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GAMETE BIOLOGY

Enhancement of the efficiency of oocyte vitrification through regulation of histone deacetylase 6 expression

Yao Wang[1,2] · Mei-lin Zhang[2,3] · Lei-wen Zhao[1] · Yan-ping Kuang[1] · Song-guo Xue[1]

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Abstract

Objective Successful oocyte vitrification (OV) is critical for cryopreservation of the oocytes from female patients with infertility, polycystic ovaries, and gynecologic cancers. Recent evidence suggests that relatively low levels of histone acetylation are critical for maintenance of the maturation capacity of cryopreserved oocytes. However, previous studies have only demonstrated a key role of histone deacetylases (HDAC) 1 and 2 in the cryopreservation of oocytes.

Methods In this study, we investigated the role of HDAC6 in these settings. We found that mouse oocytes with low HDAC6 levels decreased survival rate, cleavage rate, and blastocyst rate after OV. Bioinformatics analyses were used to predict HDAC6-targeting microRNAs (miRNAs), while the functional binding of miRNAs to HDAC6 mRNA was evaluated by a dual luciferase reporter assay.

Results Among all HDAC6-targeting miRNAs, we detected expression of miR-558, miR-527, and miR-762 in mouse oocytes. Specifically, we found that only miR-762 significantly inhibited protein translation of HDAC6 via binding to the 3′-UTR of the HDAC6 mRNA. Transfection of oocytes with HDAC6 or antisense of miR-762 significantly increased the survival rate, the cleavage rate, and blastocyst rate after OV.

Conclusion As a result, our data suggest that induction of HDAC6 levels by miR-762 suppression may improve the current protocol for OV.

Keywords miR-337-3p · Histone deacetylases 6 (HDAC6) · Oocyte vitrification (OV)

Introduction

Oocyte cryopreservation plays an important role in assisted reproductive technology, which provides benefits to patients with infertility, polycystic ovaries, and gynecologic cancers [1]. The vitrification method is superior to conventional slow freezing technologies in reducing chilling damage to oocytes during cryopreservation [1]. It is still a matter of debate whether oocytes should be vitrified before or after in vitro maturation. A previous study reported that porcine oocytes vitrified at germinal vesicle (GV) stage have a lower survival rate than those vitrified at metaphase II (MII) stage [2]. Another study showed that vitrification of in vitro matured MII human oocytes yielded a higher efficiency than GV oocytes [3]. However, several studies provide conflicting results. For example, in terms of fertilization rate, no significant difference was noted between vitrified in vitro matured MII and GV mouse oocytes [4].

MicroRNAs (miRNAs) are non-coding small RNAs that mediate post-transcriptional gene regression, mainly through Watson-Crick pairing to the 3′-untranslated region (3′-UTR) of the mRNA of a specific gene. Previous studies demonstrate that miRNAs play critical roles in a variety of physiological and pathological events [5–8]. However, the regulation of HDAC6 by miRNAs has been rarely studied [9, 10].

In this study, we assessed the role of HDAC6 in protecting mouse oocytes from chilling damage during cryopreservation. We found that oocytes with low HDAC6 levels had a decreased survival rate and blastocyst formation rate than those

Yan-ping Kuang and Song-guo Xue contributed equally to this work.

✉ Song-guo Xue
488984286@qq.com

[1] Department of Assisted Reproduction, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, China

[2] Center of Reproductive Medicine, Renji Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200135, China

[3] Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200135, China

with high HDAC6 levels. Bioinformatics analysis was performed to predict HDAC6-targeting miRNAs. miR-762 was found to suppress protein translation of HDAC6 mRNA. Transfection of oocytes with HDAC6-expressing plasmid or anti-miR-762 significantly increased the survival and blastocyst formation rates after cryopreservation.

Materials and methods

Protocol approval

All the experimental methods in the current study have been approved by the research committee at Shanghai Jiao Tong University. All the experiments have been carried out in accordance with the guidelines from the research committee at Shanghai Jiao Tong University. Mice experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (No. 2017-389).

Experimental design

Firstly, we divided some mice into two groups. In each group, we conducted a HDAC6 test as well as cryopreservation, thawing, mice IVF, and development observation. The HDAC6 level and developing potential after vitrification in each group were tested.

Secondly, we did transfection experiments. Three kinds of different miRNA and as-miRNA were microinjected into mice oocytes, followed by HDAC6 3′-UTR transfection experiment.

Thirdly, in our study, miR-762, null, and as-miR-762 were microinjected into the oocytes, then HDAC6 mRNA and protein level were tested.

Fourthly, hundreds of oocytes were recruited in this part. There were four groups in this test: untreatment, null plasmid, transfected with HDAC6, and transfected with as-miR-762. The miR-762 levels and HDAC6 protein levels were tested in four groups. The oocyte cryopreservation, thawing, mice IVF, and developing potential were observed.

There was a total of 2434 oocytes that came from 89 C57/BL6 female mice and 6 male mice being used in this study.

Mouse oocyte collection

Oocytes were collected from 16-week-old female pregnant C57/BL6 mice that were superovulated with subcutaneous injection of 10 IU (i.p.) pregnant mare's serum gonadotropin (PMSG) (Sigma-Aldrich, Shanghai, China) followed by 10 IU hCG (Sigma-Aldrich) 48 h later. Fourteen hours after hCG injection, oocytes were obtained from the oviducts and put into M2 medium. The cumulus-oocyte complexes (COCs) were released by tearing the ampullae of the oviducts. The cumulus cells were removed enzymatically using 75 U/mL hyaluronidase and by mechanical dissociation using a glass pipette. The denuded oocytes were then washed with m-HTF-HEPES at room temperature before being submitted to cryopreservation experiments. Only morphologically normal metaphase II (MII) stage oocytes, judged by the presence of a visible first polar body (PB1), were selected for the study. All the mice came from pathogen-free laboratory of Shanghai Animal Research Center. The mice were housed in a temperature- and light-controlled room with free access to food and water under a photoperiod of 12 h in the light and 12 h in the dark. The experimental protocols and animal handling procedures were reviewed and approved by Animal Ethics Committee of Jiao Tong University. All animals received humane care during the study protocol and euthanasia.

Cryopreservation of oocytes

Vitrification solutions have been described previously in another article by Dr. Wang [11]. Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) was diluted to 15 and 15% (*v*/*v*) in Ca2+-free M2 medium containing 30% (*w*/*v*) Ficoll (FW 70000) and 0.5 M sucrose. Oocyte freezing was performed within 30 min after collection. Cryotop (KITAZATO corporation, Japan) was used for vitrification, 5–6 oocytes per straw, 40–50 oocytes per load. Vitrification media and oocytes were maintained at 37 °C on a warming plate (Wenesco, Inc., Chicago, IL, USA). Oocytes were vitrified in vitrification solutions, and the straws were then immediately plunged into liquid nitrogen (LN2). After storage for no less than 24 h in LN2, oocytes were removed for warming. The oocytes were released and kept in 0.5 mol/L sucrose for 5 min. Subsequently, the oocytes were placed into 100 μl of M2 droplets in a petri dish (35 × 10 mm, Corning Incorporated, Corning, NY, USA) and incubated in a CO_2 incubator for 30 min before fertilization procedure. The protocol was the same as before which was mentioned by Prof. RC Chian [12].

Part I

A total of 605 mouse oocytes from 25 mice were used in this first part of experiment. The mice were separated into two groups randomly, group 1 (12 mice) and group 2 (13 mice). Each mice's oocytes were divided into two parts, one section for the cryopreservation, thawing, mice IVF, and developing observation (203 oocytes) (Fig. 1), and the other section for HDAC6 test (402 oocytes). Four hundred two oocytes were extracted and analyzed by Western blot for HDAC6. We compared HDAC6 levels within two groups. Based on median HDAC6 levels, the oocytes had different HDAC6 levels, we named them as HDAC6-high group and HDAC6-low group, p value was 0.031 between two groups. In this section, we proceeded vitrification for all 203 oocytes right after we sacrificed all the female mice. When we got the HDAC6 results, we did thawing procedure for the two groups, following

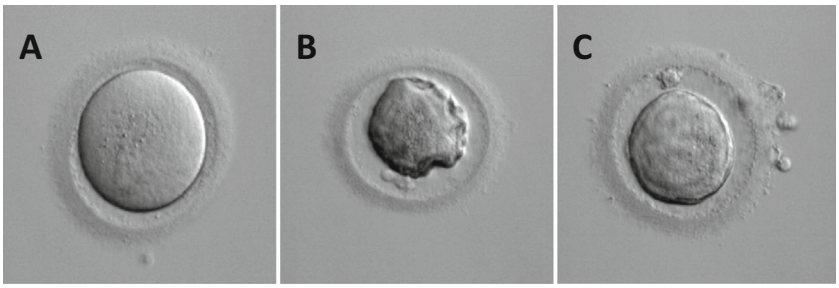

Fig. 1 Photographs of oocytes fresh before vitrification (**a**), during vitrification procedure (**b**), and frozen-thawed (**c**)

by mice IVF. The survival oocytes, cleavage, and blastocyst formation results were collected.

Part II

Plasmids transfection

Nine mice were recruited in a transfection experiment. There were totally 277 oocytes in this study, and 185 oocytes were microinjected with different miRNA and also as-miRNA. Eight hours later, all oocytes were tested. Three miRNAs (miR-558, miR-527, and miR-762) and as-miRNA (as-miR-558, as-miR-527, and as-miR-762) were transfected and tested in levels by RT-qPCR. HDAC6 coding sequence and a null control (null) were cloned into backbone vector (Clontech, Mountain View, CA, USA) to generate corresponding plasmids. Anti-miR-558, anti-miR-527, and anti-miR-762 inhibitors were purchased from Exiqon (Woburn, MA, USA). Sequencing was performed to confirm the correct orientation of the plasmids, which were then used to transfect the cells at a concentration of 50 nmol/l using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). To monitor transfection efficiency, Cy3-labeled negative control miRNA (Invitrogen) was transfected in parallel.

Quantitative real-time PCR

Total RNA was extracted from oocytes with miRNeasy mini kit (Qiagen, Hilden, Germany) for cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data was collected and analyzed using $2^{-\Delta\Delta}$Ct method. Values of genes were first normalized against α-tubulin and compared to experimental controls.

Western blot

Proteins were isolated from oocytes. Primary antibodies were rabbit anti-HDAC6 and anti-α-tubulin (Cell Signaling, Carpinteria, CA, USA). Secondary antibody was HRP-conjugated anti-rabbit (Dako, Carpinteria, CA, USA). Figure images were representative from five repeats. α-tubulin was used as a protein loading control.

Luciferase-reporter activity assay

There were totally 92 oocytes that came from three mice used in HDAC6 3′-UTR transfection experiment. The intact 3′-UTR of HDAC6 mRNA (HDAC6 3′-UTR), together with a 3′-UTR with mutation at either miRNA binding site of HDAC6 mRNA (HDAC6 3′-UTR mut), were cloned into luciferase reporter plasmids. Oocytes were co-transfected with 1 μg as-miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids or co-transfected with 1 μg miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids. The target gene of miRNAs was predicted by TargetScan, using the context++ score system, as described [13, 14]. The dual-luciferase reporter plasmids, p3′-UTR-HDAC6 (containing the wild-type HDAC6 3′-UTR binding site in luciferase reporter plasmid (RiboBio Co., Ltd., Shanghai, China) and p3′-UTR-HDAC6-mut (containing the mutated binding site AGGGGCACCACUACUAAGAUUAA; mut) were constructed. For the luciferase assay, the 500 ng constructed plasmid and 100 nmol/l miRNAs were co-transfected into oocytes using Lipofectamine™ 3000 Reagent (Invitrogen). The luciferase activity was detected with the dual-luciferase reporter assay system (Promega, Shanghai, China) after co-transfection cells for 48 h, with compliance of the manufacturer's protocol.

Part III

The study aimed to determine the effects of miR-762 expression on the cellular HDAC6 levels in oocytes. There were 88 oocytes that came from three mice used in RT-qPCR to investigate HDAC6 level in different miR-762-modified oocytes. There were 605 oocytes that came from 20 mice used in Western blot for HDAC6 level in miR-762-modified oocytes.

Part IV

In this part, 1372 oocytes came from 49 mice which were recruited in the test of overexpression of HDAC6 or depletion of miR-762 in oocytes for cryopreservation. We separated all the oocytes into four groups. Group 1, 335 untreated oocytes (UT); group 2, 341 oocytes transfected with null plasmid (null); group 3, 350 oocytes transfected with HDAC6 plasmids (HDAC6); and group 4, 346 oocytes transfected with as-

miR-762 (as-miR-762). We extracted RNA and protein from the oocytes. The miR-762 levels were tested by RT-qPCR, and HDAC6 levels were tested by Western blot.

Statistical analysis

SPSS version 19.0 (IBM, Armonk, NY, USA) was used in this study. All values represent the mean ± standard deviation (SD). Statistical analysis of group differences was carried out using a one-way analysis of variance (ANOVA) test, and the χ^2 Fisher's exact test was used to compare two groups of data. A value of $p < 0.05$ was considered statistically significant after Bonferroni's correction.

Results

Oocytes with low HDAC6 levels have a decreased survival rate, cleavage rate, and blastocyst rate after OV

We found that oocytes coming from the same mice group with low HDAC6 levels had significantly lower survival rates (93.5 vs 99.1%), cleavage rates (54.0 vs 73.6%), and blastocyst rates (39.7 vs 69.6%) after OV, compared to those with high HDAC6 levels (Table 1).

miR-762 targets 3′-UTR of HDAC6 mRNA to inhibit its translation in oocytes

We further explored whether miRNAs regulated HDAC6 in oocytes. We performed bioinformatics analysis to identify the candidate miRNAs that bound to the 3′-UTR of HDAC6 mRNA. Based on current progress in oocyte and embryo cryopreservation by slow freezing and vitrification [1], we found three miRNAs (miR-558, miR-527, and miR-762) that have binding sites on HDAC6 mRNA and are also expressed in oocytes. To determine whether the binding of these three miRNAs to HDAC6 mRNA may functionally inhibit protein translation of HDAC6, we transfected oocytes with miR-558, miR-527, miR-762, or their antisense inhibitors. Changes in the miRNA levels in cells were confirmed by RT-qPCR (Fig. 2a–c). Then, the intact 3′-UTR of HDAC6 mRNA (HDAC6 3′-UTR), together with a 3′-UTR with mutant at either miRNA binding site of HDAC6 mRNA (HDAC6 3′-UTR mut), was cloned into luciferase reporter plasmids. Oocytes were co-transfected with 1 μg as-miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids or co-transfected with 1 μg miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids. The results suggest that among all three miRNAs, only miR-762 may specifically target 3′-UTR of HDAC6 mRNA to inhibit its translation in oocytes (Fig. 2df), as shown by bioinformatics analyses, which suggests that miR-762 binds to 3′-UTR of HDAC6 mRNA at 254–261 base site (Fig. 2g).

miR-762 decreases HDAC6 protein levels in oocytes

We aimed to determine the effects of miR-762 expression on the cellular HDAC6 levels in oocytes. We found that changes in miR-762 levels did not alter the mRNA levels of HDAC6 (Fig. 3a), but significantly altered the HDAC6 protein (Fig. 3b). Overexpression of miR-762 reduced the cellular levels of HDAC6 by Western blot, while depletion of miR-762 augmented the cellular levels of HDAC6 (Fig. 3b).

Overexpression of HDAC6 or depletion of miR-762 improves survival rate, cleavage rate, and blastocyst rate of oocytes after OV

We examined whether overexpression of HDAC6 or depletion of miR-762 may affect the oocyte survival and developmental potential after OV. First, we prepared plasmids expressing HDAC6 or as-miR-762 or control null constructs. Secondly, we separated oocytes into four groups. Group 1, 335 untreated oocytes (UT); group 2, 341 oocytes transfected with null plasmid (null); group 3, 350 oocytes transfected with HDAC6 plasmids (HDAC6); and group 4, 346 oocytes transfected with as-miR-762. In total, 407 oocytes from four groups underwent the OV procedure, thawing, mice IVF, and developing observation. The data in regard to survival, cleavage, and blastocyst formation were collected. We extracted RNA and protein from the oocytes and confirmed the effects on miR-762 levels by RT-qPCR (Fig. 4a) and HDAC6 levels with Western blot (Fig. 4b). Moreover, we found that although neither overexpression of HDAC6 nor depletion of miR-762 in oocytes had significant effect on oocyte survival rate versus null control, either treatment significantly increased the cleavage rate (HDAC6, 74.8 vs 67.4%; as-miR-762, 74.3 vs 67.4%) and blastocyst rate (HDAC6, 70.0 vs

Table 1 Relationship of HDAC6 levels and survival/developmental potential of cryopreserved oocytes

	No. of oocytes	No. of survival oocytes (%)	No. of cleavage (%)	No. of blastocyst (%)
HDAC6-high	101	99(98)	75(74)	70(69)
HDAC6-low	102	95(93)	54(53)	41(40)
X^2		2.7491	4.4802	5.3804
p value		0.0472	0.0358	0.0204

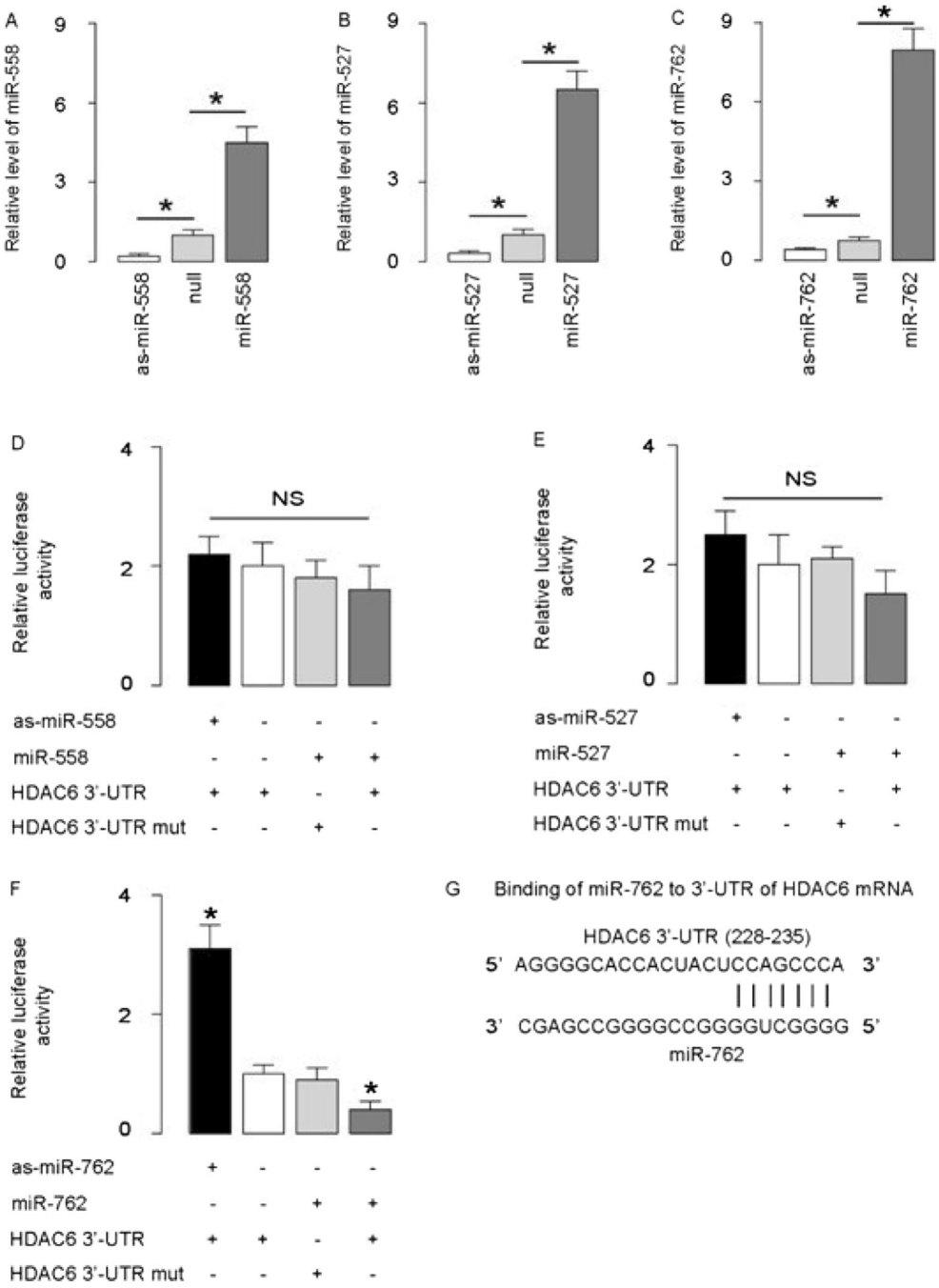

Fig. 2 miR-762 targets 3′-UTR of HDAC6 mRNA to inhibit its translation in oocytes (**a**–**c**). Oocytes were transfected with indicated oligonucleotides. RT-qPCR showed changes in the miR-558 levels in miR-558-modified cells (**a**), changes in the miR-527 levels in miR-527-modified cells (**b**), and changes in the miR-762 levels in miR-762-modified cells (**c**). **d**–**f** The intact 3′-UTR of HDAC6 mRNA (HDAC6 3′-UTR), together with a 3′-UTR with mutant at either miRNA binding site of HDAC6 mRNA (HDAC6 3′-UTR mut), was then cloned into luciferase reporter plasmids. Oocytes were co-transfected with 1 μg as-miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids or co-transfected with 1 μg miRNA/null plasmids and 1 μg HDAC6 3′-UTR or HDAC6 3′-UTR mut plasmids. **d** Assay for miR-558. **e** Assay for miR-527. **f** Assay for miR-762. **g** Bioinformatics analysis for binding site ofmiR-762 in 3′-UTR of HDAC6 mRNA. *$p < 0.05$. NS non-significant. The values obtained came from five replicates

49.9%; as-miR-762, 69.7 vs 49.9%) after OV, compared to null treatment (Table 2).

Discussion

Previous studies have shown that histone acetylation levels in vitrified-warmed oocytes increased significantly and contributed to poor survival and loss of developmental potential of oocytes after OV [2]. However, those studies mostly focused on the roles of HDAC1 and HDAC2, which belong to class I HDACs [4]. Compared with freshly derived ovine embryos, vitrified counterparts displayed reduced expression of HDAC1 [15]. Histone acetylation is critical for proper cellular functions including chromosome condensation, DNA double-strand breakage repair, and mRNA transcription. Sperm chromatin is de-condensed followed by recondensation in the early fertilization, in which histone acetylation alters accordingly. Therefore, improper histone acetylation activity may affect the process of meiosis of oocytes, and subsequently affect preimplantation of the embryos [16–18].

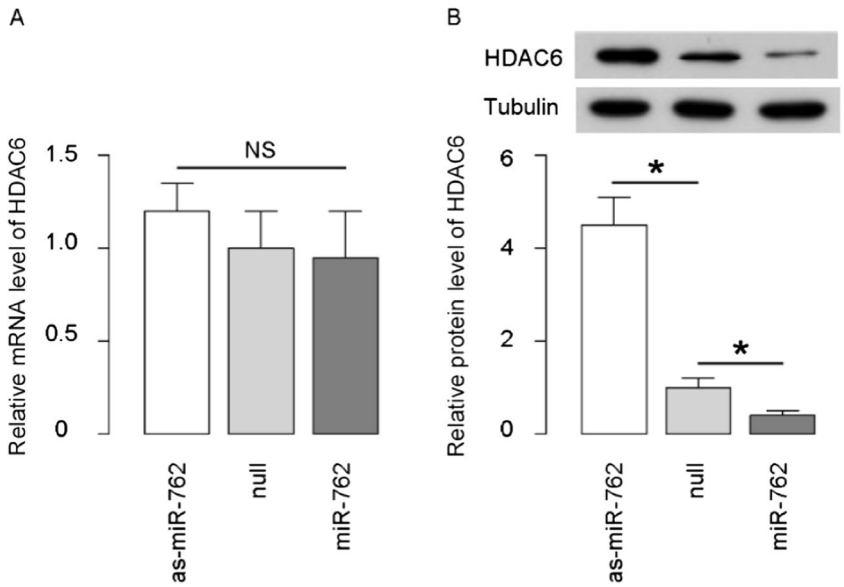

Fig. 3 MiR-762 decreases HDAC6 protein levels in oocytes. **a** RT-qPCR for HDAC6 in miR-762-modified oocytes. **b** Western blot for cellular HDAC6 in miR-762-modified oocytes. *$p < 0.05$. NS non-significant. The values obtained came from five replicates

In this study, we explored the involvement of HDAC6 in the regulation and control of oocyte survival and maintenance of developmental potential. The data from Fig. 1 suggest that HDAC6 may be critical for oocyte survival and maintenance of developmental potential after OV. Also, the data suggest that depletion of miR-762 in oocytes may increase HDAC6 levels in oocytes (Fig. 3). We adopted the potential effects of miRNA intervention on HDAC6 protein translation to increase cellular HDAC6 levels in oocytes, resulting in improved preservation of developmental potential of oocytes after OV.

The data from Fig. 4 suggest that overexpression of HDAC6 or depletion of miR-762 may improve survival and developmental potential of oocytes after OV. Regarding overexpression of HDAC6 using as-miR-762 to increase HDAC6 levels, it is largely free of significant side effects when compared to either traditional gene therapy and protein intervention. Overexpression of HDAC6 in oocytes may increase the burden of massive protein translation to the oocyte and may result in an increase in the levels of miR-762 as a feedback, to level down the systemic efficacy. The size of as-miR-762 is rather small, and its effects on protein profile of the oocytes are much more modest than direct alteration of expression of a protein.

It should be mentioned that there is no evidence for the effect of HDAC6 on in vivo development of the blastocysts from cryopreserved oocytes. This issue would be addressed in future work.

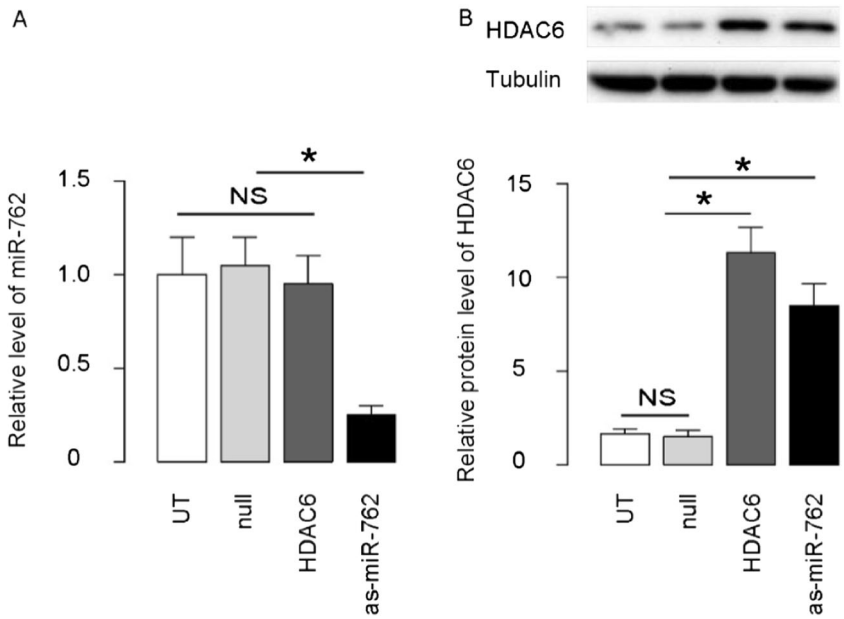

Fig. 4 Overexpression of HDAC6 or depletion of miR-762 in oocytes for cryopreservationmiR-762 levels by RT-qPCR (**a**). HDAC6 levels by Western blot (**b**). *$p < 0.05$. NS non-significant

Table 2 The survival/developmental potential of HDAC6 or as-miR-762-expressing cryopreserved oocytes

	No. of oocytes	No. of survival (%)	No. of cleavage (%)	No. of blastocyst (%)
Untreated (UT)	103	99(96)	66(64)	54(52)
Null-transfected	101	96(95)	68(67)	54(53)
HDAC6-transfected	101	100(99)	78(77)	70(69)
As-miR-762-transfected	102	100(98)	76(75)	71(70)
X^2(HDAC6 vs null)		1.2479	3.0276	5.4296
X^2(as-miR-762 vs null)		1.3323	2.9544	6.1023
p value (HDAC6 vs null)		0.2934	0.0418	0.0277
p value (as-miR-762 vs null)		0.2484	0.0476	0.0135

In conclusion, it is evidenced that overexpression of as-miR-762 leads to an increase in the protein levels of HDAC6 in mouse oocytes. Induction of HDAC6 promotes survival, cleavage, and blastocyst development of cryopreserved oocytes after recovery. Therefore, re-expression of HDAC6 may confer benefits in improving mouse oocyte vitrification.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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