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GAMETE BIOLOGY

Downregulation of gene expression and activity of GRIM-19 affects mouse oocyte viability, maturation, embryo development and implantation

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Abstract
Purpose To investigate the expression of GRIM-19 (Gene associated with retinoid-interferon-induced mortality 19) in mouse oocytes and preimplantation embryos, and to study the effect of GRIM-19 on the developmental competence of mouse oocytes and embryos.
Methods GRIM-19 was evaluated at both mRNA and protein levels. The expression of GRIM-19 gene was downregulated in mouse oocytes cultured in vitro by specific small interfering RNA (siRNA) injection, while the activity of GRIM-19 was decreased by microinjection of a GRIM-19 antibody into the cytoplasm of germinal vesicle (GV) oocytes. Oocytes matured in vitro were then fertilized by intracytoplasmic sperm injection (ICSI), followed by observation and evaluation of fertilization rate, cleavage rate, blastocyst formation rate and implantation rate.
Results GRIM-19 is expressed throughout oocyte maturation and preimplantation embryo development stages. GRIM-19 was localized primarily in the cytoplasm of all cells examined. Downregulation of gene expression and activity of GRIM-19 resulted in decreased oocyte viability, potency of oocyte maturation, embryo development and implantation.
Conclusions GRIM-19 may play important roles in mouse oogenesis and early embryonic development and implantation.

Keywords GRIM-19 · Oocyte · Preimplantation embryo · Mitochondria

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Introduction

Oocyte quality, which accounts for developmental competence, is the most crucial factor affecting the chance of success in the treatment course of in vitro fertilization and embryo transfer (IVF-ET) [1–3]. It has been demonstrated that the abnormal structure and function of oocyte's mitochondria could reduce the quality and the fertilization competence of oocytes, one of the key reasons for infertility and low reproductive ability [4–7]. Mitochondria are maternally inherited and their replication does not begin until after implantation [8]. In order to improve oocyte quality, cytoplasm and mitochondria transfer have been reported to be an effective treatment procedure. And, more than 30 babies have been born with this technique [9, 10]. However, the use of this method raises concerns including mitochondrial heteroplasmy and the spread of mitochondrial genetic disease [11]. In addition, increased mitochondria number in oocytes may also influence the development of embryo [12]. Thus, a major problem revolves around improving mitochondrial function of oocytes.

Grim-19 (Gene associated with retinoid-interferon-induced mortality 19) was originally identified as a critical regulatory protein for interferon-beta and retinoic acid-induced cell death [13–16]. It has been mapped to human chromosome 19p13.2, and codes for a novel 16 kDa protein. It was also demonstrated

that GRIM-19 is a functional subunit of mitochondrial respiratory chain complex I, and plays an essential role in the assembly and enzymatic activity of complex I [17–19]. Therefore, GRIM-19 plays a dual protein function involved in cell death and mitochondrial metabolism. Huang et al. [18] also demonstrated that homozygous deletion mutation of the *Grim-19* gene causes embryonic lethality at embryonic day 9.5. GRIM-19 −/− blastocysts showed retarded growth in vitro and display abnormal mitochondrial structure, morphology and cellular distribution. However, the expression and function of GRIM-19 during oogenesis and early embryogenesis is still unknown.

The present study aims to: 1) investigate the expression of GRIM-19 in mice oocytes and preimplantation embryos; 2) evaluate the function of GRIM-19 on mice oocytes maturation, early embryo development and embryo implantation.

Materials and methods

Animals and experiment protocols

All experimental protocols and animals used were approved by the Animal Research Ethical Committee, Qilu Hospital of Shandong University. All experiments were performed on Kunming mice, aged between 8 and 12 weeks and weighing 30–35 g, purchased from Shandong University (scxk LU 20090001), and bred in our facilities (which were maintained at 21±2 °C and 55±10 % relative humidity on a 12 h light/dark cycle). All groups with sample size in this study are listed in Table 1.

Ovary stimulation, oocyte and embryo harvesting

Ovaries were stimulated with FSH (purified follicle stimulating hormone; Livzon Group, Zhuhai, China; 10 IU per dose) to superovulate followed by 10 IU human chorionic gonadotrophin (hCG; Livzon Group, Zhuhai, China) 46–48 h later. Germinal vesicle (GV) oocytes were collected from ovaries 46–48 h after FSH injection. Milrinone, which increases cAMP, was used to inhibit germinal vesicle breakdown (GVBD). MII oocytes (with the first polar body) and MI oocytes (with no PB or nuclear structure) were collected 12 h post hCG injection. Embryos at 2-cell, 4-cell, 8-cell, morula, and blastocyst stages were obtained after successful mating with males at 46–48, 55, 65, 75, and 84–86 h after post-hCG, respectively. Pregnancy was confirmed the following morning by the presence of a vaginal plug. Mice were sacrificed by cervical dislocation for oocytes and embryos collecting.

Realtime PCR

mRNA expression of grim-19 was detected using realtime PCR. Oocytes (GV, MI and MII) and embryos of each stage (2- to 8-cell, morula, blastocyst) were pooled in groups for total RNA extraction (n=50 for each stage). The zona pellucida was removed chemically by exposure to Tyrode's solution (Irvine Scientific Santa Ana, California, USA). Total RNA was isolated using the RNeasy micro kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol.

cDNA was produced and amplified using the cDNA synthesis kit (Takara, DaLian, China) by random hexamers according to the manufacturer's instructions. The nucleotide sequences of primer pairs for GRIM-19 were designed as follows: Forward-5′-TCGGGGACTGTCGGGGTAC-3′, Reverse-5′- AGGGTCCTCCGGTCCTTCT-3′. Histone H2a family member Z (*H2afz*) was used as a normalizer, because its expression pattern has been previously measured and established to be stable in mouse oocytes and preimplantation embryos under different conditions [20, 21]. All PCR reactions were performed in triplicate in a 20 μl reaction volume on the quantitative real-time PCR StepOne plus system (ABI, Carlsbad, California, USA) using the SYBR Premix Ex Taq™ II (Takara, DaLian, China). The reaction mixtures were subjected to an initial denaturation of 95 °C for 10 min, followed by 36 cycles of 95 °C for 5 s, 60 °C for 30 s, and during which fluorescence was measured and was followed by a melt curve analysis to confirm a single gene-specific peak and to detect primer/dimer formation. A negative template control of DEPC water was used to ensure that there was no nucleotide contamination. The relative amount of each mRNA was determined using the $2^{-\Delta(\Delta CT)}$ method and calculated by dividing

Table 1 All groups with sample size

SiRNA injection		Antibody injection	
Groups	Sample size	Groups	Sample size
Group I, GRIM-19 siRNA(1) injected group;	1000	Group I, GRIM-19 antibody injected group	1000
Group II, GRIM-19 siRNA(2) injected group	1000	Group II, Rabbit normal IgG injected group	200
Group III, control siRNA injected group	200	Group III, untreated control group	200
Group IV, untreated control group	200		

the intensity. Data were normalized based on *H2afz* transcript levels, and three independent experiments were carried out.

Western blotting

Protein expression was analyzed by western blotting. Western blot analysis was performed as described previously [18]. Briefly, embryos (n=200 for each stage) were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1 % deoxycholic acid, 1 % Triton X-100, 0.1 % SDS, 0.25 mM EDTA) with Phenylmethanesulfonyl fluoride. The lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with phosphate-buffered saline (PBS) containing 0.1 % Tween 20 and 5 % skimmed milk before it was incubated with the appropriate primary (rabbit polyclonal antibody against GRIM-19, Santa, California, USA, diluted to 1:2000) and secondary antibodies (HRP-labeled Goat Anti-Rabbit, Beyotime, Nantong, China, diluted to 1:500). Immunoreactivity was detected using the appropriate peroxidase-linked secondary antibody and enhanced chemiluminescence (ECL) detection reagent. Three independent experiments were carried out.

Mitotracker staining

The mitochondria were visualized by Mitotracker® Red (Invitrogen, California, USA). Embryos were incubated with 400 nM Mitotracker® Red for 20 min. After staining, embryos were washed in fresh, pre-warmed G1. Then the medium was removed carefully, and replaced with freshly prepared, pre-warmed buffer containing 4 % formaldehyde. The embryos were fixed at room temperature for 30 min.

Immunohistochemistry

The subcellular localization of GRIM-19 was investigated by immunofluorescent staining. Embryos were fixed in 4 % paraformaldehyde for 30 min at room temperature and permeabilized for 20 min in 0.5 % Triton X-100. After being blocked with 5 % goat serum (Zhongshan, Beijing, China) in PBS overnight at 4 °C, oocytes and embryos were exposed to primary antibodies (rabbit polyclonal antibody against GRIM-19, Santa, America, diluted to 1:100) for 2 h followed by washed 3 times in PBS for 10 min each. Then oocytes and embryos were incubated for 2 h at room temperature in the presence of 1:100 diluted secondary antibodies (Fluorescein-Conjugated AffiniPure Goat Anti-Rabbit IgG, zhongshan, Beijing, China). After rinsing 3 times in PBS for 10 min each, oocytes and embryos were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Beyotime, Nantong, China) for 3 min. For negative controls, oocytes and embryos incubated with only the secondary primary antibody.

Microinjection of GRIM-19 siRNA into GV oocytes

Oocytes with germinal vesicle were microinjected with siRNAs using a micromanipulation operating system (RI, Research Instrument Company, England) within 30 min. siRNA duplex oligoribonucleotides targeting the coding region of GRIM-19 (GenBank Accession no: NM023312) were obtained from Invitrogen (Carlsbad, California, USA). 5–10 pl of 50 μM control siRNA or specific GRIM-19 siRNA (GRIM-19 siRNA (1): GCAATGCCAACTTCGGCTT, GRIM-19 siRNA (2): GGCTTCACCTGGTACACTT) was microinjected into the cytoplasm. Non-silencing siRNA nucleotides were used as a negative control. After microinjection, the oocytes were arrested at the GV stage for 16 h in G1-containing 2.5 μM milrinone. Real time PCR and western blotting were performed to detect the reduction degree of GRIM-19 mRNA and protein in mouse oocytes after injection, and *Gapdh* was used as a normalizer. Then, the oocytes were transferred to IVM medium (Quinn's, USA) at 37 °C with 5 % CO_2.

Microinjection of GRIM-19 antibody into GV oocytes

Oocytes with germinal vesicle were microinjected with antibodies using a micromanipulation operating system (RI, Research Instrument Company, England). GRIM-19 antibody (stock solution, 200 ug/ml, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, California, USA) was microinjected into the cytoplasm of GV oocytes as described by Dai et al. [22]. Rabbit normal IgG-injected (Santa Cruz, California, USA) oocytes and untreated oocytes were used as controls to assess injection damage. The microinjection experiments were done 20 replicates. Microinjection was completed in 30 min, with volume of about 7 pl per oocyte. To determine in vitro maturation rate, the microinjected GV oocytes were cultured in IVM medium in a 5 % CO_2 incubator at 37 °C for 14 h.

In vitro maturation, fertilization and blastocytst culture

GV oocytes were cultured individually in 50 μL IVM medium droplets at 37 °C with 5 % CO_2. The maturity of oocytes was evaluated after 14 h culture. In-vitro matured oocytes (MII) were inseminated by ICSI using mice spermatozoa collected from the cauda epididymidis of KM males (>12 weeks) and purified as described elsewhere [23]. After injection, oocytes were cultured individually in 50-μL droplets of embryo culture medium (G-1, Vitrolife, Sweden) at 37 °C under 5 % CO_2. Fertilization was inspected for two pronucleus formation (2PN) under an inverted microscope 16–18 h after injection and embryos were cultured until blastocyst stage at day 4.

Blastocyst transfer

To determine the influence of GRIM-19 on implantation competency, blastocysts in GRIM-19 siRNA injected group, GRIM-19 antibody injected group and control group were transferred to recipient mice. Hyperstimulation was accomplished with the same FSH and hCG as described above. Pseudopregnancy mice were obtained by witnessed copulation with vasectomied mice, and 28 oseudopregnancy mice were used. To perform the transfers, pseudopregnancy mice were anesthetized by 50 mg/kg pentobarbital sodium subcutaneous injection. Ten blastocysts were transferred to the each uterine horn of day-2.5 pseudopregnant mice [24–26]. On day 6 post-coitus, surrogate mice were injected Trypan blue through tail vein and sacrificed 10 min later to analysis the implantation ability. Recipients and blastocysts used in the blastocyst transfer experiments are list in Tables 2 and 3.

Statistical analysis

All data were presented as mean ± SEM. The SPSS 11.5 statistical software program was used for statistical analysis. Percentages were compared between experimental groups and control groups by means of χ^2 test. One-way ANOVA was used for analysis of the relative levels of GRIM-19 mRNA and protein. A value of $P<0.05$ was considered significant.

Results

Expression of GRIM-19 mRNA in mouse oocytes and preimplantation embryos

GRIM-19 mRNA was detected in all developmental stage of mouse oocytes and preimplantation embryos. The expression of GRIM-19 was not different between groups with GV, MI and MII oocytes (Fig. 1a).

The GRIM-19 mRNA quantity was significantly higher in embryos than in oocytes ($P<0.01$). In embryos, GRIM-19 mRNA level increased from 2-cell stage to 8-cell stage, then decreased progressively in morulae and blastocyst stage. The expression of GRIM-19 mRNA in the 8-cell stage was significantly higher than that in other stages (Fig. 1a).

Expression of GRIM-19 protein in mouse oocytes and preimplantation embryos

The GRIM-19 protein expression was found in all developmental stages of mouse oocytes (from GV stage to MII stage) and preimplantation embryos (from 2-cell stage to blastocyst stage). Results confirmed that GRIM-19 at protein levels increased following oocyte maturation, and the expression levels of GRIM-19 in the 8-cell stage were significantly higher than that in other stages (Fig. 1b).

Localization of GRIM-19 protein by immunofluorescence

Immunofluorescent staining at blastocyst stage showed that GRIM-19 fluorescence labeling primarily in the cytoplasm of all cells investigated, and GRIM-19 display a diffuse pattern throughout the cytoplasm. Merging of GRIM-19 with Mitotracker Red indicated that GRIM-19 is partially localized to the mitochondria (Fig. 2). These results suggested that the biological roles of GRIM-19 in embryos may associate with its function in mitochondria.

Injection of GRIM-19 siRNA into GV oocytes

To downregulate the expression of GRIM-19 in GV oocyte, we injected GRIM-19 specific siRNA into GV oocytes and evaluated the maturation rate, fertilization rate, cleavage rate, and blastocyst formation rate. After siRNA injection, GV oocytes were cultured individually in 50 μL IVM medium droplets, and the maturity of oocytes was evaluated after 14 h culture. After ICSI, fertilization was inspected for two

Table 2 Maturation, development and implantation of mouse GV oocytes after injection with GRIM-19 siRNA(1) or siRNA(2)

	GV (*n*,%)	Degeneration (*n*, %)	Maturation (*n*, %)	Fertilization (*n*, %)	Cleavage (*n*, %)	Blastocyst formation (*n*, %)	Recipient (*n*)	Pregnancy (*n*)	Implantation (*n*, %)
Group I	1000	294(29.4) ab	338(47.9) ab	111(32.8) ab	58 (52.3) ab	20(34.5) ab	2(20)	2	4(20.0) ab
Group II	1000	282(28.2) ab	363(50.6) ab	123(34.0) ab	67 (54.5) ab	28(41.8) ab	3(28)	3	6(21.4) ab
Group III	200	25(12.5)	129(73.7)	98(76.0)	70(71.4)	63(90.0)	5(50)	5	39(78.0)
Group IV	200	16(8.0)	135(73.4)	106(78.5)	84(79.2)	77(91.7)	5(50)	5	41(82.0)

Group I, GRIM-19 siRNA(1) injected group; Group II, GRIM-19 siRNA(2) injected group; Group III, control siRNA injected group; Group IV, untreated control group

a Compared to control siRNA injected group, difference significant, $P<0.05$

b Compared to Untreated group, difference significant, $P<0.05$

Table 3 Maturation, development and implantation of mouse GV oocytes after microinjecting of GRIM-19 antibody

	GV (*n*, %)	Degeneration (*n*, %)	Maturation (*n*, %)	Fertilization (*n*, %)	Cleavage (*n*, %)	Blastocyst formation (*n*, %)	Recipient (*n*)	Pregnancy (*n*)	Implantation (*n*, %)
Group I	1000	239(23.9) ab	351(46.1) ab	127(36.2) ab	63(49.6) ab	24(38.1) ab	3(24)	3	6(25.0) ab
Group II	200	23(11.5)	127(71.8)	99(78.0)	74(74.7)	67(90.5)	5(50)	5	38(76.0)
Group III	200	16(8.0)	135(73.4)	106(78.5)	84(79.2)	77(91.7)	5(50)	5	41(82.0)

Group I, GRIM-19 antibody injected group; Group II, Rabbit normal IgG injected group; Group III, untreated control group

a. Compared to Rabbit normal IgG injected group, difference significant, $P<0.05$

b. Compared to Untreated group, difference significant, $P<0.05$

pronucleus formation (2PN) under an inverted microscope 16–18 h after injection and embryos were cultured till blastocyst stage at day 4. To examine the reduction level of GRIM-19 mRNA in mouse oocytes after injection, real time PCR was performed. GRIM-19 siRNA (1) and siRNA (2) significantly reduced endogenous GRIM-19 mRNA in mouse oocytes. The suppression level of GRIM-19 at protein level was detected by Western blot, which was in accordance with the real time PCR. To confirm specificity of the signal, immunofluorescent staining was done after siRNA treatment, and the result suggested that the GRIM-19 was downregulated by GRIM-19 siRNA (1) or siRNA (2) (Fig. 3). These results suggested that the downregulation of GRIM-19 expression by GRIM-19 siRNA (1) or siRNA (2) injection was effective.

The degeneration rate in the GRIM-19 siRNA(1) and siRNA(2) injected group (Group I, 29.4 %, Group II, 28.2 %, $n=1000$) was higher than that in control siRNA injected group (Group III, 12.5 %, $n=200$) and untreated group (Group IV, 8.0 %, $n=200$) ($P<0.05$) (Table 2). After IVM, the maturation, fertilization, cleavage, and blastocyst formation rates were significantly lower in the GRIM-19 siRNA(1) and siRNA(2) injected group compared with control siRNA injected group and untreated group ($P<0.05$, Table 2, Fig. 3). These results indicated that downregulation of GRIM-19 expression impact oocyte viability, oocyte maturation and embryo development.

Microinjection of GRIM-19 antibody into GV oocytes

To investigate the function of GRIM-19 on the maturation and development of oocyte, we injected GRIM-19 antibody into GV stage oocytes and evaluated the maturation rate, fertilization rate, cleavage rate, and blastocyst formation rate. The degeneration rate in the GRIM-19 antibody-injected group

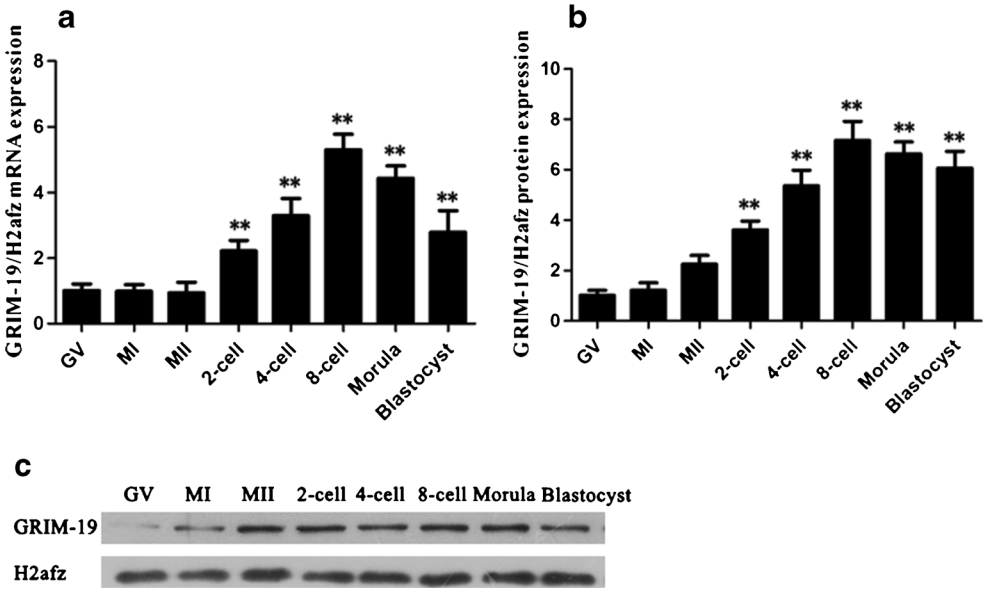

Fig. 1 Expression of GRIM-19 mRNA and protein during oogenesis and early embryogenesis. **a** GRIM-19 mRNA levels were detected by real time PCR at all stages from GV-stage oocytes to blastocyst ($n=50$ for each stage). The expression level was calculated from the Ct values by the $2^{-\triangle\triangle}$Ct method. The value of MII oocytes was assigned an arbitrary value. Three independent experiments were carried out. Values are mean ± SD. *$P<0.05$, **$P<0.01$ vs. MII oocytes. **b** Western blot analysis of GRIM-19 expression in mouse oocytes and embryos. *Bar graphs* are expressed as a ratio of GRIM-19 to H2afz for three independent experiments and the value of MII oocytes is assigned an arbitrary value. Values are mean ± SD. *$P<0.05$, **$P<0.01$ vs. MII oocytes. **c** Western blot analysis of GRIM-19 expression in mouse oocytes and embryos

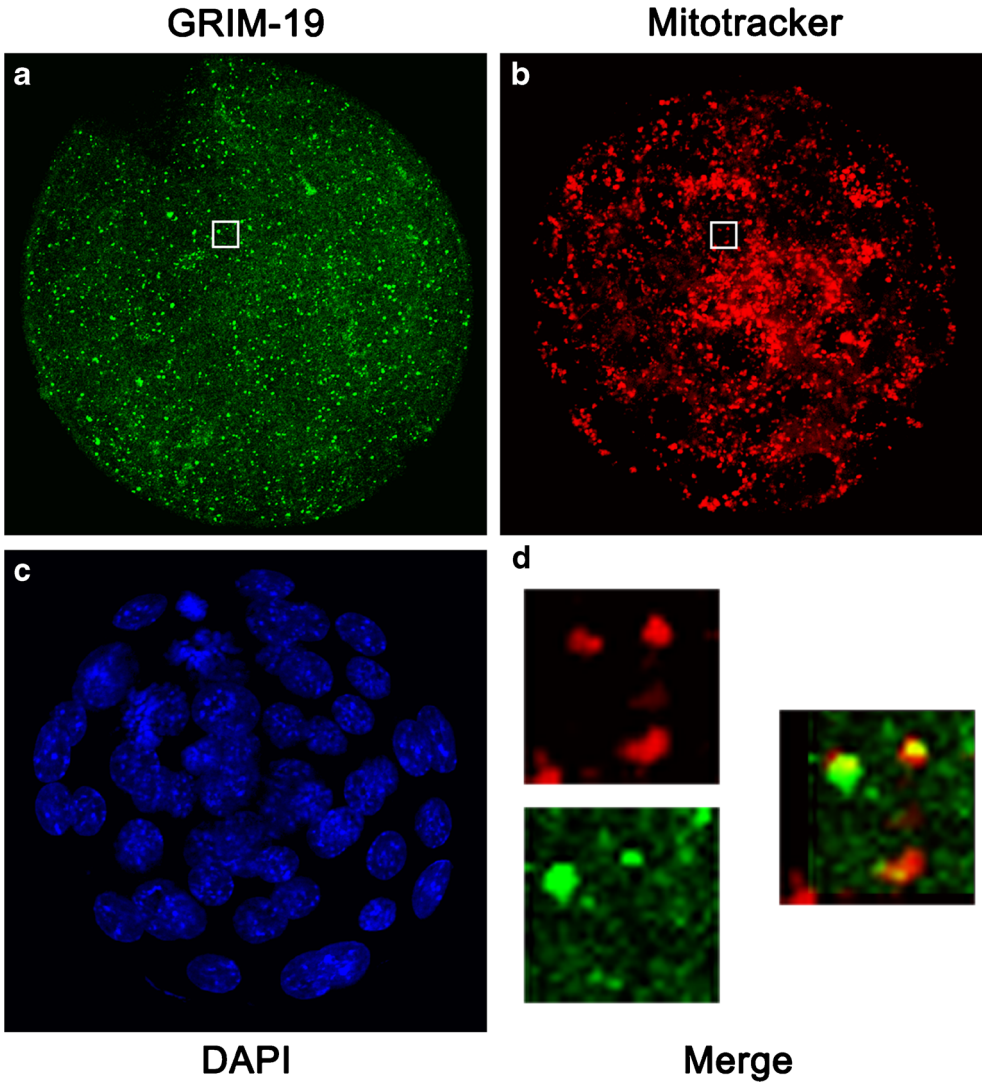

Fig. 2 Localization of GRIM-19 protein by Immunofluorescence. Blastocysts were stained with GRIM-19 antibody and DAPI. Mitochondria were stained with Mitotracker Red. Overlay shows high correlation between mitotracker and GRIM-19

(Group I, 23.9 %, n=1000) was higher than that in the rabbit normal IgG -injected group (Group II, 11.5 %, n=200) and untreated group (Group III, 8.0 %, n=200) (P<0.05) (Table 3). After IVM, the maturation, fertilization, cleavage, and blastocyst formation rates were significantly lower in the GRIM-19 antibody-injected group compared with rabbit normal IgG -injected group and untreated group (P<0.05, Table 3, Fig. 4). These results indicated that there was a decreased effect of downregulated GRIM-19 on oocyte viability, oocyte maturation and embryo development.

Potential for implantation after microinjection

Blastocysts (n=272) were transferred to the 28 pseudopregnant mice. GRIM-19 siRNA(1) injected group: 20 blastocysts transferred to 2 pseudopregnant mice; GRIM-19 siRNA(2) injected group: 28 blastocysts transferred to 3 pseudopregnant mice; control siRNA injected group: 50 blastocysts transferred to 5 pseudopregnant mice. GRIM-19 antibody injected group: 24 blastocysts transferred to 3 pseudopregnant mice; Rabbit normal IgG injected group: 50 blastocysts transferred to 5 pseudopregnant mice; and untreated control group: 50 blastocysts transferred to 5 pseudopregnant mice (Tables 2 and 3). All of 28 recipients were pregnant in at least one horn. The implantation rate of blastocysts derived from the GRIM-19 siRNA injected group was significantly lower than that of control siRNA injected group and untreated group (P<0.05, Table 2). The implantation rate of blastocysts derived from the GRIM-19 anitibody microinjected group was significantly lower than that of rabbit normal IgG microinjected group and untreated group (P<0.05, Table 3, Fig. 4). Our findings indicated that down-regulation of GRIM-19 reduced the potential of implantation.

Discussion

To date, no reports have been made about the role of GRIM-19 in oogenesis and early embryogenesis, although it has been

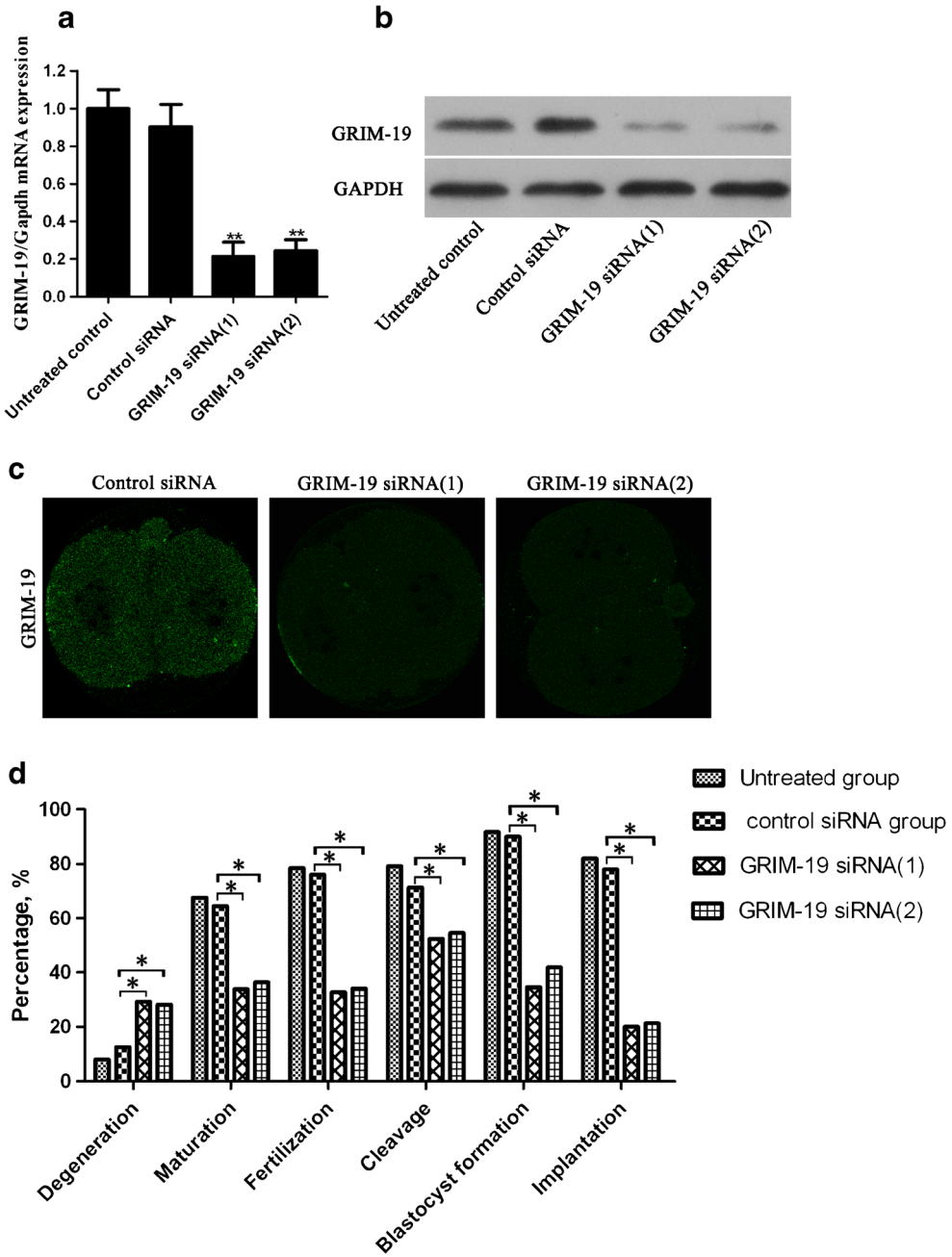

Fig. 3 Microinjection of GRIM-19 siRNA into GV oocytes. **a** Real time PCR was performed. GRIM-19 siRNA(1) and GRIM-19 siRNA(2) significantly reduced endogenous GRIM-19 mRNA in mouse oocytes. **b** The suppression degree of GRIM-19 at protein level was detected by Western blot. **c** Immunofluorescent staining of GRIM-19 after siRNA treatment. **d** The degeneration, maturation, fertilization, cleavage, and blastocyst formation rates in the GRIM-19 siRNA(1) and siRNA(2) injected group compared with control siRNA injected group and untreated group

demonstrated that homozygous deletion mutation of the *Grim-19* gene causes embryonic lethality at embryonic day 9.5. Present study provides new insights into function of GRIM-19 on mice oocytes maturation, early embryo development and embryo implantation.

As a major outcome, this study confirmed that GRIM-19 expressed in all developmental stages of mouse oocytes (from GV stage to MII stage) and preimplantation embryos (from 2-cell stage to blastocyst stage), evidently indicating the important role of GRIM-19 in oogenesis and early embryogenesis. Simultaneously, by microinjection of GRIM-19 specific siRNA and antibody, we demonstrated that down regulation the expression and activity of GRIM-19 resulted in decreased capacity of oocyte viability, oocyte maturation, embryo development and implantation. Furthermore, we demonstrated that GRIM-19 display a diffuse pattern throughout the cytoplasm, and partially localize to the mitochondria.

Our results indicated that downregulation of GRIM-19 in oocytes reduced oocyte viability and ability of maturation. Most GV oocytes injected with Grim-19 siRNA degenerate in IVM medium. Similarly, almost all the embryos, which fail to develop from Grim 19-depleted oocytes, degenerate in the culture medium.

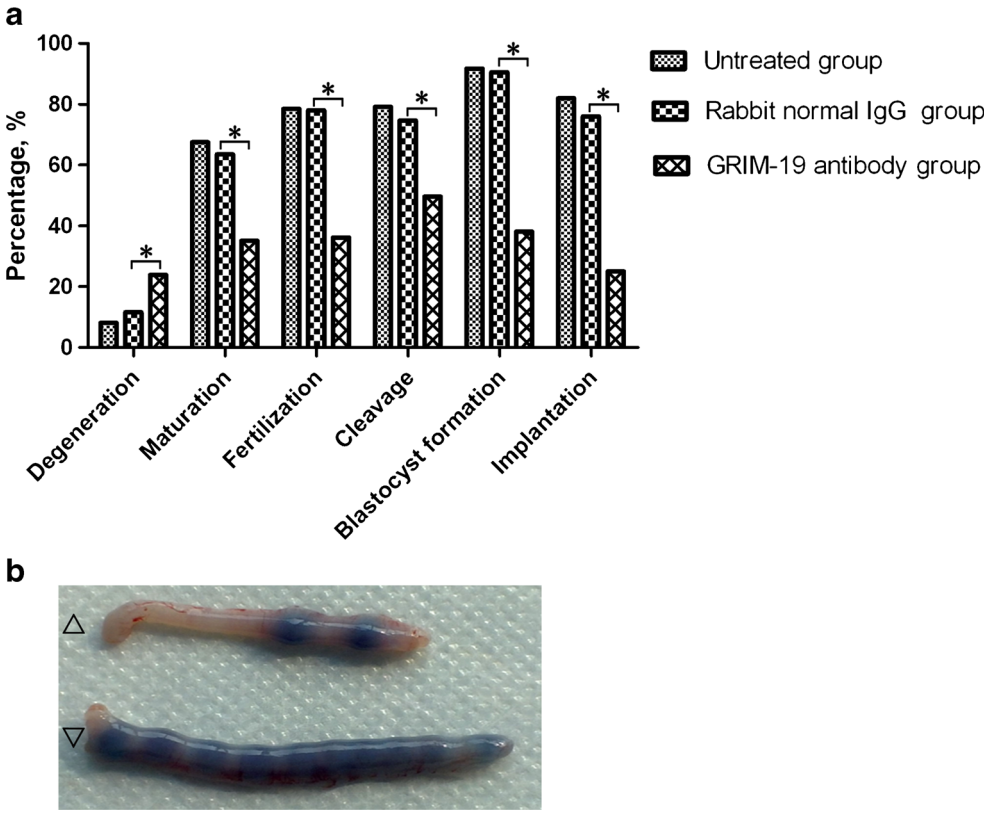

Fig. 4 Microinjection of GRIM-19 antibody into GV oocytes. **a** The degeneration, maturation, fertilization, cleavage, and blastocyst formation rates in the GRIM-19 antibody-injected group compared with control group. **b** Effect of downregulation activity of GRIM-19 on the implantation competence. The implantation rate of blastocysts derived from the GRIM-19 anitibody microinjected group (△) was significantly lower than that of rabbit normal IgG microinjected group (▽)

Mitochondria are the most abundant organelles in the mammalian oocyte and preimplantation embryo. Mitochondria are energy supplying organelles in the mammalian oocyte and early embryo, and ATP produced by mitochondria is the major energy source of oocytes and embryos [27, 28]. Mitochondria are maternally inherited, and oocytes contain a pool of non-replicating yet functional mitochondria until the late blastocyst stage [29]. GRIM-19 is one functional component of mitochondria respiratory chain complex I, and is necessary in assembling the complex I-V, transferring proton and maintaining mitochondrial membrane potential ($\triangle\Psi m$). It has been reported that the lack of GRIM-19 could lead to the abnormality of mitochondrion structure, distribution and function [19]. Loss of mitochondria function might potentially lead to cells death. Further studies are needed to detect the mitochondrial membrane potential of oocytes and preimplantation embryos after downregulation of gene expression and activity of GRIM-19.

The cellular location of GRIM-19 is a crucial factor in determining its biological function. GRIM-19 was originally reported as a nuclear protein in HeLa cells [13]. But studies performed by different groups examined the cellular location of GRIM-19 in various cell lines from different species and demonstrated that its primary localization in the cytoplasm [14, 18]. Further results confirmed that GRIM-19 is primarily localized in the mitochondrial complex I [30, 31]. In the present study, we found that GRIM-19 is diffusely distributed throughout the cytoplasm and partially co-localized with the mitochondria of mouse oocytes and preimplantation embryos. Our results confirmed that GRIM-19 was mainly located in cytoplasm of oocytes and embryos, which indicate that its biological roles may associate with its function in mitochondria.

At present, the evaluation of preimplantation embryo is mainly based on morphology [32–34]. However this evaluation system is not comprehensive. Research shows that damage of mitochondria respiratory chain might reduce ATP production, leading to aneuploidy, interfering the normal fertilization and embryonic development and influencing the development potential of embryos [12]. The low implantation rate is observed in women with advanced maternal age, and it might be related to the low content of ATP in the eggs [35]. The ability of mitochondria to supply ATP is considered the most critical factor for the developmental competence of oocytes and embryos [36]. After downregulation of GRIM-19 in GV oocytes, the in vitro maturation rate was significantly decreased. Meanwhile, the fertilization, cleavage and blastocyst formation rates were also dramatically decreased. These results suggested that downregulation of GRIM-19 in mouse oocytes might interference the oocyte viability, maturation and developmental competence. To further determine the effects of GRIM-19 on embryo implantation, we transfered blastocyst stage embryos to the uterus horns of

pseudopregnancy mice. Notably, the implantation ratio of blastocysts in the GRIM-19 downregulated group was significantly lower than in the control group. Our results demonstrated that downregulation of GRIM-19 in embryos reduced their implantation potential.

In conclusion, evidence provided in the present study indicated that GRIM-19 expressed in mouse oocytes and preimplantation embryos. GRIM-19 was mainly located in cytoplasm of oocytes and embryos. Downregulation of GRIM-19 in oocytes and embryos reduced oocyte viability, ability of maturation, further development and implantation. Thus, GRIM-19 may play important roles in mouse oogenesis, early embryonic development and implantation. Since downregulation can only provide a partial evaluation of GRIM-19 function, future studies where upregulation of the GRIM-19 is performed are needed to detect the role of GRIM-19 in oogenesis and embryo development.

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References

1. Duranthon V, Renard JP. The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. Theriogenology. 2001;55(6):1277–89.
2. Sirard MA. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology. 2001;55(6):1241–54.
3. Krey LC, Grifo JA. Poor embryo quality: the answer lies (mostly) in the egg. Fertil Steril. 2001;75(3):466–8.
4. Jansen RP, Burton GJ. Mitochondrial dysfunction in reproduction. Mitochondrion. 2004;4(5–6):577–600.
5. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell. 2003;112(4):481–90.
6. Saraste M. Oxidative phosphorylation at the fin de siecle. Science. 1999;283(5407):1488–93.
7. Coenen MJ, van den Heuvel LP, Smeitink JA. Mitochondrial oxidative phosphorylation system assembly in man: recent achievements. Curr Opin Neurol. 2001;14(6):777–81.
8. McConnell JM, Petrie L. Mitochondrial DNA turnover occurs during preimplantation development and can be modulated by environmental factors. Reprod Biomed Online. 2004;9(4):418–24.
9. Cohen J, Scott R, Alikani M, Schimmel T, Munne S, Levron J, et al. Ooplasmic transfer in mature human oocytes. Mol Hum Reprod. 1998;4(3):269–80.
10. Dale B, Wilding M, Botta G, Rasile M, Marino M, Di Matteo L, et al. Pregnancy after cytoplasmic transfer in a couple suffering from idiopathic infertility: case report. Hum Reprod. 2001;16(7):1469–72.
11. Barritt JA, Brenner CA, Malter HE, Cohen J. Mitochondria in human offspring derived from ooplasmic transplantation. Hum Reprod. 2001;16(3):513–6.
12. El Shourbagy SH, Spikings EC, Freitas M, St John JC. Mitochondria directly influence fertilisation outcome in the pig. Reproduction. 2006;131(2):233–45.
13. Angell JE, Lindner DJ, Shapiro PS, Hofmann ER, Kalvakolanu DV. Identification of GRIM-19, a novel cell death-regulatory gene induced by the interferon-beta and retinoic acid combination, using a genetic approach. J Biol Chem. 2000;275(43):33416–26.
14. Lufei C, Ma J, Huang G, Zhang T, Novotny-Diermayr V, Ong CT, et al. GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. EMBO J. 2003;22(6):1325–35.
15. Zhang J, Yang J, Roy SK, Tininini S, Hu J, Bromberg JF, et al. The cell death regulator GRIM-19 is an inhibitor of signal transducer and activator of transcription 3. Proc Natl Acad Sci U S A. 2003;100(16):9342–7.
16. Maximo V, Botelho T, Capela J, Soares P, Lima J, Taveira A, et al. Somatic and germline mutation in GRIM-19, a dual function gene involved in mitochondrial metabolism and cell death, is linked to mitochondrion-rich (Hurthle cell) tumours of the thyroid. Br J Cancer. 2005;92(10):1892–8.
17. Fearnley IM, Carroll J, Shannon RJ, Runswick MJ, Walker JE, Hirst J. GRIM-19, a cell death regulatory gene product, is a subunit of bovine mitochondrial NADH: ubiquinone oxidoreductase (complex I). J Biol Chem. 2001;276(42):38345–8.
18. Huang G, Lu H, Hao A, Ng DC, Ponniah S, Guo K, et al. GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I. Mol Cell Biol. 2004;24(19):8447–56.
19. Huang G, Chen Y, Lu H, Cao X. Coupling mitochondrial respiratory chain to cell death: an essential role of mitochondrial complex I in the interferon-beta and retinoic acid-induced cancer cell death. Cell Death Differ. 2007;14(2):327–37.
20. Mamo S, Gal AB, Bodo S, Dinnyes A. Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. BMC Dev Biol. 2007;7:14.
21. Jeong YJ, Choi HW, Shin HS, Cui XS, Kim NH, Gerton GL, et al. Optimization of real time RT-PCR methods for the analysis of gene expression in mouse eggs and preimplantation embryos. Mol Reprod Dev. 2005;71(3):284–9.
22. Dai Y, Lee C, Hutchings A, Sun Y, Moor R. Selective requirement for Cdc25C protein synthesis during meiotic progression in porcine oocytes. Biol Reprod. 2000;62(3):519–32.
23. Stewart BM, Block J, Morelli P, Navarette AE, Amstalden M, Bonilla L et al. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced in vitro with sex-sorted semen. J Dairy Sci 94(7):3437–45
24. Yamagata K, Suetsugu R, Wakayama T. Assessment of chromosomal integrity using a novel live-cell imaging technique in mouse embryos produced by intracytoplasmic sperm injection. Hum Reprod. 2009;24(10):2490–9.
25. Yamagata K, Suetsugu R, Wakayama T. Long-term, six-dimensional live-cell imaging for the mouse preimplantation embryo that does not affect full-term development. J Reprod Dev. 2009;55(3):343–50.
26. Ohta H, Sakaide Y, Wakayama T. Long-term preservation of mouse spermatozoa as frozen testicular sections. J Reprod Dev. 2008;54(4):295–8.
27. Hiendleder S, Wolf E. The mitochondrial genome in embryo technologies. Reprod Domest Anim. 2003;38(4):290–304.
28. Kondo I, Suganuma N, Ando T, Asada Y, Furuhashi M, Tomoda Y. Clinical factors for successful cryopreserved-thawed embryo transfer. J Assist Reprod Genet. 1996;13(3):201–6.
29. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, et al. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro

maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod. 2001;64(3):904–9.
30. Carroll J, Fearnley IM, Skehel JM, Shannon RJ, Hirst J, Walker JE. Bovine complex I is a complex of 45 different subunits. J Biol Chem. 2006;281(43):32724–7.
31. Murray J, Zhang B, Taylor SW, Oglesbee D, Fahy E, Marusich MF, et al. The subunit composition of the human NADH dehydrogenase obtained by rapid one-step immunopurification. J Biol Chem. 2003;278(16):13619–22.
32. Sakkas D, Shoukir Y, Chardonnens D, Bianchi PG, Campana A. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum Reprod. 1998;13(1):182–7.
33. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, et al. Characterization of a top quality embryo, a step towards single-embryo transfer. Hum Reprod. 1999;14(9):2345–9.
34. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod. 2000;15(11): 2394–403.
35. Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. Reprod Biomed Online. 2004;8(1):45–58.
36. Lee SH, Han JH, Cho SW, Cha KE, Park SE, Cha KY. Mitochondrial ATPase 6 gene expression in unfertilized oocytes and cleavage-stage embryos. Fertil Steril. 2000;73(5):1001–5.