



Mitochondrial Ca²⁺ Is Related to Mitochondrial Activity and Dynamic Events in Mouse Oocytes

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Mitochondrial energy insufficiency is strongly associated with oocyte activation disorders. Ca²⁺, especially that in the mitochondrial matrix, plays a pivotal role in mitochondrial energy supplementation, but the underlying mechanisms are still only poorly understood. An encoded mitochondrial matrix Ca²⁺ probe (Mt-GCaMP6s) was introduced to observe mitochondrial Ca²⁺ ([Ca²⁺]_m) dynamic changes during oocyte maturation and activation. We found that active mitochondria surrounding the nucleus showed a higher [Ca²⁺]_m than those distributed in the cortex during oocyte maturation. During oocyte partheno-activation, the patterns of Ca²⁺ dynamic changes were synchronous in the cytoplasm and mitochondria. Such higher concentration of mitochondrial matrix Ca²⁺ was closely related to the distribution of mitochondrial calcium uptake (MICU) protein. We further showed that higher [Ca²⁺]_m mitochondria around the chromosomes in oocytes might have a potential role in stimulating mitochondrial energy for calmodulin-responsive oocyte spindle formation, while synchronizing Ca²⁺ functions in the cytoplasm and nuclear area are important for oocyte activation.

Keywords: mitochondrial Ca²⁺, oocyte activation, oocyte maturation, oocyte metabolism, Ca²⁺ oscillations

INTRODUCTION

According to the WHO 2016 report, at least one of 10 couples in developed countries cannot have children within 5 years of marriage, half of which are due to female infertility (Chow et al., 2016). Oocyte activation inefficiency is a major problem causing female infertility (Schatten et al., 2014). Oocyte activation rates in obese, diabetic and aging women are low, affecting the development of preimplantation embryos and also pregnancy (Minge et al., 2008; Igosheva et al., 2010; Jungheim et al., 2010; Shankar et al., 2011; Luzzo et al., 2012; Wang et al., 2017). Such disorders are strongly related with mitochondrial energy supply insufficiency (Gesink Law et al., 2007; Rittenberg et al., 2011). Resolving the low quality of oocytes caused by metabolic abnormalities and improving the efficiency of assisted reproduction are key issues that researchers need to address.

Abbreviations: [Ca²⁺]_i, Intracellular Ca²⁺ Concentration; no[Ca²⁺]_m, Mitochondrial Matrix Ca²⁺ Concentration; cpGFP, Circularly Permuted Green Fluorescent Protein; GV, Germinal Vesicle; GVBD, Germinal Vesicle Breakdown; MII, Metaphase II; Mt-GCaMP6s, Encoding Mitochondrial Ca²⁺ Probe Based cpGFP With A Mitochondrial Signal Peptide.

Ca²⁺ is one of the major signaling molecules regulating several cell functions, such as cell division, arrest, and apoptosis. At fertilization, oocyte activation begins with a series of crucial steps triggered by periodical repetitive increases and decreases in intracellular Ca²⁺ ([Ca²⁺]_i) concentrations known as [Ca²⁺]_i oscillations (Stricker, 1999; Wakai et al., 2013). Mitochondrial function and intracellular [Ca²⁺]_i are two critical factors for the oocyte's developmental potential (Wacquier et al., 2019). Although [Ca²⁺]_i oscillations are required for oocyte activation (Yazawa et al., 2007), very little is known about how cytoplasmic and mitochondrial Ca²⁺ regulates the energy supply mode transition during oocyte activation.

Mitochondrial Ca²⁺ is a key factor initiating mitochondrial metabolism and regulating mitochondrial activity. Mitochondrial matrix Ca²⁺ homeostasis is strictly regulated by the transmission of Ca²⁺ between the cytoplasm, ER and mitochondria (Dumollard et al., 2006), which plays important roles in cellular physiology. Mitochondrial Ca²⁺ regulates membrane potential, stimulates rate-limiting enzymes in the Krebs cycle, and accelerates ATP synthesis (Chacko and Eliceiri, 2018). Ca²⁺ oscillations triggered by sperm-oocyte fusion also affect fertilized oocyte mitochondria and promote the transition of mitochondria from the resting state to the activation state (Dumollard et al., 2006). Our previous results showed that mitochondrial activity plays a critically important role in maintaining [Ca²⁺]_i oscillations (Wang et al., 2018). Although there are some reports on the mechanisms of Ca²⁺ and mitochondria interactions during cell differentiation (Mallilankaraman et al., 2012a,b), the relationship between [Ca²⁺]_m and mitochondrial metabolism is unknown in oocytes.

In this study, we observed the dynamic [Ca²⁺]_m changes during *in vitro* maturation and activation of oocytes, and we further studied the functions of matrix Ca²⁺ on mitochondrial activity, with the ultimate goal to improve oocyte quality by modifying the metabolism of mitochondria within the oocyte.

MATERIALS AND METHODS

Ethics Statement

Female ICR mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were handled in accordance with the institutional animal care policies of the Institute of Zoology, Chinese Academy of Sciences. Mice were maintained under a 12 h light and 12 h darkness cycle in a specific pathogen-free stage at the Central Animal Laboratory of the Institute of Zoology. The Laboratory Animal Care and Use Committee of the Institute of Zoology approved this study.

Oocyte Collection and Partho-Activation

Female mice were injected with 10 IU pregnant mare serum gonadotropin (PMSG, Ningbo Hormone Product Co., Ltd., Cixi, China). After 44–48 h, GV oocytes were

collected by ovarian mincing. Female mice were injected with 10 IU PMSG followed 44–48 h later by injection of 10 IU human chorionic gonadotropin (hCG, Ningbo Hormone Product Co., Ltd., Cixi, China). After 15 h of hCG, ovulated MII oocytes were collected and denuded in 1 mg/mL hyaluronidase. Matured oocyte [Ca²⁺]_i/[Ca²⁺]_m oscillations were induced by 10 mM strontium chloride (SrCl₂, 10025-70-4, Sangon Biotech, Shanghai, China) in Ca²⁺-free CZB for 4 h. After partho-activation, eggs were transferred into KSOM (MR-106, Merck Millipore, MA, United States).

Plasmid Construction of Vectors

An encoded Ca²⁺-sensitive probe was constructed based on the cpGFP (Circularly Permuted Green Fluorescent Protein) system and calmodulin (GCaMP6s) to detect Ca²⁺ concentrations (Akerboom et al., 2012; Zhong and Schleifenbaum, 2019). The mitochondrial location probe plasmid was constructed to express the Ca²⁺ detection protein probe with mitochondrial localization signal peptide cloned from Trmt10c gene (Metodieiev et al., 2016; Oerum et al., 2018). For cloning of genes encoding MICU protein, voltage-dependent anion selective channel (VDAC) and CaMs, The coding sequences (CDS) were obtained by RT-PCR with template of ovary cDNA cloned into the pMD-18-T vector (Takara, Dalian, Liaoning, China). 18-T vector were sliced and linked CDS to upstream of mCherry. Two plasmids, p-UC57-Mt-GCaMP6s (Supplementary Figure S1A) and p-GEMHE-MICU-mCherry (Supplementary Figure S1B), were dissolved in water and stored at –20°C.

In vitro Transcription of cRNA

Templates of *in vitro* transcription from constructed plasmids were obtained by PCR with primers M13F and M13R (Supplementary Figures S1C–F). PCR products were diluted in RNase-free water. cRNA transcripts were synthesized *in vitro* with T7 RNA polymerase mMACHINE T7 kit (Ambion, Life Co., Calsbad, CA, United States). Poly(A) tail was added to the sequence end by polymerase tailing kit (PAP5104, Lucigen, Beijing, China). The RNA solutions were then stored at –80°C in a final concentration of 400 µg/mL until further use. Approximately 50 pl of RNA solution was injected into each GV oocyte.

GV Oocyte Microinjection

The M2 medium and M16 medium containing 2.5 µM milrinone were prepared and warmed to 37°C. Milrinone is a phosphodiesterase inhibitor that maintains meiotic arrest once oocytes are removed from the follicles (Stein and Schindler, 2011). Micro-drops each containing 20 µl M16 medium with milrinone were prepared in a dish and overlaid with mineral oil. Injection pipettes were made by pulling borosilicate-glass capillary with filament in a mechanical puller. We used a Flaming-Brown micropipette puller (Model P-97) with the following settings: *P* = 540, *Heat* = 300, *Pull* = 130, *Vel* = 100, *Time* = 150. Microinjection platform was prepared by placing a 10 µl micro-drop of M2 medium

with milrinone on a chamber slide, and the drop was covered with mineral oil and placed on the microscope stage. Injection and holding pipettes were placed into the drop of M2 medium with milrinone. Microinjection of cRNA was performed with Narishige micromanipulators (Narishige Inc., Sea Cliff, NY) under a Nikon TE 200 (Nikon UK Ltd., Kingston upon Thames, Surrey, United Kingdom) and finished within 30 min. After cRNA injection, oocytes were arrested at the GV stage in M16 medium containing 2.5 μM milrinone for 4 h. Then the oocytes were transferred to M16 medium and cultured under mineral oil at 37°, in an atmosphere of 5% CO₂ in air for 14 h *in vitro* maturation.

Real-Time Recording of [Ca²⁺]_i and [Ca²⁺]_m Changes

We set out to confirm [Ca²⁺]_i oscillations regulation on mitochondrial activity during oocyte activation. [Ca²⁺]_i oscillations were observed by staining with 2 μM Fluo4 AM (488 nm excitation, 525 nm emission) in the partheno-activation system. Rhod2 is a classical dye for determining mitochondrial Ca²⁺ concentration ([Ca²⁺]_m) with a higher Ca²⁺ affinity (Garcia-Perez et al., 2008; Eisner et al., 2010). We recorded [Ca²⁺]_m dynamic changes with 2 μM Rhod2 AM (561 nm excitation, 590 nm emission) in a partheno-activation system. Mt-GCaMP6s was introduced to obtain the patterns of mitochondrial matrix Ca²⁺ dynamic changes. Real-time images were obtained using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, MA, United States) and recorded at 2 frames per minute. [Ca²⁺]_i and [Ca²⁺]_m intensities were detected using an argon laser.

Real-Time Recording of Distribution of Activated Mitochondria

Activated mitochondria were imaged with 0.1 μM MitoRed (C2005, Beyotime, China), a cell permeable potential-sensitive fluorescent mitochondrial dye emitting in red (561 nm excitation, 590 nm emission) channel, which has a high affinity with higher potential mitochondria (Chazotte, 2011), by using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, United States). The GV oocyte maturation was conducted by incubation in M16 (M7167, Sigma-Aldrich, United States) with 0.1 μM MitoRed. MII oocytes were activated in Ca²⁺-free-CZB with SrCl₂, staining with 0.1 μM MitoRed. A software Volocity was used to analyze fluorescence intensity.

Statistical Analysis

All experiments were conducted at least three times. No fewer than 40 oocytes were collected and examined in each experiment and no fewer than three biological repeats for one group were conducted. We presented information of samples with Means and Standard Deviations (SD). Results were analyzed by SPSS 19.0. The significance of differences among groups was analyzed by the Chi-square test; $p < 0.05$ were considered statistically significant.

RESULTS

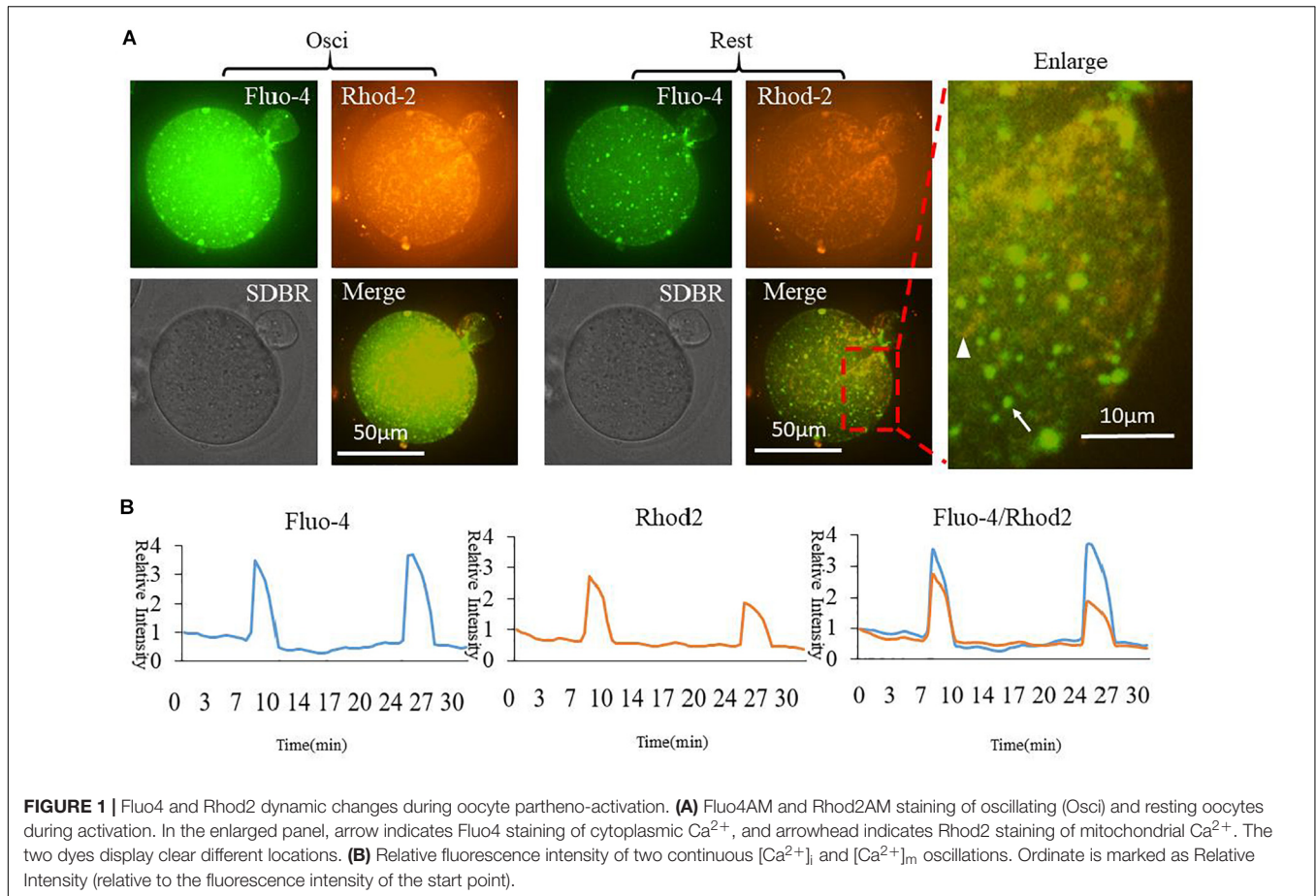
Dynamic Changes in Mitochondrial Matrix Ca²⁺ During Partheno-Activation of Oocytes

In order to compare cytoplasmic and mitochondrial Ca²⁺ dynamic changes during oocyte partheno-activation, three dyes were employed to observe the Ca²⁺ concentration patterns in PMSG/HCG primed ovulated MII oocytes. Firstly, we detected Ca²⁺ dynamic changes in the cytoplasm ([Ca²⁺]_i) and mitochondria ([Ca²⁺]_m) by co-staining with Fluo4 AM (Figure 1A arrow) and Rhod2 AM (Figure 1A arrowhead). Oocyte [Ca²⁺]_i oscillations were detected using Fluo4 AM in the partheno-activation system, while Rhod2 AM was reported as a classic dye for determining [Ca²⁺]_m in somatic cells (Garcia-Perez et al., 2008; Eisner et al., 2010). Rhod2 signal showed the same pattern as [Ca²⁺]_i oscillations (Figure 1B). Ca²⁺ concentrations in the cytoplasm and mitochondria changed synchronously during partheno-activation. We suggest that patterns of mitochondrial Ca²⁺ during oocyte activation may be the same as that of [Ca²⁺]_i oscillations.

In order to further confirm the changes of [Ca²⁺]_m during oocyte activation, we applied *in vitro* transcription and microinjection (Stein and Schindler, 2011) of cRNA encoding a cpGFP (circularly permuted green fluorescent protein) based-Ca²⁺ probe with a mitochondrial signal peptide (Mt-GCaMP6s) (Supplementary Figure S1A; Akerboom et al., 2012) to determine [Ca²⁺]_m patterns. Indeed, most of the mitochondria could be stained by Rhod2 while only some energized mitochondria were stained by GCaMP6s (Figure 2). In comparison, GCaMP6s is more suitable for detecting the dynamic changes of [Ca²⁺]_m in oocytes. Finally regardless of which dye was used, [Ca²⁺]_i and [Ca²⁺]_m oscillations had the same pattern during oocyte activation. Ca²⁺ changes in the cytoplasm and mitochondria were synchronous.

Dynamic Changes in [Ca²⁺]_m During Oocyte Meiotic Maturation

We selected Mt-GCaMP6s to observe the dynamic pattern of [Ca²⁺]_m changes in mitochondria during GV oocyte *in vitro* maturation (Figure 3 and Supplementary Video S1). Mitochondrial [Ca²⁺]_m was located to the surrounding area of the germinal vesicle, which was clearly higher than that located at the oocyte cortex. After GVBD, higher [Ca²⁺]_m mitochondria were observed to distribute around the spindle. Mitochondrial metabolism is much stronger in the energy-demanding part of the oocyte. Interestingly, there were two increasing [Ca²⁺]_m spikes during oocyte maturation (Figure 3B). The first [Ca²⁺]_m spike was observed at 9 h, before the first polar body extrusion, in about half of the GV oocytes examined (46.3 ± 8.3%). 1st polar body non-extruded oocytes did not show any Ca²⁺ spike. The second spike occurred 4–5 h after the first polar body emission, i.e., at about 14 h of culture in 33.2 ± 6.2% of oocytes which exhibited the first spike (Figure 3C). This coincided with the suitable window when the oocytes are ready for activation. These two time points were likely most critical for energy requirements.



Higher $[\text{Ca}^{2+}]_m$ and Activated Mitochondrial Distribution in Oocytes

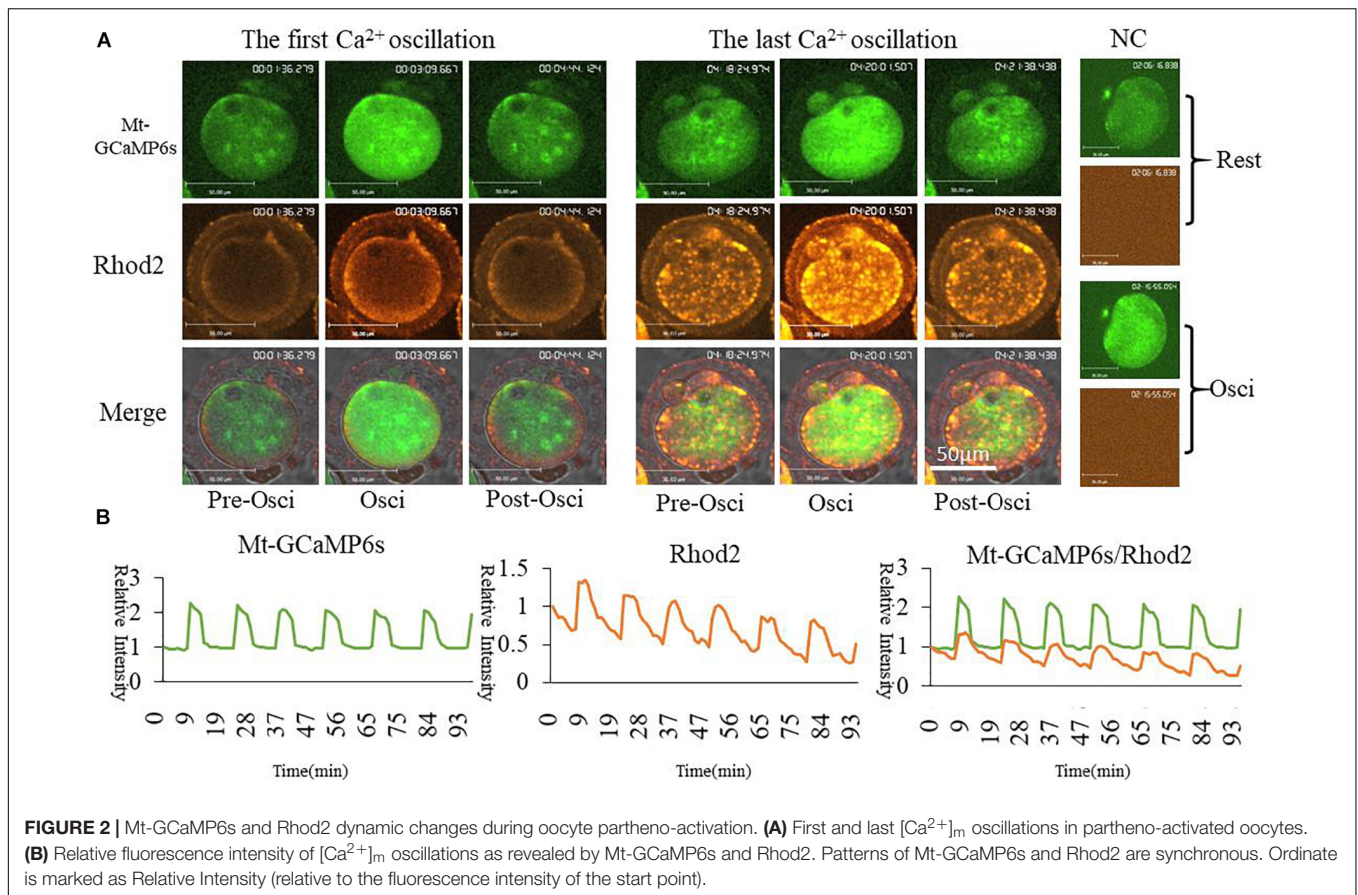
Mt-GCaMP6s was further applied to observe the dynamic relationship between $[\text{Ca}^{2+}]_m$ and activated mitochondrial distribution during oocyte maturation (Figure 4) and during a 4-h short term observation (Supplementary Figure S2). MitoRed-stained activated mitochondria surrounded the chromosomes and spindle with a higher $[\text{Ca}^{2+}]_m$. Next, we employed a partheno-activation system (Ca^{2+} -free CZB with SrCl_2) to observe the changes of $[\text{Ca}^{2+}]_m$ and activated mitochondrial distribution during oocyte activation (Figure 5). We found that there were two types of $[\text{Ca}^{2+}]_m$ distributions of different mitochondria. In one part staining was maintained for a long time. The higher $[\text{Ca}^{2+}]_m$ co-localized with the MitoRed positive mitochondria. $[\text{Ca}^{2+}]_m$ in the other part of mitochondria changed as $[\text{Ca}^{2+}]_i$ oscillation patterns rapidly increased and decreased. We found that during activation, $[\text{Ca}^{2+}]_i$ oscillations in oocytes will drive Ca^{2+} changes in the mitochondria, but it does not affect these MitoRed positive mitochondria.

Higher $[\text{Ca}^{2+}]_m$ Related to the Distribution of MICU

Mitochondrial calcium uniporter (MCU) has multiple subunits, and its Ca^{2+} influx activity is controlled by regulatory subunits,

including mitochondrial calcium uptakers (MICU) (Xing et al., 2019). *In vitro* transcription vector of MICU was constructed to confirm the relationship between $[\text{Ca}^{2+}]_m$ and MICU. MICU was found to co-distribute with higher $[\text{Ca}^{2+}]_m$ mitochondria in the GV oocytes (Figure 6). In PA oocytes, such distribution was similar as that of activated mitochondria and higher $[\text{Ca}^{2+}]_m$ mitochondria. During activation, $[\text{Ca}^{2+}]_i$ oscillations will drive Ca^{2+} changes in the mitochondria, but it did not affect these Mito-Red positive mitochondria. MICU distribution in oocytes was similar to Mito-Red positive activated mitochondria. This may indicate that MICU is related to mitochondrial matrix Ca^{2+} and mitochondrial activity.

VDACs (voltage-dependent anion channels) are a widespread on the outer mitochondrial membranes of most organisms (De Pinto et al., 2003). The OMM functions as a link between mitochondrial metabolism and the rest of the cell (Cassara et al., 2009). Some other reports also demonstrated the presence of VDACs in the plasma membrane and other organelles (Bathori et al., 2000; Shoshan-Barmatz et al., 2006). *In vitro* transcription of VDAC was constructed to observe the distribution of VDAC in mouse oocytes and to confirm the relationship between $[\text{Ca}^{2+}]_m$ and VDAC. The distribution of $[\text{Ca}^{2+}]_m$ and VDAC were obviously different. VDAC distributed in most parts of oocytes (Supplementary Figure S3).



Calmodulin and Higher [Ca²⁺]_m Mitochondria Distribution in Oocytes

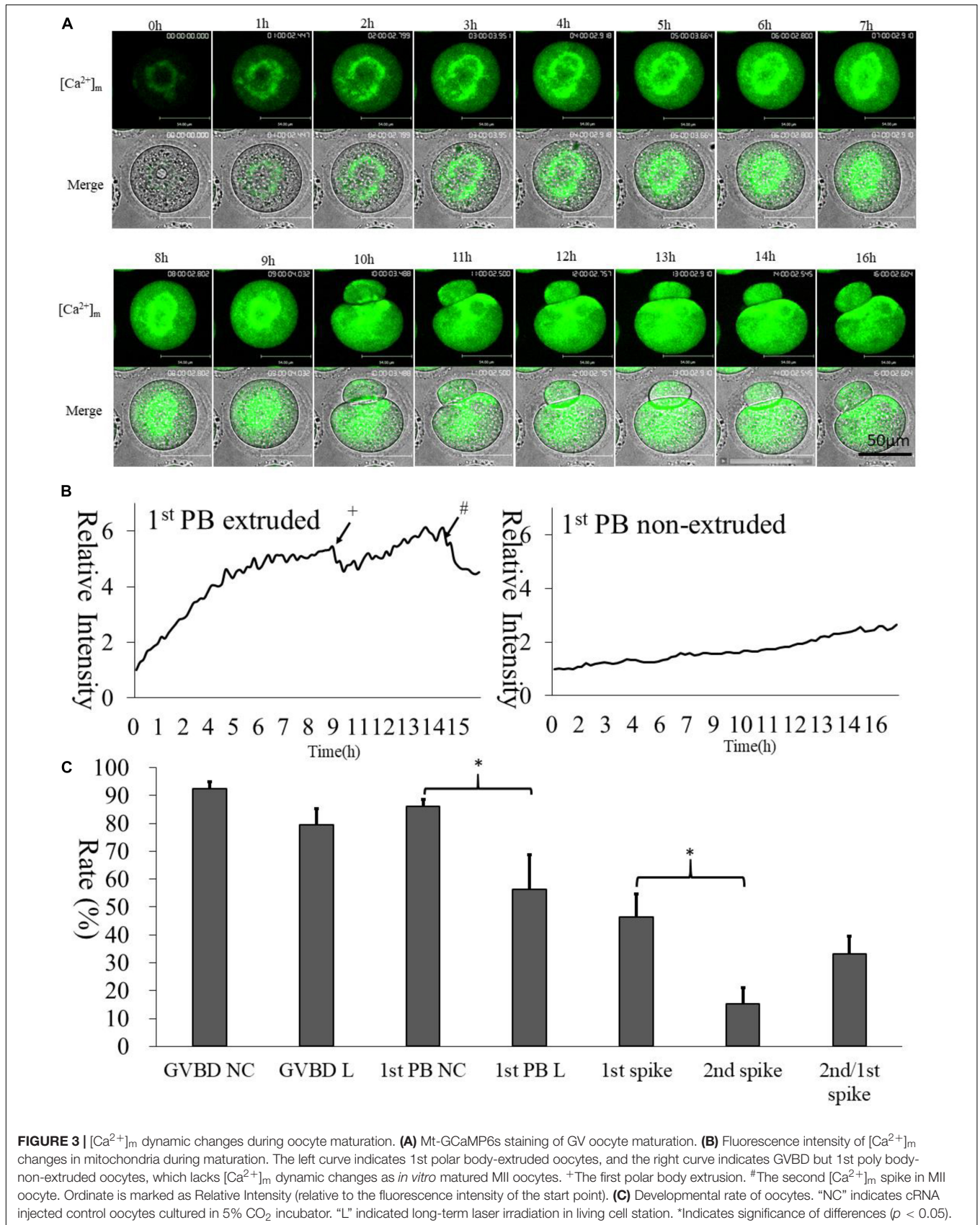
Calmodulins (CaMs), including two members termed CaM1 and CaM2, are thought to be the initial responding proteins in the cell to sense dynamic changes in Ca²⁺ concentration. CaMs are expected to distribute in oocytes around the most sensitive organelles for Ca²⁺. Firstly, we cloned the CaM1 and CaM2 cDNA sequences and linked them with mCherry as pGEMHE-CaM1-mCherry and pGEMHE-CaM2-mCherry plasmids (**Supplementary Figure S1**); then we observed the localization of CaMs during oocyte maturation. Both CaM1 and CaM2 are located at the spindle (**Supplementary Figure S4**), which indicates that CaM1 and CaM2 have similar locations and functions in spindle formation as previously reported (Xu et al., 1996; Mehta et al., 2014).

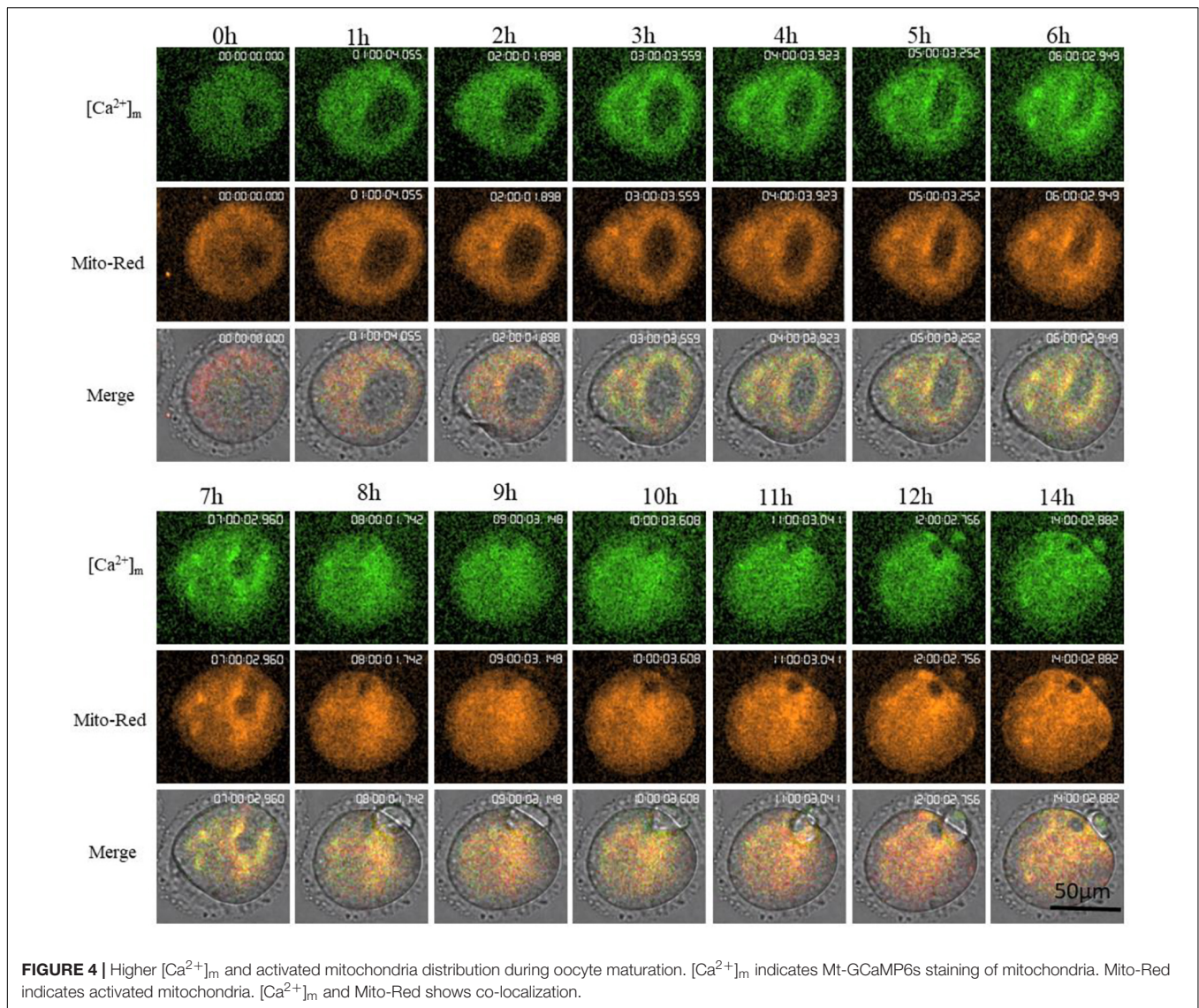
Since CaM1 and CaM2 have similar distributions, we wanted to confirm the relationship between [Ca²⁺]_m and CaM2. Higher [Ca²⁺]_m mitochondria surrounded the spindle, while CaM2 was localized on the spindle (**Figure 7**). We conclude that CaM2 is more directly involved in spindle formation, while [Ca²⁺]_m regulates mitochondrial activity for energy supplementation. [Ca²⁺]_i oscillations triggering cytoplasmic Ca²⁺ increase during oocyte activation will synchronize mitochondrial energy supplementation, spindle formation and maintenance as well as other cellular events during oocyte activation.

DISCUSSION

Abnormal metabolism such as hypertension, hyperglycemia and hyperlipidemia will cause long-term stress in the oocyte's endoplasmic reticulum and mitochondria (Wang et al., 2019), which severely damages the quality of oocytes, thereby leading to lower pregnancy rates in women (Junghem et al., 2013a). Increased Ca²⁺ signaling plays an important role in oocyte activation, which is a prerequisite for embryonic development (Dupont et al., 2010). Ca²⁺ signaling to specific organelles, such as the mitochondrial matrix for oxidative metabolism (Griffiths and Rutter, 2009), regulates organelle-specific cell functions (Marchant et al., 2002). Mitochondrial damage in oocytes reduces a woman's reproductive capacity (Junghem et al., 2013b), caused by inefficient oocyte activation (Ou and Sun, 2017).

Mitochondrial Ca²⁺ uptake has been studied for more than five decades (Rottenberg and Scarpa, 1974). Mitochondrial matrix calcium ([Ca²⁺]_m) dynamics plays vital roles in regulating fundamental cellular and organelle functions including bioenergetics (Van Blerkom et al., 1995; Dumollard et al., 2007; Fink et al., 2017). The cytosolic Ca²⁺ influx to mitochondria (Viola et al., 2009) increases [Ca²⁺]_m to stimulate important enzymes in the Krebs cycle (Rizzuto et al., 2012), which provides enough ATP to support the increased energy requirement for maintaining mammalian oocyte activation (Chacko and Eliceiri, 2018). A large amount of Ca²⁺ is accumulated in the

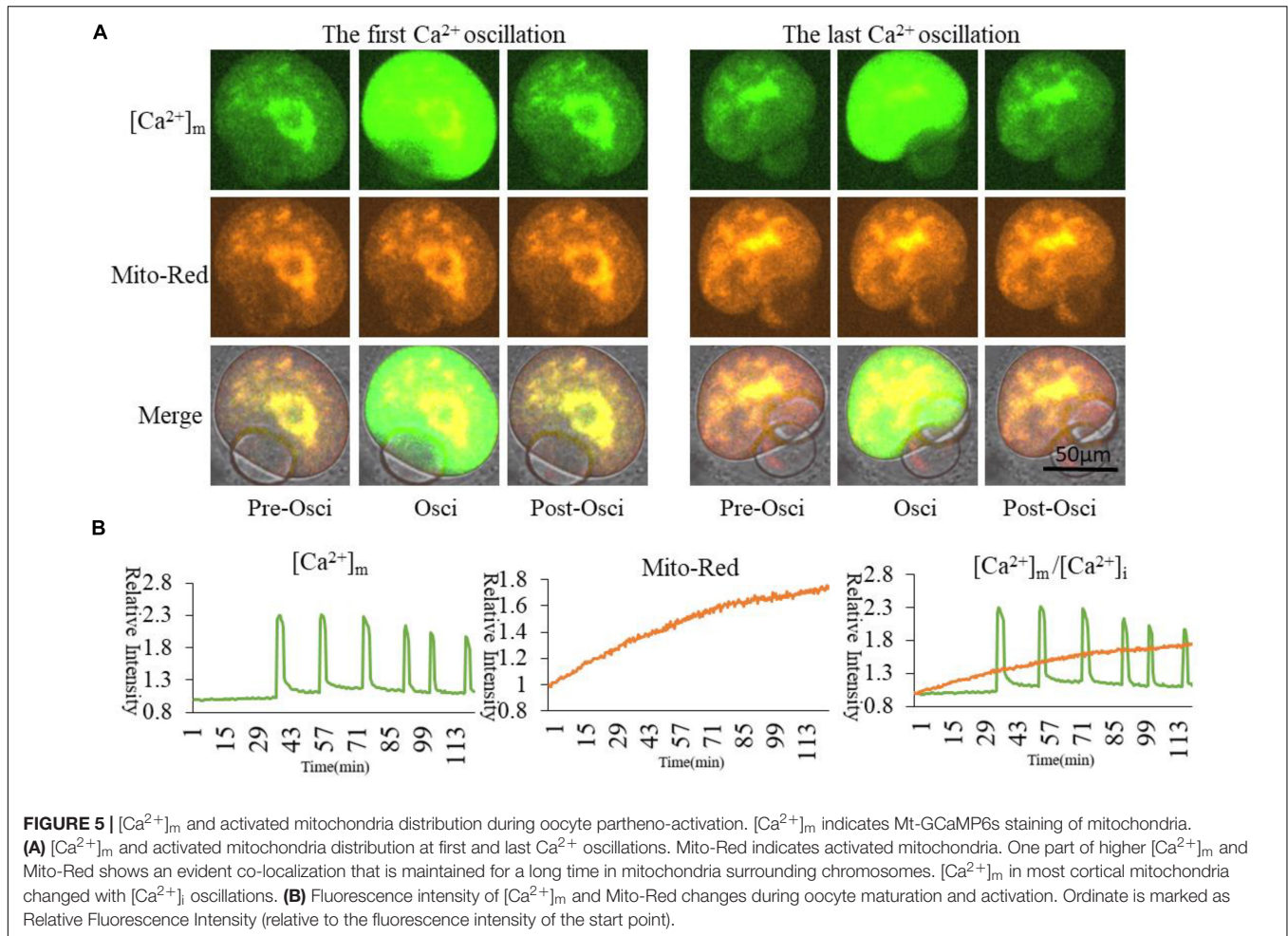




mitochondrial matrix to promote the transition of mitochondria from the resting state to the activated state (Dumollard et al., 2006), and changes in $[\text{Ca}^{2+}]_m$ will modify the mitochondrial metabolism to a certain extent. It is known that mitochondrial Ca^{2+} uptake is the basis for providing the necessary link between ATP supply and demand during cardiomyocyte contraction (Hajnoczky et al., 1995). Disturbances in Ca^{2+} fluxes lead to reduced bioenergetics of the cell or cellular death (Naon and Scorrano, 2014; Mishra, 2016; Samanta et al., 2018). In contrast, severe heart disease, such as reperfusion injury, is caused by excessive intake of mitochondria to induce Ca^{2+} overload and increase ROS production, even leading to cell apoptosis (Gordo et al., 2002; Ozil et al., 2005; Bonora et al., 2017). To prevent this, elevated $[\text{Ca}^{2+}]_m$ rapidly migrates from the mitochondria into the cytoplasm and ER (Hajnoczky et al., 1995; Berridge et al., 2000; Bootman et al., 2001) which is essential for cells to perform accurate functions. Our previous study showed that mitochondrial activity plays an important

role in the maintenance of $[\text{Ca}^{2+}]_i$ oscillations during oocyte activation (Wang et al., 2018); moreover, $[\text{Ca}^{2+}]_i$ oscillations also activate mitochondrial ATP production during fertilization (Campbell and Swann, 2006). We focused on the interplay between mitochondrial metabolism and Ca^{2+} signaling during oocyte maturation and activation in this study.

Our current understanding of $[\text{Ca}^{2+}]_m$ regulation in oocytes is still limited. In order to determine the relationship between $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_i$ dynamics, we focused on whether the Ca^{2+} changes in the mitochondria are independent or accompanied by changes in cytoplasmic $[\text{Ca}^{2+}]_i$ oscillations. Rhod2 is a dye used to detect mitochondrial Ca^{2+} (Dubouskaya et al., 2018). The localizations of Fluo-4 and Rhod2 in oocytes were significantly different. It was clearly seen that Fluo-4 was distributed in the cytoplasm (arrow in **Figure 1A**), while Rhod2 distribution was similar to that of the mitochondria (arrowhead in **Figure 1A**). From the fluorescence analysis results (**Figure 1B**), it can be seen that Fluo-4 AM and Rhod2

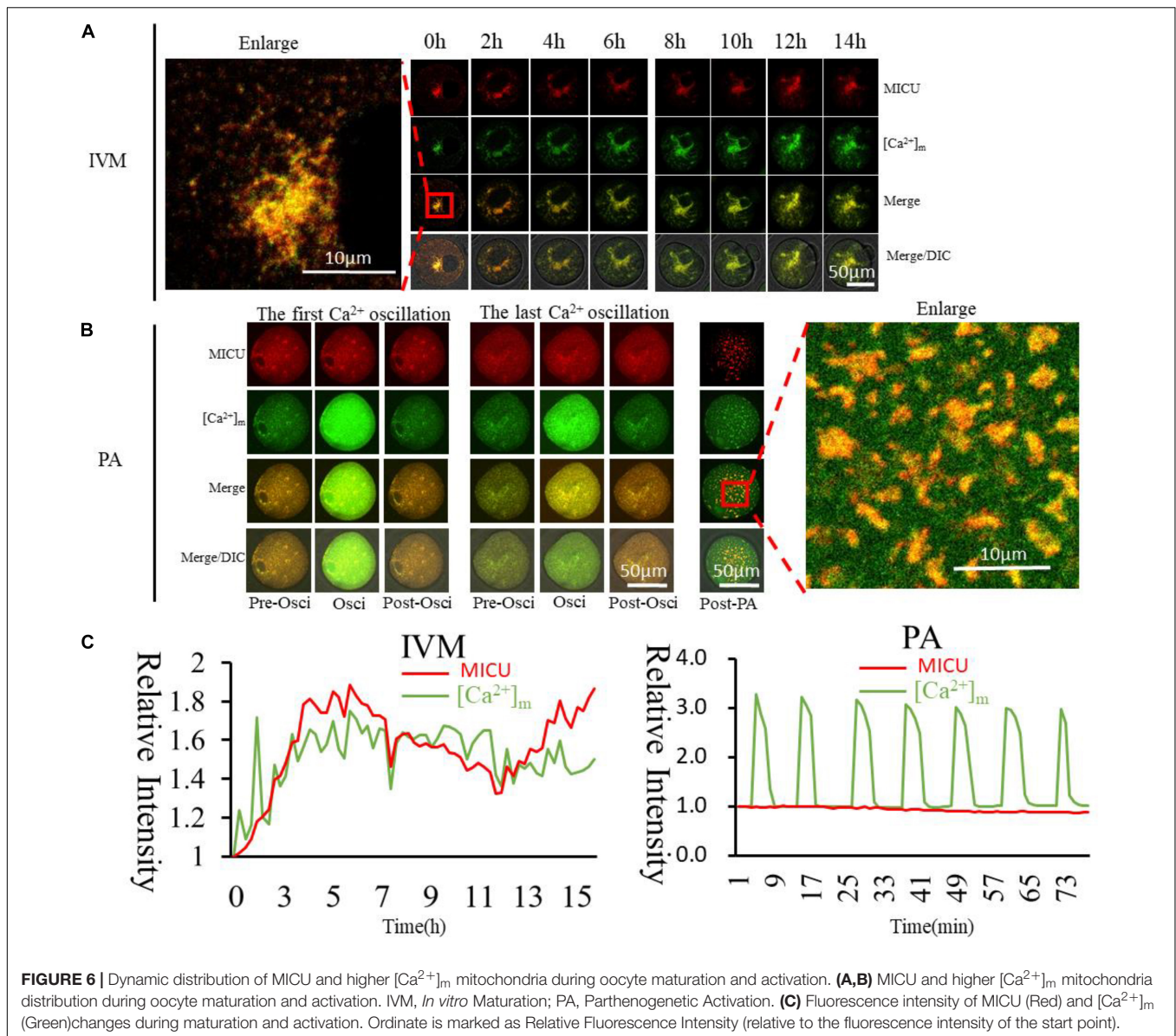


AM have the same patterns as oscillations. We thus have confirmed that Ca^{2+} in the cytoplasm and in the mitochondria change simultaneously.

Although Rhod2 is used as a classic Ca^{2+} probe in mitochondria (Dubouskaya et al., 2018), it is not widely used in oocytes. In order to ensure an accurate pattern of the mitochondrial Ca^{2+} dynamic change, synchronized with the cytoplasmic $[\text{Ca}^{2+}]_i$ oscillations, we applied an encoded Ca^{2+} probe with N-terminal mitochondrial localization signal peptide (Mt-GCaMP6s) to detect the patterns of $[\text{Ca}^{2+}]_m$ (Supplementary Figure S1A). After injection of Mt-GCaMP6s cRNA, $[\text{Ca}^{2+}]_m$ still exhibited oscillation patterns synchronized with Rhod2. Because Rhod2 displayed exactly the same pattern as $[\text{Ca}^{2+}]_i$ oscillations (Figure 1), $[\text{Ca}^{2+}]_m$ exhibited a same pattern in synchrony with $[\text{Ca}^{2+}]_i$. We also found that there are some differences in the position of Rhod2 and Mt-GCaMP6s; Rhod2-stained mitochondria are increased in quantity compared to Mt-GCaMP6s positive mitochondria. Indeed, most mitochondria can be stained by Rhod2, while only some of them displaying much higher $[\text{Ca}^{2+}]_m$ mitochondria will be stained by Mt-GCaMP6s (Figure 2). Comparative consideration of such two mitochondrial Ca^{2+} dyes, Mt-GCaMP6s may be more suitable to distinguish the changes of

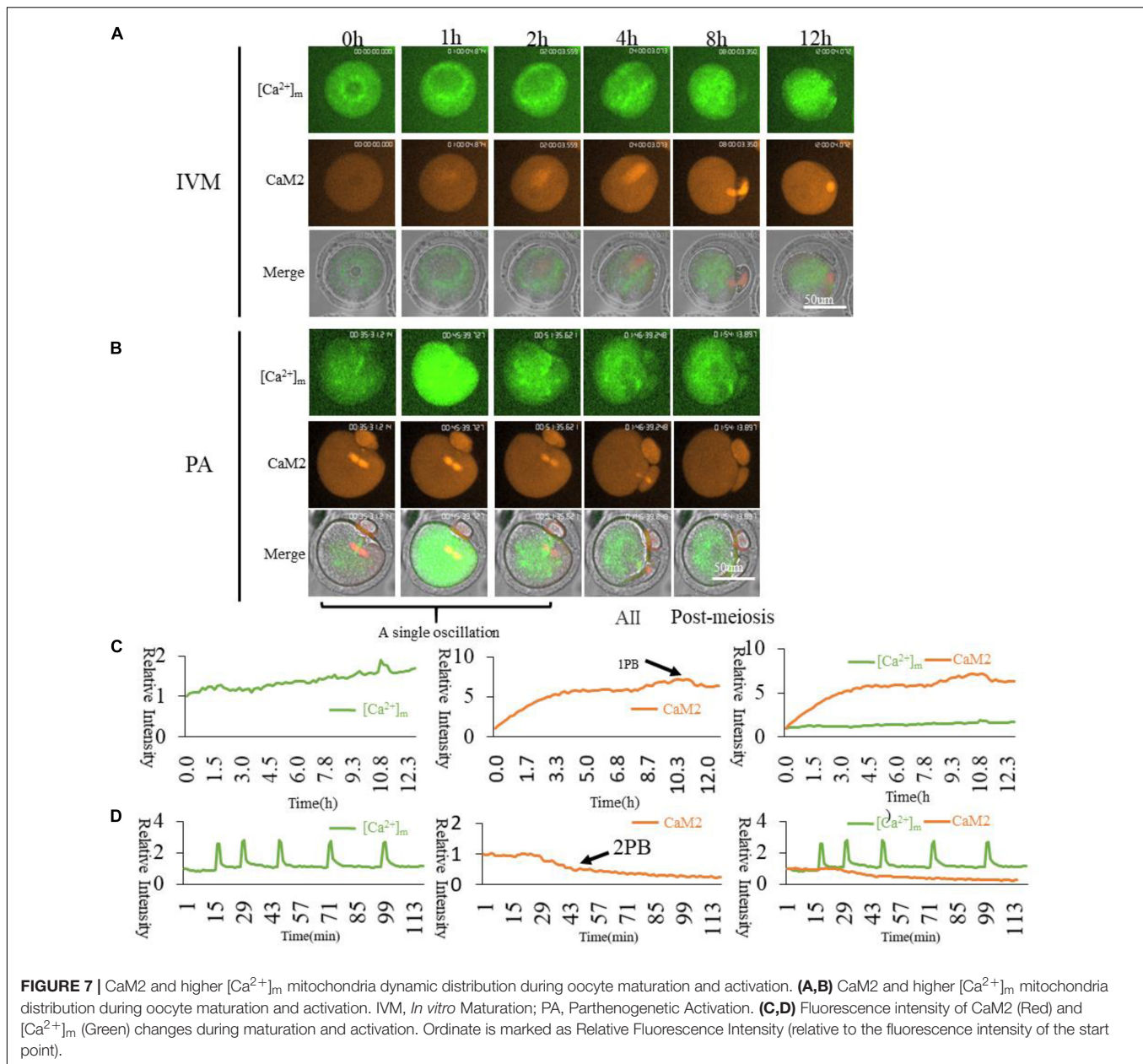
Ca^{2+} concentration in mitochondria. Further studies are needed to better understand the difference between Rhod2 and Mt-GCaMP6s positive mitochondria.

Next, we wanted to detect the dynamic changes of $[\text{Ca}^{2+}]_m$ during GV oocyte maturation. We found that higher $[\text{Ca}^{2+}]_m$ mitochondria distributed around the germinal vesicle (Figure 3 and Supplementary Video S1). After GVBD, $[\text{Ca}^{2+}]_m$ was higher in the area surrounding the spindle. During emission of the first polar body, $[\text{Ca}^{2+}]_m$ in the equatorial plate area increased significantly. Mitochondrial metabolism is much stronger in the energy-demanding part of the oocyte. Interestingly, there were two spikes of $[\text{Ca}^{2+}]_m$ around the time of first polar body extrusion (Figure 3B). Right curve indicates “GVBD but 1st poly body- non-extruded oocytes” displayed that maturation failed oocytes after GVBD, which lacks a $[\text{Ca}^{2+}]_m$ dynamic changes pattern as that matured MII oocytes. The irradiation of the laser caused failure of the first polar body extrusion in some oocytes (Figure 3C). The 2nd spike only displayed in second meiotic oocytes which had extruded 1st polar body. The highest $[\text{Ca}^{2+}]_m$ was found in MII oocytes in preparation for activation. These two time points are likely the most energy requiring critical periods for oocytes.



Is this dynamic change in mitochondrial $[\text{Ca}^{2+}]_m$ related to activated mitochondria? To address this question the relationship between $[\text{Ca}^{2+}]_m$ and the distribution of active mitochondria was studied. MitoRed is a dye localized to activated mitochondria (Chazotte, 2011). During GV oocyte *in vitro* maturation, we observed $[\text{Ca}^{2+}]_m$ by Mt-GCaMP6s and activated mitochondria by MitoRed (Figure 4), respectively. Mito-Red may have cytotoxicity for long-term observations, so that we could not obtain a clear imaging as Mt-GCaMP6s single signal (488 nm excitation) during oocyte maturation (Figure 3A). In order to obtain a clear location information of $[\text{Ca}^{2+}]_m$ and activated mitochondria, we performed a short 4-h observation with double concentration of MitoRed (Supplementary Figure S2). Double concentration of Mito-Red led to overexposure and cytotoxic effects after more than 4 h of observation. Higher $[\text{Ca}^{2+}]_m$ and activated mitochondria showed a much clearer co-localization

than during long term observation. If higher $[\text{Ca}^{2+}]_m$ was co-localized with activated mitochondria, Ca^{2+} dynamic entry into mitochondria may participate in mitochondrial metabolism transfer from the resting to the activated state. There was a clear co-localization between higher $[\text{Ca}^{2+}]_m$ and MitoRed-positive mitochondria surrounding the chromosomes (Figure 4 and Supplementary Figure S2). During GV oocyte maturation from prophase I to metaphase II, chromosomes and the spindle require energy in the entire oocyte. The co-localization of higher $[\text{Ca}^{2+}]_m$ and MitoRed-positive mitochondria indicated that higher activated mitochondria required a higher $[\text{Ca}^{2+}]_m$. The localization relationship between $[\text{Ca}^{2+}]_m$ and active mitochondria during oocyte partheno-activation was also observed next (Figure 5). $[\text{Ca}^{2+}]_m$ and MitoRed still had clear co-localizations in the resting state. With $[\text{Ca}^{2+}]_i$ oscillations, all of the mitochondria in the entire oocyte have a higher



$[\text{Ca}^{2+}]_m$, increasing in the flashing state, which was consistent with the result of **Figure 1**. While oocytes were in the cytoplasmic Ca^{2+} flashing state, the $[\text{Ca}^{2+}]_m$ increases, and the MitoRed positive mitochondria remain in a stable distribution pattern. One part of the mitochondria maintained a higher $[\text{Ca}^{2+}]_m$ around the nucleus, and the other mitochondrial $[\text{Ca}^{2+}]_m$ will increase instantaneously carried by $[\text{Ca}^{2+}]_i$, resulting in a pulse-like function enhancement. The role of $[\text{Ca}^{2+}]_m$ oscillations on mitochondrial metabolism needs to be further studied.

The MCU, which is identified as a highly selective Ca^{2+} channel (Xing et al., 2019), uptakes Ca^{2+} across the inner mitochondrial membrane, playing critical roles in various mitochondrial functions (Kirichok et al., 2004; Mammucari et al., 2017). MCU consists of multiple subunits, and its Ca^{2+}

influx activity is controlled by regulatory subunits (Yu et al., 2014), including two regulators MICU1 (Perocchi et al., 2010) and MICU2 (Patron et al., 2014). MICU1 is localized to the inner mitochondrial membrane with conformational changes upon Ca^{2+} binding (Kamer and Mootha, 2014). In our study, MICU distributed with higher $[\text{Ca}^{2+}]_m$ mitochondria in the GV oocytes (**Figure 6**), however in PA oocytes, distribution of MICU was similar as activated mitochondria with no change as $[\text{Ca}^{2+}]_i$ oscillations. This may indicate that MICU is related to mitochondrial Ca^{2+} and mitochondrial activity. We also observed that VDAC (**Supplementary Figure S4**), which is widely believed to be distributed on the outer membrane, has a different distribution from the higher $[\text{Ca}^{2+}]_m$ mitochondria, indicating that the process of regulating Ca^{2+} entry and exit from

the mitochondrial matrix into and out of the mitochondria is not related to VDAC.

Dynamic Ca²⁺ responds to its corresponding functions, and this change is determined by a protein initiation signal pathway with [Ca²⁺]_i sensing. Most studied Ca²⁺-responsive proteins are members of the calmodulin family. The calmodulin family contains CaM1 and CaM2. We examined the relationship between CaMs and higher [Ca²⁺]_m of mitochondria. We cloned CaM1 and CaM2 linked with mCherry (**Supplementary Figure S1**). In GV oocytes, two types of cRNA were injected and time-lapse confocal laser microscopy was conducted. We found both CaM1 and CaM2 in the spindle area (**Supplementary Figure S4**). CaM2 and [Ca²⁺]_m were examined to show the relationship between cytoplasmic Ca²⁺-sensor CaM2 and active mitochondria. It can be seen in **Figure 7** that higher [Ca²⁺]_m mitochondria are distributed surrounding the spindle, however, CaM2 is associated with the spindle (**Figure 7A**). When activated, CaM2 associates with the spindle until the second polar body extrusion (**Figure 7B**). The main function of the CaMs family may be directly involved in microtubule organization and spindle formation as described before (Fan and Sun, 2004). The distribution of higher [Ca²⁺]_m and activated mitochondria around the spindle also indicates that the change of calcium-regulated spindle dynamic changes requires a continuous supply of energy by higher [Ca²⁺]_m stimulated mitochondria. Cortical region Mito-Red signals are independent of [Ca²⁺]_m dynamic changes, which was not our expected result. Cortical [Ca²⁺]_m oscillations may have some other function of mitochondria regulations. There may be another possibility that another probe but not Mito-Red is suitable for detecting the mitochondrial state during partheno-activation. An accurate distribution of higher [Ca²⁺]_m mitochondria in oocytes is important for oocyte spindle reformation.

Deepening our understanding and basic knowledge about oocyte maturation/activation and energy metabolism is of great value for solving clinical problems such as insufficient oocyte cytoplasmic maturation and activation as well as insufficient energy supply caused by mitochondrial damage. Studying the regulatory mechanisms between [Ca²⁺]_m and mitochondrial metabolism in oocytes will provide more possibilities for improving the efficiency of assisted reproduction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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ETHICS STATEMENT

All mice were handled in accordance with the institutional animal care policies of the Institute of Zoology, Chinese Academy of Sciences. The Laboratory Animal Care and Use Committee of the Institute of Zoology approved this study.

AUTHOR CONTRIBUTIONS

FW, Q-YS, and X-HO conceived and designed the experiments. FW and others conducted experiments. FW and Q-YS analyzed the data. FW, HS, X-HO, and Q-YS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.585932/full#supplementary-material>

Supplementary Figure 1 | Constructed vectors for *in vitro* expression. **(A,B)** Complete cyclic plasmids maps of Mt-GCaMP6s and mCherry labeled protein. **(C–F)** Templates for cRNA *in vitro* expression by PCR with primers M13F and M13R.

Supplementary Figure 2 | Short term observation of higher [Ca²⁺]_m and activated mitochondrial distribution during oocyte maturation. Double concentration of Mito-Red led to overexposure and cytotoxic effect after more than 4 h of observation. Higher [Ca²⁺]_m and activated mitochondria show clear co-localization.

Supplementary Figure 3 | Dynamic distribution of VDAC and higher [Ca²⁺]_m mitochondria during oocyte maturation and activation. **(A,B)** VDAC and higher [Ca²⁺]_m mitochondria distribution during oocyte maturation and activation. **C:** Fluorescence intensity of VDAC (Red) and [Ca²⁺]_m (Green) changes during maturation and activation. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point).

Supplementary Figure 4 | CaM1 and CaM2 distribution in mature oocytes. Both CaM1 and CaM2 are located at the spindle.

Supplementary Video 1 | [Ca²⁺]_m dynamic changes during oocyte maturation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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