Mitochondrial dynamics controlled by mitofusins define organelle positioning and movement during mouse oocyte maturation

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ABSTRACT: Mitochondria are abundant in fully grown mammalian oocytes with a unique spherical morphology, but the mechanisms controlling mitochondrial behavior are not well understood. Here we describe for the first time the control of mitochondrial behavior in mouse oocytes by a fusion/fission mechanism. Mitofusins (Mfn1 and Mfn2) and OPA1 proteins are required for outer and inner mitochondrial membrane fusion, respectively, whereas Drp1 is the key regulator of mitochondrial fission. We show that mouse oocytes express the Mfn1, Mfn2, Opa1 and Drp1 proteins, both in immature and mature oocytes at similar levels. Overexpression of Mfn1 or Mfn2 causes marked mitochondrial aggregation, particularly in the perinuclear region during meiotic progression. Tracking of mitochondria with chromosomes or endoplasmic reticulum (ER) throughout oocyte maturation demonstrates that Mfn1 and Mfn2-promoted mitochondrial aggregation disturbs the spatiotemporal dynamic of the chromosomes and ER, respectively. Our findings suggest that organelle dynamics are co-ordinately controlled during meiotic division, and an imbalance of mitochondrial fusion/fission leads to disorganization of the organelle compartments.

Key words: endoplasmic reticulum / meiosis / mitochondrial dynamics / oocyte maturation / spindle formation

Introduction

During production of a haploid gamete, mammalian oocytes undergo two consecutive division cycles in a process called oocyte maturation, i.e. the germinal vesicle (GV) oocytes at prophase of the first meiosis progress into metaphase of the second meiosis (MII). The subcellular distribution of organelles changes dynamically during oocyte maturation, before fertilization readiness is achieved. For instance, the progression of meiosis is accompanied by reorganization of the cytoskeleton, including meiotic spindle formation, migration and cortical anchoring (Brunet and Maro, 2005; Sun and Schatten, 2006). To generate fertilization-induced Ca2+ responses, the endoplasmic reticulum (ER) increases Ca2+ stores (Mehlmann and Kline, 1994; Jones et al., 1995; Wakai and Fissore, 2013) and is redistributed during maturation, such that the ER in MII oocytes consists of a fine reticular network with a dense accumulation in the cortex (Mehlmann et al., 1995; Stricker, 2006; FitzHarris et al., 2007). Mitochondria also undergo redistribution during maturation; remarkably, they accumulate around the spindle at the metaphase of the first meiosis (MI) (Van Blerkom, 1991; Yu et al., 2010; Dalton and Carroll, 2013). Although the roles of mitochondria during oocyte maturation remain unclear, their close association with the meiotic spindle implies a requirement for ATP, as a lack of mitochondrial ATP production causes meiotic spindle abnormalities at the MII stage (Zhang et al., 2006; Johnson et al., 2007). Moreover, at fertilization, the mitochondria in the immediate vicinity of the ER in MII oocytes seem to facilitate Ca2+ oscillations via an ATP-driven Ca2+ pumping mechanism (Dumollard et al., 2004).

In mammals, mitochondria and mitochondrial DNA (MtDNA) are inherited through the female germ line. It is thought that a massive expansion of mitochondria and MtDNA occurs during oogenesis, resulting in the presence of >100,000 mitochondria and further copies of MtDNA in fully grown oocytes (Jansen, 2000; Shoubridge, 2000). Mitochondria in mammalian oocytes displayed typical spherical shapes with sparse cristae (Jansen, 2000; Sathananthan and Trounson, 2000). Several reports have demonstrated that the subcellular distribution of mitochondria during meiosis is mediated by microtubules (Van Blerkom, 1991; Yu et al., 2010; Dalton and Carroll, 2013), but little attention has been given to the involvement of mitochondrial intrinsic...
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dynamics in this process. Control over mitochondrial morphology is plastic, as the shape of the mitochondria is changed frequently by organelar fusion and fission events; the resultant morphology ranges from elongated tubules to small fragmented spheres. Accumulating data suggest that these opposing events play crucial roles in diverse cellular processes, including energy metabolism, calcium signaling and apoptosis (Chan, 2006). Yet, the physiological importance of mitochondrial morphology may differ depending on the type of cell, especially in the case of highly specialized cells such as oocytes.

Recent studies have provided molecular insights into the mechanisms underlying mitochondrial fission and fusion, as both processes are mediated by dynamin-like GTPases that are well conserved among yeast, flies and mammals (Okamoto and Shaw, 2005). In mammals, fusion between the mitochondrial outer membranes (OM) is mediated by mitofusin (Mfn) 1 and Mfn2 (Santel and Fuller, 2001; Chen et al., 2003; Santel et al., 2003), whereas fusion between the mitochondrial inner membranes (IM) is mediated by optic atrophy 1 (Opa1) (Olichon et al., 2003). Mitochondrial fusion is mediated by dynamin-related protein 1 (Drp1) (Smirnova et al., 1998; Pitts et al., 1999), which is distributed in the cytoplasm and is recruited to the mitochondrial surface.

In the present study using mouse oocytes, we explored the spatiotemporal control of mitochondrial morphology by mitochondrial fusion and fission mechanisms and the functional impact of that morphology on meiotic division.

Materials and Methods

Collection of oocytes

GV oocytes were collected from the ovaries of 8- to 10-week-old CD-1 female mice. Females were injected with 5 IU of pregnant mare serum gonadotrophin (PMSG). Cumulus cell-enclosed GV oocytes were recovered 42–46 h post-PMSG administration, and the cumulus cells were removed by repeated pipetting. Oocytes were matured in vitro for 12–15 h in M16 medium under paraffin oil, at 37°C in a humidified atmosphere containing 5% CO2. All procedures were performed according to research animal protocols approved by the Tokyo University of Agriculture Institutional Animal Care and Use Committee.

Plasmids

The coding regions of mouse Mfn1, Mfn2, Opa1 and Drp1 I were amplified by PCR and subcloned into a pcDNA6 vector (pcDNA6/Myc-His B; Invitrogen, Carlsbad, CA, USA) between the BamHI and EcoRI restriction sites (Mfn1, Mfn2 and Drp1I) and the KpnI and Ncol restriction sites (Opa1). To analyze the subcellular distribution, Mfn1 and Mfn2 were C-terminally tagged with enhanced green fluorescent protein (EGFP) between the Xhol and Xbal restriction sites. Point mutations in Mfn1 (K88T and T109A) were introduced using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). To visualize the mitochondria, the gene sequence encoding the mitochondrial targeting sequence of calreticulin, mCherry and the KDEL ER retention sequence were subcloned into pcDNA6. For visualization of DNA and microtubule growth, gene sequences encoding histones H2B and EB3 were amplified by PCR and ligated to EGFP-bearing and mCherry-bearing pcDNA6, respectively. ATP biosensor, AT1.03 and AT1.03RK in the pcDNA3.1 vector were kindly provided by Dr H. Imamura (Kyoto University).

Preparation and microinjection of cRNA

Plasmids were linearized with a restriction enzyme downstream of the insert to be transcribed. Capped DNA was transcribed in vitro using the T7 or SP6 mMESSAGE mMACHINE Kit (Ambion) using the promoter that was contained in the constructs. A poly(A)-tail was added to the transcribed RNAs using a Tailing Kit (Ambion), and poly(A)-tailed RNAs were eluted with RNase-free water and stored in aliquots at −80°C. For microinjection, cRNA solution was loaded into glass micropipettes at the concentration of 1 μg/μl (Mfn1-GFP and Mfn2-GFP), 500 ng/μg (Mfn1WT, Mfn1K88T, Mfn1T109A, Mfn2, Opa1 and Drp1I) or 100 ng/μg (Mito-GFP, EB3-GFP, Mito-Drp1, H2B-mCherry and ER-mCherry). cRNAs were delivered into GV oocytes using piezo-driven micropipette unit (Prime Tech). Manipulation was carried out in M2 medium containing 5 μg/ml cytosinol A (Sigma). The volumes injected typically ranged from 2 to 10 pl, which is 1–5% that of the egg. To allow translation of protein, oocytes were kept in media supplemented with 100 μM 3-isobutyl-1-methylxanthine (IBMX) for 3 h, after which maturation was induced by removing IBMX.

Confocal microscopy

Live cell imaging of oocytes expressing fluorescently-tagged proteins was performed using a laser-scanning confocal microscope (LSM 710, Carl Zeiss) outfitted with a 63 × 1.4 NA oil immersion objective lens. Images were acquired with LSM software (Carl Zeiss). Images were processed using Zen imaging software (Carl Zeiss). Time-lapse imaging of Mito-GFP and H2B-mCherry during oocyte maturation was performed using a Nipkow disk confocal unit (CV-1000, Yokogawa Electric Corp.) outfitted with a ×20 objective lens. For tracking, we imaged 9–10 z-confocal sections (every 7–8 μm) at intervals of 10 min for 15–17 h after the start of maturation.

Fluorescence resonance energy transfer and Ca2+ imaging

ATEam, a fluorescence resonance energy transfer-based ATP indicator, has been used successfully to measure cellular ATP levels in live somatic cells (Imamura et al., 2009) and recently in mouse oocytes (Dalton et al., 2014). To estimate the relative changes in ATP levels, the emission ratio of AT1.03 and AT1.03RK (yellow FP [YFP]/cyan FP [CFP]) was imaged using a CFP excitation filter, dichroic beam splitter, and CFP and YFP emission filters (Chroma Technology, Rockingham, VT, USA; ET436/20X, 98007bs, ET480/40 m and ET535/30 m). Oocytes were attached to glass-bottomed dishes (MatTek Corp., Ashland, MA, USA) and placed on the stage of an inverted microscope. CFP and YFP intensities were collected every 10 min using a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ, USA). The rotation of excitation and emission filter wheels was controlled using the MAC5000 filter wheel/shutter control box (Ludl) and Metamorph software (Nikon). To measure cytoplasmic Ca2+ in oocytes, we incubated with 1 μM Fura-red (Life Technologies) supplemented with 0.02% pluronic acide (Life Technologies) for 20 min at room temperature. Fura-red was excited at 405 nm wavelength every 1 min, and emitted light was collected at 480 and 580 nm wavelengths.

Western blot analysis

Cell lysates from mouse oocytes were prepared by adding 2 × sample buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking, membranes were probed with rabbit polyclonal anti-Mfn1 (1:1000, Abcam), mouse monoclonal anti-Mfn2 (1:500, Abcam), mouse monoclonal anti-Opa1 (1:500, BD Biosciences) and mouse monoclonal anti-Drp1 (1:500, BD Biosciences) antibodies for 1 h at room temperature. Species-specific secondary antibodies...
Immunofluorescent staining
Oocytes were fixed and permeabilized with 2% paraformaldehyde (Wako) in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma) for 40 min at room temperature. After washing with PBS supplemented with 1% bovine serum albumin (PBS–BSA), the oocytes were incubated overnight at 4°C with mouse anti-Drp1 antibody diluted 1:300 in PBS–BSA. Then oocytes were washed with PBS–BSA and incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG (1:300, Life Technologies) for 1 h at room temperature. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI, Wako). Samples were mounted on glass slides and examined using a laser-scanning confocal microscope (LSM 710, Carl Zeiss) outfitted with a 63 × 1.4 NA oil immersion objective lens.

Electron microscopy
Oocytes were fixed in 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 1 h and stored at 4°C until processed. After post-fixation in 2% osmium tetroxide at 4°C, the specimens were dehydrated with a graded series of ethanol and were embedded in epoxy resin Quetol-812. Semi-thin sections were cut for light microscopy and stained with toluidine blue for further sectioning of areas. Thereafter, ultra-thin sections were cut and stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) (JEM 1200EX, JEOL) at 80 kV.

Statistical analysis
Values from three or more experiments performed on different batches of oocytes/eggs were analyzed using the Student’s t-test or one-way ANOVA with post hoc Tukey–Kramer’s test, as appropriate using Ekuseru-Toukei 2010 software (Social Survey Research Information Co., Ltd). Differences were considered significant at P < 0.05. Values are presented as mean ± SEM.

Results
Mouse oocytes express mitochondrial fusion and fission proteins, Mfn1, Mfn2, Opal and Drp1
We first investigated the expression of mitochondrial fusion proteins in GV and MII oocytes by western blot analysis. Antibodies against Mfn1 and Mfn2 recognized bands of the predicted size (ca. 86 kDa) (Fig. 1A and B, left panels), and overexpression of these proteins, which was achieved by injection of cRNAs into GV oocytes, markedly increased the intensity of each target band (Fig. 1A and B, right panels). Endogenous Mfn1 and Mfn2 proteins were expressed during maturation, and levels of proteins in the GV and MII stages were comparable. We also confirmed the expression of Opal1 proteins in both GV and MII oocytes (Fig. 1C, left panel).

Mitochondrial fusion is balanced by mitochondrial fission, which is mediated by Drp1 activity. A Drp1-specific monoclonal antibody recognized a band of the expected size (ca. 80 kDa) in both GV and MII oocytes (Fig. 1D, left panel), and Drp1 band intensity was increased by overexpression (Fig. 1D, right panel).

Distribution and organization of mitochondria in oocytes are altered by overexpression of Mfn1 and Mfn2
We next addressed whether overexpression of mitochondrial fusion or fission proteins affects mitochondrial morphology. Mitochondria in GV oocytes were fixed and permeabilized with 2% paraformaldehyde (Wako) in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma) for 40 min at room temperature. After washing with PBS supplemented with 1% bovine serum albumin (PBS–BSA), the oocytes were incubated overnight at 4°C with mouse anti-Drp1 antibody diluted 1:300 in PBS–BSA. Then oocytes were washed with PBS–BSA and incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG (1:300, Life Technologies) for 1 h at room temperature. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI, Wako). Samples were mounted on glass slides and examined using a laser-scanning confocal microscope (LSM 710, Carl Zeiss) outfitted with a 63 × 1.4 NA oil immersion objective lens.

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oocytes were distributed diffusely throughout the cytoplasm (Fig. 2; control, GV). The majority of oocytes (67%) overexpressing Mfn1 exhibited marked mitochondrial aggregation throughout the cytoplasm (Fig. 2; Mfn1, GV), although mitochondria tended to migrate toward the chromosomes over time. During maturation of these oocytes, mitochondrial aggregation was particularly concentrated around the chromosomes (Fig. 2; Mfn1, MII). Overexpression of Mfn2 in GV oocytes also induced mitochondrial aggregation (87% of the oocytes) (Fig. 2; Mfn2, GV). In this case, mitochondrial aggregation appeared to be localized preferentially in the perinuclear area at the MII stage; nonetheless, mitochondrial clusters remained throughout the cytoplasm (Fig. 2; Mfn2, MII).

In contrast to Mfn overexpression, there were no apparent changes in mitochondrial morphology in GV oocytes overexpressing Opa1 (Fig. 2; Opa1, GV). Despite a modest concentration around the spindle area, most mitochondria in MII oocytes were dispersed in the cytoplasm (Fig. 2; Opa1, MII). There were no detectable differences in mitochondrial morphology between cells overexpressing Drp1 and control oocytes (Fig. 2; Drp1, GV and MII).

**Figure 2** Mitochondrial morphology in mouse oocytes overexpressing Mfn1, Mfn2, Opa1 and Drp1. The distribution of mitochondria in GV and MII oocytes was examined using confocal microscopy. Control oocytes and oocytes expressing Mfn1 and Mfn2, Opa1 and Drp1 were stained with MitoTracker, and representative images taken at the equatorial plane are shown (n > 50). Scale bar, 20 μm.
Perinuclear mitochondrial aggregation is associated with microtubules

From the GV to MII stages, the spatiotemporal changes in the expression of mitochondria tagged with green fluorescent protein (GFP) were analyzed at 0, 4, 8 and 12 h of maturation, which corresponded with GV, GV breakdown (GVBD), MI and MII stages of meiotic progression, respectively (Supplementary data, Fig. S1, control). Remarkably, mitochondria accumulated around the MI spindle, although a large portion of the accumulated mitochondria were released at the MII stage (Fig. 3, upper right). We investigated if the persistence of mitochondrial aggregation around the chromosomes at the MII stage, typically seen in Mfn1-overexpressing oocytes, was associated with the microtubule network. During maturation, the treatment of oocytes with nocodazole, a microtubule inhibitor, prevented MI spindle formation, and mitochondria were dispersed throughout the cytoplasm (Fig. 3, upper right). As expected, in the presence of nocodazole, Mfn1-overexpressing oocytes formed conspicuous mitochondrial clusters, but these clusters failed to surround the chromosomes (Fig. 3, lower left).

Mfn1-induced mitochondrial aggregation is dependent on GTPase activity

Expression of Mfn1 and Mfn2 as GFP-tagged fusion proteins demonstrated that Mfn1 and Mfn2 were localized to the mitochondrial aggregation sites, suggesting that these proteins contributed to the aggregation event (Fig. 4A). To address whether mitochondrial aggregation is specifically associated with the enzymatic activity of Mfn, we expressed mutant Mfn proteins. Mutations in the GTPase domains (Mfn1K88T and Mfn1T109A) caused a loss of GTPase activity, and the Mfn1T109A mutant, in particular, appeared to have a dominant negative effect (Santel et al., 2003; Park et al., 2010), resulting in mitochondrial fragmentation. The formation of perinuclear mitochondrial clusters in MII oocytes was inhibited (Mfn1T109A) or greatly reduced (Mfn1K88T) by expression of these mutant proteins (Fig. 4B), indicating that the aggregation phenotype is dependent on the GTPase activity of Mfn1. Similar or higher levels of expression of these mutant proteins relative to wild-type Mfn1 were confirmed by western blot analysis (Fig. 4B).

Mfn-mediated mitochondrial aggregation alters cellular ultrastructure

It is noteworthy that, even when z-axis stack images of mitochondria in a whole oocyte were reconstructed, the elongated tubular network was not observed in oocytes (Supplementary data, Fig. S2, control), regardless of Mfn1 or Mfn2 overexpression (Supplementary data, Fig. S2; Mfn1 and Mfn2). Moreover, injection of neither higher nor lower cRNA concentrations failed to induce the tubular network (data not shown), suggesting tubulation-incompetence in the oocyte mitochondria.

To clarify whether Mfn-induced mitochondrial aggregation alters the mitochondrial morphology at an ultrastructural level, TEM images for control and Mfn-overexpressing oocytes were compared. The control MII oocytes showed spherical mitochondria dispersed throughout the cytoplasm. Mitochondrial shape was round to oval, with a dense matrix and occasional vacuolated structures (Fig. 5A, left). A higher-magnification view showed irregularly localized cristae, and the OM of some mitochondria was sometimes in contact with that of other mitochondria and with the smooth ER (Fig. 5A, right).

In MII oocytes overexpressing Mfn1, the contact sites between mitochondrial OMs were increased visibly (Fig. 5B, left—black arrowheads; Fig. 5D), whereas no apparent changes were found in the shape of individual mitochondria. As expected, no mitochondrial tubules were found at the aggregation sites. Remarkably, the ER accumulated at the sites where mitochondria were concentrated, and the OMs appeared to be cross-linked by the ER (Fig. 5B, right—white arrowheads). These features were also observed in oocytes overexpressing Mfn2 (Fig. 5C).

Mfn2 expression affects redistribution of the ER during maturation and affects Ca2+ stores

TEM analysis revealed an enhanced ER accumulation at mitochondrial aggregation sites. Consistent with this observation, the co-expression of GFP-tagged mitochondria and mCherry-tagged ER demonstrated that Mfn-induced mitochondrial aggregation interferes with ER distribution, as discrete regions of the ER were associated with mitochondrial clusters (Fig. 6A). In the case of Mfn2 overexpression in particular, the ER network was markedly disintegrated, compared with that observed in the control. The 3D time-lapse imaging of GFP-tagged mitochondria and mCherry-tagged ER demonstrated the precise movement of two organelles during maturation in control oocytes (Supplementary data, Movie S1) and oocytes overexpressing Mfn2 (Supplementary data, Movie S2). In control oocytes, the ER accumulated around chromosomes at the GVBD stage. During the transition from the MI to MII stages, ER migrated toward the cortical regions surrounding the meiotic spindle. In the case of Mfn2-overexpressing oocytes, with progression of maturation,
the ER forms clusters at the sites where mitochondria were concentrated. Further, we found that Mfn2 overexpression causes a severe loss of Ca^{2+} stores in the ER (Fig. 6B and C), suggesting that the disorganization of ER impaired Ca^{2+} homeostasis in the cell.

**Mfn1-induced mitochondrial aggregation disrupts MII chromosome alignment and spindle bipolarity**

During oocyte maturation, the maternal genome is segregated into highly asymmetric partitions of the cytoplasm, leading to the formation of polarized eggs and small polar bodies. Positioning of the MII spindle near the cortex with aligned chromosomes is required for the emission of the second polar body and cortical reorganization at fertilization. We found that ~30% of Mfn1-overexpressing oocytes produced a large polar body (Fig. 7A, left panel) that was seldom observed in control and Mfn1-GTPase mutants (Fig. 7B). Remarkably, even in the presence of a comparatively normal-sized polar body (Fig. 7A, right panel), the chromosomes were dispersed and frequently were located away from the plasma membrane.

We postulated that the disorganization of chromosomes is due to marked perinuclear aggregation of mitochondria in Mfn1-overexpressing oocytes. To track the precise movement of mitochondria and chromosomes during maturation, we performed 3D time-lapse imaging of GFP-tagged mitochondria and mCherry-tagged histone H2B (H2B) in control (Fig. 7C, control; Supplementary data, Movie S3) and Mfn1-overexpressing oocytes (Fig. 7C, Mfn1; Supplementary data, Movie S4). After the GVBD, mitochondria in control oocytes moved toward chromosomes, forming small clusters (see Supplementary data, Movie S3; 01:00–06:00). Chromosomes migrated into the...
cortex and aligned with the surrounding mitochondria at the metaphase plate (Supplementary data, Movie S3; 06:00–09:00). After releasing the first polar body (1PB), the spindle area anchored to the cortex domain, with chromosome congression (Supplementary data, Movie S3; 09:00–17:00). Mitochondria in Mfn1-overexpressing oocytes accumulated more intensely around the chromosomes area (Supplementary data, Movie S4; 01:00–07:00). Chromosomes appeared to segregate without formation of a clear metaphase plate (Supplementary data, Movie S4; 08:00–10:00). Importantly, after releasing the 1PB, most oocytes (67%) lost chromosome congression in the MII plate, and the clustered mitochondria remained in the center of the cytoplasm, along with the dispersed chromosomes (Supplementary data, Movie S4; 10:00–17:00).

As expected, formation of abnormal MII spindles that were surrounded tightly by mitochondria was frequently observed in oocytes overexpressing Mfn1 (78% of oocytes) (Fig. 8A, left panels). Live-cell imaging of microtubule dynamics, using the plus-end probe EB3-GFP, also demonstrated that spindle elongation was disorganized in Mfn1-overexpressing oocytes (68% of oocytes) (Fig. 8A, right panels).

A tight association between mitochondria and microtubules implies a requirement for ATP by the developing spindle. We hypothesized that highly aggregated mitochondria may compromise mitochondrial function and may reduce ATP levels, leading to aberrant spindle formation.

Maturating oocytes exhibited dynamic changes in ATP levels, as a sharp decrease and a small increase were observed around 2 and 8 h after the start of maturation, respectively (Fig. 8B, control). Unexpectedly, Mfn1 overexpression gave rise to no statistically significant differences in ATP levels at each time point during maturation, albeit with a slight delay in the increase in ATP levels (Fig. 8B, Mfn1). We also confirmed that overexpression of Mfn2 did not alter ATP levels (data not shown).

**Discussion**

The present study in mouse oocytes demonstrates the spatiotemporal mitochondrial dynamics controlled by fusion and fission mechanisms, in addition to the resulting functional impact on meiotic division. We found that an altered balance among the mitochondria fusion proteins Mfn1 and Mfn2 changed the mitochondrial distribution and cellular ultrastructure in oocytes. We also provide precise 3D mitochondria-tracking datasets with chromosomes and ER throughout the first meiotic division.

In mammalian cells, mitochondria show a wide variety of shapes depending on the cell type. In many adherent cell types, such as fibroblasts, mitochondria form a dynamic interconnected network such that short and long tubular mitochondria continuously divide and fuse (Chen and Chan, 2004). At steady state, the overall morphology of the mitochondrial population is determined by the balance between fusion and fission mechanisms.
Mitochondrial fusion in oocytes

When this balance is experimentally altered, mitochondrial morphology changes. A high fusion-to-fission ratio results in long and highly interconnected tubules (Santel and Fuller, 2001; Smirnova et al., 2001; Yoon et al., 2003; Cipolat et al., 2004; Otera et al., 2010), whereas a low fusion-to-fission ratio results in small fragmented spheres (Chen et al., 2003; Olichon et al., 2003; Park et al., 2010). Despite the expression of both fusion and fission proteins, mitochondrial morphology in oocytes is intrinsically fragmentary throughout the cytoplasm. These results are contrasted with the case of somatic cells, such that the tubular network is enhanced by overexpression of mitochondrial fusion proteins (Santel and Fuller, 2001; Legros et al., 2002; Cipolat et al., 2004). Promotion of fusion by Mfn overexpression did not induce tubule formation, but Mfn overexpression did induce mitochondrial aggregation that consisted of contact between mitochondrial OMs; hence, mitochondria in oocytes might lack complete-fusion competence. There were no apparent changes in mitochondrial morphology between control and Drp1-overexpressing oocytes. Fragmented mitochondrial morphology in control oocytes implies active mitochondrial fission at the steady state and thereby might attenuate the increased levels of Drp1, presumably because the endogenous binding sites on mitochondria become saturated. The dominant negative mutant of Mfn1 also gave rise to no detectable alterations in mitochondrial morphology. In somatic cells, expression of Mfn1 mutants in the GTPase domains (Mfn1K88T and Mfn1T109A) result in the different mitochondrial morphologies (Santel et al., 2003). Mfn1K88T modestly induced mitochondrial aggregation, whereas Mfn1T109A apparently acts as dominant negative and blocks endogenous mitochondrial fusion, leading to the increase in mitochondrial fragmentation. Our data are roughly line with these previous observations; nonetheless, it is still unclear whether Mfn1T109A shows the dominant negative effect, because mitochondria display the fragmented morphology in control oocytes. Further studies are thus needed to address the precise balance of mitochondrial fusion and fission in oocytes.

Ultrastructural analysis has shown that oocyte mitochondria have the shape of a small sphere with a dense matrix and irregular cristae located peripherally or transversely. These structural features might be the cause of insensitivity or resistance to IM fusion. This insensitivity might be the reason why overexpression of Opa1 had no significant effect on the mitochondrial morphology. Opa1 is present as multiple splice variants, each of which undergoes proteolysis according to the presence of exons containing photolytic cleavage sites (Ishihara et al., 2006; Griparic et al., 2007), which results in multiple bands on western blots. Overexpression of Opa1 (mouse variant 1) increased levels of long-isofrom Opa1 (Fig. 1C, right panel), which is thought to be important for fusion competence (Ishihara et al., 2006). We propose that Mfn-mediated fusion in oocytes occurs between adjacent mitochondria as a focal and transient event under physiological conditions. This OM fusion form could be maintained for a short time before dividing again via fission mechanisms, without the formation of complete fusion, possibly because fewer IM fusion mechanisms exist. This incomplete fusion would allow small, fragmented mitochondria to disperse into the cytoplasm at the MII stage, despite the microtubule-associated accumulation around the MI spindle, which might be also important for the control of mitochondrial segregation between the oocyte and polar body.

The close association between mitochondria and ER involves diverse cellular processes, including regulation of lipid biosynthesis and metabolic

Figure 6 Discrete ER network in Mfn-overexpressing mouse MII oocytes. (A) The subcellular distribution of mitochondria and ER in control oocytes (n = 22), oocytes overexpressing Mfn1 (n = 11) and Mfn2 (n = 26) was analyzed using GFP- and mCherry-tagged fusion proteins, respectively. Scale bar, 20 μm. (B and C) The ER Ca²⁺ stores were estimated from the Ca²⁺ responses induced by the addition of 1 μM ionomycin (B) and 20 μM thapsigargin (C) in Ca²⁺ free medium and traces of Fura-red emission ratio are shown. The comparisons of fluorescent Ca²⁺ peaks in control oocytes and oocytes overexpressing Mfn2 and/or Mfn1 are shown in the bar graph to the right of the traces. Error bars represent SEM and bars with asterisk are significantly different between the groups (***P < 0.01; analysis of variance with Tukey-Kramer’s test and *P < 0.05; t-test).
energy, Ca2+ homeostasis, and apoptosis (Pizzo and Pozzan, 2007). The reciprocal and functional interaction between these two organelles was also reported in mouse oocytes, as the mitochondria seem to facilitate Ca2+ oscillations at fertilization via the sarcoER Ca2+-ATPase-driven Ca2+ pumping mechanism (Dumollard et al., 2004; Wakai et al., 2013).

The ER forms fine reticular network with large ER-rich clusters in the cortex in MII oocytes. We found that the ER is attracted to mitochondrial aggregation sites in Mfn-overexpressing oocytes, using both TEM and live cell fluorescence imaging analysis, suggesting that Mfn contributes to the bridge connecting the mitochondria and ER. In particular, Mfn2-induced mitochondrial aggregation led to discrete ER networks and a decrease in ER Ca2+ stores. Remarkably, Mfn2 is enriched at contact sites between mitochondria and ER, and Mfn2 deficiency disrupts ER morphology and the physical/functional mitochondria–ER interactions (de Brito and Scorrano, 2008); as the distance between the mitochondria and ER increased, there was a decrease in mitochondrial Ca2+ uptake. Although the molecular mechanisms responsible for the physical connection between mitochondria and ER are largely unknown, our observation supports the notion that Mfn is one of the membrane-tethering molecules that mediates mitochondria–ER interaction (de Brito and Scorrano, 2010). We speculate that overexpression of Mfn2 enhances ER–mitochondria tethering. The tight interaction of two organelles might disturb their reorganization during maturation, thereby leading to an irregular localization throughout the cytoplasm. The reason why Mfn2 overexpression results in loss of ER Ca2+ remains unclear. The aberrant ER morphology may compromise ER Ca2+ homeostasis via ER–mitochondria communication.

The cytoskeletal network is another regulator that defines the positioning and movement of organelles. The accumulation of mitochondria around the MI spindle is associated with microtubule organization (Van Blerkom, 1991; Dalton and Carroll, 2013). Transport of mitochondria along microtubules is mediated by the motor protein kinesin family members and cytoplasmic dynein, which move toward the plus and minus ends of microtubules, respectively (Hirokawa, 1998). During maturation, the activity of both motors is required for the modest mitochondrial accumulation in the spindle region (Dalton and Carroll, 2013), as an inhibition of dynein reduced the mitochondrial association with the spindle, whereas an inhibition of kinesin-1 activity resulted in an increase of mitochondrial accumulation. Even though we showed that mitochondria displayed marked and persistent aggregation around the spindle in Mfn1-overexpressing oocytes, mitochondria were dispersed throughout the cytoplasm with cluster formation upon treatment with nocodazole, suggesting the subcellular compartmentalization of mitochondria is primarily directed by the microtubule network.

Importantly, Mfn1-induced mitochondrial aggregation disrupts spindle formation, resulting in chromosome misalignment. Since mammalian oocytes lack centrosomes, microtubule-organizing centers (MTOCs) substitute as centrosomes and contribute to the acentriolar bipolar spindle formation. The multipolar MTOCs are organized into a bipolar spindle through kinesin-5 activity, thereby establishing chromosome bi-orientation (Schuh and Ellenberg, 2007). The participation of ATP-driven motors in mitochondria and spindle organization likely stimulates a high demand for ATP by the developing spindle. In fact, dysfunction in mitochondrial ATP production leads to meiotic spindle abnormalities (Zhang et al., 2006; Johnson et al., 2007). Nonetheless, we did not observe a significant reduction of ATP levels during maturation of Mfn1-overexpressing oocytes, suggesting that, despite the conspicuous aggregation, mitochondria could maintain ATP production. Although further studies are needed to determine the roles of mitochondria in

Figure 7  Chromosome misalignment at the MII stage in Mfn1-overexpressing mouse oocytes. (A) Representative images of mitochondria and chromosomes in Mfn1-overexpressing MII oocytes producing a large polar body (LPB) and normal polar body (NPB). Mitochondria and chromosomes are labeled by GFP- and mCherry-tagged fusion proteins, respectively. Scale bar, 20 μm. (B) Percentages of oocytes producing a LPB 15 h after start of maturation (n = 50). (C) Representative sequence of images of mitochondria (GFP) and chromosomes (mCherry) during maturation from control oocytes (n = 9) and oocytes overexpressing Mfn1 (n = 18). Times after the start of maturation are denoted in hours and minutes. Scale bar, 20 μm.
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In conclusion, we describe for the first time the control of mitochondrial behavior in mouse oocytes by a fusion/fission mechanism. Impaired balance of mitochondrial fusion and fission protein functions perturbs the spatiotemporal dynamics of organelles, which would almost certainly lead to embryos with decreased developmental competence. The disorganization of organelles in mature oocytes, including human oocytes, is a hallmark of poor developmental competence of oocytes, which is commonly associated with diseases (Wang et al., 2009; Grindler and Moley, 2013), maternal age (Tarin et al., 2001; de Bruin et al., 2004) or post-ovulatory aging (Tarin et al., 2000; Van Blerkom, 2011). Our findings provide a foundation for further studies to elucidate the molecular mechanisms underlying the coordination of organelles that regulate the functions of oocytes.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles

T.W. and T.K. designed study and wrote the manuscript. T.W. executed all of the experiments and analysis. Y.H. and K.M. performed the 3D time-lapse imaging. All authors contributed to the discussion of results and participated in manuscript preparation.

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Conflict of interest

None declared.

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