Mfn2 regulates mitochondria-associated ER membranes to affect PCOS oocyte development

Running Head: Mfn2 affect PCOS oocyte development

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Abstract

This study aimed to investigate the role of mitochondrial-related protein Mfn2 in polycystic ovary syndrome (PCOS) and its impact on oocyte development. The pathological features of PCOS model mice were confirmed by HE staining and immunohistochemistry. The expression of Mfn2 and mitochondrial-related proteins in PCOS oocytes and granulosa cells was detected by qRT-PCR and Western blot. Mitochondrial quantity was measured by mito-tracker staining, and the structure of Mitochondria-associated ER membranes (MAMs) was observed by transmission electron microscopy. The results showed that Mfn2 was significantly downregulated in PCOS oocytes and granulosa cells, and its expression was inhibited in oocytes at different developmental stages. Moreover, the structure of MAMs was also disrupted. Downregulation of Mfn2 expression led to a reduction in mitochondrial quantity in oocytes and granulosa cells, as well as disruption of MAMs structure, while overexpression of Mfn2 had the opposite effect. In conclusion, this study indicates that Mfn2 affects the development of PCOS oocytes by regulating MAMs and may be involved in maintaining the stability of MAMs structure and function, thereby affecting mitochondrial quantity and function. These findings provide new insights into the pathogenesis and treatment of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent reproductive endocrine disorders affecting women of reproductive age, with a prevalence of 5-10%(1). PCOS
is characterized by pathological and physiological changes, including abnormal follicular development, insulin resistance, and excess androgen production (2). Among these changes, abnormal follicular development and insulin resistance are more commonly observed. However, the pathogenesis and molecular regulatory pathways of PCOS remain unclear and are deemed to be multifactorial, involving genetic, environmental, and lifestyle factors. Recent research has suggested that mitochondrial dysfunction may also play a role in the pathogenesis of PCOS (3, 4).

Mitochondria are important organelles that play a crucial role in energy production, metabolism, and cell apoptosis. Mitochondrial dynamics, including mitochondrial fusion and fission, are essential for maintaining normal mitochondrial function. Moreover, mitochondria are dynamically linked with other organelles, especially the endoplasmic reticulum (ER), and their signaling and coordinated function affect the entire cell life cycle. Mitochondria-associated ER membranes (MAMs) refer to the close contacts established between mitochondria and ER through a series of proteins, which form the physical basis for communication between these organelles (5). The coordinated stability of MAMs' structure and function is the material basis for maintaining normal cell physiological function (6, 7). Recent studies have shown that abnormal MAMs' structure and function are closely related to the occurrence and development of many clinical diseases, such as metabolic syndrome (8), cancer (9), and neurodegenerative diseases (10).

Mfn2 is present in the outer mitochondrial membrane and on the surface of the ER connected to mitochondria. It is a crucial participant in the ER-mitochondria interaction.
and is involved in the construction of MAMs (11). Mfn2 regulates mitochondrial morphology, inter-organelle Ca²⁺ transport (12, 13), lipid transportation, and participates in ER stress (14) and mitophagy. It is a key mediator of mitochondrial fusion, and its expression is decreased in PCOS patients (15). Mfn2 has been implicated in various physiological processes such as insulin sensitivity, adipocyte differentiation, and vascular function, and has been shown to regulate the formation and function of MAMs (16).

Taken together, mitochondria (17) and ER (18) play important roles in the pathological process of PCOS. As significant subcellular structures, the ER and mitochondria are closely related to the cellular functional state, with MAMs strengthening the connection between the two. By altering the protein components and structure fixed on MAMs, the fate of the cell is determined in physiological and pathological conditions. Nonetheless, there is currently a gap in the existing research regarding the role of Mfn2 in MAMs in the context of PCOS or oocyte development. Therefore, we intend to establish a PCOS mouse model and selectively modulate Mfn2 to regulate the growth and development of oocyte cells. This research aims to elucidate the mechanism of Mfn2 in the MAMs during oocyte development in mice with PCOS. Such endeavors hold the potential to yield novel insights into the pathophysiology of PCOS and advance female fertility preservation studies.

**Methods**

**Animal**

Seventy female C57BL/6J mice at 3-4 weeks of age were purchased from SiPeiFu
(Beijing) Biotechnology Co., Ltd. All animals were maintained under a 12-hour light/dark cycle with constant temperature and humidity, and had access to standard food and water without restriction.

Construction of PCOS model

The C57BL/6J mice were randomly divided into two groups: the model group (n=20) and the control group (n=20). Mice in the model group received daily subcutaneous injections of 0.1 mL of 0.6 mg/kg DHEA (androgen hormone) (Solarbio, Beijing, ID0220) dissolved in sesame oil with 95% ethanol (volume ratio 9:1) for 20 consecutive days to establish the PCOS model. The control group received an equal volume of solvent injections. At the end of the experiment, mice were euthanized using an overdose of isoflurane (RWD lifescience, Shenzhen). Three mice from each group were selected for ovarian tissue embedding and histological examination using HE staining to confirm the successful establishment of the model for subsequent experiments. The in vivo experiment was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (Approval No. 2020-2026).

Collection of immature oocytes and granulosa cells

For this experiment, 30 female C57BL/6 mice at around 4 weeks of age were used. They were injected with 5 IU of PMSG (pregnant mare serum gonadotropin) intraperitoneally, and after 23 hours, they were euthanized by cervical dislocation. The ovaries were dissected and placed in a pre-equilibrated oocyte retrieval medium
overnight. The surface impurities and bloodstains were washed away. The pipette was prepared and the oocytes were transferred to a pre-IVM medium under a microscope. The oocytes were then transferred to an IVM medium various times to obtain oocytes at the GV, GVBD, MI, and MII stages. Oocytes and granulosa cells were mechanically separated for subsequent testing.

Preparation of oocytes and granulosa cells with Mfn2 underexpression or overexpression

Oocytes and granulosa cells were collected and suspended in DMEM/F12 culture medium containing 10% fetal bovine serum. Mfn2 overexpression vector and Mfn2 siRNA were synthesized by Guangzhou Ruibo Biotechnology Co., Ltd. The Mfn2 overexpression vector or Mfn2 siRNA was mixed with a transfection reagent according to the manufacturer's instructions to prepare the transfection mixture. The mixture was incubated at room temperature for 10-20 minutes. The collected oocytes and granulosa cells were separately placed in M2 medium containing the transfection mixture and incubated for 4-6 hours in a CO2 incubator. After transfection, the oocytes and granulosa cells were washed with fresh M2 medium to remove the transfection mixture and transferred to a new M2 medium for further culture. The efficiency of Mfn2 overexpression or knockdown was verified by qPCR.

HE staining

The experimental procedures for HE staining were carried out according to the
instructions of the Hematoxylin-Eosin staining kit (Solarbio, G1120). The mouse ovarian tissue was embedded in paraffin and cut into sections, which were then dried and dewaxed in xylene for 30 minutes. Subsequently, the sections were immersed in a gradient of ethanol concentrations for 5 minutes each, with the ethanol concentration decreasing from 100% to 30%, followed by immersion in pure water. The sections were stained in Hematoxylin solution for 4-6 minutes and then rinsed in running water for 5 minutes. To remove excess Hematoxylin, the sections were dipped in 1% hydrochloric acid and 70% alcohol for 5-10 seconds. After rinsing in running water again, the sections were stained in Eosin solution for 1 minute and then rinsed until the color no longer bled. Following dehydration and transparency treatment, the sections were mounted with neutral gum, air-dried, and observed under a microscope.

Immunohistochemistry

The paraffin-embedded ovarian tissue sections were immersed in 1× citrate buffer repair solution and heated in a microwave oven for 10 minutes, followed by 30 minutes of cooling at room temperature. After washing three times, the sections were incubated in 3% hydrogen peroxide solution for 10 minutes, followed by blocking with blocking solution for 1 hour at room temperature. Then, diluted Anti-Mitofusin 2 primary antibody (1:100, Abcam, ab124773) was added and incubated at 4°C overnight. After removal of the primary antibody, SignalStain® Boost Detection Reagent (HRP, Mouse #8125) was added and incubated for 30 minutes. The sections were then washed with buffer solution and stained using SignalStain® DAB substrate. The staining intensity
was observed, and the sections were dehydrated, mounted with neutral gum, and observed under a microscope.

MAMs Separation

After washing the collected oocytes with PBS, they were homogenized in a 50 mL glass/Teflon Potter-Elvehjem homogenizer containing buffer at 4°C. The resulting homogenate was centrifuged at 740×g for 5 minutes to remove cell debris and nuclei, and the supernatant was collected and centrifuged again at 9,000×g for 10 minutes. After discarding the supernatant, the pellet was resuspended in 20 mL of homogenization buffer, and all centrifugation steps were carried out at 4°C. Next, crude mitochondria were isolated from the resuspended mixture using a high-speed centrifuge and a Percoll gradient solution. The resulting MAMs were located at the top layer, and the purified mitochondria were located at the bottom of the tube.

Quantitative Real-time PCR

In this study, we employed qRT-PCR to examine the expression levels of Mfn2, IP3R, VDAC, and Drp1 in MAMs from PCOS model and control groups, as well as the levels of Mfn2 in oocytes and granulosa cells at different developmental stages. Additionally, we identified the expression of Mfn2 in oocytes from the sh-NC, shMfn2, OE-NC, OE-Mfn2, and Control groups using microinjection. Cells were lysed and RNA was extracted using Trizol, and then cDNA was synthesized from the RNA. The resulting cDNA was diluted and added to a reaction mixture containing primers for amplification.
Primer sequences are provided in Table 1.

Western Blot

We performed Western Blot analysis to verify the expression levels of Mfn2, IP3R, VDAC, and Drp1 in the PCOS model and normal MAMs, as well as the Mfn2 content in oocytes and granulosa cells at different developmental stages, to ensure the rigor of our experiments. The cells or tissues to be analyzed were lysed using Lysis buffer (Beijing Biocytogen, P0013B) at 4°C for 30 minutes, and the total protein was obtained by sonication using an ultrasonic homogenizer. After measuring the protein concentration with a BCA protein concentration assay kit (Beijing Biocytogen, P0010), the protein was denatured by boiling for 10 minutes. Once the SDS-PAGE gel was prepared, the protein of interest was loaded for electrophoresis separation, then transferred onto a PVDF membrane, and blocked in a 5% non-fat milk TBST solution. The PVDF membrane was then immersed in a primary antibody incubation solution, including Anti-Mitofusin 2 antibody (1:1000, Abcam, ab219730), Anti-IP3R1 antibody (1:1000, Abcam, ab264281), Anti-VDAC1/Porin antibody (1:1000, Abcam, ab306581), and Anti-DRP1 antibody (1:1000, abcam, Ab184247), and incubated overnight at 4°C. After thorough washing with TBST, the corresponding HRP was used for incubation at 37°C for 2 hours, followed by washing and colorimetric exposure.

Mito-Tracker Staining

Mito-Tracker Red CMXRos (Beyotime, C1035) was used to detect mitochondrial
distribution and content in oocytes and granulosa cells. Following the instructions, the working solution was prepared and added to cells at a certain density. After incubating at 37°C for 30 minutes, the working solution was removed, and fresh cell culture medium was added. The cells were observed under a fluorescence microscope.

Transmission Electron Microscopy (TEM)

TEM was used to observe the structure of MAMs in oocytes and granulosa cells. The cultured cells were centrifuged and fixed in a solution of 4% formaldehyde and 2% glutaraldehyde overnight at 4°C, followed by secondary fixation with 1% osmium tetroxide for 2 hours. After initial staining with 1% uranyl acetate, the samples were dehydrated using a gradient concentration of ethanol and embedded in epoxy resin blocks. The blocks were then dried in an oven and sliced into sections. The sections were placed on carbon support film and stained with 50% citric acid lead for 20 minutes, followed by 8% uranyl acetate for 30 minutes. Finally, the samples were imaged using TEM.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8.0 software. The data were presented as mean ± standard deviation. Multiple group comparisons were conducted using one-way ANOVA, and statistical significance was defined as p < 0.05.

Results
PCOS (Polycystic Ovary Syndrome) Model Establishment

HE staining results (Figure 1A, B) showed that the ovaries of the control group mice were normal in shape, with visible follicles at different stages and multilayered granulosa cells. Compared with the control group, the model group showed more cystic and atretic follicles, more small follicles and dilated large follicles, a significant decrease in the number of granulosa cell layers in the inner layer of the follicles, and less distinct corpora lutea.

Expression of Mfn2 and three mitochondrial-related proteins in MAMs of PCOS model

Immunohistochemical results (Figure 2A) showed that Mfn2 was widely expressed in ovarian granulosa cells, follicular membrane, and inner layers of follicular membrane cells. Compared with the control group, the expression of Mfn2 was downregulated in the granulosa cells of the model group. The mRNA expression levels and protein abundance of Mfn2 and mitochondrial-related proteins were detected by qRT-PCR (Figure 2B) and Western blot (Figure 2C) in the PCOS model group and control group, and the differences between the two groups were compared. The results showed that the mRNA expression levels and protein abundance of Mfn2 (p<0.001), IP3R (p<0.001), VDAC (p<0.001, p<0.01), and Drp1 (p<0.001) were significantly lower in the PCOS model group compared to the control group.

The content of Mfn2 varies among oocytes and granulosa cells at different developmental stages

The results of qPCR (Figure 3A) and WB (Figure 3B, C) showed that the content of
Mfn2 in granulosa cells was higher in the control group than in the model group (p<0.05). However, for the expression and protein abundance of Mfn2 in oocytes at different developmental stages, the results of qPCR and WB were inconsistent. In qPCR results, there was no significant difference in Mfn2 content among different developmental stages in the model group, while in the control group, the Mfn2 content was the lowest in the GV stage and the highest in the MII stage. However, in the WB results, the trend in both the control and model groups was that the Mfn2 protein abundance was the highest in the MI stage and the lowest in the GV stage. Meanwhile, the results of qPCR (Figure 3D) and WB (Figure 3E, F) showed that the content of Mfn2 in the control group was higher than that in the model group at different stages of oocyte development (p<0.001).

**The effect of PCOS on mitochondrial quantity and distribution**

Mitochondrial distribution was observed using mito-tracker staining in the oocytes and granulosa cells extracted from the two groups of mice. In the control group, mitochondria were evenly distributed throughout the cell, with strong fluorescence intensity indicating a greater number of mitochondria. Compared with the control group, the fluorescence intensity of mitochondria in the model group was significantly reduced (p<0.001), signifying a decreased quantity (Figure S1). Additionally, mitochondria were concentrated in the cytoplasmic region, with fewer mitochondria in the nucleus (Figure 4A). TEM revealed abnormal mitochondrial structures in the model group, including disorganized matrix and absence of mitochondrial cristae (Figure 4B). By analyzing the interaction between mouse ER and mitochondria using TEM, it was
observed that the contact between the ER and mitochondria was significantly reduced in the model group, which may have an impact on the stability of their function.

**Downregulation of Mfn2 leads to reduced mitochondrial quantity and and declining functionality**

This was confirmed by qPCR analysis of Mfn2 expression in oocytes from the sh-NC, shMfn2, OE-NC, OE-Mfn2, and Control groups. The qPCR results (Figure 5) showed a significant decrease in Mfn2 mRNA levels in oocytes from the shMfn2 group (p<0.001) and a significant increase in Mfn2 mRNA levels in oocytes from the OE-Mfn2 group, confirming the success of transfection (p<0.001). Mito-tracker staining results (Figure 6A and S2) showed a significant decrease in mitochondrial quantity in granulosa cells (p<0.05) and oocytes (p<0.001) with Mfn2 knockdown, while the opposite was observed in the Mfn2 overexpression group with significantly increased mitochondrial quantity (p<0.001). TEM analysis of the contact between mitochondria and ER was used to evaluate MAM formation. The results showed a significant decrease of MAMs in the shMfn2 group, while in the OE-Mfn2 group, the distance between the ER and mitochondria was similar to that of the control group, indicating that MAM formation was not affected (Figure 6B).

**Discussion**

The MAMs are specific domains between the ER and mitochondria, closely linked to various biological processes such as cellular metabolism, apoptosis, and stress response (19). Recent studies indicate that MAMs play a critical role in regulating ovarian
function. PCOS is a complex endocrine-metabolic disorder with unclear pathogenesis. In this study, we examined the structure of MAMs and the expression of Mfn2 in PCOS oocytes, and found that Mfn2 was significantly downregulated in both oocytes and granulosa cells, along with decreased expression of mitochondrial-related proteins. Additionally, Mfn2 expression was inhibited at different developmental stages of oocytes, and MAMs structure was also damaged. Further experiments revealed that Mfn2 expression is closely associated with mitochondria, and its downregulation affects the number of mitochondria and the structure of MAMs in oocytes and granulosa cells, while upregulation of Mfn2 leads to an increase in the number of mitochondria and a stable structure. These findings suggest that Mfn2 regulates PCOS oocyte development by modulating MAMs.

The results of both qRT-PCR and Western Blot analyses have revealed significantly lower mRNA and protein expression levels of Mfn2, IP3R, VDAC, and Drp1 in the PCOS model group compared to the control group. Among these, Mfn2 is a fusion protein between mitochondria and the ER, whose downregulation may result in mitochondrial dysfunction and metabolic disorders (15). On the other hand, IP3R and VDAC are respectively located in the ER and outer membrane, and their downregulation could affect calcium ion transfer and energy metabolism between mitochondria and the ER (20-22). Drp1 is a protein involved in mitochondrial fission and its downregulation may affect mitochondrial division and repair (23). Therefore, we speculate that Mfn2 is involved in regulating the Ca2+ transfer between the ER and...
mitochondria, maintaining the stability of MAMs' structure and function, and thus affecting the quantity and function of mitochondria. When the expression of Mfn2 is downregulated, the stability of MAMs is compromised, leading to interference with the Ca2+ transfer between the ER and mitochondria, and thus impacting PCOS oocyte development.

Previous studies have shown that MAMs play an important role in regulating ovarian function, and Mfn2, as a structural protein of MAMs, is involved in regulating the fusion of the outer mitochondrial membrane and plays a critical role in ovarian development (24, 25). The downregulation of Mfn2 can affect the in vitro maturation and fertilization of immature oocytes by regulating meiosis and mitochondrial function (26, 27). Studies have shown that Mfn2 gene knockout mice exhibit insulin resistance and mitochondrial dysfunction (28), which is consistent with the results of this study and indicates that the regulation of MAMs by Mfn2 is an important mechanism affecting the development of PCOS oocytes. Moreover, Mfn2 has been found to play a critical role in various diseases, such as obesity and type 2 diabetes (15), indicating that Mfn2 may be an important therapeutic target with broad clinical application prospects.

This study has several limitations. Firstly, it is based on cellular experiments, and further animal experiments and clinical studies are needed to validate our findings. Secondly, this study has not explored in depth the molecular mechanisms of how Mfn2 exerts its effects on oocyte development through the regulation of MAMs. Future
studies can use more advanced techniques in bioinformatics and molecular biology, such as RNA sequencing, proteomics, and metabolomics, to further investigate this question. In addition, while this study provides new insights into the treatment of PCOS, the specific treatment methods and drugs have not been determined. Future research can further explore how to treat PCOS by regulating MAMs and developing relevant treatment methods and drugs.

Conclusion

This study has revealed that Mfn2 is significantly downregulated in PCOS oocyte and granulosa cells, which also results in a decrease in mitochondrial-related proteins and destabilization of MAMs structure during different stages of oocyte development. Further experiments indicate that Mfn2 expression is closely related to mitochondria, and its downregulation affects the number of mitochondria and the structure of MAMs in oocyte and granulosa cells. Therefore, this study suggests that Mfn2 plays a crucial role in regulating PCOS oocyte development through MAMs. These findings not only enhance our understanding of the pathogenesis of PCOS but also provide important clues for the identification of new therapeutic targets for PCOS.

Declarations

Ethical Approval

This study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (Approval No. 2020-2026).

Competing Interests

The authors declare there is no potential conflict of interest.
Authors' contribution

All authors contributed to the study conception and design. Xiuhua Liao prepared the materials, analyzed the data and wrote the first draft of the manuscript. Suqin Zhu, Shumin Qiu, Yan Sun and Beihong Zheng performed the experiment. Suqin Zhu, Hua Cao, Wenwen Jiang and Huiling Xu collected and analyzed the data. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. All authors agree to be accountable for all aspects of the work.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Reference


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**Figure legend**

Figure 1. Histological analysis of PCOS model. (A) HE staining of the ovaries, 100×; (B) HE staining of the ovaries, 200×. CL, corpus luteum; ANF, antral follicle; ATF, atretic follicle; CF, cystic follicle.
Figure 2. The proteins abundance and genes expression in the PCOS mouse model. (A) Immunohistochemistry was applied to assess Mfn2 in ovarian tissue sections (200×). Mfn2 and mitochondrial-related proteins expression in MAMs, including IPR3, DRP1 and VDAC, were analyzed by (B) qRT-PCR and (C) Western blot in the control group and model group, **p<0.01, ***p<0.001, compare with control.

Figure 3. The contents of Mfn2 in oocytes and granulosa cells at different developmental stages. Mfn2 in granulosa cells was detected by (A) qRT-PCR and (B, C) Western blot in the control group and model group. Mfn2 in oocytes at different stages was detected by (D) qRT-PCR and (E, F) Western blot in the control group and model group, *p<0.05, **p<0.01, ***p<0.001, compare with control.

Figure 4. The effect of PCOS on mitochondrial quantity and distribution. (A) Mitochondrial distribution was visualized by mito-tracker staining in the oocytes and granulosa cells isolated from both groups of mice. (B) The interaction between ER and mitochondria was observed using TEM in the oocytes and granulosa cells. The red square presents MAMs.

Figure 5. Validation of transfection in oocytes with Mfn2 overexpression vector or Mfn2 siRNA. Mfn2 in oocytes was detected by qRT-PCR in different transfection groups, ***p<0.001, compare with control.
Figure 6. The influence of Mfn-2 on mitochondrial quantity and distribution in oocytes and granulosa cells. (A) Mitochondrial quantity in oocytes and granulosa cells transfected with different miR-542 levels was demonstrated by mitochondrial tracking staining. (B) The situation of MAMs in oocytes and granulosa cells transfected with different miR-542 levels was observed through TEM. The red square presents MAMs.

Figure S1. The effect of PCOS on mitochondrial quantity. Fluorescent intensities of mitochondria in (A) oocyte and (B) granulosa cells, ***p<0.001, compare with control.

Figure S2. The influence of Mfn-2 on mitochondrial quantity in (A) oocytes and (B) granulosa cells, *p<0.05, ***p<0.001, compare with control.
Histological analysis of PCOS model. (A) HE staining of the ovaries, 100; (B) HE staining of the ovaries, 200. CL, corpus luteum; ANF, antral follicle; ATF, atretic follicle; CF, cystic follicle.
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The contents of Mfn2 in oocytes and granulosa cells at different developmental stages. Mfn2 in granulosa cells was detected by (A) qRT-PCR and (B, C) Western blot in the control group and model group. Mfn2 in oocytes at different stages was detected by (D) qRT-PCR and (E, F) Western blot in the control group and model group, *p<0.05, **p<0.01, ***p<0.001, compare with control.
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175x83mm (300 x 300 DPI)
Mfn2 in oocytes was detected by qRT-PCR in different transfection groups, ***p<0.001, compare with control.
The influence of Mfn-2 on mitochondrial quantity and distribution in oocytes and granulosa cells. (A) Mitochondrial quantity in oocytes and granulosa cells transfected with different miR-542 levels was demonstrated by mitochondrial tracking staining. (B) The situation of MAMs in oocytes and granulosa cells transfected with different miR-542 levels was observed through TEM. The red square presents MAMs.
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The effect of PCOS on mitochondrial quantity. Fluorescent intensities of mitochondria in (A) oocyte and (B) granulosa cells, ***p<0.001, compare with control.
The influence of Mfn-2 on mitochondrial quantity in (A) oocytes and (B) granulosa cells, *p<0.05, ***p<0.001, compare with control.

175x90mm (300 x 300 DPI)