 1,2-propanediol during slow freezing alters the proteome. Fertil Steril 2008; 89(5 Suppl): 1441–1447
26. Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. Fertil Steril 2006; 86(1): 70–80
27. Borini A, Coticchio G. The efficacy and safety of human oocyte cryopreservation by slow cooling. Semin Reprod Med 2009; 27(6): 443–449
28. Ubaldi F, Anniballo R, Romano S, Baroni E, Albricci L, Colamaria S, Capalbo A, Sapienza F, Vajta G, Rienzi L. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. Hum Reprod 2010; 25(5): 1199–1205
29. Cobo A, Diaz C. Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials. Fertil Steril 2011; 96(2): 277–285
30. Rienzi L, Cobo A, Paffoni A, Scarduelli C, Capalbo A, Vajta G, Remohí J, Ragni G, Ubaldi FM. Consistent and predictable delivery rates after oocyte vitrification: an observational longitudinal cohort multicentric study. Hum Reprod 2012; 27(6): 1606–1612
31. Yan L, Yang M, Guo H, Yang L, Wu J, Li R, Liu P, Lian Y, Zheng X, Yan J, Huang J, Li M, Wu X, Wen L, Lao K, Li R, Qiao J, Tang F. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol 2013; 20(9): 1131–1139
32. Tang F, Barbacioru C, Nordman E, Li B, Xu N, Bashkirov VI, Lao K, Surani MA. RNA-Seq analysis to capture the transcriptome landscape of a single cell. Nat Protoc 2010; 5(3): 516–535
33. Zhou F, Li X, Wang W, Zhu P, Zhou J, He W, Ding M, Xiong F, Zheng X, Li Z, Ni Y, Mu X, Wen L, Cheng T, Lan Y, Yuan W, Tang F, Liu B. Tracing haematopoietic stem cell formation at single-cell resolution. Nature 2016; 533(7604): 487–492
34. Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL, Zhou P. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. Fertil Steril 2009; 92(4): 1306–1311
35. Fadini R, Brambillasca F, Renzini MM, Merola M, Comi R, De Ponti E, Dal Canto MB. Human oocyte cryopreservation: comparison between slow and ultrarapid methods. Reprod Biomed Online 2009; 19(2): 171–180
36. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. Hum Reprod Update 2012; 18(5): 536–554
37. Parmegiani L, Garello C, Granella F, Guidetti D, Bernardi S, Cognigni GE, Revelli A, Filicori M. Long-term cryostorage does not adversely affect the outcome of oocyte thawing cycles. Reprod Biomed Online 2009; 19(3): 374–379
38. Goldman KN, Kramer Y, Hodes-Wertz B, Noyes N, McCaffrey C, Grifo JA. Long-term cryopreservation of human oocytes does not increase embryonic aneuploidy. Fertil Steril 2015; 103(3): 662–668
39. Qian SB, McDonough H, Boellmann F, Cyr DM, Patterson C. CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. Nature 2006; 440(7083): 551–555
40. Chen YJ, Lai KC, Kuo HH, Chow LP, Yih LH, Lee TC. HSP70 colocalizes with PLK1 at the centrosome and disturbs spindle dynamics in cells arrested in mitosis by arsenic trioxide. Arch Toxicol 2014; 88(9): 1711–1723
41. Fang CT, Kuo HH, Pan TS, Yu FC, Yih LH. HSP70 regulates the function of mitotic centrosomes. Cell Mol Life Sci 2016; 73(20): 3949–3960
42. Kishor A, White EJF, Matsangos AE, Yan Z, Tandukar B, Wilson GM. Hsp70's RNA-binding and mRNA-stabilizing activities are independent of its protein chaperone functions. J Biol Chem 2017; 292(34): 14122–14133
43. Mišunová M, Svitálková T, Pleštilová L, Kryštufková O, Tegzová D, Svobodová R, Hušáková M, Tomčík M, Bečvář R, Závada J, Mann H, Kolesár L, Slavčev A, Vencovský J, Novota P. Molecular markers of systemic autoimmune disorders: the expression of MHC-located HSP70 genes is significantly associated with autoimmunity development. Clin Exp Rheumatol 2017; 35(1): 33–42
44. Takahashi S, Andreoletti G, Chen R, Munehira Y, Batra A, Afzal NA, Beattie RM, Bernstein JA, Ennis S, Snyder M. *De novo* and rare mutations in the HSPA1L heat shock gene associated with inflammatory bowel disease. Genome Med 2017; 9(1): 8
45. Le Masson F, Christians E. HSFs and regulation of Hsp70.1 (Hspa1b) in oocytes and preimplantation embryos: new insights brought by transgenic and knockout mouse models. Cell Stress Chaperones 2011; 16(3): 275–285
46. Lelièvre JM, Peynot N, Ruffini S, Laffont L, Le Bourhis D, Girard PM, Duranthon V. Regulation of heat-inducible HSPA1A gene expression during maternal-to-embryo transition and in response to heat in *in vitro*-produced bovine embryos. Reprod Fertil Dev 2017; 29(9): 1868–1881
47. Schumacher C, Wang H, Honer C, Ding W, Koehn J, Lawrence Q, Coulis CM, Wang LL, Ballinger D, Bowen BR, Wagner S. The SCAN domain mediates selective oligomerization. J Biol Chem 2000; 275(22): 17173–17179
48. Edelstein LC, Collins T. The SCAN domain family of zinc finger transcription factors. Gene 2005; 359: 1–17

49. Carneiro FR, Silva TC, Alves AC, Haline-Vaz T, Gozzo FC, Zanchin NI. Spectroscopic characterization of the tumor antigen NY-REN-21 and identification of heterodimer formation with SCAND1. Biochem Biophys Res Commun 2006; 343(1): 260–268
50. Oka T, Krieger M. Multi-component protein complexes and Golgi membrane trafficking. J Biochem 2005; 137(2): 109–114
51. Martianov I, Ramadass A, Serra Barros A, Chow N, Akoulitchev A. Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. Nature 2007; 445(7128): 666–670
52. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007; 129(7): 1311–1323
53. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 2009; 106(28): 11667–11672
54. Loewer S, Cabili MN, Guttman M, Loh YH, Thomas K, Park IH, Garber M, Curran M, Onder T, Agarwal S, Manos PD, Datta S, Lander ES, Schlaeger TM, Daley GQ, Rinn JL. Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. Nat Genet 2010; 42(12): 1113–1117
55. Li J, Gao Z, Wang X, Liu H, Zhang Y, Liu Z. Identification and functional analysis of long intergenic noncoding RNA genes in porcine pre-implantation embryonic development. Sci Rep 2016; 6 (1): 38333
56. Guttman M, Donaghey J, Carey BW, Garber M, Grenier JK, Munson G, Young G, Lucas AB, Ach R, Bruhn L, Yang X, Amit I, Meissner A, Regev A, Rinn JL, Root DE, Lander ES. lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature 2011; 477(7364): 295–300
57. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL, Lander ES. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009; 458(7235): 223–227
58. Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986; 327(8486): 884–886