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Effect of vitrification on biogenesis pathway and expression of development-related microRNAs in preimplantation mouse embryos

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Abstract Vitrification of embryos has been known as the most efficient cryopreservation method in assisted reproductive technology clinics. Vitrification of preimplantation embryo might be associated with altered gene expression profile and biochemical changes of vitrified embryos. Stringent regulation of gene expression in early embryonic stages is very critical for normal development. In the present study, we investigated the effect of vitrification on the canonical miRNA biogenesis pathway, and also the expression of developmental related miRNAs, in 8-cell and blastocyst mouse embryos. Although the expression pattern of the miRNA biogenesis pathway genes differed between 8-cell and blastocyst mouse embryos, vitrification did not affect the expression level of these genes in preimplantation embryos. The expression levels of miR-21 and let-7a were significantly decreased in vitrified 8-cell embryos and fresh blastocysts when compared with fresh 8-cell embryos. The expression of Stat3 was significantly reduced in blastocysts after vitrification. The alteration in the expression pattern of miRNAs, due to their mode of action, can affect broad downstream key developmental signaling pathways. Therefore, the blastocyst stage

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is the preferred point for embryo vitrification as they are less susceptible to cryo-damages regarding the stability of miRNAs related to the developmental and implantation competence of embryo.

Keywords Preimplantation embryo · Vitrification · microRNA expression · microRNA biogenesis

Abbreviations

ART	Assisted reproductive technology
IVF	In vitro fertilization
miRNAs	microRNAs
DGCR8	DiGeorge Syndrome Critical Region 8
pre-miRNA	Precursor miRNA
XPO5	Exportin-5
AGO	Argonaute
RISC	RNA-induced silencing complex
PMSG	Pregnant mare serum gonadotropin
hCG	Human chorionic gonadotropin
ES	Equilibrium solution
EG	Ethylene glycol
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
HTF	Human tubal fluid
VS	Vitrification solution
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
DAVID	Visualization and Integrated Discovery
Itgb3	integrin β3
Cdx2	Caudal-type homeobox 2
Stat3	Signal transducer and activator of transcription 3

Introduction

Assisted reproductive technology (ART) has vastly advanced in the treatment of infertile patients during recent years. Embryos produced in-vitro were traditionally transferred to the uterus at the 8-cell stage on day 3, but nowadays, with the progress of embryo culture media, the possibility of embryo transfer at the blastocyst stage on day 5 is also provided. Since ovarian stimulation in in-vitro fertilization (IVF) cycles leads to an increased number of available embryos, supernumerary embryos are cryopreserved for use in future treatment cycles (Gerris et al. 2003). Vitrification is the most common cryopreservation method for this purpose, and it can be applied at both of 8-cell and blastocyst embryos (Rienzi et al. 2017). Nevertheless, it remains controversial that which embryo developmental stage, 8-cell or blastocyst, is better for vitrification.

MicroRNAs (miRNAs) are small (20–24 nucleotides), non-coding RNAs. The canonical miRNA biogenesis pathway, which main miRNAs are synthesized via this pathway (Paul et al. 2018), initiates from the nucleus. RNase III enzyme DROSHA and DiGeorge Syndrome Critical Region 8 (DGCR8) recognize and cleave the stem-loop of the primary miRNAs to produce the precursor miRNA (pre-miRNA). Pre-miRNA is recognized by Exportin-5 (XPO5) and exported to the cytoplasm. In the cytoplasm, pre-miRNA hairpin is cleaved by another RNase III enzyme DICER into the mature length of miRNA duplex. The mature miRNA binds with Argonaute (AGO) proteins in the RNA-induced silencing complex (RISC), to participate in target mRNAs degradation or inhibition of translation (Paul et al. 2018), and thereby control gene expression at the post-transcriptional level (Bartel 2004).

Recent studies have shown that miRNAs play substantial roles in fertility and preimplantation development of embryo (Bartel 2004; Virant-Klun et al. 2016), As Spatio-temporal pattern of gene expression in early embryonic stages is very vital for normal development, miRNAs have been demonstrated to possess indispensable role in the early embryonic development (Hossain et al. 2012). Differential expression of miRNAs in different stages of preimplantation embryos may reflect their specific functionality in each stage through modulation of gene expression (Yang et al. 2008). Furthermore, miRNAs have been illustrated to regulate the expression of critical genes related to blastocyst implantation (Galliano and Pellicer 2014). Recently, it has been suggested that blastocysts, via secreted miRNAs, could alter the endometrial adhesion and initiate implantation (Cuman et al. 2015). Disruption of the miRNA biogenesis pathway resulted in the early embryonic developmental arrest, depletion of stem cells in mouse and zebrafish (Bernstein et al. 2003; Wienholds et al. 2003), and impairment of implantation process in mouse blastocyst (Cheong et al. 2014).

In our previous study we noticed that the IVF procedure could alter the expression of let-7a, which plays crucial roles in the development and implantation in mouse blastocyst (Azizi et al. 2019). Although embryo vitrification is the most accepted procedure for embryo cryopreservation in ART worldwide, some studies have reported structural and biochemical alterations following vitrification of 8-cell and blastocyst embryos, which could reduce the developmental and implantation potential (Chatzimeletiou et al. 2011; Fabian et al. 2005; Gómez et al. 2009; Li et al. 2012; Stinshoff et al. 2011). Besides, the altered gene expression profile of vitrified embryos was revealed in preceding studies (Mamo et al. 2006; Yao et al. 2017). It has been reported that the vitrification of blastocyst could lead to altered miRNAs profiles, which could be related to the reduced developmental potential of mouse embryos (Zhao et al. 2015). Each miRNA holds a great ability to target numerous genes through complementary sequences (Selbach et al. 2008). So, dysregulation of miRNAs in vitrified embryos may influence broad downstream signaling pathways. Nevertheless, the information regarding the effect of vitrification on the expression of miRNAs in preimplantation embryos is very scanty.

Hence, in this study, we aimed to investigate whether vitrification could alter miRNAs canonical biogenesis pathway and also the expression of developmental and implantation related miRNAs (miR-21, miR-93, miR-24 and let-7a) and their presumptive target genes, in 8-cell and blastocyst mouse embryos. We also tried to specify which embryo developmental stage, 8-cell or blastocyst, is less prone to the deregulation of miRNAs and their canonical biogenesis pathway components after vitrification.

Materials and methods

Animals

All animal procedures were conducted according to the guidelines of Shahid Beheshti University of Medical Science, and the study was approved by the ethics committee at Deputy of Research and Technology (IR.SBMU. MSP.REC.1396.220). NMRI mice (Pasteur Institute, Tehran, Iran) were maintained for two weeks in the 12-h light-dark cycle, with free access to water and food. Superovulation of 6 weeks old female mice was induced by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Gonaser®, Laboratorios Girona, Spain) and then, 48 h later, 10 IU of human chorionic gonadotropin (hCG; choriomon; IBSA, Switzerland). Afterward, superovulated female mice were mated with fertile males. Female mice with a vaginal plug were isolated and caged until euthanization. All females without a vaginal plug and male mice at the endpoint of the study transferred to another project. Euthanization of female mice was individually done by cervical dislocation.

Embryo collection

8-cell embryos and blastocysts were collected from excised oviducts and uterine, 65–66, and 92–94 h after hCG injection, respectively. The embryo Flushing from oviducts and uterine were performed by phosphate-buffered saline (PBS) (pre-warmed in a humidified incubator with an atmosphere of 5% CO_2 at 37 °C for 1 h). Only morphologically normal embryos were used. The embryos were placed into the droplets of KSOM covered with mineral oil in a petri dish and cultured into an incubator at 37 °C with 5% CO_2 for 1 h.

All embryos used in this study were randomly divided into four groups: (1) fresh 8-cell, (2) fresh blastocyst, (3) vitrified 8-cell, (4) vitrified blastocyst. For 8-cell and blastocyst groups, 30 and 50 embryos were pooled, respectively, and eight pooled samples (n = 8) were analyzed in each group.

Vitrification and warming of embryos

Embryos were equilibrated in equilibrium solution (ES), 7.5% ethylene glycol (EG), 7.5% dimethyl sulfoxide (DMSO), and 10% Bovine serum albumin (BSA; Sigma, Louis, MO, USA) in HEPES-buffered human tubal fluid (HTF-HEPES; Irvine Scientific) medium, for 10 minutes. The embryos were subsequently transferred to vitrification solution (VS), 15% EG, 15% DMSO, 0.5 mol/L sucrose, and 10% BSA in HTF for 45–60 s. The embryos were loaded onto a CryoTop (Kitazato), and then immediately plunged into liquid nitrogen and stored for 1 week.

For warming, embryos on CryoTops were immersed in a drop of warming solution 1 (1.0 mol/L sucrose and 20% BSA in HTF) for 1 minute. The

procedure was followed by transferring embryos to warming solution 2 (0.5 mol/L sucrose and 20% BSA in HTF) for 3 minutes, and then into warming solution 3 (20% BSA in HTF containing no sucrose) for 5 minutes.

After the warming, the embryos were placed into the droplets of KSOM (Millipore, Billerica, MA) covered with mineral oil in a petri dish and cultured into an incubator at 37 °C with 5% CO_2 for 1 h to recover in the culture.

RNA isolation and cDNA synthesis

Total RNA was extracted from embryos using 0.5 µl 6 M guanidine hydrochloride reagent (Sigma-Aldrich Inc., USA) (Liu et al. 2012a, b). RNA samples were treated with DNase I (RNase-free) (Thermo Scientific, Waltham, MA, USA) for removing genomic DNA contamination, and quantified by WPA spectrophotometer (Biochrom). Reverse transcription of mRNAs (500 ng RNA/reaction) and miRNAs (25 ng RNA/reaction) were performed according to RevertAid first-strand cDNA synthesis kit instruction (Thermo Scientific, Waltham, MA, USA) using random hexamer and stem-loop RT primers (0.375 µM), respectively. All primers and probes used for RT–PCR were designed by AlleleID 6 software (Table 1) and synthesized by Macrogen (Macrogen, South Korea).

Analysis of expression of mRNAs and miRNAs by qRT-PCR

The qRT-PCRreaction of mRNAs consisted of following components: 10 µl 2X RealQ Plus MasterMix Green (Ampliqon, Denmark), 0.8 µl of each primer, 2 µl first-strand cDNA template and 6.4 µl distilled water. The thermocycling parameters were as follows: 15 min at 95 °C for enzyme activation and 40 cycles of 95 °C for 20 s, followed by 60 °C for 60 s using the Rotor-Gene Q instrument (Qiagen).

For quantification of miRNAs, each qRT-PCRreaction (20 µl) contained 10 µl of 2X RealQ Plus MasterMix for Probe (Ampliqon, Denmark), 0.8 µl of each primer, 0.5 µl probe, 2 µl first-strand cDNA template and 5.9 µl distilled water. The thermocycling parameters were set as follows: 95 °C for 15 min to activate the enzyme, 45 cycles of 95 °C for 25 s followed by 60 °C for 60 s. RNU6-1 RNA (U6) was considered as a housekeeping gene for the quantification of miRNAs. Expression of Ppia and H2A histone family, member Z were compared to select the most stable gene as housekeeping. Ppia was used for the normalization of expression of mRNAs. Using the $2^{-\Delta Ct}$ formula, the relative expression of genes was calculated.

Gene ontology and pathway enrichment

Presumptive targets of let-7a and miR-21 were predicted by four algorithms: TargetScan (http://www.targetscan.org), Mirwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) and miRDB (http://www.mirdb.org/miRDB/), MicroT-CDs (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index). Targets of each miRNA (retrieved from different algorithms) were unified, and duplicates were omitted. Transcriptome profiles of pre-implantation mouse embryos from two previously published studies (Hamatani et al. 2004; Zeng et al. 2004) were integrated and utilized as a background list. Common genes between presumptive targets and background lists were utilized for gene ontology (GO) annotation and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis by Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov). The Benjamini correction was applied to adjust the *P*-value for multiple comparisons. The network of the miRNAs with genes of crucial signaling pathways, proposed in KEGG analysis, was drawn by Cytoscape (version 3.4.0, Cluepedia plugin).

Statistical analysis

Kruskal–Wallis test was utilized for comparison of all groups. Pairwise comparisons were performed by Mann–Whitney U test and Bonferroni correction was considered for estimation of significance threshold. All data were presented as mean ± standard error of the mean. GraphPad Prism 6.01 was used for statistical analysis and presentation of data.

Results

In this study, 500 and 850 embryos (8-cell and blastocysts, respectively) were used. The survival rates after warming were about 93% and 89% for

Table 1 Sequence of oligonucleotides

Gene	Oligonucleotide type	Sequence (5′–3′)
mmu-miR-21-5p	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
	Forward primer	GCCCTAGCTTATCAGACTG
	Reverse primer	TGCAGGGTCCGAGGTA
	Taq-Man probe	FAM-TGTTGAGTCGTATCCAGTGCG-BHQ1
mmu-miR-93-5p	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT
	Forward primer	GGGCAAAGTGCTGTTCG
	Reverse primer	TGCAGGGTCCGAGGTA
	Taq-Man probe	FAM-CAGGTAGGTCGTATCCAGTGCG-BHQ1
mmu-miR-24-3p	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGTTC
	Forward primer	GCTTGTGGCTCAGTTCAG
	Reverse primer	GCAGGGTCCGAGGTATTC
	Taq-Man probe	FAM-AGGAACAGGTCGTATCCAGTGCG-BHQ1
mmu-let-7a-5p	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTATAC
	Forward primer	GGTCACTGAGGTAGTAGG
	Reverse primer	CAGGGTCCGAGGTATTC
	Taq-Man probe	FAM-TGTATAGTTGTCGTATCCAGTGCG-BHQ1
U6	Forward primer	GCTTCGGCAGCACATATAC
	Reverse primer	ATTTGCGTGTCATCCTTGC
	Taq-Man probe	FAM-CAGG**G**GCCATGCTAATCTTCTCT-BHQ1
Ago2	Forward primer	CCAAAGAAATCAAAGGTCTAAAGG
	Reverse primer	ACTGTCTGCCCACTCTCC
Cd44	Forward primer	GCTCTGATTCTTGCCGTCTG
	Reverse primer	CACTGGGTTTCCTGTCTTCC
Cdx2	Forward primer	GAAACCTGTGCGAGTGGATG
	Reverse primer	AGCCGCTGATGGTCTGTG
Dgcr8	Forward primer	TGGGAAGTCTGAGGTTTGC
	Reverse primer	CCGTAAGTCACACCATCAATG
Dicer1	Forward primer	GCGGCTTGAGATGCTTGG
	Reverse primer	AGGCGATACAGGTTACAGTTG
Drosha	Forward primer	GCCTACCAACTTCTTAACTGAC
	Reverse primer	ATCGTGTAGTCTATGTTGAATCG
Xpo5	Forward primer	TCTATGCTTCGTGTCTTCGTG
	Reverse primer	GATGCTCCTCTGGTTGATGAC
Itgb3	Forward primer	AGTGTCTGGCTGTGAGTCC
	Reverse primer	GGTTCTCCTTCAGGTTACATCG
Stat3	Forward primer	CCTGGCACCTTGGATTGAG
	Reverse primer	TGCTGATAGAGGACATTGGAC
H2afz	Forward primer	GCTGGTGGTGGTGTCATC
	Reverse primer	GCCTCCAACTTGCTCAAATAG
Ppia	Forward primer	CAGACAAAGTTCCAAAGACAGC
	Reverse primer	GCCGCCAGTGCCATTATG

Ago2, Argonaute 2; Cdx2, Caudal-type homeobox 2; Dgcr8, DiGeorge Syndrome Critical Region 8; Xpo5, Exportin-5; Itgb3, integrin β3; Stat3, signal transducer and activator of transcription 3; H2afz, H2A histone family, member Z; Ppia, peptidylprolyl isomerase A; **G** was replaced by deoxyinosine during synthesis

8-cell embryos and blastocysts. So, 480 and 800 embryos (8-cell and blastocysts, respectively) were selected for RNA isolation.

Expression of miRNA biogenesis genes in vitrified and fresh mouse embryos

No difference in expression levels of miRNA biogenesis genes was observed in fresh embryos compared with vitrified ones in both 8-cell and blastocysts groups. Comparing fresh 8-cell embryos and blastocysts, *Xpo5* showed significantly decreased levels ($P = 0.015$) in blastocysts versus 8-cell embryos (Fig. 1). The expression of *Dicer* and *Ago2* in blastocyst embryos was reduced but did not reach the significance threshold. *Dgcr8* and *Drosha* expression did not reveal any significant changes (Fig. 1).

Expression of miRNAs and their target genes in vitrified and fresh mouse embryos

Expression levels of miR-21 and let-7a were significantly down-regulated in vitrified 8-cell embryos compared with fresh 8-cell embryos ($P = 0.007$ and $P = 0.014$, respectively). Relative expression of miR-93 was increased in vitrified blastocysts versus fresh ones but did not reach the significance threshold ($P = 0.08$). Comparing fresh 8-cell embryos and blastocysts, miR-21, and let-7a showed significantly reduced expression levels in blastocysts ($P = 0.0003$ and $P = 0.004$, respectively) (Fig. 2).

No expression of *Cd44* and integrin β3 (*Itgb3*) was detected in 8-cell embryos and blastocysts. Caudal-type homeobox 2 (*Cdx2)* expression level did not show any difference between studied groups, but the relative expression of the signal transducer and activator of transcription 3 (*Stat3)* was significantly decreased in vitrified blastocysts versus fresh blastocysts ($P = 0.002$) (Fig. 3).

Functional analysis of predicted targets of altered miRNAs

Predicted targets of miRNAs were retrieved from four databases (Targetscan, Mirtarget2, Mirwalk, and MicroT-CDs). After the integration of predicted targets and omission of duplicates, a list of targets was allocated to each miRNA. Predicted targets of miR-21 and let-7a were compared with common expressed transcripts in mouse embryos from two reports (background gene list) (Hamatani et al. 2004;

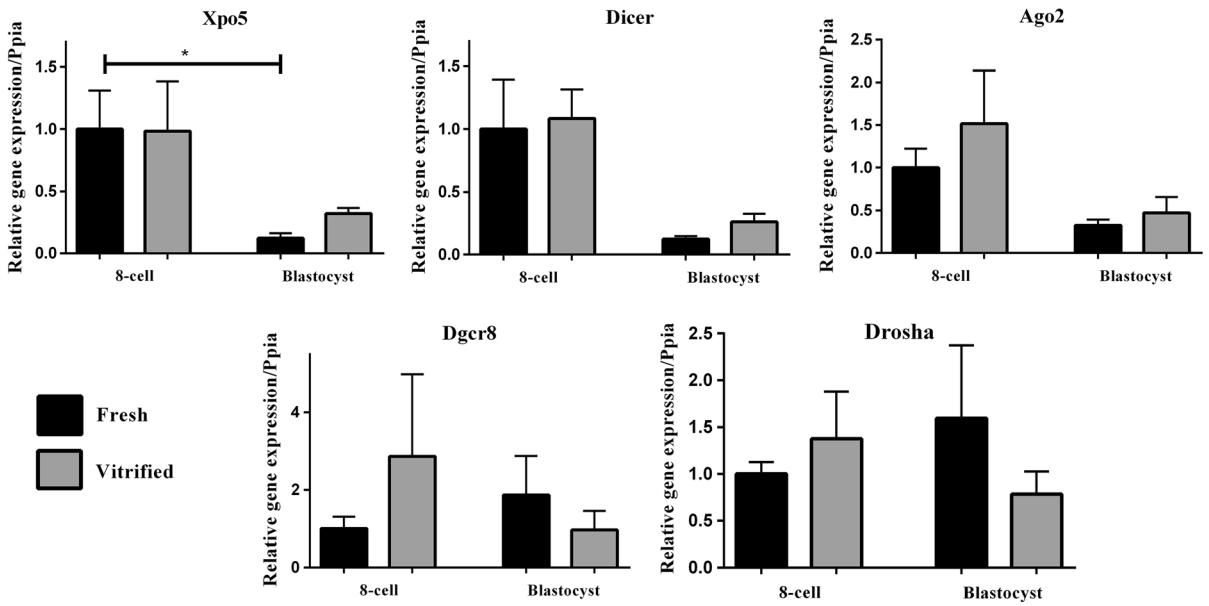

Fig. 1 Expression of miRNA biogenesis genes in fresh and vitrified 8-cell embryos and blastocysts. No difference significant was observed between fresh and vitrified groups. Exportin-5 (*Xpo5*) was significantly decreased in fresh blastocysts versus fresh 8-cell embryos ($P = 0.015$)

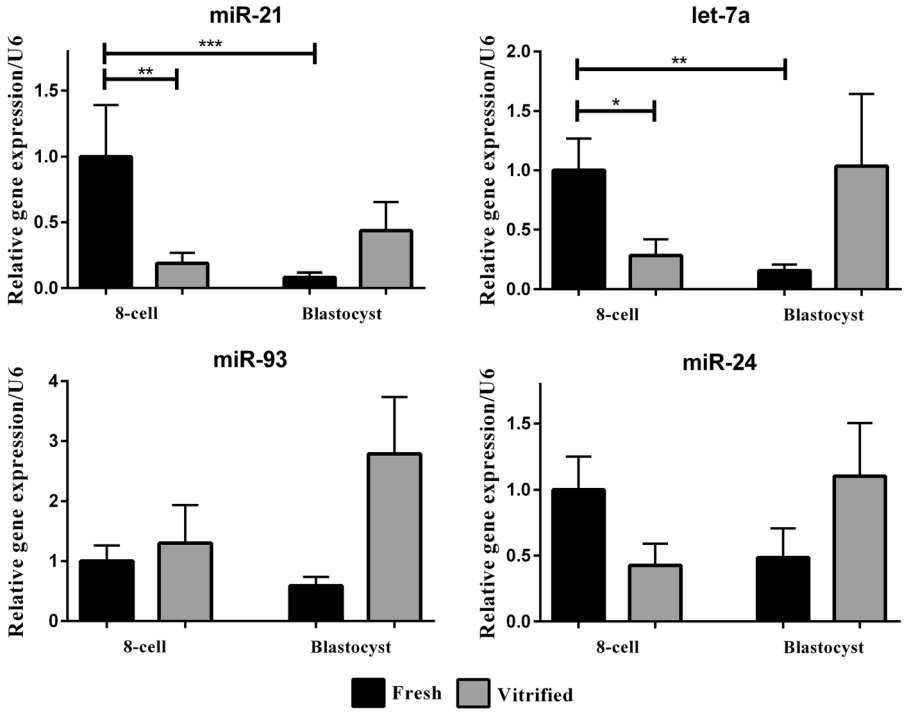

Fig. 2 Expression of miR-21, let-7a, miR-93, and miR-24 in fresh and vitrified 8-cell embryos and blastocysts. Relative expression of miR-21 and let-7a was significantly decreased in vitrified 8-cell embryos compared with fresh 8-cell embryos ($P = 0.007$ and $P = 0.014$, respectively). Relative expression of miR-21 and let-7a was significantly reduced in fresh blastocysts versus fresh 8-cell embryos ($P = 0.0003$ and $P = 0.004$, respectively)

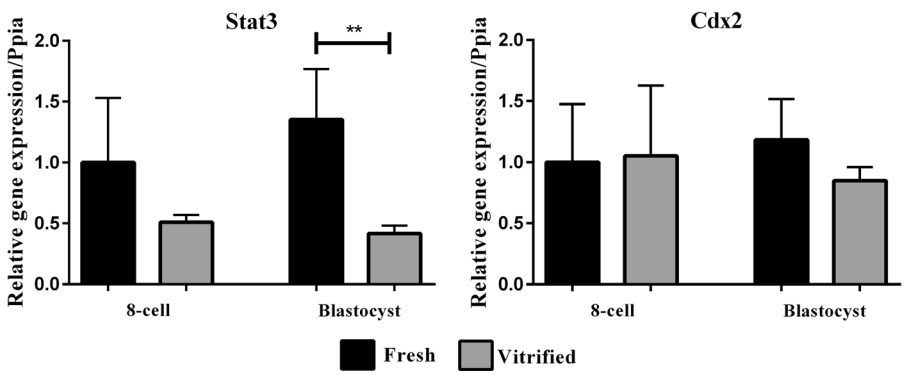

Fig. 3 Expression of presumptive targets of miRNAs in fresh and vitrified 8-cell embryos and blastocysts. Relative expression of the *Stat3* was significantly decreased in vitrified blastocysts versus fresh blastocysts ($P = 0.002$)

Zeng et al. 2004), and only common genes were used for functional analysis.

Using DAVID, gene ontology (GO) analysis of predicted targets of each miRNA for biological processes was performed. The top ten biological processes of miRNAs were listed in Table 2 after the exclusion of obsolete and redundancy terms. KEGG pathway enrichment analysis proposed several signaling pathways with essential roles in implantation and early development, such as MAPK signaling pathway, Neurotrophin signaling pathway, PI3K-Akt signaling pathway, mTOR signaling pathway, Wnt signaling pathway, Hippo signaling pathway (Table 3). The network of differentially expressed miRNAs with predicted targets belonging to these signaling pathways could reveal the immense

Table 2 Gene Ontology analysis of putative target genes for miR-21 and let-7a

microRNA	GO term	Gene count	*P*-value
miR-21	Regulation of transcription, DNA-templated	58	8.40E−05
	Protein phosphorylation	20	2.80E−02
	Blood vessel remodeling	6	4.30E−02
	Heart development	12	8.50E−02
	Outflow tract morphogenesis	6	9.60E−02
	Positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	4	1.20E−01
	Regulation of translation	8	1.10E−01
	T cell differentiation	5	2.10E−01
	Covalent chromatin modification	11	2.10E−01
	Cell fate commitment	6	2.10E−01
let-7a	Regulation of transcription, DNA-templated	102	1.80E−03
	Covalent chromatin modification	22	1.60E−02
	Protein phosphorylation	35	2.30E−02
	mRNA transport	11	8.00E−02
	Regulation of translation	12	1.80E−01
	Protein transport	32	1.80E−01
	Multicellular organism development	45	5.40E−01
	Stem cell population maintenance	8	5.20E−01
	In utero embryonic development	18	5.50E−01
	Negative regulation of stem cell differentiation	4	6.30E−01

GO analysis was performed by DAVID. The top ten GO terms were listed in the table. *P*-value was depicted after adjustment with the Benjamini method

GO, Gene ontology

capability of a few miRNAs for targeting and modulating different signaling pathways (Fig. 4).

Discussion

In this study, to our knowledge, we reported the first experimental investigation of the influence of vitrification as a widely used procedure in ART, on the expression of the miRNA biogenesis pathway in preimplantation mouse embryos. It has been shown that the expression levels of most miRNAs and also the canonical pathway of miRNA biogenesis in preimplantation embryos were stage-dependent (García-López and Del Mazo 2012). So, it seems that the proper function of miRNAs processing machinery plays an essential role in embryonic development and differentiation. In this study, we found that the mRNA expression levels of miRNA biogenesis pathway elements in fresh 8-cell embryos and blastocysts were different, while vitrification could not affect the miRNA biogenesis pathway in both developmental stages of preimplantation mouse embryos. Nevertheless, the expression of miR-21, and let-7a, which play important roles in embryo development and implantation, were changed after vitrification and warming of preimplantation embryos.

We did not observe any specified changes in the expression levels of *Drosha* and *Dgcr8* in 8-cell embryos and blastocysts, but the expression of *Dicer*, *Xpo5*, and *Ago2* was decreased in blastocysts compared with 8-cell embryos. There is only one previous study that reported the expression of the miRNA biogenesis pathway in preimplantation mouse embryos (García-López and Del Mazo 2012). Similar to the findings of García-López and colleagues, we found similar alterations in the expression pattern of miRNA biogenesis genes, except for *Dgcr8*, in 8-cell

Table 3 KEGG pathway analysis of putative target genes for miR-21 and let-7a

miRNAs	KEGG pathways	Gene count	*P*-value
miR-21	MAPK signaling pathway	14	2.70E−03
	Rap1 signaling pathway	10	1.30E−01
	Endocytosis	10	2.30E−01
	Ras signaling pathway	9	2.70E−01
	Neurotrophin signaling pathway	6	3.60E−01
	Chemokine signaling pathway	7	5.10E−01
	T cell receptor signaling pathway	5	4.90E−01
	Dorso-ventral axis formation	3	4.70E−01
	Prolactin signaling pathway	4	4.90E−01
	PI3K-Akt signaling pathway	9	5.20E−01
let-7a	MAPK signaling pathway	16	7.30E−01
	Wnt signaling pathway	11	5.60E−01
	FoxO signaling pathway	10	5.70E−01
	Lysine degradation	6	5.20E−01
	Signaling pathways regulating pluripotency of stem cells	10	4.90E−01
	Lysosome	9	4.90E−01
	mTOR signaling pathway	6	4.90E−01
	Hippo signaling pathway	10	4.90E−01
	Dorso-ventral axis formation	4	5.00E−01
	Progesterone-mediated oocyte maturation	7	5.20E−01

KEGG analysis was performed by DAVID. Top ten KEGG results after the omission of cancer-related pathways were listed in the table. *P*-value was depicted after adjustment with the Benjamini method

KEGG, Kyoto Encyclopedia of Genes and Genomes

embryos compared to blastocysts. Although they only detected *Drosha* in blastocysts, our results in conjunction with some previous reports reinforce the presence of mRNAs of miRNA biogenesis genes in blastocysts (Cheong et al. 2014; Cui et al. 2007; Shen et al. 2010). These discrepancies may originate from the increased number of embryos allocated to each pool for qRT-PCR (50 blastocysts in the current study) or different efficiencies of PCR reactions. We also showed that the mRNA expression levels of miRNA biogenesis genes were not significantly altered in vitrified 8-cell embryos and blastocysts. So, cryopreservation of embryos by vitrification method did not influence the canonical biogenesis pathway of miRNAs in 8-cell embryos and blastocysts. In our previous study, we also showed that the IVF procedure did not change the canonical biogenesis pathway of miRNAs in preimplantation mouse embryos (Azizi et al. 2019).

In our study, we selected four important miRNAs (miR-21, let-7a, miR-93, and miR-24), which have previously characterized roles in mouse embryo development and differentiation. We noticed that the expression of miR-21 was significantly higher in 8-cell embryos compared to blastocysts. Moreover, the vitrification of 8-cell embryos led to the down-regulation of the expression of miR-21. It has been reported that miR-21 has essential roles in DNA repair (Tulay et al. 2015), trophectoderm specification (Viswanathan et al. 2009), and apoptosis (Zhang et al. 2015). We found that the expression level of let-7a was higher in 8-cell embryos versus blastocysts. Vitrification of blastocysts did not exert any effect on the expression level of let-7a, but vitrified 8-cell embryos showed down-regulation of let-7a when compared with fresh 8-cell embryos. Consistently, expression of let-7a was revealed to be diminished in blastocysts compared to previous developmental stages (Cheong et al. 2014; García-López and Del Mazo 2012). It was shown that the expression of let-7a deceased in IVF blastocysts compared to in vivo embryos (Azizi et al. 2019). Let-7a may regulate mouse embryo implantation in blastocysts (Cheong et al. 2014). Besides, the let-7 family of miRNAs has been suggested to be effective factors in trophectoderm specification (Viswanathan et al. 2009). The reduction of let-7a levels in blastocyst may promote the activation of blastocyst and initiation of implantation (Liu et al. 2012a). Hence, premature reduction of let-7a in 8-cell embryos after vitrification may disturb the prerequisite molecular events for proper

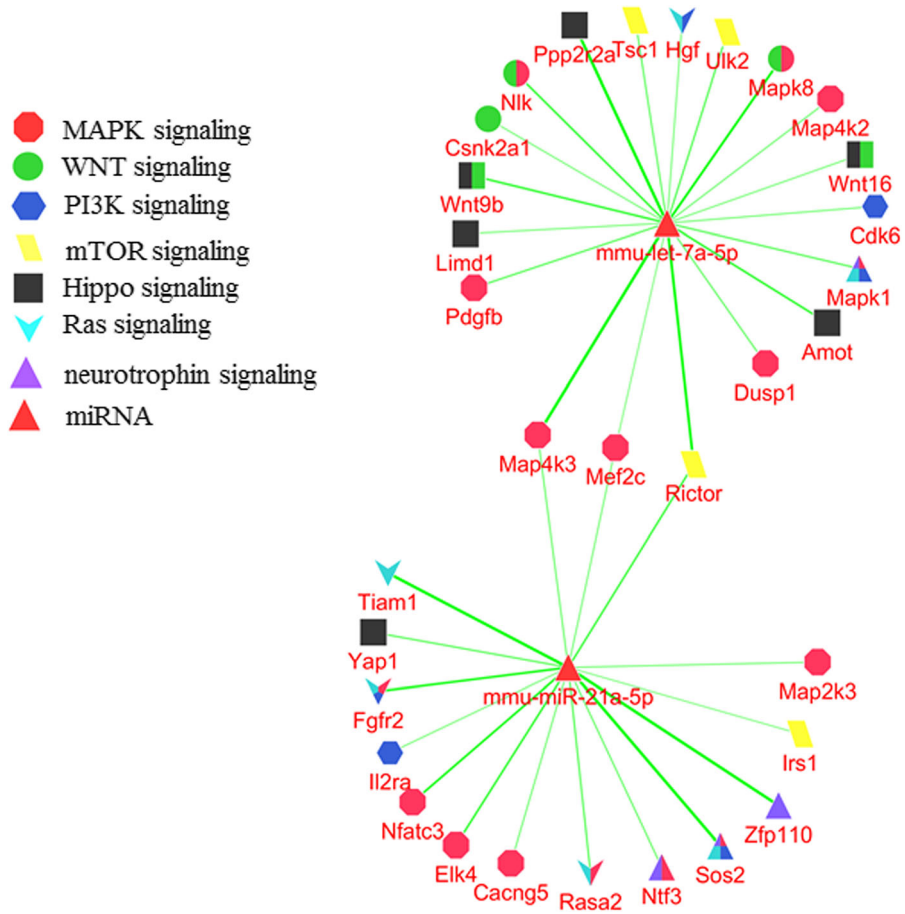

Fig. 4 Network of miR-21 and let-7a with key signaling pathways from KEGG enrichment analysis. The network was made using Cluepedia plugin in Cytoscape software. Nodes represent octagon for MAPK signaling pathway, ellipse for WNT signaling pathway, hexagon for PI3K-Akt signaling pathway, parallelogram for mTOR signaling pathway, rectangle for Hippo signaling pathway, V for Ras signaling pathway, purple triangle for neurotrophin signaling pathway, red triangle for miRNAs and edges for interactions. (Color figure online)

implantation. Moreover, the expression level of miR-93 was similar in 8-cell embryos and blastocysts. Vitrification did not affect the expression level of miR-93 in 8-cell embryos, but vitrified blastocysts expressed higher level of miR-93 in comparison to fresh blastocysts. The up-regulation of miR-93 in vitrified blastocysts was associated with down-regulation of *Stat3*. In agreement with our findings, previous study by Foshy and co-workers has experimentally verified that *Stat3* mRNA is a target of miR-93, and miR-93 may down-regulate *Stat3* in blastocysts (Foshay and Gallicano 2009). *Stat3*, a component of the JAK-STAT signaling pathway, plays significant roles in embryonic development and differentiation. In this regard, *Stat3* has been demonstrated as an, essential transcription factor in early development of mouse embryo, as its disruption was reported to be associated with embryonic lethality (Takeda et al. 1997). In this study, we observed changes in the expression of mature miRNAs in vitrified embryos compared to fresh ones while the expression levels of miRNA biogenesis genes remained unchanged. Regulatory mechanisms may directly control the level of mature miRNAs (Lee et al. 2008). So, the expression of mature miRNAs in a tissue- or stage-specific manner may not be associated with concordant changes in the expression level of pri- and pre-miRNAs. Various environmental and cellular conditions may affect decay processes of mature miRNAs which uncouple their cellular levels with

their corresponding precursors' expression (Rüegger and Großhans 2012).

KEGG analysis of predicted targets of miR-21 and let-7a indicated several critical signaling pathways. Among all the enriched pathways, MAPK signaling pathway (Maekawa et al. 2005; Zhang et al. 2007), Neurotrophin signaling pathway (Kawamura et al. 2007, 2009), PI3K-Akt signaling pathway (Riley et al. 2005; Zhang et al. 2007), mTOR signaling pathway (Gangloff et al. 2004; Murakami et al. 2004), Wnt signaling pathway (Xie et al. 2008; Zhang et al. 2007), Hippo signaling pathway (Nishioka et al. 2009; Sasaki 2017), and Ras signaling pathway (Lu et al. 2008; Yang et al. 2006), have been proposed to play essential roles in implantation and early development of embryos.

Conclusions

In this study, we found that the vitrification of preimplantation mouse embryos could deregulate the expression of miRNAs, while their biogenesis pathway elements were unaffected. More noticeable changes were observed in the expression of miRNAs (miR-21, let-7a) in 8-cell embryos. Deviation of miRNAs' expression, due to their mode of action, can be associated with dramatic downstream changes in critical developmental signaling pathways. Therefore, vitrification in blastocysts may lead to less detrimental effects on the developmental and implantation competence of embryo, comparing to 8-cell embryos.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate No human participants or human tissue were involved in this study. All animal procedures were performed according to the guidelines of Shahid Beheshti University of Medical Science. This study was approved by Shahid Beheshti University of Medical Science ethics committee at Deputy of Research and Technology (IR.SBMU. MSP.REC.1396.220).

References

Azizi E, Ghaffari Novin M, Naji M, Amidi F, Shams Mofarahe Z (2019) Dose in vitro fertilization affect the expression of miRNAs and their biogenesis pathway in preimplantation mouse embryos? Birth Defects Res 112:62–70

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

Bernstein E et al (2003) Dicer is essential for mouse development. Nat Genet 35:215

Chatzimeletiou K et al (2011) Cytoskeletal analysis of human blastocysts by confocal laser scanning microscopy following vitrification. Hum Reprod 27:106–113

Cheong AW, Pang RT, Liu W-M, Kottawatta KSA, Lee K-F, Yeung WS (2014) MicroRNA Let-7a and dicer are important in the activation and implantation of delayed implanting mouse embryos. Hum Reprod 29:750–762

Cui X-S, Shen X-H, Kim N-H (2007) Dicer1 expression in preimplantation mouse embryos: Involvement of Oct3/4 transcription at the blastocyst stage. Biochem Biophys Res Commun 352:231–236

Cuman C et al (2015) Human blastocyst secreted microRNA regulate endometrial epithelial cell adhesion. EBioMedicine 2:1528–1535

Fabian D, Gjørret JO, Berthelot F, Martinat-Botté F, Maddox-Hyttel P (2005) Ultrastructure and cell death of in vivo derived and vitrified porcine blastocysts . Mol Reprod Dev Inc Gamete Res 70:155–165

Foshay KM, Gallicano GI (2009) miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. Dev Biol 326:431–443

Galliano D, Pellicer A (2014) MicroRNA implantation. Fertil Steril 101:1531–1544

Gangloff Y-G et al (2004) Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol Cell Biol 24:9508–9516

García-López J, Del Mazo J (2012) Expression dynamics of microRNA biogenesis during preimplantation mouse development . Biochim Biophys Acta (BBA) Gene Regul Mech 1819:847–854

Gerris J, De Neubourg D, De Sutter P, Van Royen E, Mangelschots K, Vercruyssen M (2003) Cryopreservation as a tool to reduce multiple birth. Reprod Biomed Online 7:286–294

Gómez E, Muñoz M, Rodríguez A, Caamaño J, Facal N, Díez C (2009) Vitrification of bovine blastocysts produced in vitro inflicts selective damage to the inner cell mass. Reprod Domest Anim 44:194–199

Hamatani T, Carter MG, Sharov AA, Ko MS (2004) Dynamics of global gene expression changes during mouse preimplantation development. Dev Cell 6:117–131

Hossain M, Salilew-Wondim D, Schellander K, Tesfaye D (2012) The role of microRNAs in mammalian oocytes and embryos. Anim Reprod Sci 134:36–44

Kawamura K, Kawamura N, Fukuda J, Kumagai J, Hsueh AJ, Tanaka T (2007) Regulation of preimplantation embryo development by brain-derived neurotrophic factor. Dev Biol 311:147–158

Kawamura K, Kawamura N, Sato W, Fukuda J, Kumagai J, Tanaka T (2009) Brain-derived neurotrophic factor promotes implantation and subsequent placental development by stimulating trophoblast cell growth survival. Endocrinology 150:3774–3782

Lee EJ, Baek M, Gusev Y, Brackett DJ, Nuovo GJ, Schmittgen TD (2008) Systematic evaluation of microRNA processing patterns in tissues, cell lines and tumors. RNA 14:35–42

Li L, Zhang X, Zhao L, Xia X, Wang W (2012) Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing . Mol Reprod Dev 79:229–236

Liu W-M, Pang RT, Cheong AW, Ng EH, Lao K, Lee K-F, Yeung WS (2012a) Involvement of microRNA lethal-7a in the regulation of embryo implantation in mice. PLoS ONE 7:e37039

Liu W-M, Pang RT, Chiu PC, Wong BP, Lao K, Lee K-F, Yeung WS (2012b) Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci 109:490–494

Lu C-W, Yabuuchi A, Chen L, Viswanathan S, Kim K, Daley GQ (2008) Ras-MAPK signaling promotes trophectoderm formation from embryonic stem cells and mouse embryos. Nat Genet 40:921

Maekawa M, Yamamoto T, Tanoue T, Yuasa Y, Chisaka O, Nishida E (2005) Requirement of the MAP kinase signaling pathways for mouse preimplantation development. Development 132:1773–1783

Mamo S, Bodo S, Kobolak J, Polgar Z, Tolgyesi G, Dinnyes A (2006) Gene expression profiles of vitrified in vivo derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. Mol Reprod Dev Inc Gamete Res 73:1380–1392

Murakami M et al (2004) mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol 24:6710–6718

Nishioka N et al (2009) The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell 16:398–410

Paul AB, Sadek ST, Mahesan AM (2018) The role of microRNAs in human embryo implantation: a review. J Assist Reprod Genet 36:1–9

Rienzi L et al (2017) Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update 23:139–155

Riley JK, Carayannopoulos MO, Wyman AH, Chi M, Ratajczak CK, Moley KH (2005) The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo. Dev Biol 284:377–386

Rüegger S, Großhans H (2012) MicroRNA turnover: when, how, and why. Trends Biochem Sci 37:436–446

Sasaki H (2017) Roles and regulations of Hippo signaling during preimplantation mouse development. Dev Growth Differ 59:12–20

Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008) Widespread changes in protein synthesis induced by microRNAs. Nature 455:58

Shen X-H, Han Y-J, Cui X-S, Kim N-H (2010) Ago2 and GW182 expression in mouse preimplantation embryos: a link between microRNA biogenesis and GW182 protein synthesis. Reprod Fertil Dev 22:634–643

Stinshoff H, Wilkening S, Hanstedt A, Brüning K, Wrenzycki C (2011) Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level. Theriogenology 76:1433–1441

Takeda K et al (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci 94:3801–3804

Tulay P, Naja R, Cascales-Roman O, Doshi A, Serhal P, Sen-Gupta S (2015) Investigation of microRNA expression and DNA repair gene transcripts in human oocytes and blastocysts. J Assist Reprod Genet 32:1757–1764

Virant-Klun I, Ståhlberg A, Kubista M, Skutella T (2016) MicroRNAs: from female fertility, germ cells, and stem cells to cancer in humans. Stem Cells Int 2016:1–17

Viswanathan SR, Mermel CH, Lu J, Lu C-W, Golub TR, Daley GQ (2009) microRNA expression during trophectoderm specification. PLoS ONE 4:e6143

Wienholds E, Koudijs MJ, van Eeden FJ, Cuppen E, Plasterk RH (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet 35:217

Xie H et al (2008) Inactivation of nuclear Wnt-β-catenin signaling limits blastocyst competency for implantation. Development 135:717–727

Yang W et al (2006) An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival. Dev Cell 10:317–327

Yang Y et al (2008) Determination of microRNAs in mouse preimplantation embryos by microarray . Dev Dyn Off Publ Am Assoc Anat 237:2315–2327

Yao J et al (2017) Effect of vitrification on in vitro development and imprinted gene Grb10 in mouse embryos. Reproduction 154:197–205

Zeng F, Baldwin DA, Schultz RM (2004) Transcript profiling during preimplantation mouse development. Dev Biol 272:483–496

Zhang C et al (2015) The anti-apoptotic role of berberine in preimplantation embryo in vitro development through regulation of miRNA-21. PLoS ONE 10:e0129527

Zhang Y, Yang Z, Wu J (2007) Signaling pathways and preimplantation development of mammalian embryos. FEBS J 274:4349–4359

Zhao X, Hao H, Du W, Zhu H (2015) Effect of vitrification on the microRNA transcriptome in mouse blastocysts. PLoS ONE 10:e0123451