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ORIGINAL RESEARCH

Dynamic interaction of formin proteins and cytoskeleton in mouse oocytes during meiotic maturation

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ABSTRACT: Formin-2 (Fmn2) nucleates actin filaments required for spindle migration during the metaphase of meiosis I in mouse oocytes. While recent studies showed that Fmn2 is involved in the formation of a dynamic actin meshwork on meiotic spindle and the migration of chromosomes, the precise location and the mechanism of action of Fmn2 in the mouse oocyte is not known. In this work, we show that Fmn2 is colocalized with spindle during metaphase I (MI) and this pattern is lost in nocodazole-treated oocytes. Fmn2 directly interacts with polymerized microtubules (MTs) in vitro via a well-conserved domain called formin homology 2 (FH2). Microinjection of mRNA encoding formin homology 1 (FH1) FH2 domains of Fmn2 into Fmn2−/− oocytes partially rescued the defect of polar body extrusion, while mRNAs encoding FH2 domain alone could not rescue the defect. mDia1 and mDia2, Diaphanous (Dia) subfamily of formin proteins, exhibit unique patterns of expression in mouse oocytes. While mDia1 is localized on meiotic spindle, mDia2 localization is confined to spindle poles similar to γ-tubulin. Collectively, our results suggest that the ability of Fmn2 to directly interact with MTs and to polymerize actins via the conserved FH1FH2 domains is crucial for chromosomes migration in MI oocytes. We also show that mDia1 and mDia2 are dynamic components of meiotic spindle and pole complex during meiotic maturation of oocytes.

Key words: oocyte maturation / animal model / cytoskeleton / formin / mouse

Introduction

The process of oocyte maturation during mammalian development requires intricate coordination of many signaling molecules (Carabatsos et al., 2000). In mammals, oocytes remain arrested at the prophase of meiosis I (PI) prior to birth. Two rounds of meiosis to complete oogenesis resume when the cyclic release of pituitary hormones initiates a signaling cascade in the ovary, stimulating follicular growth and oocyte maturation. Each round of meiosis generates an oocyte and a polar body (PB), which degenerates during early embryogenesis. The meiotic divisions involve extensive reorganization of microtubules (MTs) and actin filaments (Schatten et al., 1986; Albertini, 1992; Brunet et al., 1999). Chromosomal abnormalities due to meiotic errors are a leading cause of embryonic lethality and pregnancy losses (McFadden and Kalousek, 1991; Hassold and Hunt, 2001).

Formins are multi-domain actin nucleators (Higgs, 2005). In mice, at least 15 members of the formin gene family have been identified, and the list includes formin-1 (Fmn1) and formin-2 (Fmn2), mDia1, mDia2 and mDia3. Formins have highly conserved domains called formin homology (FH) domains. Closely situated FH1 and FH2 domains within a formin protein together mediate actin nucleation, and N-terminal region in some formins seem to be responsible for the interaction with small GTPase binding proteins (Faix and Grosse, 2006). The roles for formins in polarized morphogenesis in various species were demonstrated in mutant studies of several model organisms (Emmons et al., 1995; Swan et al., 1998). In mice, a role for formin in polarized morphogenesis is exemplified in Fmn2-deficient mice (Leader et al., 2002). In mice, Oocytes from Fmn2−/− mice show defective chromosomal migration and cytokinesis during meiosis I (Dumont et al., 2007). This defect leads to fertilization of tetraploid oocytes and recurrent abortions of multiploid embryos (Leader et al., 2002). In humans, expression of FMN2 and DIAPH2 (the human homolog of mDia3) is four to eight times higher in oocytes from polycystic ovarian syndrome (PCOS) patients (Wood et al., 2007), suggesting that formins may play important roles in maintaining oocyte quality both in mice and humans. Several cellular functions of formins in mitotic cells have been investigated. During mitosis, mDia1 is localized in mitotic spindles in HeLa cells (Kato et al., 2001; Rundle et al., 2004). mDia2 directly binds to MTs and is able to induce the actin scaffold.
at the contractile ring during cytokinesis in NIH 3T3 cells (Watanabe et al., 2008). mDia2 is also implicated in the enceluation of mouse fetal erythroblasts (Ji et al., 2008). Likewise, mDia3 is shown to regulate attachment of MTs to kinetochores and may behave like a chromosomal passenger protein in HeLa cells (Yasuda et al., 2004). While several studies have shown that mDia proteins function during cell polarization and migration (Yamaoka et al., 2006; Brandt et al., 2007; Gupton et al., 2007; Yang et al., 2007), the mechanism of action in a specific developmental context including meiosis has not been explored.

Recent works using Fmn2-deficient oocytes have demonstrated that Fmn2 is involved in actin polymerization which is required for the migration of chromosome-spindle complex during meiosis I (Azoury et al., 2008; Li et al., 2008; Schuh and Ellenberg, 2008). Fmn2-dependent actin polymerization occurs dynamically near metaphase I (MI) spindle and thus it is expected that Fmn2 protein is localized near spindle structure (Li et al., 2008). However, the precise localization of Fmn2 protein in MI oocyte has not been shown. In this work, we show that Fmn2 is localized in a spindle-like structure during metaphase and anaphase of Meiosis I and that Fmn2 interacts with the MTs via FH2 domain. We also show that mDia1 and mDia2 exhibit specific colocalization with spindle components during meiosis I.

Materials and Methods

Materials

pcS2-Fmn2-Myc Plasmid was a generous gift from Dr B. Leader. Rabbit polyclonal anti-Fmn2 antibody was custom-raised (3-R2) (Young-In Fron-tier, Seoul, Korea). The following antibodies were purchased from Santa Cruz Technologies (Santa Cruz, CA, USA): Fmn-2 (+H-100), mDia1 (C-20), mDia2 (M-16) and mDia3 (C-15, with epitopes from mDia3 protein ID_060879) were used as antigens. Rabbit monoclonal (YL 1/2) anti-tubulin antibody was purchased from Abcam (Cambridge, MA, USA) and mouse monoclonal anti-α-tubulin and anti-β-tubulin antibodies were purchased from Sigma (St Louis, MO, USA). Mouse monoclonal anti-γ-tubulin antibody was a generous gift of Dr Kunsoo Rhee (Seoul National University, Seoul, Korea).

Cell culture

NIH 3T3 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded onto coverslips in 6-well plate. To enrich mitotic cells, 100 ng/ml nocodazole was treated for 8 h. Cells were then subjected to immunofluorescence staining with anti-mDia1 antibody. NIH 3T3 and HeLa cells were grown in 4-well culture plate (SPL Life-science) and were fluorescently stained with anti-mDia1 antibody. Cells were washed twice with phosphate-buffered saline (PBS) and were subjected to immunofluorescence staining with anti-mDia1 antibody.

Mice and oocyte handling

Mice were maintained in accordance with the policies of the Konkuk University Institutional Animal Care and Use Committee. Mice were cared in a controlled barrier facility within College of Veterinary Medicine, Konkuk University. Temperature, humidity and photoperiod (12-h light and 12-h dark cycle) were kept constant. Fmn2-deficient mouse line was a generous gift from Drs B. Leader and P. Leder (Harvard Medical School, Boston, MA, USA). Fmn2+/− female mice were bred with Fmn2−/− male mice to maintain the colony. Fmn2+/− and Fmn2−/− female mice were used in the experiments. Five-week-old Fmn2-deficient or 4-week-old ICR mice (Orient-Bio, Kyunggi-do, Korea) were injected with 5 IU. PMSG (Sigma) to induce folliculogenesis. At 48 h post-injection, fully grown cumulus–oocyte complexes (COCs) were collected by ovary puncture. Cumulus cells were removed by gentle pipetting. Collected oocytes were cultured in M16 media and processed for immunofluorescence staining at 3 (prometaphase I, PMI), 8 (MI) and 12–16 h (PB). COCs were also processed for RNA isolation. A diagram depicting meiosis in the mouse oocyte is shown in Supplementary data, Fig. S1.D. When Fmn2−/− oocytes were used for the staining, a few oocytes were taken out for the batch and cultured overnight to confirm the failure of PB extrusion.

RT–PCR

Total RNA was extracted by using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer’s protocol. To isolate minute amounts of RNA from cumulus cells or oocytes, glycogen was used in a modified protocol. Resuspended RNA samples were then treated with ribonuclease A-free deoxyribonuclease (Takara, Japan) for 1 h at 37°C to remove any contaminated genomic DNA. RNA concentration and purity were evaluated by the ratio of optical density (OD)260/(OD)280 by a spectrophotometer. RNA samples (0.8 µg each) were subjected to RT using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for cDNA synthesis. Samples were either used directly for PCR or stored at −20°C. PCR was carried out using Prime Taq Premix (2×) (Genet Bio, Korea) and reactions were run on a PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). Following initial denaturation at 95°C for 5 min, PCR was performed at 95°C for 30 s at a specific annealing temperature (55–62°C) for 30 s and at 72°C for 30–60 s. Amplified products were analyzed on 1.5% agarose gel electrophoresis. PCR of glyceraldehyde-3-phosphate dehydrogenase was performed as internal control. To perform RT–PCR with RNA samples from mouse organs, 2 µg of total RNA in water was used. Other procedures were same as above. Sequences of primers used for PCR analysis are given in Supplementary data, Table S1.

Tissue preparation for paraffin sections

Some ovaries were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Tissues were then dehydrated and embedded in paraffin. Paraffin sections of ovary were cut at 8 µm and mounted onto poly-L-lysine-coated slides (Polysciences, Inc., Washington, PA, USA). Tissue sections were deparaffinized and rehydrated before being subjected to immunofluorescence staining.

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed as described previously (Eo et al., 2008). Denuded oocytes were spun down onto poly-L-lysine-coated slides (Polysciences, Inc.) and fixed in cold 3.7% formaldehyde in PBS. For immunofluorescence staining, samples were first permeabilized in 0.25% Triton X-100 for 10 min. For double labeling of tubulin and a formin protein, oocytes were fixed and extracted in MT stabilizing buffer (2% formaldehyde, 0.5% Triton X-100, 1 µM Taxol, 10 U/ml aprotinin and 50% deuterium oxide in PBS) for 20 min at 37°C (Leader et al., 2002). After washings, non-specific antiseraum binding was blocked with 2% bovine serum albumin (BSA)/PBS for 40–60 min. Slides were
incubated for 60–90 min with custom-generated polyclonal anti-Fmn2 (1.7 μg/ml). mDia primary antibodies were used at 1:50–1:100 in 2% BSA/PBS. Following three washes with 2% BSA/PBS, rabbit anti-goat or chick anti-rabbit Alex-quant conjugate (Invitrogen) secondary antibody (1:250 in 2% BSA/PBS) was applied for 40 min. Subsequently, slides were washed with 2% BSA/PBS and nuclei were stained with TO-PRO-3 iodide (Invitrogen, 1:500). After a final wash in PBS coverslips were mounted using ProFold Gold antifade reagent (Invitrogen). As a mock control, immunoglobulin G from host species of the primary antibody was used at each staining (representative figures shown in Supplementary data, Fig. S1C). Images were obtained using the Olympus FluoviewTM FV1000 Confocal Microscope (Tokyo, Japan) equipped with multi Argon-ion (457, 488 and 515 nm), He-Ne (green, 543 nm) and He-Ne (red, 633 nm) lasers. Images were analyzed using the software Fluoview version 1.5, a platform associated with the confocal microscope.

Plasmid construction and in vitro translation
From the full-length mouse Fmn2 cDNA (NM_019445.2), four deletion constructs were prepared as follows. Fmn2 ΔC of amino acid 1–734, FH1 of amino acid 735–1138, FH2 of amino acid 1139–1578 and Fmn2 FH1/FH2 of amino acid 735–1578 were amplified by PCR and cloned into Xhol and KpnI sites of pRSET vector (Invitrogen) (see Fig. 1 constructs I–IV). In vitro translation was performed by using TNT® T7 Coupled Reticulocyte Lysate System kit following the manufacturer’s instruction (Promega, Madison, WI, USA).

Expression and purification of FH2 fragment
FH2 of amino acid 1139–1578 in pRSET vector was expressed and purified as follows. An overnight culture of 3 ml [BL21 (DE3), Novagen] was added to 200 ml of LB medium containing 100 μg/ml of ampicillin and incubated at 37°C and in a shaking incubator at 200 rpm to an OD600 of 0.5. Protein expression was induced by adding Isopropyl beta-D-1-thiogalactopyranoside solution (final concentration at 1 mM) and incubated at 37°C for 6 h. The cell pellet was resuspended in 4 ml of the lysis buffer (100 mM Tris-Cl, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 20 mM Taxol and 0.05% NP-40) and 50 μl of nuclei were added to the supernatant and the mixture was shaken at 25°C for 1 h. The lysate-Nickel-Nitrilotriacetic Acid (Ni-NTA) mixture was loaded on a Poly-Prep column (Bio-Rad, Hercules, CA, USA), drained, and then washed three times with 4 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The proteins were eluted from the column in 2 ml of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The buffer of the protein solution was exchanged to 500 μl of PBS buffer using a centrifugal device (Amicon Ultra-15, Millipore, Billerica, MA, USA). mDia primary antibodies were used at 1:50–1:100 in 2% BSA/PBS and nuclei were stained with TO-PRO-3 iodide (Invitrogen). As a mock control, immunoglobulin G from host species of the primary antibody was used at each staining (representative figures shown in Supplementary data, Fig. S1C). Images were obtained using the Olympus FluoviewTM FV1000 Confocal Microscope (Tokyo, Japan) equipped with multi Argon-ion (457, 488 and 515 nm), He-Ne (green, 543 nm) and He-Ne (red, 633 nm) lasers. Images were analyzed using the software Fluoview version 1.5, a platform associated with the confocal microscope.

MT interaction assay
Tubulins from the bovine brain (Cytoskeletons, Inc., Denver, CO, USA) at 5 μg/ml were polymerized at 37°C and diluted with the dilution buffer (80 mM PIPES, pH 7.0, 1 mM MgCl2, 1 mM EGTA, 20 mM Taxol and 0.05% NP-40) to 0.2 μg/ml (Zhou et al., 2006). For each reaction set, the total (pellet + supernatant) chemiluminescence was given 1, and the relative amounts of FH2 domain (measured by LAS-3000) in pellet and supernatant was determined as percentages of that reaction. These experiments were performed three times and the average and the SD were shown in barograms.

Western blotting
Protein extracts from cell lysates of NIH 3T3 and HeLa cells were prepared in solubilization buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol and 1 mM EGTA]. All buffers contained a aliquot of Complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Tissues were homogenized with Polytron homogenizer (Brinkmann, Westbury, NY, USA) and centrifuged at 12 000 rpm. Cells were sonicated and centrifuged at 12 568 g. Oocytes were directly collected in 1× sample buffer. The supernatants were subjected to BCA assays (Thermo Scientific, Rockford, IL, USA) for quantification. Approximately 50–100 μg proteins were loaded onto 7.5% SDS–PAGE gels. After transferring into nitrocellulose membranes, the membranes were subjected to western blotting with specific antibodies. Intensity of anti-tubulin is compared among loaded samples. For abundant proteins, Intron enhanced chemiluminescence (ECL) reagent (Intron Biotechnology, Sungnam, Korea) was used. Proteins with low abundance were detected with SuperSignal West Femto ECL reagent (Thermo Scientific, Rockford, IL, USA) for quantification. Approximately 50–100 μg proteins were loaded onto 7.5% SDS–PAGE gels. After transferring into nitrocellulose membranes, the membranes were subjected to western blotting with specific antibodies. Intensity of anti-tubulin is compared among loaded samples. For abundant proteins, Intron enhanced chemiluminescence (ECL) reagent (Intron Biotechnology, Sungnam, Korea) was used. Proteins with low abundance were detected with SuperSignal West Femto ECL reagent (Thermo Scientific, Rockford, IL, USA) for quantification. Approximately 50–100 μg proteins were loaded onto 7.5% SDS–PAGE gels. After transferring into nitrocellulose membranes, the membranes were subjected to western blotting with specific antibodies. Intensity of anti-tubulin is compared among loaded samples. For abundant proteins, Intron enhanced chemiluminescence (ECL) reagent (Intron Biotechnology, Sungnam, Korea) was used. Proteins with low abundance were detected with SuperSignal West Femto ECL reagent (Thermo Scientific, Rockford, IL, USA) for quantification.
Results

**Fmn2 is localized in the meiotic spindle during oocyte maturation**

Fmn2 is 1578-amino acid long and has well-defined FH1 and FH2 domains (Fig. 1). The critical role for Fmn2 in the migration of chromosome-spindle complex in mouse oocytes was recently shown (Azoury et al., 2008; Li et al., 2008; Schuh and Ellenberg, 2008) but the subcellular localization of this protein is not clear. Thus, we raised polyclonal antibodies against different regions of Fmn2, and an antibody (3-R2) recognizing an epitope near the C-terminal end (amino acid 1551–1565; Fig. 1) was used for immunofluorescence staining of mouse oocytes during meiosis.

As shown in Fig. 2, anti-Fmn2 antibody (3-R2) detected immunoreactive Fmn2 scattered in the ooplasm at PI (Fig. 2A). At PMI, Fmn2 begins to accumulate near the forming meiotic spindle (Fig. 2B), and seems to overlap with migrating spindle from MI until the completion of meiosis (Fig. 2C and D). Similar localization of Fmn2 at spindle is noted in MII oocytes (Fig. 2E). To examine if Fmn2 is co-localized with meiotic spindle, we performed co-staining of Fmn2 and tubulin. As shown in Fig. 3, Fmn2 localization in MI or AI oocytes overlaps with that of spindle (anti-tubulin staining, shown in red), giving out a yellowish signal.

Notably, anti-Fmn2 antibody (3-R2) also showed localized signal in MTs in ~20% of Fmn2+/− oocytes (Supplementary data, Fig. S1A). Another anti-Fmn2 antibody from a commercial source showed localization on meiotic spindle in all Fmn2+/− oocytes we examined (Supplementary data, Fig. S1A). This antibody, H-100, uses an internal region of amino acids 531–619 as the epitope. Fmn2+/− mice were produced by replacing FH1 domain with the neomycin cassette, and a read-through mRNA of the same size as the original full-length mRNA had been observed (Leader et al., 2002). Thus, positive signal given by anti-Fmn2 antibodies in Fmn2+/− oocytes could be due to the production of read-through products containing the neo cassette instead of FH1 domain. Western blot analysis with H-100 showed the presence of weak immunoreactive band of slightly smaller size in the brain lysate from Fmn2+/− mice (Supplementary data, Fig. S1B).

Nocodazole treatment to depolymerize spindle disrupted Fmn2 localization, suggesting that Fmn2 localization requires the presence of a fully formed spindle structure. In contrast, when oocytes were treated with cytochalasin D, an inhibitor of actin polymerization, the localization of Fmn2 on meiotic spindle was not altered. This indicates that Fmn2 localization on spindle does not depend on actin filaments on meiotic spindle (Supplementary data, Fig. S2).

**Fmn2 interacts with MTs via FH2 domain in vitro**

To examine if Fmn2 physically interacts with spindle, we performed an *in vitro* MT interaction assay (Zhou et al., 2006). Constructs I–IV in pRSET vector shown in Fig. 1 were first subjected to *in vitro* transcription and the products were run on formaldehyde gel. All four constructs gave rise to RNA of the expected sized. They were subjected to *in vitro* transcription/translation, and products were confirmed in SDS–PAGE followed by western blotting with anti-Xpress antibody. Among these, construct IV encoding FH2 did not produce detectable amounts of protein (Supplementary data, Fig. S3B). Thus, it was omitted in this set of experiments. Translated products were mixed with polymerized MTs and subjected to differential centrifugation at 100 000g to precipitate polymerized MTs along with interacting proteins. Among three fragments, FH1FH2 fragment showed a slight tendency to aggregate, and a small amount appeared in the pellet section. When MT was added, a significantly higher amount of FH1FH2 fragment was detected in the pellet (Fig. 4A), suggesting that FH1FH2 fragment could interact with polymerized MT. In contrast, ΔC (construct I) did not seem to interact with polymerized MT (see Supplementary data, Fig. S3C showing reactions with higher amount of *in vitro* translated product).

As we could not detect any product from the construct IV containing FH2 domain after *in vitro* translation, this construct was subjected to *in vitro* transcription/translation, and products were confirmed in SDS–PAGE followed by western blotting with anti-Xpress antibody. Among these, construct IV encoding FH2 did not produce detectable amounts of protein (Supplementary data, Fig. S3B). Thus, it was omitted in this set of experiments. Translated products were mixed with polymerized MTs and subjected to differential centrifugation at 100 000g to precipitate polymerized MTs along with interacting proteins. Among three fragments, FH1FH2 fragment showed a slight tendency to aggregate, and a small amount appeared in the pellet section. When MT was added, a significantly higher amount of FH1FH2 fragment was detected in the pellet (Fig. 4A), suggesting that FH1FH2 fragment could interact with polymerized MT. In contrast, ΔC (construct I) did not seem to interact with polymerized MT (see Supplementary data, Fig. S3C showing reactions with higher amount of *in vitro* translated product).

As we could not detect any product from the construct IV containing FH2 domain after *in vitro* translation, this construct was subjected
The equilibrium between FH2 and MT was 1:1 molar ratio (m.w. of tubulins associated from MT and detected in the supernatant. Roughly the equi-

The result shows that Fmn2 interacts with spindle via FH2 domain. Thus, as we have shown above, interacts with spindle via FH2 domain. Thus, we investigated which fragment of Fmn2 is sufficient for PB extrusion by microinjecting sense RNA encoding different fragment of Fmn2. Constructs ∆C encoding N-terminal half, FH2 and FH1FH2 in pRSET vector were in vitro transcribed and used for RNA microinjection. For each group, 80–100 GV oocytes were injected in the presence of IBMX, given a recovery time of 2–3 h, and observed 16 h later. As shown in Fig. 5, ∼40% of oocytes receiving RNA encoding FH1FH2 fragment extruded PB. The FH2 domain, which interacts with spindle alone is insufficient for PB extrusion, suggesting that the actin polymerization capacity of FH1FH2 fragment is required for spindle migration and cytokinesis.

mDia1 exhibits a similar localization with Fmn2 in mouse oocytes

Recent studies reported that mDia1 and mDia2 mediate polymerization of cortical F-actin in various cell types (Yamana et al., 2006; Gupton et al., 2007). mDia1 and mDia3 are also localized in mitotic spindle and kinetochores in cultured cells, respectively (Kato et al., 2001; Rundle et al., 2004; Yasuda et al., 2004). While roles for mDia proteins in cultured cell systems have been studied, expression of these genes in the reproductive system has not been investigated. Thus, we set out to examine expression of mDia genes in oocytes. First, we performed RT–PCR analyses of mDia1, mDia2 and mDia3 in multiple mouse tissues (Supplementary data, Fig. S4A). While these genes are expressed in multiple tissues, all three are expressed in the ovary. Then we examined expression of mDia genes in mature mouse oocytes obtained from PMSG-treated ovary and cumulus cells which surround mature oocytes. While mDia1 and mDia2 are detected in both oocytes and cumulus cells, mDia3 expression is weakly noted only in cumulus cells (Supplementary data, Fig. S4B).

We then examined mDia1 localization in mouse oocytes. The antibody we used detects a single band of ∼180 kDa in mouse oocytes and other samples (Supplementary data, Fig. S5). In oocytes, mDia1 is shown scattered in ooplasm during PI and starts to accumulate near condensing chromosomes at PMI (Fig. 6A). At MI, mDia1 is co-localized in meiotic spindle, and nocodazole treatment abolished this localization (Fig. 6B and C). This pattern is reminiscent of Fmn2 localization. In Fmn2−/− oocytes, similar pattern of mDia1 localization is observed (Supplementary data, Fig. S6). In the mouse ovary, mDia1 is shown in subsets of granulosa and cumulus cells of some follicles (Fig. 7). Notably, mDia1 in these cells seems to exhibit asymmetric cytoplasmic localization (Fig. 7A, arrows). As it is reported in other cells that mDia1 is also involved in cell migration, we cultured GCs in vitro and examined mDia1 localization. mDia1 is shown scattered in the cytoplasm of GCs, and also in cell edges of some cells (Fig. 7B, arrowheads).

mDia2, a potential component of spindle pole complex in mouse oocytes

In mitotic cells, mDia2 induces formation of an actin scaffold at the contractile ring during cytokinesis (Watanabe et al., 2008), and is also implicated in the enucleation process of mouse fetal erythroblasts. The actin polymerization capacity of FH1FH2 fragment is required for spindle migration and cytokinesis.

Figure 3 Co-localization of Fmn2 and meiotic spindle in mouse oocytes. MI or AI mouse oocytes were stained with rabbit anti-Fmn2 and mouse monoclonal anti-tubulin antibodies and probed with Alexa Fluor 488- and 568-conjugated secondary antibodies, respectively. DNA was counter-stained with TO-PRO-3-iodide (shown in blue). Nocodazole was treated to some oocytes 6–8 h post-germinal vesicle stage at 10 μg/ml for 2 h to depolymerize spindle. Similar results were obtained from oocytes prepared from at least three to four batches of mice. Red scale bar, 30 μm; white scale bar, 10 μm.

Microinjection of mRNA encoding FH1FH2 fragment partially restores defective PB extrusion in Fmn2−/− oocytes

Oocytes from Fmn2−/− mice show defective PB extrusion because chromosome-spindle complex fails to migrate to the cortex during MI (Leader et al., 2002; Dumont et al., 2007). Fmn2 bears the capacity to polymerize actin filament with FH1FH2 fragment (Li et al., 2008), and as we have shown above, interacts with spindle via FH2 domain. Thus, we investigated which fragment of Fmn2 is sufficient for PB extrusion by microinjecting sense RNA encoding different fragment of Fmn2. Constructs ∆C encoding N-terminal half, FH2 and FH1FH2 in pRSET vector were in vitro transcribed and used for RNA microinjection. For each group, 80–100 GV oocytes were injected in the presence of IBMX, given a recovery time of 2–3 h, and observed 16 h later. As shown in Fig. 5, ∼40% of oocytes receiving RNA encoding FH1FH2 fragment extruded PB. The FH2 domain, which interacts with spindle alone is insufficient for PB extrusion, suggesting that the actin polymerization capacity of FH1FH2 fragment is required for spindle migration and cytokinesis.

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mDia2, a potential component of spindle pole complex in mouse oocytes

In mitotic cells, mDia2 induces formation of an actin scaffold at the contractile ring during cytokinesis (Watanabe et al., 2008), and is also implicated in the enucleation process of mouse fetal erythroblasts.
Interestingly, mDia2 exhibits a unique localization during meiosis. As shown in Fig. 8A, mDia2 at PI shows a punctuate pattern of localization which resembles MT organizing centre (MTOC) distribution. At PMI, concentrated spots of mDia2 are observed near condensing chromosomes. From MI when meiotic spindle is fully formed, it is localized at spindle poles. Nocodazole treatment did not alter focal concentration of mDia2 in presumptive pole areas, suggesting that a fully formed spindle is not required for mDia2 localization (Fig. 8B). mDia2 localization overlaps with that of γ-tubulin (Fig. 8C), a component of spindle pole complex (Gueth-Hallonnet et al., 1993). As this unique localization was not reported previously, we examined localization of mDia2 in mitotic NIH 3T3 cells. As shown in Supplementary data, Fig. S7, mDia2 is localized in spindle poles along with γ-tubulin in mitotic cells.

Discussion

The ability of Fmn2 to drive spindle migration resides in the formation of spindle-like actin cables during MI (Azoury et al., 2008; Li et al., 2008; Schuh and Ellenberg, 2008) (Fig. 3). In the present investigation,
Defective PB extrusion in Fmn2−/− oocytes was partially rescued by microinjecting sense RNA encoding FH1FH2 domain. About 80–100 Fmn2−/− oocytes were used in each group, except for the control group where 40 oocytes were used. Oocytes were stained with Syto Green fluorescent nucleic acid dye (green) at the time of scoring (16 h in culture). Percentages of oocytes in MI stage (yellow), 2-cell-like morphology (purple) and PB stage (blue) in each group is shown. Representative figures of oocytes after microinjecting FH1FH2 RNA are shown.

mDia1 is co-localized with meiotic spindle in MI and AI mouse oocytes. (A) Oocytes at PI, PMI, MI or MII stage were stained with goat polyclonal anti-mDia1 (green). (B and C) MI or Al oocytes were co-stained with anti-mDia1 (green) and rat monoclonal anti-tubulin (red) antibodies. DNA was counter-stained with TO-PRO-3-iodide (shown in blue). Nocodazole was treated at 10 μg/ml for 2 h to depolymerize spindle. Yellowish fluorescence is an overlay of mDia1 localization (green) and spindle (red). Nocodazole treatment abolished spindle and also mDia1 localization. Similar results were obtained from oocytes prepared from at least three to four batches of mice. Red scale bar, 30 μm; white scale bar, 10 μm.
we provide evidence that FH2 domain of Fmn2 directly interacts with MT, and this supports that Fmn2 potentially functions as a scaffold for cytoskeletal organization and function during meiosis. The role for actin cables in pulling chromosome-spindle complex during MI in mouse oocytes had long been suspected, but only recently the presence of actin-like filaments and cables linking the oocyte cortex and spindle has been clearly visualized (Azoury et al., 2008; Li et al., 2008; Schuh and Ellenberg, 2008). Localization of Fmn2 on meiotic spindle is reminiscent of F-actin cage on chromosome-spindle complex, which is not observed in Fmn2−/− oocytes (Azoury et al., 2008). Thus, this strongly suggests that Fmn2 may be responsible for formation of F-actin cage required for chromosome-spindle migration during meiosis. As we have shown herein, although FH2 bears an intrinsic activity to interact with MTs, it alone is not sufficient to rescue Fmn2−/− oocytes from defective PB extrusion (Fig. 5). The activity of FH1FH2 fragment as actin nucleator is required for PB extrusion. Thus, it is likely that Fmn2, while interacting with the spindle, forms actin cables and establishes a link with the cortex for spindle migration. Drosophila formin, Cappuccino, also interacts with MT via FH2, while Fmn1 binds to MTs through a domain in N-terminus (Rosales-Nieves et al., 2006; Zhou et al., 2006). It remains to be investigated if MT-interacting activity endowed by FH2 is needed for PB extrusion by FH1FH2 fragment.

By using two antibodies, we obtained evidence that Fmn2−/− mice may express an attenuated level of the full-length immuno-reactive product (Supplementary data, Fig. S1). There is a possibility that the replacement of FH1 domain with the neomycin cassette during the initial production of gene-targeted mice (Leader et al., 2002) could have enabled some read-through production of full-length mRNA and proteins. While this possibility requires further investigation, low level production of FH1-deleted full-length product nonetheless does not seem to hinder the expressivity of the gene deficiency phenotype.

While investigating the role for Fmn2 has been somewhat confined to the oocyte system due to the strong phenotype of female hypofertility in Fmn2−/− mice (Leader et al., 2002), functional studies of mDia proteins have been performed in a variety of systems. Fmn1 and all three mDia proteins have actin polymerization activity (Gavin et al., 2002; Li and Higgs, 2003; Kobielak et al., 2004; Bartolini et al., 2008). mDia1 is involved in phagocytic cup formation and cell migration and is under the regulation of Ras GTPase-activating like protein in these processes (Brandt et al., 2007). During mitosis, mDia1 is localized in three types of spindle MTs, kinetochore MT, non-kinetochore MT and astral MT in HeLa cells (Kato et al., 2001). As we show herein, mDia1 is also present in spindle of oocytes, thus the function of mDia1 in mitosis and meiosis may be similar. The subcellular localization of mDia1 and Fmn2 in oocytes is also analogous to each other. Considering the strong phenotype of Fmn2−/− oocytes, mDia1 in the oocyte may be involved in a function not associated with PB extrusion. Furthermore, mDia1-deficient female mice do not show any fertility defect (Sakata et al., 2007). In HeLa cells, depletion of mDia1 in mitotic cells does not hinder cytokinesis (Rundle et al., 2004).

FH1FH2 fragment of mDia2 stabilizes MT in vitro, and this activity is independent of actin polymerization (Bartolini et al., 2008). During mitosis, levels of mDia2 protein are under the regulation of ubiquitin-mediated proteolysis (DeWard and Alberts, 2009). Furthermore, failure to regulate levels of mDia2 results in multinucleation, because
of aberration in contractile ring formation (Watanabe et al., 2008). Thus, tight regulation of the level of mDia2 protein is crucial during mitotic cell division. We show that in meiotic oocytes and in mitotic NIH 3T3 cells, mDia2 exhibits a unique pattern of localization on spindle poles. This pattern was not previously noted in a study using a different antibody (Watanabe et al., 2008), and further investigation is needed to address this discrepancy of whether it is associated with distinct nature of antibodies used.

Oocytes in many species, such as Caenorhabditis elegans, Xenopus and mammals, undergo meiotic division by using acentrosomal spindle assembly driven by chromosomes (Compton, 2000). In the absence of centrioles, vertebrate oocytes produce acentriolar MTOCs which contain the pericentriolar material components γ-tubulin (Schuh and Ellenberg, 2007). In prophase stage of oocytes, MTOCs form de novo from MT network, and MTOC foci appear at network junctions (Schuh and Ellenberg, 2007). In mouse oocytes, mDia2 is initially scattered about in the ooplasm in a punctuate pattern at PI and is then translocated to the site of γ-tubulin localization at MI (Fig. 8). Thus, it is possible that mDia2 is a component of MTOC and spindle pole complex. As we showed herein (Supplementary data, Fig. S7), NIH 3T3 cells during mitosis also express mDia2 in spindle poles along with γ-tubulin. Thus, mDia2 may be a common member of acentrosomal and centrosomal spindle pole complexes. Further studies are forthcoming to reveal the role of this formin protein in the formation and function of the spindle pole complex.

In a study which compared gene expression profiles of oocytes from normal and PCOS patients, not only the presence of FMN2 and DIAPH2 (a homolog of mDia3) mRNAs was confirmed in human oocytes, but also an aberrant up-regulation of both genes was detected (Wood et al., 2007). In cultured HeLa cells, the endogenous mDia3 was shown localized in kinetochores complex during mitosis, and mDia3 and CDC42 together regulate MT attachment to kinetochores on mitotic spindles. Furthermore, depletion of mDia3 with siRNA causes the chromosomes to remain scattered at the poles (Yasuda et al., 2004). In mouse oocytes, however, mDia3 expression does not seem to be abundant and we failed to detect mDia3 localization with two commercially available antibodies. Both antibodies detect a single band of ~130 kDa in whole lysates of mouse ovary, brain, NIH 3T3 and HeLa cells (Supplementary data, Fig. S5B). mDia3 may be of low abundance in the ovary or may exhibit a highly dynamic expression pattern which makes the detection of subcellular localization difficult. Further investigation is warranted in regard to expression and function of mDia3 in meiosis.

Figure 8 mDia2 is localized in spindle poles in mouse oocytes. (A) Oocytes at PI, PMI, MI or MII stage were stained with goat polyclonal anti-mDia2 (green). DNA was counter-stained with TO-PRO-3-iodide (shown in blue). mDia2 is localized in spindle poles of MI and MII mouse oocytes. (B) MI oocytes were co-stained with anti-mDia2 (green) and rat monoclonal anti-tubulin (red) antibodies. Nocodazole treatment abolished spindle but mDia2 remains as dots near chromosomes (arrowhead). (C) MI oocytes were co-stained with anti-mDia2 and mouse monoclonal anti-γ-tubulin antibodies. Yellowish fluorescence is an overlay of mDia2 localization (green) and γ-tubulin (red). Similar results were obtained from oocytes prepared from at least three to four batches of mice. Red scale bar, 30 μm; white scale bar, 10 μm.
For Fmn2, binding proteins regulating its activity is yet to be determined. mDia proteins have a well-defined small GTPase-binding domain in the N-terminal half of the protein, and several Rho family proteins have been shown to regulate activity of these proteins (reviewed in Lammers et al., 2008; Chesarone et al., 2010). Other proteins are also involved in targeting mDia protein to a specific subcellular compartment, or regulate their activity. Cdc42 is one of small GTPase-binding proteins, and plays a crucial role during PB extrusion in meiotic oocytes (Ma et al., 2006). Whether Cdc42 in mouse oocytes mediates interaction with a formin remains to be determined.

In conclusion, the significance of this work is summarized as follows. First, while Fmn2 interacts with MTs via FH2, FH1FH2 fragment is required for spindle migration for the emission of PB. This result, along with published data by others, shows dual interaction of Fmn2 with actin and MT. It remains to be studied how the activity of Fmn2 as a scaffold for cytoskeletons is executed and regulated. Designing a detailed domain-based approach would help address the regulatory mechanism of Fmn2. Secondly, we report that mDia1 and mDia2 are components of meiotic spindles and poles, respectively. While it is estimated that mDia1 is not needed for meiosis, the localization of mDia2 in spindle poles during both mitosis and meiosis warrants further investigation to examine the function of mDia2 in spindle pole dynamics. Considering that aneuploidy due to meiotic errors is one of the leading causes of embryonic lethality and pregnancy loss (McFadden and Kalousek, 1991; Hassold and Hunt, 2001), this work advances our understanding of cellular events which govern the process of meiotic maturation.

**Author’s roles**

S.K., H.S. and H.J.L. designed research; S.K. and H.S. performed research; S.K., H.S. and H.J.L. analyzed data and S.K., H.S. and H.J.L. wrote the paper.

**Supplementary data**

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

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**Supplementary data**

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