Biochimica et Biophysica Acta 1853 (2015) 317–327

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Formin mDia1, a downstream molecule of FMNL1, regulates Profilin1 for actin assembly and spindle organization during mouse oocyte meiosis ☆

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ARTICLE INFO

Article history:
Received 26 August 2014
Received in revised form 6 October 2014
Accepted 4 November 2014
Available online 15 November 2014

Keywords:
mDia1
Profilin1
Actin
Spindle formation
Polar body extrusion
Oocyte

ABSTRACT

Mammalian diaphanous1 (mDia1) is a homologue of Drosophila diaphanous and belongs to the Formin-homology family of proteins that catalyze actin nucleation and polymerization. Although Formin family proteins, such as Drosophila diaphanous, have been shown to be essential for cytokinesis, whether and how mDia1 functions during meiosis remain uncertain. In this study, we explored possible roles and the signaling pathway involved for mDia1 using a mouse oocyte model. mDia1 depletion reduced polar body extrusion, which may have been due to reduced cortical actin assembly. mDia1 and Profilin1 had similar localization patterns in mouse oocytes and mDia1 knockdown resulted in reduced Profilin1 expression. Depleting FMNL1, another Formin family member, resulted in reduced mDia1 expression, while RhoA inhibition did not alter mDia1 expression, which indicated that there was a FMNL1-mDia1-Profilin1 signaling pathway in mouse oocytes. Additionally, mDia1 knockdown resulted in disrupting oocyte spindle morphology, which was confirmed by aberrant p-MAPK localization. Thus, these results demonstrated indispensable roles for mDia1 in regulating mouse oocyte meiotic maturation through its effects on actin assembly and spindle organization.

1. Introduction

The meiosis and mitosis, both including DNA replication and cytokinesis events, are essential processes that emerge in mammal development. The mitosis, a kind of continuous symmetric division, encompasses a round of DNA replication and cytokinesis. Eventually, the mitotic cell divides into two identical size daughter cells, which contain the same genome as the parental cells. Unlike the mitosis, firstly, mammalian oocyte meiosis is characterized by a unique asymmetric division, as the spindle migrates to the cortex and extrudes a small polar body. Secondly, during meiosis, the mammalian oocyte undergoes two consecutive rounds of asymmetric divisions while only one round of DNA replication, generating a totipotent haploid egg. This brief period, so called 'meiotic maturation', is essential for maintaining female genome integrity. Oocytes are arrested at the diplotene stage of the first meiotic prophase within ovarian follicles, which is also defined as the germinal vesicle (GV) stage. During oocyte maturation, fully grown oocytes reinitiate meiosis, as indicated by germinal vesicle breakdown (GVBD). Finally, an oocyte proceeds through the first meiosis, and then transforms into a metaphase II (MII)-arrested oocyte with an extruded small first polar body (pbI) until it is fertilized [1]. Unlike the mitotic division that the actomyosin furrow ingression mainly depends on the actomyosin ring contraction, interestingly, the polar body extrusion is a specialized cytokinesis, coordinated by the cortical membrane protrusion and actomyosin ring contraction [2]. Additionally, in contrast to the mitosis during which a centrally positioned spindle midzone leads to the bilateral furrowing from the center of the somatic cells, the meiotic spindle midzone-induced membrane furrow changes from the initial unilateral to the eventual bilateral during oocyte polar body extrusion [3].

Polar body extrusion is a highly coordinated process that depends on the dynamic coordination of microtubules and actin filaments related events during divisions [4,5]. During meiosis I, microtubules form a specialized bipolar-shaped spindle at the metaphase I (MI) stage. Accurate spindle assembly is required for orderly meiosis during oocyte maturation [6]. In addition to the induction of actin organization by chromatin, spindle assembly is also attributed to this process. During cell cycle progression, the polymerized actin distributes parallel to the plasma membrane at the oocyte cortex, and plays a critical role in the establishment of cortical rigidity to act as a foundation for proper

☆ Funding: This work was supported by the National Basic Research Program of China (2014CB138503), the Natural Science Foundation of Jiangsu Province (BK20140030), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20130097120055), and the Biogreen 21 Program (PJ009594, PJ009080 and PJ00909801), RDA, Republic of Korea.

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http://dx.doi.org/10.1016/j.bbamcr.2014.11.005
0167-4889/

spindle assembly [7,8]. This regulation may be due to the physical interactions between the plus ends of astral microtubules with a rigid actin cortex that facilitate placing, shortening, and focusing of the nascent spindle [9,10]. Also, actin filaments ensure the targeting of microtubule spindle positioning at the periphery of an oocyte concomitant with chromosome segregation [11,12]. Additionally, a fine network of microfilaments is found throughout the entire cortex and is responsible for constructing the classical contractile ring, which constricts at the base of the protrusion for pinching off a polar body [2,13]. Although several molecules have been proposed to contribute to cytoskeletal regulation during oocyte meiosis in mammals, the molecular mechanisms that modulate the meiotic apparatus remain to be determined.

Formin family proteins are essential for diverse cellular processes, including vesicle trafficking [14,15], cell migration [16,17], and microtubule stabilization [18,19]. Formins also have highly conserved, critical functions for cytokinesis [20–22]. Additionally, Formins are conserved actin nucleators that are involved in promoting the assembly of actin filaments [23]. Furthermore, a previous study has shown that a Formin protein, Formin2 is responsible for correct positioning of the metaphase spindle and final abscission of the first polar body during meiosis [24]. mDia proteins are the mammalian homologues of Drosophila diaphanous, which is a member of the Formin-homology family of proteins and is a downstream effector of the small GTPase Rho.

In mammals, the mDia family comprises 3 isoforms, mDia1 (diaphanous1), mDia2 (diaphanous2), and mDia3 (diaphanous3) [23,25], which vary in their subcellular localization, enzymatic activity, and their targets. mDia1 and mDia2 are crucial for cell migration and cell adhesion [26]. In addition, mDia proteins are strongly implicated in cell division. Several lines of evidence indicate that an activated mDia and diaphanous are essential for catalyzing linear actin nucleation and polymerization during cytokinesis [27,28]. In addition to their functions for actin assembly, mDia proteins reportedly stabilize and align microtubules in mitotic cells [18,28,29]. Among these, mDia2 has been the most extensively investigated. Studies have indicated that mDia2 was involved in actin assembly regulation during cytokinesis [30,31]. mDia3, which is found at the kinetochores of condensed chromosomes in HeLa cells, might be essential for microtubule-mediated chromosome segregation during mitosis [29]. mDia1 is also crucial to a large variety of cellular and morphogenetic functions via its effects on actin assembly [32,33]. Although mDia proteins have been implicated in multiple critical biological processes during mitosis, their physiological functions and the signaling cascade involved during oocyte meiosis have not been investigated.

For this study, we proposed that mDia1 would have significant roles during mouse oocyte maturation. To confirm our hypothesis, we used RNA interference (RNAi) to deplete the mDia1 isoform in mouse oocytes and assessed mDia1 effects with regard to cytoskeleton-mediated dynamic events during mouse oocyte meiotic maturation. Our results indicated a direct contribution of mDia1 in mouse oocyte meiosis in that mDia1-induced actin assembly and spindle organization were critical for polar body extrusion by dividing oocytes.

2. Materials and methods

2.1. Antibodies and chemicals

Rabbit monoclonal anti-mDia1, mouse monoclonal anti-Profilin1, and rabbit polyclonal anti-FMNL1 antibodies were from Abcam (Cambridge, MA, USA). Phalloidin-TRITC and mouse monoclonal anti-α-tubulin-FITC antibodies were from Sigma-Aldrich Corp. (St. Louis, MO, USA). FITC-conjugated and TRITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG were from Zhongshan Golden Bridge Biotechnology, Co., Ltd. (Beijing). All other chemicals and reagents were from Sigma-Aldrich Corp., unless otherwise stated.

2.2. Oocyte harvest and culture

ICR mice were used for all experiments. Our experiments were approved by the Animal Care and Use Committee of Nanjing Agriculture University and were performed in accordance with Animal Research Institute Committee guidelines. Mice were housed in a temperature-controlled room with an appropriate light: dark cycle, fed a regular diet, and maintained under the care of the Laboratory Animal Unit, Nanjing Agricultural University. Young female mice (6–8 weeks) were used for oocyte collection. For in vitro maturation, germinal vesicle-intact oocytes were harvested from female ovaries and cultured in M2 medium under paraffin oil at 37 °C in a 5% CO_2 atmosphere. Oocytes were removed from culture at different times for microinjection, real-time RT-PCR, and immunofluorescent staining and Western blot analysis.

2.3. Nocodazole treatment of oocytes

For nocodazole treatment, 10 mg/ml nocodazole in DMSO stock was diluted in M2 medium to give a final concentration of 20 μg/ml. After incubating in M2 medium supplemented with nocodazole for 10 min, the oocytes were collected for immunofluorescence microscopy after 9 h culture.

2.4. Real-time quantitative pcr analysis

Real-time quantitative PCR analysis was used to examine mDia1 mRNA expression in oocytes. Total RNA was extracted from 30 oocytes using a Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS), and single-stranded cDNA was generated using a PrimeScript™ RT reagent kit (Takara, Dalian, China) with Oligo (dT) 12–18 primers (Invitrogen). The cDNA products were then diluted 6-fold with deionized water before use as a template for real-time PCR. A cDNA mDia1 fragment was amplified using the following primers: forward, ACG CCA TCC TCT TCA AGC TA; reverse, TGG AGC CCG CAT TCA TAT AG. Each reaction mixture included 10 μl of FastStart Universal SYBR Green Master (Rox; Roche Applied Science, Mannheim, Germany), 0.6 μl each of the forward and reverse primers, and 2 μl of template cDNA. The total reaction volume was 20 μl. Real-time quantitative PCR (qPCR) was done using a StepOne plus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), and amplification was done using a 7500 Fast Dx Real-Time PCR instrument (Life Technologies, Carlsbad, CA, USA), according to the manufacturers' protocols. Cycling conditions were: initial incubation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 31 s. A final extension step at 60 °C for 1 min was added at the end of the last cycle. Amplification for each sample was done in triplicate. qRT–PCR analyses were done 3 times with independent RNA samples. The fold-change in mRNA expression was determined with the reference gene GAPDH by using the $2^{-\triangle\triangle}$Ct method.

2.5. mDia1 siRNA, mDia1 antibody and FMNL1 morpholino (MO) injection

Three target-specific short interfering double-stranded RNA oligomers (siRNAs) (sc-35191; Santa Cruz) were used for mDia 1 RNAi. Stealth RNAi negative control duplexes were used as a control. For mDia1 knockdown (KD) experiments, mDia1 siRNA was diluted with RNAase free water to give a 50 nM stock solution, and ≈5–10 pl of mDia1 siRNA solution was microinjected into the cytoplasm of a fully grown GV oocyte using an Eppendorf FemtoJet (Eppendorf AG) with a Nikon Diaphot ECLIPSE TE300 inverted microscope (Nikon U.K. Ltd) equipped with a Narishige MM0-202N hydraulic three-dimensional micromanipulator (Narishige Inc. Tokyo, Japan). Negative control siRNA (5–10 pl) was microinjected into oocytes as a control. For antibody injection, 5–10 pl of an mDia1 antibody was microinjected and the same volume of water was injected as a control.