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Increase of mitochondria surrounding spindle causes mouse oocytes arrested at metaphase I stage

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ABSTRACT

During oocyte meiosis, mitochondria usually surround spindle to meet the energy demand of spindle migration and chromosome segregation. Therefore, the mitochondrion surrounding spindle is widely accepted as an important indicator to demonstrate the mitochondrial function in oocyte studies. However, the role of mitochondria surrounding spindle in oocyte quality is not exactly addressed. Mitofusin-2 (MFN2) is a mitochondrial outer membrane GTPase that mediates mitochondrial clustering and fusion. Here, we increased the mitochondria surrounding spindle by overexpression of MFN2 in mouse oocytes. Results indicate that the increase of mitochondria surrounding spindle has little effect on germinal vesicle breakdown (GVBD), spindle migration, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) production and Endoplasmic reticulum (ER) distribution, while blocks chromosome segregation, destroys the spindle, and finally causes most of the oocytes to arrest at metaphase I stage. Collectively, our results demonstrate the mitochondria surrounding spindle is precisely regulated during oocyte maturation, while too much of it may cause abnormal oocyte meiosis. Therefore, although mitochondrion surrounding spindle is a typical biological event during oocyte maturation, utilizing it to demonstrate the mitochondrial function and oocyte quality should be much careful.

1. Introduction

Meiosis is the unique mechanism to halve genome and produce haploid cells. During mammalian oocyte maturation, the cells experience two consecutive asymmetric divisions, namely meiosis I and II, to produce one mature oocyte and three polar bodies [1]. The maintenance of correct chromosome number in germ cells is necessary for reproduction; its abnormity usually causes miscarriage or serious disorders such as Down syndrome in human beings [2]. It is well known that the defect of meiosis I is the most important factor to cause aneuploidy oocytes [3,4].

After fully grown, oocyte resumes meiosis due to the decline of cyclic AMP (cAMP) [5], and then experiences a series of important events, including germinal vesicle breakdown (GVBD), chromosome condensation and alignment, meiotic spindle assembly and migration, chromosome segregation, and lastly extrudes the first polar body to complete meiosis I [6]. During this process, a great amount of mitochondria translocate from cytoplasm to perinuclear region, and then surround the meiotic spindle [7,8]. It is widely believed that the mitochondria surrounding spindle is helpful for meiosis by providing sufficient energy to support the meiosis spindle migration and faithful chromosome segregation [9]. Therefore, people usually detect the mitochondria surrounding spindle to evaluate mitochondrial function during oocyte meiosis [7]. However, the exact role of it is still poorly understood.

Mitochondria are highly dynamic and multiple function organelles, and they usually remodel their conformations to be granular, network or clustering to fulfill variable functions [10]. In fully grown oocytes, most of the mitochondria are granular and dispersed evenly in cytoplasm, but the mitochondria surrounding spindle is clustering [11]. Mitofusin-2 (MFN2) is a highly conserved transmembrane GTPase which mediates mitochondrial clustering and fusion [12]. Absence of functional MFN2 in cells evokes mitochondrial fragmentation and depolarization [13,14], while overexpression of it induces mitochondrial aggregation [15]. In this study, we overexpressed MFN2 in GV oocyte and investigated its effects on oocyte maturation. Results indicated that overexpression of MFN2 in oocyte had little effects on mitochondrial membrane

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potential (MMP), reactive oxygen species (ROS) production and Endoplasmic reticulum distribution (ER) distribution, but increased the mitochondria surrounding spindle, caused spindle fragment and blocked chromosome migration. Finally, the oocytes with MFN2 overexpression were arrested at metaphase I.

2. Materials and methods

2.1. Plasmids construction and mRNA preparation

Total RNA and complementary DNA (cDNA) from mouse ovary was prepared by phenol-chloroform extraction and a cDNA Synthesis Kit (QuantScript RT Kit, KR103, TIANGEN Biotech(Beijing) Co.,Ltd., Nanjing, China), respectively. *Mfn2* was cloned from the mouse ovary cDNA and inserted into a vector with mCherry fluorescent protein by cloning recombination (ClonExpress II One Step Cloning Kit, C112, Vazyme Biotech Co.,Ltd., Nanjing, China). The plasmids labeled ER and tubulin were gifts from Addgene (mOrange2-ER-5, #57956; mTFP1-Tubulin-6, #55512). Furthermore, T7 promoter was inserted into these plasmids to allow mRNA transcription *in vitro*. And, the mRNAs were prepared with an mRNA synthesis kit (HiScribe™ T7 ARCA mRNA Kit (with tailing), E2065S, New England Biolabs, US).

2.2. Cell culture and transfection

HeLa cells were purchased from national institute for food and drug control of China and cultured in DMEM/F-12 (DMEM/F-12 Hyclone, SH30023.01, Thermo Fisher scientific, US) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. To image the fluorescence with oil objective lens, cells were cultured in an 8-chamber plate with glass bottoms and transfected with corresponding plasmids after culture 24 h (Lipofectamine™ 3000 Transfection Reagent, L3000015, Thermo Fisher scientific, US).

2.3. Mouse oocyte collection and culture

All mice were treated as the guidelines of the Animal Experiment Standard of Guangdong Second Provincial General Hospital. Briefly, ICR female mice were purchased from Beijing HFK Bioscience Co., Ltd and housed to 8–10 weeks old. The mice were sacrificed by cervical dislocation, and GV oocytes released from ovary were collected in M2 medium and cultured in fresh M16 medium (M16 Medium, M7292, Sigma-Aldrich, US) droplets covered by paraffin oil at 37 °C in a 5% CO_2 atmosphere.

2.4. Microinjection

Microinjection needles and holding pipette were produced from capillary tubes (out diameter 1 mm, inner diameter 0.8 mm) by a micropipette puller (P-97, Sutter Instrument, California, US) and microforge (MF-900, NARISHIGE, Tokyo, Japan). GV oocytes were placed in M2 medium droplet covered by paraffin oil at room temperature, and then microinjected with mRNA by piezo (Eppendorf PiezoXpert®, Eppendorf, Hamburg, Germany) under the inverted microscope (Ti2-U, Nikon Corporation, Tokyo, Japan), which equipped with a NT-88-V3 micromanipulator System.

2.5. Fluorescence imaging

Besides fluorescent protein labeling, the mitochondrial tracker (Mito-Tracker Green, C1048, Beyotime Biotechnology Co., Ltd., Shanghai, China) and ER tracker (ER-Tracker Blue-White DPX, for live-cell imaging, 40761ES50, Yeasen Biotech Co., Ltd., Shanghai, China) were used to label mitochondria and ER in this study, respectively. Briefly, the samples were stained with mito-tracker and ER-tracker for 20 min and washed with PBS for 3 times, and then imaged with a high-speed spinning disk confocal microscopy (Andor Dragonfly 200, Andor Technology, Belfast, UK).

2.6. MMP and ROS detection

DCFH-DA (2,7-Dichlorodi-hydrofluoresceindiacetate, S0033,Beyotime Biotechnology Co., Ltd., Shanghai, China) and Rhodamine 123 (2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester, C2007, Beyotime Biotechnology Co., Ltd., Shanghai, China) were used to determine the ROS production and MMP in this study. HeLa cells or oocytes incubated with the probes for 30 min in darkness, and then were washed three times with PBS before imaging. Utilizing DCFA and Rhodamine 123 or other dyes to monitor ROS and MMP is very unstable due to these dyes characters. To decrease undesired impacts from laser intensity, exposure time, fluorescence bleaching and focal plane differences, the control and experimental samples were usually mixed together and then imaged simultaneously.

2.7. Data analysis

The level of ROS content and MMP were demonstrated by fluorescence intensity for each cell and oocyte. And, the co-localizations between mitochondria and ER were evaluated by Pearson Correlation Coefficients. The contour dimension of tubulin fragments were calculated by data of Z axis and 3D model, the model was made by Imaris. All the fluorescence data were obtained by ImageJ, and the results were analyzed by Origin2019, P value was calculated by GraphPad. The results were expressed as mean ± SEM and analyzed by two-tailed Student's t-test. Statistically significant values of $P > 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$ are indicated as n.s., asterisks (*), (**) and (***), respectively.

3. Results

3.1. Overexpression of MFN2 increases the mitochondria surrounding spindle in oocytes

MFN2 as a mitochondrial fusion protein is involved in mouse oocyte meiosis maturation [16]. Therefore, we increased mitochondria surrounding perinuclear region by overexpression of MFN2. Since it is lacking of RNA transcription in GV oocyte, *Mfn2* mRNA was transcribed *in vitro* and introduced into GV oocytes through microinjection. Results shown in Fig. 1A indicate that in the control oocytes, most of mitochondria disperse evenly in cytoplasm at GV stage, and then relocate to perinuclear region and surround spindle after GVBD. While in the MFN2overexpression oocytes, mitochondria aggregate at GV stage, and surround the perinuclear region and spindle tightly (Fig. 1B). Results shown in Fig. 1C indicate that the mitochondrial width surrounding spindle in the control oocytes is about 13 μm on average, while that in the MFN2 overexpression oocytes is about 7 μm (Fig. 1C).

Mitochondria and ER usually interact with together through the mitochondria-associated ER membranes (MAM) to regulate calcium homeostasis and lipid metabolism. Thus, the mitochondria clustering and ER tethering usually emerge simultaneously in cell. Here, by labeling ER-Tracker, we showed that most of ER in HeLa cell is reticulum and the overexpression of MFN2 clearly causes it tethering (Fig. 1D), while it is punctum in GV oocyte and the MFN2 overexpression has little effect on its shape and distribution (Fig. 1E).

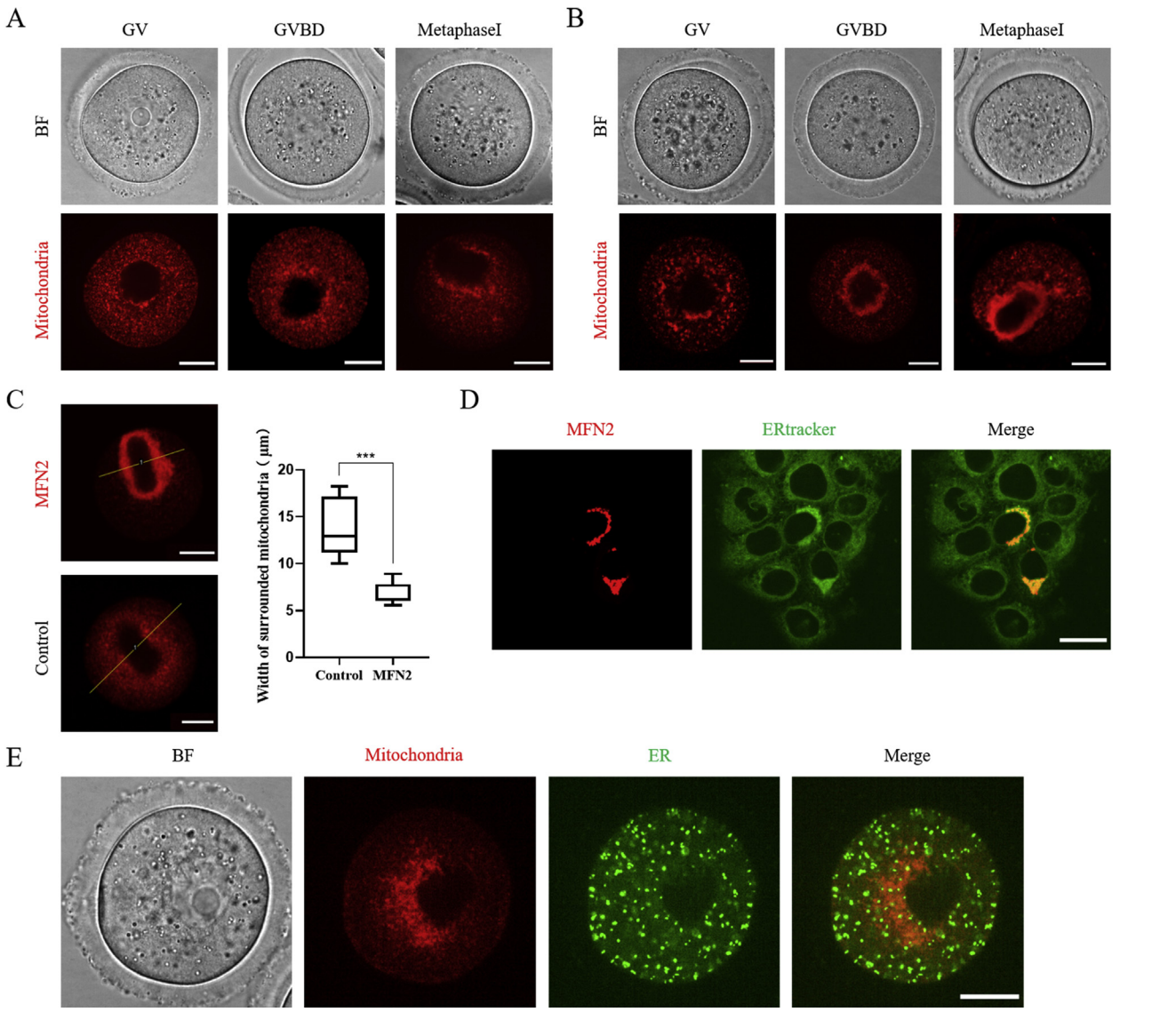

Fig. 1. Effects of MFN2 overexpression on mitochondrial and ER distribution in mouse oocyte. (A) Mitochondria redistribute during meiosis; (B) Overexpression of MFN2 induces mitochondria aggregated and increases the mitochondria surrounding perinuclear region and spindle; (C) The width of mitochondria surrounding spindle in control and MFN2 overexpression oocytes. n = 8 for each group; (D) MFN2 overexpression causes ER tethered in HeLa cells; (E) MFN2 overexpression doesn't cause ER tethering in GV oocytes. Scale bars = 20 μm.

3.2. Overexpression MFN2 has little effects on MMP and ROS production

Besides the morphology and distribution, MMP and ROS are two other most important indicators widely used to evaluate mitochondrial functions *in vivo*. Generally, the high MMP and low ROS content demonstrate that the mitochondrial function is normal, and the low MMP and high ROS content indicate that the mitochondrial function is damaged. Rhodamine 123 and DCFA are two classical molecular probes widely used to detect MMP and ROS, respectively, but their results are prone to be affected by manipulation. Therefore, we mixed the control and MFN2 overexpression oocytes together to label and image the fluorescence simultaneously, because the results between the control and experiment groups are much more comparable. Results shown in Fig. 2A–B indicated that overexpression of MFN2 had little effect on the MMP and ROS production in oocytes, respectively.

3.3. Overexpression of MFN2 causes oocyte to arrest at metaphase I

The mitochondrion surrounding spindle is involved in oocyte meiosis, and thus, people usually use it to evaluate the oocyte quality during research. However, the effect on oocyte development is unclear. Here, MFN2 was overexpressed in GV oocyte to enhance mitochondria surrounding spindle, and the subsequent development was investigated. Results shown in Fig. 3A and B indicate that compared with the control samples, overexpression of MFN2 has little effects on GVBD, while causes most of the oocytes to arrest subsequently. To determine which stage of the oocyte arrested, the spindle and chromosome in the MFN2 overexpression oocytes were analyzed. The results indicated that most of the oocytes completed spindle migration, while were failure to initiate the chromosome segregation, and thus arrested at metaphase I (Fig. 3C).

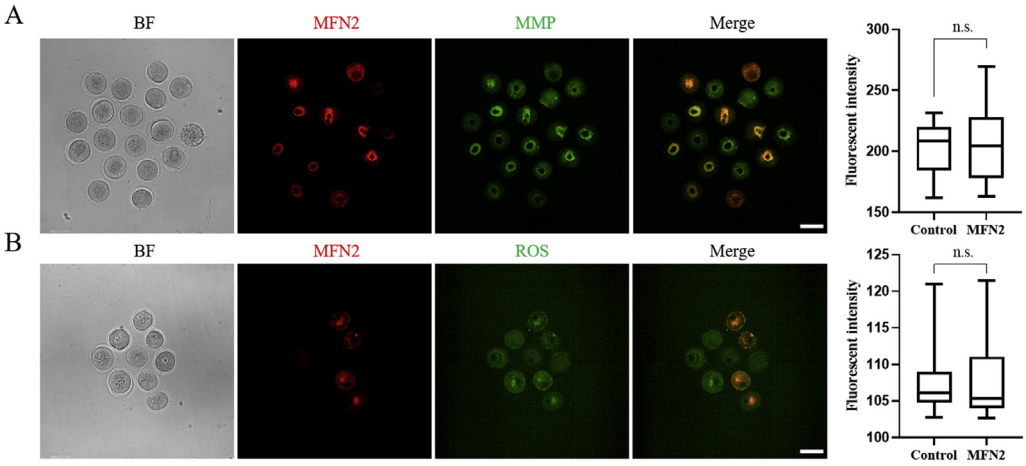

Fig. 2. MFN2 overexpression has little effect on MMP and ROS production. (A) Overexpression of MFN2 has little effect on MMP compared to the control oocytes; (B) Overexpression of MFN2 has little effect on ROS production. Scale bars = 20 μm.

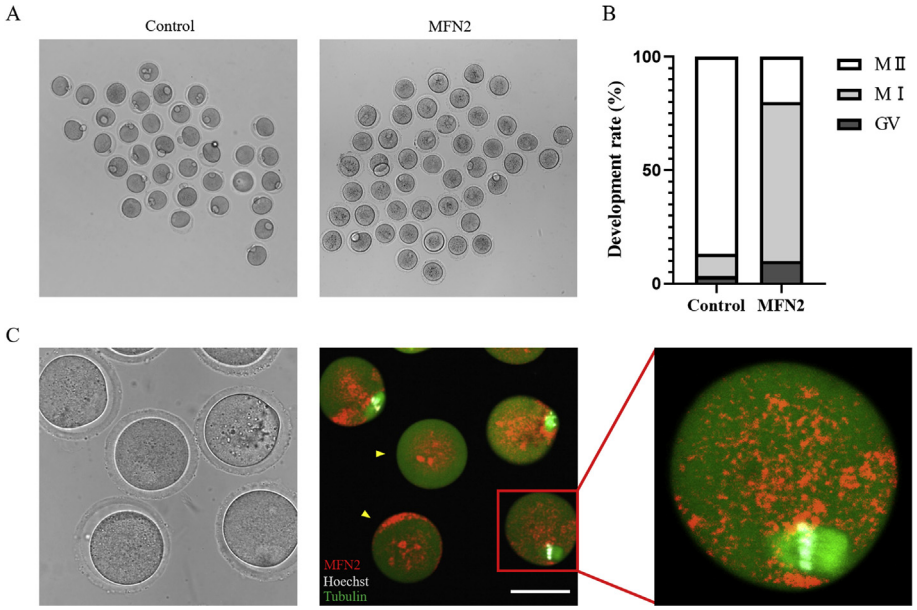

Fig. 3. Overexpression of MFN2 causes oocyte to arrest at metaphase I. (A) Representative images of developmental results for control and MFN2 overexpression oocytes; (B) Statistical analysis of the developmental results for control and MFN2 overexpression oocytes as image A; n = 160 for each group; (C) Representative images of the spindle position and chromosome in the arrested MFN2 overexpression oocytes, arrowheads indicate the spindle and chromosome of these oocytes are not in the focus planes. Scale bar = 40 μm.

3.4. Meiotic spindle was damaged in MFN2 overexpression oocytes

As described above, overexpression of MFN2 has little effects on MMP, ROS production and ER distribution in oocytes. Therefore, we asked if the overexpression of MFN2 may cause abnormal meiosis spindle. Results shown in Fig. 4A–C indicate that before and after the spindle migration, the distance between mitochondria and spindle is 3.7 μm and 5.8 μm, respectively. This suggests that the mitochondrion surrounding spindle is gradually released to allow the spindle movement and chromosome segregation. While as shown in Fig. 4D, the distance between mitochondria and spindle in the MFN2 overexpression oocytes is much smaller than in the control, which is only 2 μm after spindle migration (Fig. 4E). Furthermore, as the culture time prolonged, the aggregations of some mitochondria and tubulin appeared in the arrested oocytes (Fig. 4F and G). And moreover, results shown in Fig. 4H clearly indicate that the aggregations of mitochondria and tubulin were indeed escaped from the spindle, which caused the spindle damaged (Fig. 4I). Therefore, the release of mitochondria surrounding spindle is involved in the chromosome segregation, while MFN2 overexpression tightens and tears the spindle, which damages chromosome segregation and causes oocyte to arrest at metaphase I stage.

4. Discussion

In cells, mitochondria are not only responsible for energy production, but also play important roles in many other important biological events such as calcium homeostasis, phospholipid synthesis, and apoptosis [17]. In matured oocytes and early embryos, the number of mitochondrion DNA (mtDNA) is about 10^5 copies, and won't increase until blastocyst [18]. Mitochondrial defect usually causes the failure of oocyte and embryo development, which may be rescued by supplement of normal mitochondria

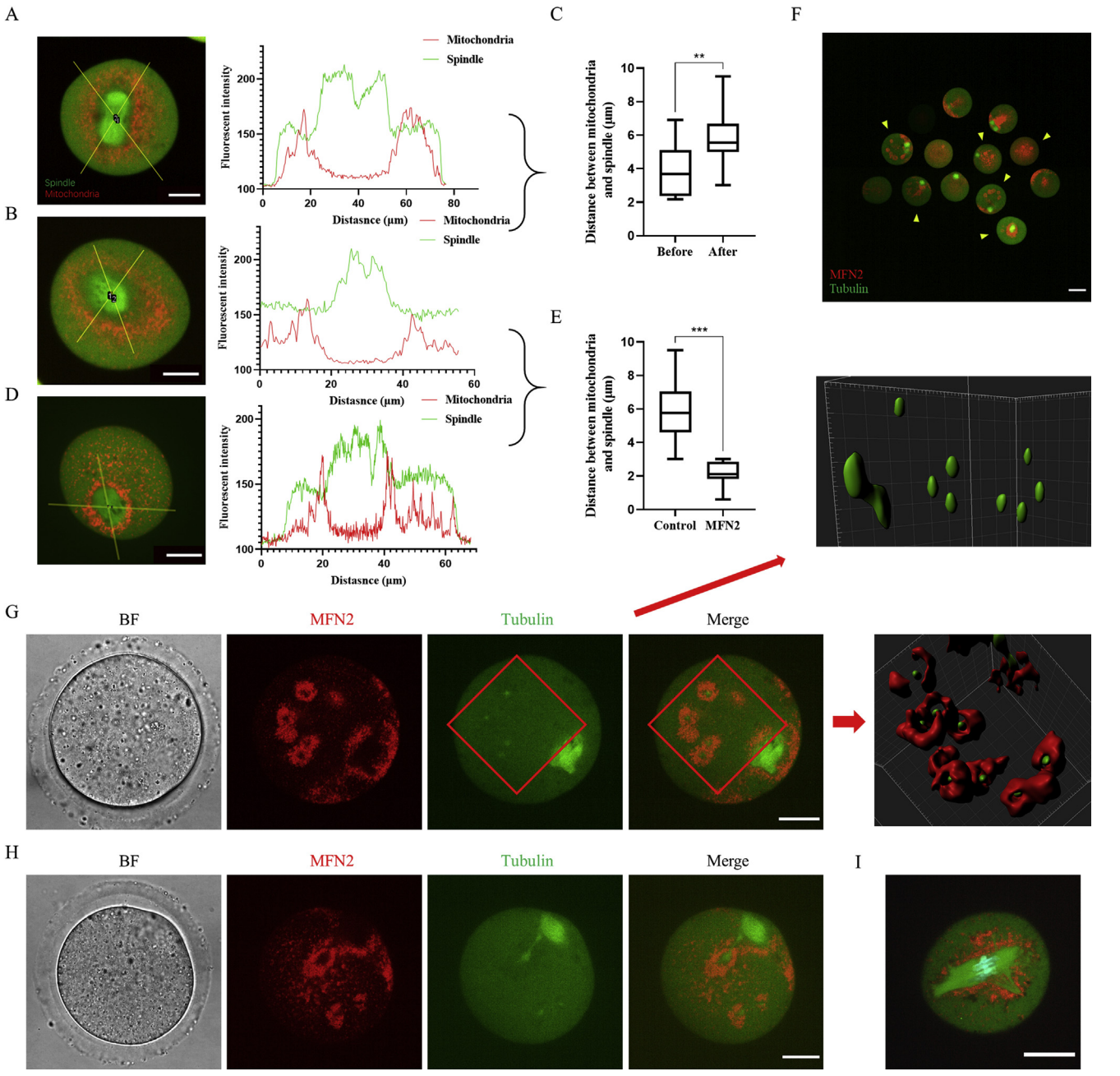

Fig. 4. Overexpression of MFN2 destroys meiosis spindle. (A) Representative image and analysis of the distance between spindle and mitochondria before spindle migration; (B) Representative image and analysis of the distance between spindle and mitochondria after spindle migration; (C) Statistical analysis of the distance between spindle and mitochondria before and after spindle migration as image A and B; (D) Representative image and analysis of the distance between spindle and mitochondria after spindle migration in MFN2 overexpression oocytes; (E) Statistical analysis of the distance between spindle and mitochondria in MFN2 overexpression and control oocytes after spindle migration as image B and C; (F) MFN2 overexpression causes mitochondria and tubulin aggregates in oocyte (arrowheads labeled), Scale bar = 40 μm; (G) Representative image of the mitochondria and tubulin aggregates in MFN2 overexpression oocytes as image F; (H) Representative image of the formation of mitochondria and tubulin aggregates; (I) Representative image of the damaged spindle due to MFN2 overexpression. Except for image F, Scale bars = 20 μm.

[19,20]. In addition, some studies also suggest that utilizing mitochondria as a biomarker for *in vitro* fertilization (IVF) outcome [21]. Therefore, mitochondria are usually used as an important indicator to evaluate oocyte and embryo quality during research. In this study, we primarily studied the effects of increased mitochondria surrounding spindle on meiosis I in mouse oocyte.

First, we determined to build a model which could increase the mitochondria surrounding spindle during meiosis I. Mitochondria are highly dynamic organelles and their morphology is usually determined by the equilibrium between mitochondrial fusion and fission proteins. MFN1 and MFN2 are two important mitochondrial fusion proteins expressed in mouse oocytes. The *Mfn1* deletion causes female mouse infertile [22], while *Mfn2* deletion has little effects on female mouse reproduction [16,23]. Therefore, we overexpressed MFN2 in GV oocytes and confirmed it can increase the mitochondria surrounding perinuclear region and spindle.

It is widely believed that the most important role of mitochondria surrounding spindle is to supply energy for spindle migration and chromosome segregation [9]. High MMP represents high energy production while high ROS represents mitochondrial

defect. Therefore, we examined the MMP and ROS production in oocytes with or without MFN2 overexpression. Currently, DCFA, Rhodamine 123 and JC-1 are the most widely used molecular probes to detect ROS and MMP in cells, respectively. However, these fluorescence dyes are prone to be variable due to operation. Therefore, we mixed the control and MFN2 overexpression oocytes together to detect the DCFA and Rhodamine 123 signal simultaneously, which promise the results much more objective. Results indicated that the increase of mitochondria surrounding spindle by overexpression of MFN2 has little effects on MMP and ROS production. Therefore, except for the increase of mitochondria surrounding spindle, overexpression of MFN2 in oocyte has little effects on mitochondrial function, which is useful to study the role of increased mitochondria surrounding spindle in meiosis.

Therefore, we determined to investigate the effects of mitochondria surrounding spindle on oocyte development. Since MFN2 overexpression has little effects on MMP and ROS production, and the increase of mitochondria surrounding spindle may provide more energy during meiosis, we presumed that MFN2 overexpression is able to facilitate meiosis and has little effects on mouse oocyte development. However, the results indicated that MFN2 overexpression had little effects on GVBD and spindle migration, while caused chromosome segregation failure and the oocytes finally arrested at metaphase I. Therefore, MFN2 overexpression may affect oocyte development through an unknown mechanism rather than abnormal mitochondrial function.

During mammalian oocyte meiosis, meiosis I and II are responsible for the homologous chromosome and sister chromosome segregation, respectively [24]. In some extent, the meiosis II indeed is much similar to mitosis. Regardless of meiosis I, meiosis II or mitosis, spindle movement is the main driving force for chromosome segregation. Therefore, we next examined the spindle during meiosis I with or without MFN2 overexpression. Results indicated that in the control oocytes, after spindle migration, the mitochondria surrounding spindle were gradually released which provided the space for spindle movement. While, in the MFN2-overexpressed oocytes, these mitochondrial release and spindle movement were absent. If continue to culture these oocytes, the delayed mitochondrial release were forced to escape from the spindle (which dilacerate the spindle as aggregations finally). Thus, mitochondria surrounding spindle are dynamic, which may be involved in the spindle formation and migration, while is harmful for spindle movement. Therefore, it should be precisely regulated to support the oocyte meiosis.

Furthermore, mitochondrial aggregation is another widely used indicator to demonstrate abnormal mitochondrial functions in oocytes, while its mechanism is usually attributed to the mitochondrial function such as insufficient energy production. MFN2 overexpression causes mitochondrial aggregation in GV oocytes, and has little effects on MMP and ROS production. These results suggest that mitochondrial aggregation may not directly affect the mitochondrial function and meiotic progression. However, the mitochondrial aggregation can inhibit chromosome segregation by dilacerating the spindle. Therefore, our results suggest a unique mechanism of mitochondrial aggregation affecting oocyte maturation.

Collectively, in this study, we built a model to increase the mitochondria surrounding spindle and carefully evaluated its role during oocyte meiosis. We found that the increase of mitochondria surrounding spindle has little effects on MMP and ROS production, while causes oocyte arrested at metaphase I by inhibiting spindle movement and chromosome segregation. Furthermore, we confirmed that the mitochondria surrounding spindle is dynamic; after spindle migration, it should be released to allow the spindle movement and chromosome segregation. Results in this study suggest that utilizing MMP, ROS production and mitochondria surround spindle may be useful to evaluate mitochondrial function and the oocyte quality, while should be much careful. Mitochondria may affect the oocyte development through other ways besides the energy production and distribution.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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