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Low MFN2 expression related to ageing in granulosa cells is associated with assisted reproductive technology outcome

BIOGRAPHY

Wenpei Xiang is Professor of Reproductive Medicine at the Family Planning Research Institute. She focuses on oogenesis, POF and female reproductive endocrinology. Her team tries to address the effect and mechanism of mitofusin 2 (MFN2) on oogenesis and explore the pathogenesis of premature ovarian failure.

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KEY MESSAGE

MFN2 expression decreased with age, which may be related to assisted reproductive technology treatment outcome by promoting cell apoptosis and affecting mitochondrial function. Oocyte quality and embryonic development could be regulated by the MFN2/mitochondrial pathway.

ABSTRACT

Research question: Is low MFN2 expression associated with ageing in granulosa cells as well as assisted reproductive technology (ART) outcome, and what is the underlying mechanism of action of MFN2?

Design: In a prospective study, fresh granulosa cells were obtained from 161 women aged 20–40 years who underwent IVF with embryo transfer and who were divided into two groups: the diminished ovarian reserve (DOR) group (n = 51) and the control group (n = 110). Patient characteristics including age, infertility duration, body mass index, FSH, anti-Müllerian hormone (AMH), antral follicle count (AFC) and husband's semen parameters and granulosa cell MFN2 expression levels, cell apoptosis, mitochondrial membrane potential ($\Delta\Psi m$) and ATP levels were analysed.

Results: There were no significant differences between the DOR and control groups in terms of age, infertility duration and husband'' semen parameters; however, significant ($P < 0.05$) changes were found between the two groups in FSH, AMH and AFC levels. MFN2 expression was remarkably lower in granulosa cells from the DOR group and decreased in both groups as age increased. Furthermore, among young patients, MFN2 levels significantly increased in patients with pregnancy. MFN2 protein levels and cell apoptosis were lower in the MFN2 knockdown (MFN2-siRNA) group than in the control (Cy3-siRNA) group. $\Delta\Psi m$ and ATP levels were reduced in the MFN2-siRNA group compared with the Cy3-siRNA group.

Conclusions: Low MFN2 expression levels in granulosa cells were related to ageing, which may be involved in the clinical outcome of ART by promoting cell apoptosis and affecting mitochondrial function.

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KEYWORDS

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INTRODUCTION

Mitochondria have an autocephalous system for RNA transcription and protein translation with their own circular mitochondrial DNA (mtDNA) as the genetic code. As the production centres of energy in eukaryotic cells, mitochondria play a vital role in ATP synthesis, oxidative stress, apoptosis, calcium homeostasis and other unexplored fields via oxidative phosphorylation and the electron respiratory chain (*Dumollard et al., 2007*). At present, there is concern that the failure of ATP production, oxidative stress injury, apoptosis and imbalances in Ca^{2+} release may cause a decline in ovarian function, which is related to mitochondrial swelling, cristae disruption, CoQ10 deficiency, and mtDNA mutation and deletion (*Chen et al., 2013; Sai et al., 2011; Wang et al., 2013; Zhang et al., 2006*). Reduced ATP production and mtDNA copy number may be responsible for aberrations in meiotic spindle assembly, abnormal chromosome segregation and the generation of aneuploidy. According to many studies, reactive oxygen species disorders, which result in decreased ATP levels and elevated concentrations of calcium ions, may cause changes in the cytoskeleton, including structural abnormalities of the spindle, aneuploidy and embryonic aberrations (*Zhang et al., 2006*). Moreover, granulosa cell apoptosis is the key factor in follicular atresia, and calcium ion imbalance decreases fertilized egg competence during embryogenesis, which reduces the likelihood of achieving pregnancy (*Chen et al., 2013; He et al., 2014; Sai et al., 2011; Wang et al., 2013*).

Accumulating in-depth studies of mitochondria have revealed a tight interdependence between normal mitochondrial function, as indicated by the mitochondrial membrane potential ($\Delta\Psi m$) and changes in GTP levels, and normal ovarian function, oocyte maturation and embryonic development. Based on recent literature, mitochondria in oocytes are extremely enriched during the maturity stage, with a content of approximately 0.15 million copies of mtDNA, which is far greater than the content in most somatic cells (*Wai et al., 2010*). Therefore, normal mitochondrial function is critical for oocyte development and maturation, which play key roles in fertilization and assisted reproductive technology (ART) outcome, and mitochondrial dysfunction may result in reduced blastocyst and embryo numbers associated with decreased oocyte quality (*Otera et al., 2013; Schatten et al., 2014; Van Blerkom, 2011*).

MFN2 (mitofusin 2), which encodes the MFN2 protein located on the outer mitochondrial membrane, is a conserved dynamin-like GTPase that affects normal mitochondrial structure and function by regulating mitochondrial fusion and fission with three other proteins (MFN1, OPA1 and DRP1) (*Lee and Yoon, 2016*). MFN2 plays a crucial role in sustaining the remodelling of mitochondrial morphology and maintaining the integrity of mitochondrial function (*Chen et al., 2003; Wakai et al., 2014*). Our research team showed that low MFN2 expression in placental villous cells results in spontaneous abortions in reproductive-aged women (*Pang et al., 2013*). It has also been suggested that MFN2 expression levels are related to oocyte quality and affect the rate of fertilization by modulating meiosis, spindle morphogenesis, chromosome separation and mitochondrial function (*Liu et al., 2016; Zhang et al., 2016*). In short, overexpression or deletion of *MFN2* may be involved in embryonic development, oogenesis and oocyte maturation, in addition to cardiovascular disease, insulin resistance-related disease, genetic kinesigenic disorders and cancer (*Burte et al., 2015; Hall et al., 2016; Ozcan, 2013; Zhao et al., 2013*). However, there is no comprehensive and meaningful clinical research profiling of the relationship between MFN2 expression and ageing in granulosa cells or ART outcome. Therefore, we determined the expression levels of MFN2, a key protein that influences mitochondrial function, and its effect on clinical pregnancy rate, and further verified the findings with granulosa cells, which play an important role in follicular development and maturation.

MATERIALS AND METHODS

Patient characteristics and granulosa cells collection

This study was approved by the Ethics Committee of the Centre of Reproductive Medicine of Tongji Medical College of Huazhong University of Science and Technology in China on 28 April 2016 (ethical approval document (03) number). Written informed consent was obtained from each participant before the start of the investigation. Fresh granulosa cells were collected from 161 women aged 20 to 40 years who underwent IVF with embryo transfer (IVF–ET) and these women were then divided into two groups: the diminished ovarian reserve (DOR) group (n = 51), the diagnosis of DOR according to the ESHRE standard (antral follicle count [AFC] <5–7, anti-Müllerian hormone [AMH] ~0.5–1.1 ng/ml, 2011, Bologna); and the control group (n = 110). The samples were collected on the basis of the following eligibility criteria: the women should not have any endocrine disease, genetic disease, autoimmune disease or anatomic abnormalities; they should not have premature ovarian failure; and their husbands should not have any disease. Short-acting decapeptyl in long-term protocol on pituitary down-regulation was used for all the patients undergoing IVF–ET, and high-quality embryos were defined as embryos with six or more uniform blastomeres and <20% fragments; these were chosen for transplantation on Day 3 after fertilization. Clinical pregnancy was defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy; miscarriage was defined as the spontaneous loss of a clinical pregnancy prior to 22 completed gestational weeks. Relevant information concerning the samples from all women is included in TABLE 1.

Granulosa cell isolation and culture

Within 3 h of oocyte retrieval, the granulosa cells were isolated from follicular fluid with 50% Percoll by centrifugation (716*g*, 20 min). Isolated granulosa cells were seeded into six-well cell culture plates (Corning, USA) at a density of 1×10^6 cells/ml in DMEM/F12 (1:1) containing 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2.

siRNA-mediated MFN2 knockdown

The chemically modified *MFN2* (5′-chol + 2′OMe) and control (5′-chol + 2′OMe + Cy3) siRNA were synthesized by RUIBO Biotechnology (Guangzhou, China). The protocol for knocking down MFN2 expression has been described previously (*Zhao et al., 2015*). Briefly, the granulosa cells isolated from young patients with normal ovarian function (≤30 years, n = 24) were

TABLE 1 CHARACTERISTICS OF THE ENROLLED PARTICIPANTS

Groups	Age (years)	Infertility duration (years)	Body mass index (kg/m^2)	FSH (mIU/ml)	AMH (ng/ml)	AFC (number)	Husband's semen parameters
Control group (n = 110)	29.9 ± 5.2	5.0 ± 4.1	21.5 ± 3.7	6.67 ± 2.30	3.23 ± 2.50	13.0 ± 3.4	Normal
DOR group (n = 51)	30.4 ± 5.0	5.1 ± 3.9	20.9 ± 3.1	8.72 ± 2.60[a]	0.67 ± 0.28[a]	3.2 ± 1.8[a]	Normal

AFC = antral follicle count; AMH = anti-Müllerian hormone; DOR = diminished ovarian reserve.

[a] $P < 0.05$ versus the control group.

cultured in DMEM/F12 containing 10% FBS and transfected with MFN2-siRNA (50 nM) or Cy3-siRNA (50 nM) for 16 h. PCR and Western blotting were utilized to identify siRNA knockdown efficiency, with each sample replicated three times.

Western blotting analysis

Proteins (50 μg) were separated by SDS-PAGE and transferred to 0.45 μm PVDF membranes. Non-specific binding was blocked with 5% non-fat milk for 1 h at room temperature. The membranes were hybridized overnight with rabbit polyclonal anti-MFN2 antibody (1:1000, Santa Cruz Biotechnology, CA), rabbit polyclonal anti-Bcl-2 antibody (1:1000, Cell Signaling, USA), rabbit polyclonal anti-Bax antibody (1:1000, Cell Signaling, USA), rabbit polyclonal anti-Caspase 3 antibody (1:1000, Cell Signaling, USA), rabbit polyclonal anti-Caspase 9 antibody (1:1000, Cell Signaling, USA) or rabbit polyclonal anti-β-actin antibody (1:1000, Santa Cruz Biotechnology, CA, USA) in TBST containing 1% non-fat milk. Membranes were washed with TBST four times for 10 min each, followed by incubation with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 1 h (1:3000, Invitrogen, USA). After four washes with TBST, immunoreactive bands were visualized by the enhanced chemiluminescence system (P1108, Beyotime Institute of Biotechnology, China). Band intensity was quantified by densitometry using Quantity One 4.5.2 analysis software (Bio-Rad, USA). The results were normalized to β-actin signal intensity.

Flow cytometry assay

Annexin V-FITC/propidium iodide (PI) double staining was used to detect cell apoptosis. In detail, granulosa cells cultured in six-well plates were collected and stained with 5 μl Annexin V-FITC for 15 min and then with 10 μl PI in the dark for 10 min at room temperature. Subsequently, the stained granulosa cells were analysed by FACS (Fluorescence-Activated Cell Sorting, BD Biosciences, USA) after being washed and resuspended. Cells incubated with PBS (Phosphate Buffer Saline) alone were used as the negative control. Both Q4 (early stage apoptotic cells) and Q2 (non-viable apoptotic cells) were defined as apoptotic cells in FACS groups.

Mitochondrial membrane potential detection

Changes in the granulosa cell $\Delta\Psi$m were monitored by incubating granulosa cells in culture medium at 37°C for 20 min with JC-1 (Beyotime, China). After being thoroughly washed, granulosa cells were imaged using a scanning microscope in selective channels for green and red (485 nm and 590 nm). Mitochondria in granulosa cells were labelled with MitoTracker Green (MTG, Beyotime, China) in culture medium at 37°C for 30 min and measured by laser scanning confocal microscopy.

Quantification of ATP levels

The supernatant from the two groups of cells was analysed using an ATP assay kit (Nanjing Jiancheng Bioengineering Institute, China) after centrifugation at 2191g for 15 min. The absorbance values of all samples were analysed by a UV/visible spectrophotometer (Perkin Elmer, USA), and the ATP content was calculated using the general formula.

Statistical analysis

Each experiment was performed three times. Data are presented as the mean ± SEM and were analysed with SPSS ver. 18.0. The normally distributed numerical variance was performed by Student's t-test, with one-way analysis for continuous variance, and chi-squared tests were executed for the differences between two or more rates. The statistical significance was set at $P < 0.05$.

RESULTS

Characteristics of the enrolled participants

A total of 161 patients were enrolled, and age, infertility duration, genetic history, FSH levels, LH levels, AMH levels, AFC, and the husband's semen parameters were compared between the two groups. There were no significant differences between the two groups in average age, infertility duration or husband's semen parameters; however, significant differences between the two groups were found in FSH levels, AMH levels and AFC (all $P < 0.05$).

MFN2 levels were decreased in granulosa cells from DOR patients and related to age

MFN2 expression in granulosa cells was determined by Western blotting to assess whether MFN2 levels were different between DOR patients and the control group. MFN2 protein levels in three different samples from each group are shown in FIGURE 1A. The relative amount of MFN2 in granulosa cells from the DOR group (0.163 ± 0.056) was remarkably lower than that in granulosa cells from the control group (0.583 ± 0.136) (FIGURE 1B, $P < 0.01$). It was found that MFN2 expression levels were significantly lower in the 36–40 years group compared with the two younger age groups in both the control and DOR groups, and further compared with the control group, the MFN2 expression was markedly decreased in the DOR group (FIGURE 1C and 1D; $P < 0.05$).

MFN2 expression in granulosa cells and clinical outcome according to age in the control group

To determine the function of MFN2, the relationship between MFN2 protein levels in granulosa cells and clinical outcomes in the control group was analysed. It was found that MFN2 protein levels were highest in the youngest patients (≤30 years) and lowest in the oldest patients (36–40 years); the number of oocytes obtained, number of high-quality embryos and clinical pregnancy rate showed the same trend, but the miscarriage rate was highest in the oldest age group (TABLE 2). To further address the relationships between MFN2 protein levels and clinical outcome, patients aged

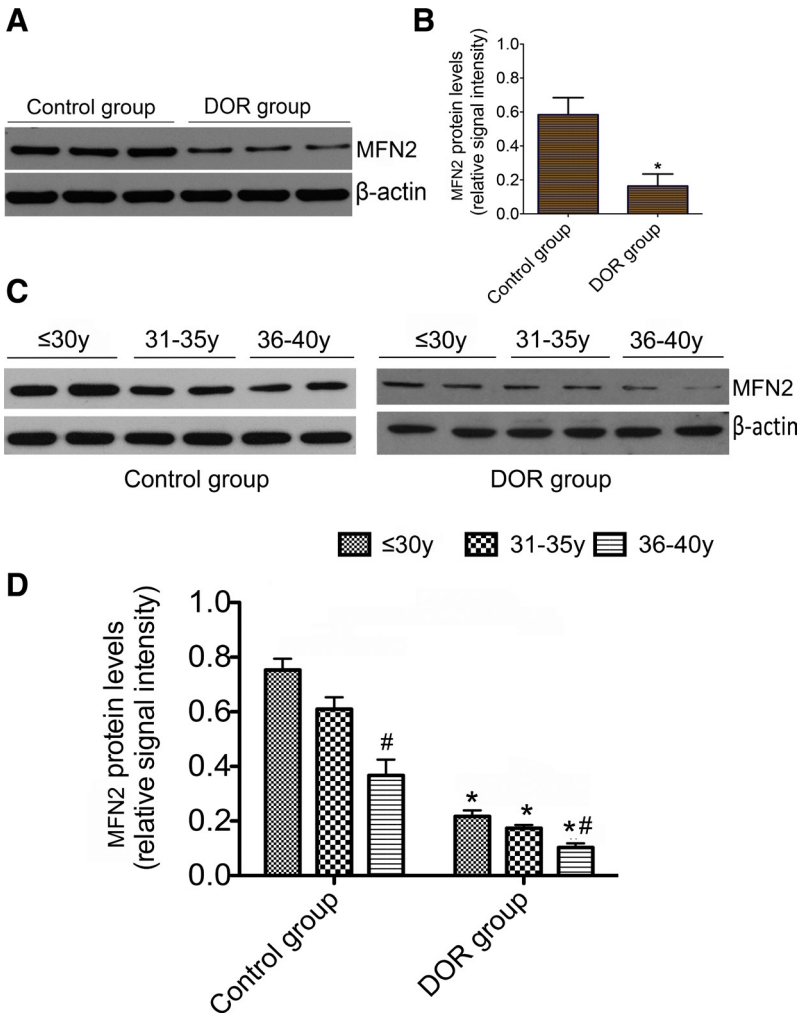

FIGURE 1 MFN2 levels in granulosa cells from the two groups assessed by Western blotting. (A) The most representative image of MFN2 expression in the diminished ovarian reserve (DOR) and control groups. (B) Relative amounts of MFN2 protein in the two groups (control group, $n = 110$; DOR group, $n = 51$), normalized to β-actin. (C) The most representative image of MFN2 expression in different age groups of patients within the DOR and control groups. (D) The relative amounts of MFN2 protein in different age groups of control patients (≤30 years, $n = 42$; 31–35 years, $n = 39$; 36–40 years, $n = 29$) and DOR patients (≤30 years, $n = 22$; 31–35 years, $n = 16$; 36–40 years, $n = 13$). *$P < 0.05$ versus the control group, #$P < 0.05$ versus ≤30 years and 31–35 years. Results shown are mean ± SEM of three experiments.

≤30 years were divided into two groups according to pregnancy status; the results showed that MFN2 levels were significantly higher in pregnant women than in non-pregnant women, and the numbers of obtained oocytes and high-quality embryos were higher in pregnant women (all $P < 0.05$; TABLE 3). These results indicate that MFN2 expression levels are related to ART clinical outcome.

Low MFN2 expression induced the apoptosis of granulosa cells from young patients

To clarify the mechanism of action of MFN2, granulosa cells from young patients were transfected with MFN2 siRNA (MFN2-siRNA group) or Cy3 siRNA (control group). Flow cytometric assays and Western blotting were used to analyse cell apoptosis and the expression of MFN2, the anti-apoptotic protein Bcl-2, the pro-apoptosis protein Bax and the apoptosis-related factors Caspase 3 and Caspase 9. Western blotting indicated that MFN2 protein levels were

TABLE 2 MFN2 LEVELS AND CLINICAL OUTCOME IN THE CONTROL GROUP ACCORDING TO AGE ($N = 110$)

Groups (years)	Number	MFN2 protein levels	No. of oocytes obtained	No. of embryos per transfer	No. of high-quality embryos	Clinical pregnancy rate (%)	Miscarriage rate (%)
≤30	42	0.67 ± 2.34	14.14 ± 6.73	2.06 ± 0.42	5.90 ± 4.49	64.28	7.41
31–35	39	0.60 ± 2.03	12.89 ± 6.19	2.05 ± 0.29	4.77 ± 4.14	61.53	8.33
36–40	29	0.36 ± 1.68*	10.64 ± 6.99[a]	2.02 ± 0.28	4.09 ± 3.81[a]	34.48[a]	11.11[a]

[a] $P < 0.05$ versus 31–35 years and ≤30 years.

TABLE 3 MFN2 LEVELS AND CLINICAL OUTCOME IN CONTROL PATIENTS AGED ≤30 YEARS (*N* = 42)

Patients	Number	MFN2 protein levels	No. of oocytes obtained	No. of high-quality embryos
Pregnant	27	0.64 ± 2.58	15.14 ± 6.08	5.89 ± 4.56
Non-pregnant	15	0.39 ± 1.37[a]	11.97 ± 5.76[a]	3.09 ± 3.58[a]

[a] $P < 0.05$ versus pregnant.

reduced in the MFN2-siRNA group, and Bax levels were significantly increased ($P < 0.05$) in the MFN2-siRNA group compared with the Cy3-siRNA group. In addition, Caspase 3 and Caspase 9 levels showed the same trend of Bax levels ($P < 0.05$, FIGURE 2A and 2B). However, Bcl-2 expression levels were reduced in the MFN2-siRNA group compared with the Cy3-siRNA group (FIGURE 2A and 2B). In addition, the apoptosis index (AI) was 30.89 ± 6.38% in the MFN2-siRNA group and 9.92 ± 2.36% in the Cy3-siRNA group ($P < 0.05$, FIGURE 2C and 2D). The study revealed that low MFN2 levels promoted apoptosis.

Low MFN2 expression caused mitochondrial function decline

To further address the mechanism of MFN2, mitochondrial function in granulosa cells from young patients that had been transfected with MFN2-siRNA and Cy3-siRNA was determined. JC-1 was used to detect the ΔΨm. The red/green fluorescence intensity ratio was apparently lower in the MFN2-siRNA group than in the Cy3-siRNA group (FIGURE 3A and 3B, $P < 0.05$). In addition, ATP levels in granulosa cells in the MFN2-siRNA and Cy3-siRNA groups were measured. ATP levels in the MFN2-siRNA group were significantly lower than those in the Cy3-siRNA group (FIGURE 3C, $P < 0.05$).

DISCUSSION

Normal mitochondrial structure, which is important for the balance of mitochondrial fusion and fission, is crucial for cell viability and senescence (*Schrepfer and Scorrano, 2016*). Some reports have shown that advanced age is characterized by a gradual decline in MFN2 expression related to mitochondrial fusion (*Picca et al., 2016*). The results of this study also showed that MFN2 expression levels in the human

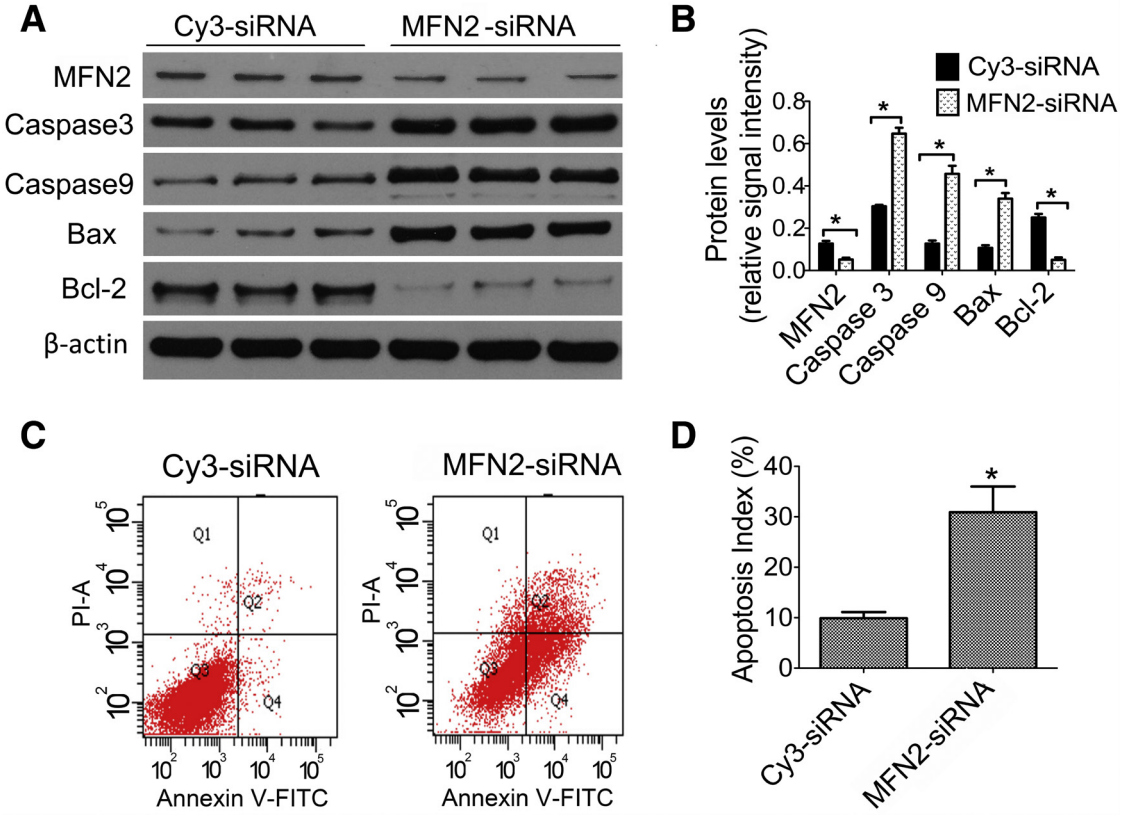

FIGURE 2 MFN2 was knocked down by transfecting granulosa cells from young patients with normal ovarian function (≤30 years, *n* = 24) with MFN2-siRNA and granulosa cells were transfected with Cy3-siRNA for the control. The AI (Q2+Q4) was assessed by FACS and the expression of apoptosis-related factors was investigated using Western blotting in both groups. (A) The most representative images of Western blotting for MFN2, Caspase 3, Caspase 9, Bax and Bcl-2 in the two groups. (B) Comparisons between MFN2, Caspase 3, Caspase 9, Bax and Bcl-2 protein levels. (C) The most representative images of apoptosis analysis in the two groups. (D) Change in the apoptosis index in the two groups. *$P < 0.05$ versus the control (Cy3-siRNA) group, results shown are mean ± SEM of three experiments.

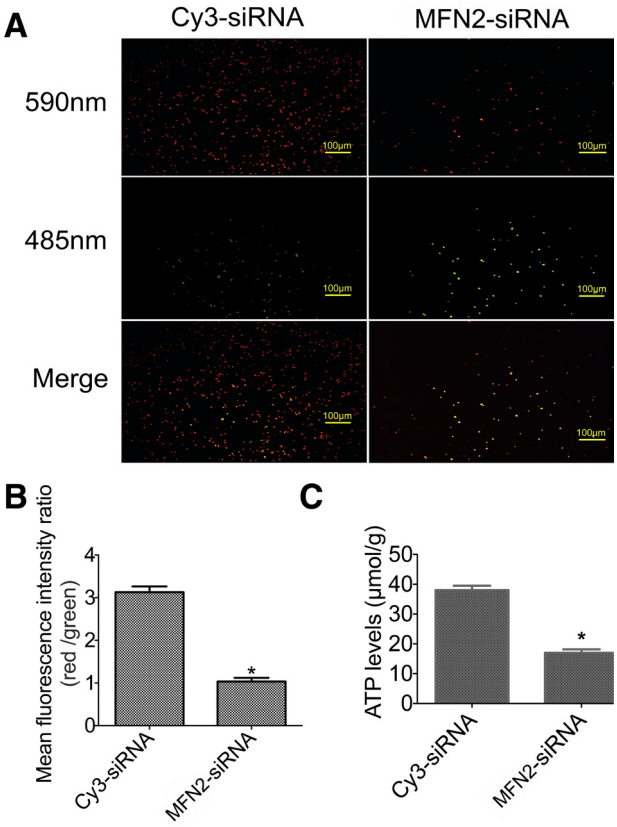

FIGURE 3 MFN2-siRNA affected the mitochondrial function of granulosa cells from young patients with normal ovarian function (n = 24). (A) The most representative fluorescent images of mitochondrial membrane potential (ΔΨm) was detected by JC-1 in Cy3-siRNA (control) and MFN2-siRNA groups (red, high ΔΨm; green, low ΔΨm). (B) The mean intensity ratio of red and green fluorescence in Cy3-siRNA and MFN2-siRNA groups. (C) Quantity of ATP in the two groups. *P < 0.05 versus Cy3-siRNA groups, results shown are mean ± SEM of three experiments.

granulosa cell lineage decreased with increasing age; furthermore, MFN2 protein expression appeared to be lower in patients with DOR regardless of age. We postulated that MFN2 protein levels in human granulosa cells were related to ovarian reserve.

In the clinic, ovarian reserve is defined as the ability to retrieve oocytes or high-quality oocytes from a patient in connection with ART clinical outcome. A massive loss of follicles occurs from birth to old age (~1–2 million at birth declines to ~30,000–40,000 at puberty), and ovarian reserve declines gradually (*McGee and Hsueh, 2000; Vaskivuo et al., 2001*). In this study, a comparative analysis of ART clinical outcomes of the enrolled participants was initially performed. It was found that the numbers of obtained oocytes and high-quality embryos and the clinical pregnancy rate decreased gradually as patient age increased; the miscarriage rate showed the opposite trend. Interestingly, the results also showed that MFN2 protein levels were predominantly decreased in patients with advanced age in the control group. More interesting is the relationship between MFN2 protein levels and clinical outcome, which was further expounded in young patients (≤30 years); the results further verified that granulosa cells from pregnant women expressed significantly higher MFN2 levels (0.64 ± 2.58) than those from non-pregnant women (0.39 ± 1.37), and higher numbers of obtained oocytes and high-quality embryos had been obtained from these pregnant women compared with non-pregnant women. It was therefore speculated that MFN2 expression levels in ovarian granulosa cells were associated with ART clinical outcome.

Declining oocyte quantity and quality both categorically affect ART clinical outcomes. A previous study suggested that normal follicular development and maturation rely on complex crosstalk between oocytes and surrounding granulosa cells (*Hsueh et al., 2015*). *Matsuda et al. (2012)* reported that follicular growth or atresia can be regulated, at least in part, by ovarian granulosa cell survival or apoptosis, which has potential implications for the maintenance of ovarian reserve associated with ART outcome (*Gaytan et al., 2018*). In this study, it was found that MFN2 protein expression in granulosa cells was notably related to ART clinical outcome. However, the underlying mechanisms by which granulosa cell MFN2 protein expression regulates ART clinical outcomes are not fully understood. To clarify the mechanism of MFN2, we knocked down MFN2 expression in human granulosa cells separated from follicular fluid. The results revealed that low MFN2 expression in granulosa cells promoted cell apoptosis. Moreover, the ΔΨm and ATP levels in granulosa cells with lower MFN2 expression both decreased significantly. We speculated that lower MFN2 expression related to age or other reasons could have a marked impact on mitochondrial function, as shown by the results of mitochondrial membrane potential (ΔΨm) and ATP levels in our study (*Chen et al., 2017; Wang et al.,*

2015), which is consistent with previous results reported by our research team (*Liu et al., 2016; Pang et al., 2013; Zhao et al., 2015*).

In summary, the above results suggested that MFN2 expression is potentially involved in the series of events by which granulosa cells are related to changes in biological function. We presume that low MFN2 expression may be one of the reasons for younger DOR patients with unsatisfactory ART outcome, at least in part, by affecting mitochondrial function and controlling the apoptosis of human ovarian granulosa cells. Therefore, low MFN2 expression could be one of the crucial aetiologies of poor ART outcomes, and further studies will be conducted to clarify the mechanism of MFN2.

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REFERENCES

Burté, F., Carelli, V., Chinnery, P.F., Yu-Wai-Man, P. **Disturbed mitochondrial dynamics and neurodegenerative disorders.** Nat. Rev. Neurol. 2015; 11: 11–24

Chen, H., Detmer, S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E., Chan, D.C. **Mitofusins Mfn1 and MFN2 coordinately regulate mitochondrial fusion and are essential for embryonic development.** J Cell Biol. 2003; 160: 189–200

Chen, H., Kui, C., Chan, H.C. **Ca(2+) mobilization in cumulus cells: role in oocyte maturation and acrosome reaction.** Cell Calcium. 2013; 53: 68–75

Chen, X.L., Zhang, G.P., Guo, S.L., Ding, J.Q., Lin, J.J., Yang, Q., Li, Z.Y. **Mfn2-Mediated Preservation of Mitochondrial Function Contributes to the Protective Effects of BHAPI in Response to Ischemia.** J. Mol. Neurosci. 2017; 63: 267–274

Dumollard, R., Duchen, M., Carroll, J. **The role of mitochondrial function in the oocyte and embryo.** Curr. Top Dev. Biol. 2007; 77: 21–49

Gaytan, F., Morales, C., Roa, J., Tena-Sempere, M. **Changes in keratin 8/18 expression in human granulosa cell lineage are associated to cell death/survival events: potential implications for the maintenance of the ovarian reserve.** Hum. Reprod. 2018; 33: 680–689

Hall, A.R., Burke, N., Dongworth, R.K., Kalkhoran, S.B., Dyson, A., Vicencio, J.M., Dorn, G.W.II, Yellon, D.M., Hausenloy, D.J. **Hearts deficient in both Mfn1 and Mfn2 are protected against acute myocardial infarction.** Cell Death Dis. 2016; 7: e2238

He, Y.H., Lu, X., Wu, H., Cai, W.W., Yang, L.Q., Xu, L.Y., Sun, H.P., Kong, Q.P. **Mitochondrial DNA content contributes to healthy ageing in Chinese: a study from nonagenarians and centenarians.** Neurobiol. Ageing. 2014; 35: 1779

Hsueh, A.J., Kawamura, K., Cheng, Y., Fauser, B.C. **Intraovarian control of early folliculogenesis.** Endocr. Rev. 2015; 36: 1–24

Lee, H., Yoon, Y. **Mitochondrial fission and fusion.** Biochem. Soc. Trans. 2016; 44: 1725–1735

Liu, Q., Kang, L., Wang, L., Zhang, L., Xiang, W. **Mitofusin 2 regulates the oocytes development and quality by modulating meiosis and mitochondrial function.** Sci. Rep. 2016; 6: 30561

Matsuda, F., Inoue, N., Manabe, N., Ohkura, S. **Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells.** J. Reprod. Dev. 2012; 58: 44–50

McGee, E.A., Hsueh, A.J. **Initial and cyclic recruitment of ovarian follicles.** Endocr. Rev. 2000; 21: 200–214

Otera, H., Ishihara, N., Mihara, K. **New insights into the function and regulation of mitochondrial fission.** Biochim. Biophys. Acta. 2013; 1833: 1256–1268

Ozcan, U. **Mitofusins: mighty regulators of metabolism.** Cell. 2013; 155: 17–18

Pang, W., Zhang, Y., Zhao, N., Darwiche, S.S., Fu, X., Xiang, W. **Low expression of Mfn2 is associated with mitochondrial damage and apoptosis in the placental villi of early unexplained miscarriage.** Placenta. 2013; 34: 613–618

Picca, A., Pesce, V., Sirago, G., Fracasso, F., Leeuwenburgh, C., Lezza, A.M.S. **'What makes some rats live so long?' The mitochondrial contribution to longevity through balance of mitochondrial dynamics and mtDNA content.** Exp. Gerontol. 2016; 85: 33–40

Sai, T., Goto, Y., Yoshioka, R., Maeda, A., Matsuda, F., Sugimoto, M., Wongpanit, K., Jin, H.Z., Li, J.Y., Manabe, N. **Bid and Bax are involved in granulosa cell apoptosis during follicular atresia in porcine ovaries.** J. Reprod. Dev. 2011; 57: 421–427

Schatten, H., Sun, Q.Y., Prather, R. **The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility.** Reprod. Biol. Endocrinol. 2014; 12: 111

Schrepfer, E., Scorrano, L. **Mitofusins, from Mitochondria to Metabolism.** Mol. Cell. 2016; 61: 683–694

Van Blerkom, J. **Mitochondrial function in the human oocyte and embryo and their role in developmental competence.** Mitochondrion. 2011; 11: 797–813

Vaskivuo, T.E., Anttonen, M., Herva, R., Billig, H., Dorland, M., te Velde, E.R., Stenback, F., Heikinheimo, M., Tapanainen, J.S. **Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4.** J. Clin. Endocrinol. Metab. 2001; 86: 3421–3429

Wai, T., Ao, A., Zhang, X., Cyr, D., Dufort, D., Shoubridge, E.A. **The role of mitochondrial DNA copy number in mammalian fertility.** Biol. Reprod. 2010; 83: 52–62

Wakai, T., Harada, Y., Miyado, K., Kono, T. **Mitochondrial dynamics controlled by mitofusins define organelle positioning and movement during mouse oocyte maturation.** Mol. Hum. Reprod. 2014; 20: 1090–1100

Wang, W., Zhang, F., Li, L., Tang, F., Siedlak, S.L., Fujioka, H., Liu, Y., Su, B., Pi, Y., Wang, X. **MFN2 couples glutamate excitotoxicity and mitochondrial dysfunction in motor neurons.** J. Biol. Chem. 2015; 290: 168–182

Wang, Y., Kong, N., Li, N., Hao, X., Wei, K., Xiang, X., Xia, G., Zhang, M. **Epidermal growth factor receptor signaling-dependent calcium elevation in cumulus cells is required for NPR2 inhibition and meiotic resumption in mouse oocytes.** Endocrinology. 2013; 154: 3401–3409

Zhang, J.H., Zhang, T., Gao, S.H., Wang, K., Yang, X.Y., Mo, F.F., Yu, N., An, T., Li, Y.F., Hu, J.W., Jiang, G.J. **Mitofusin-2 is required for mouse oocyte meiotic maturation.** Sci. Rep. 2016; 6: 30970

Zhang, X., Wu, X.Q., Lu, S., Guo, Y.L., Ma, X. **Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles.** Cell. Res. 2006; 16: 841–850

Zhao, J., Zhang, J., Yu, M., Xie, Y., Huang, Y., Wolff, D.W., Abel, P.W., Tu, Y. **Mitochondrial dynamics regulates migration and invasion of breast cancer cells.** Oncogene. 2013; 32: 4814–4824

Zhao, N., Zhang, Y., Liu, Q., Xiang, W. **Mfn2 Affects embryo development via mitochondrial dysfunction and apoptosis.** PLoS One. 2015; 10:e0125680

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