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Vesicular transport protein Arf6 modulates cytoskeleton dynamics for polar body extrusion in mouse oocyte meiosis

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ABSTRACT

Arf6 (ADP-ribosylation factor 6) is known to play important roles in membrane dynamics through the regulation of actin filament reorganization for multiple cellular processes such as cytokinesis, phagocytosis, cell migration and tumor cell invasion. However, the functions of Arf6 in mammalian oocyte meiosis have not been clarified. In present study we showed that Arf6 expressed in mouse oocytes and was mainly distributed around the spindle during meiosis. Depletion of Arf6 by morpholino microinjection caused oocytes failing to extrude first polar body. Further analysis indicated that Arf6 knock down caused the aberrant actin distribution, which further induced the failure of meiotic spindle movement. And the loss of oocyte polarity also confirmed this. The regulation of Arf6 on actin filaments in mouse oocytes might be due to its effects on the phosphorylation level of cofilin and the expression of Arp2/3 complex. Moreover, we found that the decrease of Arf6 caused the disruption of spindle formation, indicating the multiple roles of Arf6 on cytoskeleton dynamics in meiosis. In summary, our results indicated that Arf6 was involved in mouse oocyte meiosis through its functional roles in actin-mediated spindle movement and spindle organization.

1. Introduction

During mammalian oocyte meiosis, oocyte undergoes two round cell divisions without an intermediate replication of genetic material. The consecutive two meiotic divisions are highly asymmetric, generating a large oocyte and two small polar bodies, which enable the oocyte to preserve a substantial cytoplasmic store of maternal factors, which is critical for early embryo development [1]. After nuclear membrane (germinal vesicle) breaks down, microtubules assemble to form the specialized bipolar meiotic spindle with all the chromosomes aligning at the spindle equator; meanwhile, the actin filaments form a meshwork surrounding oocyte spindle. Then the spindle migrates to the oocyte cortex in an actin filament dependent manner [2]. As the meiotic spindle migrating and anchoring to the oocyte cortex, cortical actin filaments are enriched to form an actin cap overlying the spindle and cortical granules (CGs) are redistributed to form a cortical granules free domain (CGFD) [3,4], which are essential for polar body extrusion.

The actin filaments play important roles in mediating cortical reorganization and spindle relocation during mammalian oocyte meiosis [5–7]. Three types of actin nucleators Arp2/3 complex, Formin2 and Spire1/2 are shown to be required for spindle migration and asymmetric division during oocyte meiosis: inhibition of Arp2/3 complex activity disrupts spindle migration and oocyte polarity during mouse oocyte meiosis. Meanwhile, depletion of Formin2 and Spire1/2 also causes aberrant spindle location and the failure of asymmetric division in mouse oocyte meiosis [8–10]. While small GTPase Rho-GTPases regulate various cellular functions primarily through their ability to modulate microtubule dynamics and the actin dynamics in mitosis [11]. Recent works suggest that Rho-GTPases (RhoA, Cdc42, and Rac1) play essential roles in oocyte meiosis: disruption of RhoA and Cdc42 activities causes the aberrant actin assembly and spindle organization during oocyte meiosis, while inhibition of Rac1 activity disrupts spindle positioning and oocyte cortical polarity in mouse oocyte meiosis [12–14]. Rab11, which belongs to the Rab GTPases, is implicated in regulating vesicular trafficking through the recycling of endosomal compartment; it is also required for cell adhesion, maintenance of cell shape and actin cytoskeleton organization [15]. And Rab11a-positive vesicles are shown to mediate actin network and asymmetric spindle migration during mouse oocyte meiosis. This evidence indicates that the vesicle is essential for actin network modulation in oocyte meiosis [16]. However, the underlying molecules for vesicle controlling actin dynamics in oocyte meiosis remain to be explored.

The ADP-ribosylation factor proteins Arf1-6 belong to the Ras superfamily of small GTPases and play essential roles in vesicular transport, while Arf6 is a member of the ARF family, which is thought to regulate endocytosis and endosomal recycling at cell surface [17–19].

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In addition to the roles in vesicles, Arf6 is implicated in actin cytoskeleton dynamic and involved in several of actin-based processes, including cell adhesion and migration, cell morphology and cytokinesis [20]. Previous work suggests that Arf6 modulates actin cytoskeleton through its effect on lipid metabolism. And Arf6 can activate PI(4,5)P2 accumulation and further regulates actin capping and the activities of several actin-binding proteins, such as WASP family proteins, cofilin, proflin and Arp2/3 [21]. Previous works demonstrate that cofilin plays essential role in regulating actin dynamics and reorganization by stimulating the depolymerization of actin filaments, and the activity of cofilin is inhibited by the phosphorylation and dephosphorylation at Ser-3 domain [22–24]. Meanwhile, profilin1 is a widespread actin-binding protein that regulates actin polymerization [25]. Profilin could bind more strongly to ATP-actin than ADP-actin, which blocks the nucleation of actin filament [26,27]. Our recent work shows that knockdown of profilin1 affects the oocyte polar body extrusion by increasing the actin polymerization. Although Arf6 has been showed to participate in multiple cellular processes, but its function in mammalian oocyte has not been addressed.

In this study, we hypothesized that Arf6 might play important roles during mouse oocyte maturation. To demonstrate our hypothesis, we used morpholino to inhibit the translation of Arf6 protein and then examined the potential functions of Arf6 in cytoskeleton dynamics in mammalian oocyte meiosis. Our results indicated that Arf6 was essential for polar body extrusion through mediating actin assembly and spindle organization during mouse oocyte meiosis.

2. Material and methods

2.1. Antibodies and chemicals

A rabbit monoclonal anti-Arf6 and anti-profilin antibody were from Abcam (Cambridge, UK); anti-Arp2 was purchased from Santa Cruz (Santa Cruz, CA); anti-α-tubulin, anti-p-MAPK and anti-p-cofilin was from Cell Signaling Technology (Danvers, MA); Phalloidin-TRITC and mouse monoclonal anti-α-tubulin-FITC antibody were from Sigma (St Louis, MO); Alexa Fluor 488, 594 antibodies were from Invitrogen (Carlsbad, CA).

2.2. Oocyte harvest and culture

All procedures with mice were conducted according to the Animal Research Institute Committee guidelines of Nanjing Agriculture University, China. The experimental protocols were approved by Nanjing Agriculture University Animal Research Institute Committee. Mice were housed in a temperature controlled room with appropriate dark-light cycles, fed a regular diet, and maintained under the care of the Laboratory Animal Unit, Nanjing Agricultural University, China. Germinal vesicle-intact oocytes were harvested from ovaries of 4–6 week-old ICR mice and cultured in M16 medium (Sigma, MO) under paraffin oil at 37 °C in a 5% CO_2 atmosphere. Oocytes were removed from culture at different times for microinjection, immunofluorescent staining and western blot.

2.3. Arf6 morpholino (MO) injection

For Arf6 knock-down in mouse oocyte, Arf6 morpholino 5′-TCT TGG ATA GCA CCT TCC CCA TCGC-3′ (Gene Tools, Philomath, OR) was diluted with reagent grade water (Sigma) to give a 1 mM stock concentration. Each fully grown GV oocyte was microinjected with 5-10pl of Arf6 morpholino using an Eppendorf FemtoJet (Eppendorf AG, Hamburg, Germany) under an inverted microscope (Olympus IX71 Japan). After injection, oocytes were cultured in M16 medium that contained 5 μM milrinone for 24 h, and then washed three times (2 min each wash) in fresh M16 medium. Oocytes were then transferred to fresh M16 medium and cultured under mineral oil at 37 °C in a 5% CO2 atmosphere. Each control oocyte was microinjected with 5-10pl of standard control oligo. For Arf6 antibody injection, each fully grown GV oocyte was microinjected with 5-10pl of Arf6 antibody, and then cultured in fresh M16 medium.

2.4. Confocal microscopy

For staining of Arf6 or p-MAPK, oocytes were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min and then transferred to a membrane permeabilization solution (0.5% Triton X-100) for 20 min. After 1 h in blocking buffer (1% BSA-supplemented PBS), oocytes were incubated at 4 °C overnight with rabbit anti-p-Arf6 (1:100) or p-MAPK (1:100). After three washes in wash buffer (0.1% Tween 20 and 0.01% Triton X-100 in PBS), oocytes were labeled with Alexa Fluor 488 goat-anti-rabbit IgG (1:100; for Arf6 staining) or Alexa Fluor 596 goat-anti-rabbit IgG (1:100; for p-MAPK staining) at room temperature for 1 h. After three washes in wash buffer, samples were co-stained with Hoechst 33,342 for 10 min. For double staining of spindle and actin, oocytes were stained with anti-α-tubulin-FITC antibody 3 h in room temperature and then labeled with Phalloidin-TRITC for 1 h, washed three times in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 2 min, and stained with Hoechst 33,342 (10 μg/ml in PBS) for 10 min. Samples were mounted on glass slides and examined with a confocal laser-scanning microscope (Zeiss LSM 700 META). At least 30 oocytes were examined for each experimental group.

2.5. Western blot analysis

A total of 150 mouse oocytes were placed in LDS sample buffer (Life technologies) and heated at 100 °C for 10 min. Proteins were separated by NUPAGE 12% Bis-Tris Gel (200 V, 1 h) and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (30 V, 1 h). After transfer, membrane was blocked in TBST (Solarbio) containing 5% non-fat milk for 1 h, followed by incubation at 4 °C overnight with a rabbit monoclonal anti-Arf6 (1:500), rabbit monoclonal anti-p-cofilin (1:1000), rabbit polyclonal Arp2 antibody (1:200), rabbit monoclonal profilin (1:5000) and rabbit monoclonal anti-α-tubulin antibody (1:2000), (for p-cofilin, incubation buffer was 5% BSA in TBST). After washing 3 times in TBST (10 min each), membranes were incubated at 37 °C for 1 h with HRP conjugated Pierce Goat anti-Rabbit IgG (1:2000). Finally, the protein bands were visualized using an ECL Plus Western Blotting Detection System (Tanon, China).

2.6. Fluorescence and western band intensity analysis

Fluorescence intensity was assessed using Image J software (NIH). For fluorescence intensity analysis, samples of control and treated oocytes were mounted on the same glass slide. And we used the same parameters to normalize across replicates. After immunofluorescent staining, the average fluorescence intensity per unit area within the region of interest (ROI) of immunofluorescence images was examined. Independent measurements using identically sized ROIs were taken for the oocyte cytoplasm. Average values of all measurements were used to determine the final average intensities for control and treated oocytes.

For quantification of the western blot results, intensity values of bands were measured using the Image J. Three different replicates were used for the analysis.

2.7. Statistical analysis

At least three biological replicates were used for each analysis. Each replicate was done by an independent experiment at the different time. Results are given as means ± SEM. Statistical comparisons were made by independent-sample *t*-tests using SPSS 12.0 statistical software. A *P*-value of < 0.05 was considered significant.