Rho-GTPase Effector ROCK Phosphorylates Cofilin in Actin-Meditated Cytokinesis During Mouse Oocyte Meiosis

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Rho-GTPase Effector ROCK Phosphorylates Cofilin in Actin-Meditated Cytokinesis During Mouse Oocyte Meiosis[1]

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ABSTRACT

During oocyte meiosis, a spindle forms in the central cytoplasm and migrates to the cortex. Subsequently, the oocyte extrudes a small body and forms a highly polarized egg; this process is regulated primarily by actin. ROCK is a Rho-GTPase effector that is involved in various cellular functions, such as stress fiber formation, cell migration, tumor cell invasion, and cell motility. In this study, we investigated possible roles for ROCK in mouse oocyte meiosis. ROCK was localized around spindles after germinal vesicle breakdown and was colocalized with cytoplasmic actin and mitochondria. Disrupting ROCK activity by RNAi or an inhibitor resulted in cell cycle progression and polar body extrusion failure. Time-lapse microscopy showed that this may have been due to spindle migration and cytokinesis defects, as chromosomes segregated but failed to extrude a polar body and then realigned. Actin expression at oocyte membranes and in cytoplasm was significantly decreased after these treatments. Actin caps were also disrupted, which was confirmed by a failure to form cortical granule-free domains. The mitochondrial distribution was also disrupted, which indicated that mitochondria were involved in the ROCK-mediated actin assembly. In addition, the phosphorylation levels of Cofilin, a downstream molecule of ROCK, decreased after disrupting ROCK activity. Thus, our results indicated that a ROCK-Cofilin-actin pathway regulated meiotic spindle migration and cytokinesis during mouse oocyte maturation.

actin, cytokinesis, oocyte polarization, ROCK, spindle migration

INTRODUCTION

Mammalian oocyte maturation involves a unique asymmetric division. During this process, an oocyte is transformed into a highly polarized metaphase II (MII)-arrested oocyte and a small polar body, which is critical for achieving haploidy while retaining maternal components [1]. Oocyte polarization, which includes spindle migration, spindle anchoring, and cortical reorganization, is critical for oocyte asymmetric division [2]. Disrupting oocyte polarization usually results in polar body extrusion defects. Oocyte polarization is controlled by the microtubule and actin cytoskeletons [2, 3].

After germinal vesicle breakdown (GVBD), a spindle forms in the central cytoplasm and migrates to the cortex of an oocyte in an actin-dependent manner. In addition, cortical granules (CGs) become redistributed to form a CG-free domain (CGFD), actin becomes enriched to form an actin cap, and microvilli are lost in the region overlying the spindle [4–6]. All of these events are regarded as cortical reorganization. Subsequently, cytokinesis begins. In mammalian cells, cytokinesis depends on the formation of a contractile ring that constricts an ooctye in an actomyosin-based manner at the division plane [7]. Finally, the oocyte extrudes the first polar body, which results in a highly polarized oocyte [8].

Rho-kinase (ROCK) is an effector of the small GTPase Rho and has important roles in the formation of actin fibers and actin dynamics [9], which coordinate multiple processes, including cell morphology, cytokinesis, and motility [3]. ROCK is a serine/threonine kinase that belongs to the AGC family of protein kinases, which consists of ROCK1 and ROCK2. ROCK1 and ROCK2 are actin-related proteins that play key roles in actin organization [10]. ROCK promotes actin organization by phosphorylating several downstream target proteins during mitosis, including myosin light chain (MLC), LIM kinase, and Cofilin. MLC phosphorylation is necessary for the formation and maintenance of stress fibers and focal adhesions [11]. LIM kinase 1 and LIM kinase 2 are phosphorylated by ROCK, which results in increased Cofilin phosphorylation [12]. Cofilin is an actin-depolymerizing factor whose activity is inhibited by phosphorylation [13]. In many species, inhibiting ROCK activity results in significantly impaired cell migration [14], cytokinesis [13], actin fiber network disruption [15], centrosome positioning [16], and spindle orientation in mitotic cells [17].

Recent studies that used mouse oocytes showed that Rho GTPase was necessary for oocyte polar body emission and spindle rotation during meiosis [18]. However, there is no direct evidence that links oocyte polarization to ROCK. Thus, in this study, we investigated whether ROCK was involved in oocyte polarization during meiosis. Our results indicate that ROCK may be involved in spindle migration and cytokinesis by regulating Cofilin-related actin dynamics in mouse oocytes.

MATERIALS AND METHODS

Antibodies and Chemicals

A rabbit polyclonal anti-ROCK antibody was from Santa Cruz. Phalloidin-TRITC, Phalloidin-FITC, Lectin-FITC, and a mouse monoclonal anti-α-tubulin-FITC antibody were from Sigma. Alexa Fluor 488 and 594 antibodies

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were from Invitrogen. A rabbit monoclonal anti-p-Cofilin antibody was from Cell Signaling Technology. Y-27632 was from Calbiochem. Mito Tracker Red was a gift from Prof. Qiang Wang of Nanjing Medical University, China.

Oocyte Harvest and Culture

Animal care and use were in accordance with the Animal Research Institute Committee guidelines of Nanjing Agriculture University, China. 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 Agriculture University, China. Germinal vesicle-intact oocytes were harvested from ovaries of 6- to 8-wk-old ICR mice and cultured in M16 medium (Sigma) 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.

Real-Time Quantitative PCR Analysis

ROCK gene expression was determined by real-time quantitative PCR and the $\Delta\Delta C_T$ method. Total RNA was extracted from 50 oocytes using a Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS), and first-strand cDNA was generated with a cDNA synthesis kit (Takara) using Oligo (dT)12–18 primers (Invitrogen). A cDNA ROCK1 fragment was amplified using the following primers: forward, AAT GGC TTT GCA GTA ATG TGT ATC; reverse, TAT CCA TCA GTG CGG CTT TCA. We used a DyNAmo HS SYBR Green qPCR kit (FINNZYMES) with an Applied Biosystems 7500 Real-Time PCR System with the following conditions: 95°C for 10 sec and 38 cycles of 95°C for 5 sec and 59°C for 32 sec.

ROCK siRNA Injection

Approximately 5–10 pl of ROCK siRNA (sc-36432; Santa Cruz) was microinjected into the cytoplasm of a fully grown germinal vesicle (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.). After injection, oocytes were cultured in M16 medium that contained 5 μM milrinone for 24 h and then washed five times (2 min each wash) in fresh M16 medium. Oocytes were then transferred to fresh M16 medium and cultured under paraffin oil at 37°C in a 5% CO_2 atmosphere. Control oocytes were microinjected with 5–10 pl of negative control siRNA. Spindles, actin cap phenotypes, and chromosome localizations were examined using a confocal microscope (Carl Zeiss LSM 700 META).

Y-27632 Treatment

A solution of Y-27632 in water (5 mM) was diluted in M16 medium to concentrations of 25 or 50 μM. Oocytes were then cultured in this medium for varying amounts of time and used for live cell imaging and immunofluorescence microscopy. Controls were cultured in fresh M16 medium.

Time-Lapse Microscopy

To image spindle and chromosome dynamics during oocyte maturation, oocytes that had been injected with H2B-mCherry mRNA were incubated in M16 medium with Y-27632. Images were acquired with a 20×0.5 objective lens (Carl Zeiss) using a computer-controlled video microscope (Zeiss LSM 780 META). The exposure time was 300 msec every 20 min. ZEN (Carl Zeiss) and Volocity 6 software were used to analyze the video files.

Confocal Microscopy

For single staining of ROCK, actin, and CGs, 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 or at room temperature for 4 h with rabbit anti-ROCK (1:50), 10 μg/ml of Phalloidin-TRITC, or 100 μg/ml of Lectin-FITC. 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 ROCK staining) at room temperature for 1 h. Samples were costained with propidium iodide (PI) or Hoechst 33342 for 10 min and then washed three times in wash buffer. For double staining of ROCK and actin, oocytes were stained with an anti-ROCK antibody and Alexa Fluor 488 goat-anti-rabbit IgG. They were then labeled with Phalloidin-TRITC for 30 min, washed three times in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 2 min, and stained with Hoechst 33342 (10 μg/ml in PBS) for 10 min.

For mitochondria staining, oocytes were cultured in M16 medium containing 200 nM MitoTracker Red (Molecular Probes) at 37°C for 30 min. After washing, oocytes were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min and the same steps used above.

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.

Western Blot Analysis

A total of 200 mouse oocytes were placed in Laemmli sample buffer (SDS sample buffer and 2-Mercaptoethanol) and heated at 100°C for 5 min. Proteins were separated by SDS-PAGE and then electrophoretically transferred to polyvinylidene fluoride membranes. After transfer, membranes were blocked in PBST (PBS containing 0.1% Tween 20) containing 5% nonfat milk for 1 h, followed by incubation at 4°C overnight with a rabbit monoclonal anti-p-Cofilin or polyclonal ROCK antibody (1:1000) and a rabbit monoclonal anti-β-actin or tubulin antibody (1:2500; for p-Cofilin, incubation buffer was 5% BSA in PBST). After washing three times in PBST (10 min each), membranes were incubated at 37°C for 1 h with HRP conjugated Pierce Goat anti-Rabbit IgG (1:10 000). Finally, membranes were processed using an enhanced chemiluminesence detection system (Amersham).

Statistical Analysis

At least three replicates were used for each treatment with results expressed as means ± SEM. Statistical comparisons were made by ANOVA, followed by Duncan multiple comparisons test. A P-value of < 0.05 was considered significant.

For the analysis of fluorescence intensity of actin, the samples of the control oocytes and treated oocytes were mounted on the same glass slide. Image J software was used to define a region of interest (ROI), and the average fluorescence intensity per unit area within the ROI was determined. Independent measurements using identically sized ROIs were taken of the membrane and cytoplasm, and the average values of all measurements were used to determine the final average intensity between control and treatment groups.

For the analysis of band intensity, Image J software was used to define Plot lanes for the control and treatment band. The intensity of control band (ROCK:Tubulin or p-Cofilin:β-actin) was defined as standard 1. Three replicates were used for the analysis.

RESULTS

ROCK Expression and Localization During Mouse Oocyte Meiosis

Samples were taken after culture for 0, 4, 8, 9, or 12 h, which reflected the time points for the GV, GVBD, metaphase I (MI), anaphase/telophase (ATI), and MII stages, respectively. The subcellular localizations of ROCK at different stages of meiotic maturation were determined by immunofluorescent staining. As shown in Figure 1A, ROCK was localized in germinal vesicles during the GV stage. After GVBD, ROCK became localized around chromosomes. In the MI stage, when a spindle formed in the central cytoplasm and migrated to the cortex, ROCK was found around the spindles. When chromosomes segregated away from the equatorial plate and oocytes reached anaphase I, ROCK accumulated around the chromosomes. ROCK was found around the spindles again at MII. The localization pattern of ROCK was similar to that of cytoplasmic actin. As shown in Figure 1B, actin colocalized with ROCK.

Disrupting ROCK Activity Results in Polar Body Extrusion Defects

To investigate a role for ROCK during mouse oocyte meiosis, we used RNAi for ROCK and also inhibited ROCK activity using an inhibitor Y-27632. After ROCK siRNA

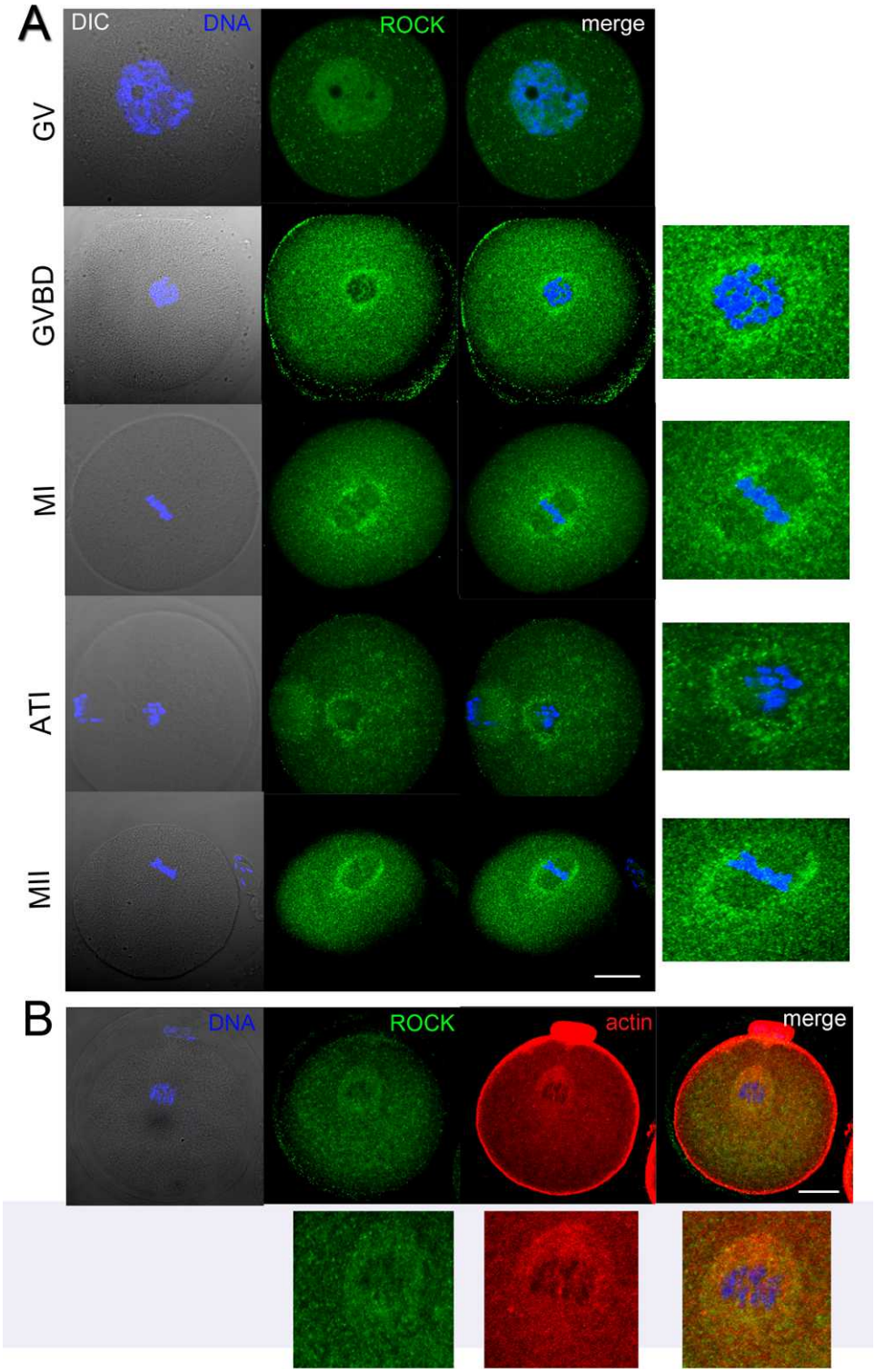

FIG. 1. **A**) Subcellular localization of ROCK during mouse oocyte meiotic maturation. ROCK antibody staining was used to determine the subcellular localization of ROCK in mouse oocytes. During the germinal vesicle (GV) stage, ROCK was primarily in germinal vesicles. After germinal vesicle breakdown (GVBD), ROCK was distributed around spindles. Green, ROCK; blue, chromatin. Bar = 20 μm. **B**) Colocalization of ROCK and actin. ROCK colocalized with actin in the cytoplasm. Green, ROCK; red, actin; blue, chromatin. Bar = 20 μm.

injection, ROCK mRNA expression was significantly decreased (29.4 ± 15.2% vs. 100%; $P < 0.05$; Fig. 2A). ROCK protein expression was also significantly decreased as compared with controls (relative ROCK/Tubulin intensity of 1 vs. 0.484 ± 0.052; $P < 0.05$; Fig. 2B). Immunofluorescent staining results showed that, after inhibitor treatment, there was minimal specific localization of ROCK around spindles (Fig. 2C). After RNAi and inhibitor treatment, a large proportion of oocytes had not extruded their polar bodies (Fig. 2D).

Next, we used live cell imaging by time-lapse microscopy to examine the dynamic changes that occurred in maturing oocytes. As shown in Figure 2E, in a control, the spindles

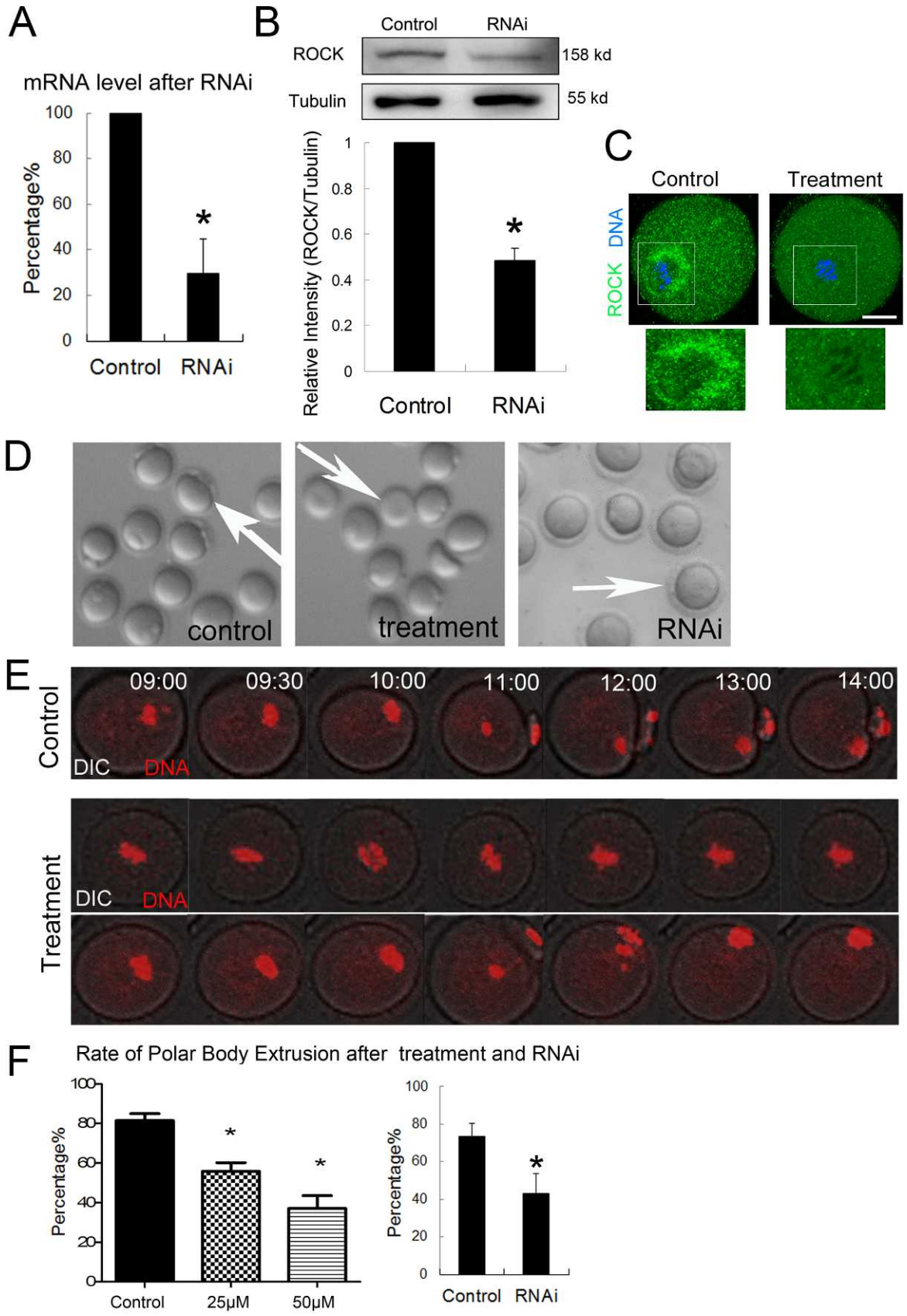

FIG. 2. Effects of disrupting ROCK activity on meiotic cell cycle progression. **A**) ROCK mRNA levels after siRNA injection. **B**) ROCK protein expression after siRNA injection. **C**) Immunofluorescent staining results showed that after inhibitor treatment, ROCK was minimally expressed around spindles. Green, ROCK; blue, DNA. Bar = 20 μm. **D**) Polar body extrusion failure after ROCK RNAi or inhibition. Images were acquired with a camera on a stereomicroscope. Arrows showed that the control oocytes extruded the polar body while the treated oocytes failed. Original magnification ×56. **E**) Time-lapse microscopy of maturing oocytes after treatment with Y-27632. In the controls, spindles moved to the cortex and extruded polar bodies; however, in

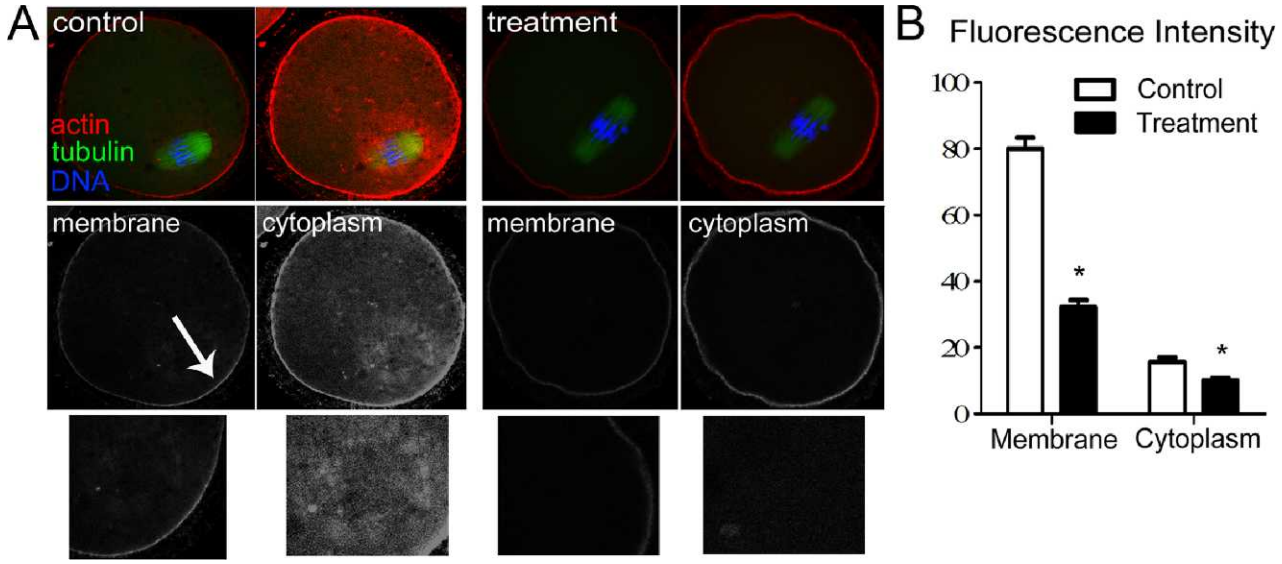

FIG. 3. Effects of disrupting ROCK activity on actin expression. **A**) Immunofluorescence microscopy of actin expression in oocyte membranes and cytoplasm after treatment. During the MI stage, actin caps (arrow) formed in the control group, whereas no actin caps formed in treated oocytes. In the cytoplasm, actin was localized around spindles in the control group, whereas no actin signals were found around spindles in treated oocytes. Bar = 20 μm. Green, tubulin; blue, chromatin; red, actin. **B**) Average actin fluorescence intensities in the membrane and cytoplasm were determined in mouse oocytes. *Significantly different ($P < 0.05$).

moved to the cortex, and the oocyte extruded a polar body. However, with inhibitor treatment, the oocytes could not extrude the polar body. Two phenotypes were observed: 1) the oocyte failed to extrude the polar body, while there was no specific signal of actin in the cytoplasm, and 2) although the chromosomes normally segregated after MI, the polar body emission failed, and the chromosome realigned together again. Only 43.1 ± 10.5% (n = 90) of oocytes extruded polar bodies after ROCK RNAi treatment as compared with 73.6 ± 6.9% of controls (n = 87; $P < 0.05$). These effects were also sensitive to the different concentrations of the inhibitor that was used. The rates of first polar body extrusion after 12 h culture with different treatments (control, 25 μM, and 50 μM) were 81.5 ± 6.1% (n = 165), 55.8 ± 7.7% (n = 174), and 37.1 ± 11.2% (n = 187), respectively ($P < 0.05$; Fig. 2F).

Disrupting ROCK Activity Results in Reduced Actin Expression in Mouse Oocytes

To further investigate the relationship between ROCK and actin expression, we examined actin expression in both membranes and the cytoplasm by immunofluorescent straining. As shown in Figure 3A, during the later MI stage, spindles localized near the cortex, and actin caps formed in the controls group. However, in the treatment group, no actin caps were observed, and actin expression on membranes was significantly decreased. Similar results were found for cytoplasmic actin; in control oocytes, actin accumulated around spindles, while there was no specific accumulation of actin around spindles in treated oocytes.

To confirm this, we determined the average actin fluorescence intensity in oocytes. As shown in Figure 3B, the average fluorescence intensity of membrane actin in the control group (80.03 ± 13.51; n = 26) was significantly higher than that in the treatment group (32.17 ± 8.83; n = 26; $P < 0.05$). We also determined the average actin fluorescence intensity in the cytoplasm. In control oocytes, the average fluorescence intensity of cytoplasmic actin (15.59 ± 5.83; n = 26) was also significantly higher compared to that of treated oocytes (10.34 ± 2.75; n = 26; $P < 0.05$; Fig. 3B). These results showed that inhibiting ROCK activity resulted in decreased actin expression and that this affected mouse oocyte cytokinesis.

Disrupting ROCK Activity Results in a Failure of Spindle Migration and Oocyte Polarization

To determine the cause of polar body extrusion failure, we investigated those processes that lead to extrusion, including spindle migration and cortical reorganization. After culture for 9 h, the locations of spindles could be categorized into three phenotypes, as shown in Figure 4A: 1) spindles that localized in the center of cytoplasm, 2) spindles that localized between the cortex and the center of oocytes, and 3) spindles that moved to the cortex.

In the control group, cell cycle progression was MI-1: 2.23 ± 1.99%; MI-2: 27.97 ± 8.86%; M-3: 43.87 ± 2.21%; and ATI/MII: 18.3 ± 3.64% (n = 89). In contrast, in the treatment group, these phenotypes were MI-1: 12.03 ± 9.29%; MI-2: 51.37 ± 4.79%; MI-3: 22.83 ± 7.56%; and ATI/MII: 8.96 ± 6.26% (n = 135). The proportions of MI-1 and MI-2 stage oocytes after treatment were significantly higher than in the control group, whereas the proportions of MI-3 and ATI/MII oocytes after treatment were significantly lower than in the control group ($P < 0.05$).

We also measured the distances of spindle poles to the cortex. As shown in Figure 4A, in the control group, this average distance was 4.76 ± 6.71 μm (n = 15), whereas in the

the treatment group, oocytes did not extrude polar bodies. Original magnification ×200. **F**) Rates of first polar body extrusion after 12 h culture in different treatment groups (control, 25 μM, and 50 μM). *Significantly different ($P < 0.05$).

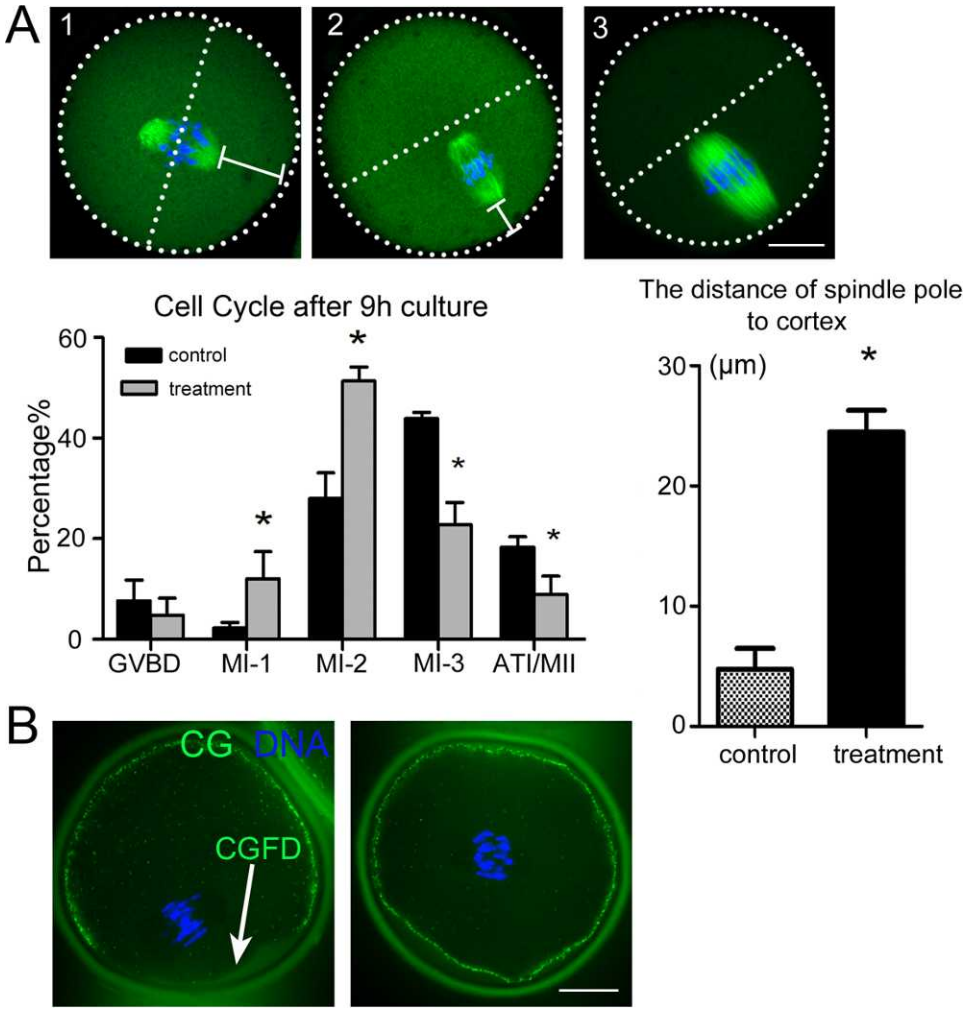

FIG. 4. Effects of disrupting ROCK activity on spindle migration in mouse oocytes. **A**) Proportions of oocytes in different cell cycle stages after 9 h of culture with or without Y-27632 treatment and distances of spindle poles to the cortex. In the control group, the spindles in most oocytes migrated to the cortex, whereas in the Y-27632 treated group, the spindles of most oocytes remained at the center of the cytoplasm. *Significantly different ($P < 0.05$). **B**) Effects of Y-27632 treatment on cortical granule-free domain formation in mouse oocytes. In the control group, cortical granules were absent at the cortex near the chromosomes at the metaphase I (MI) stage. However, in the treatment group, cortical granules were distributed uniformly across the entire cortex. Arrow shows a cortical granule-free domain. Green, cortical granules; blue, chromatin. Bar = 20 μm.

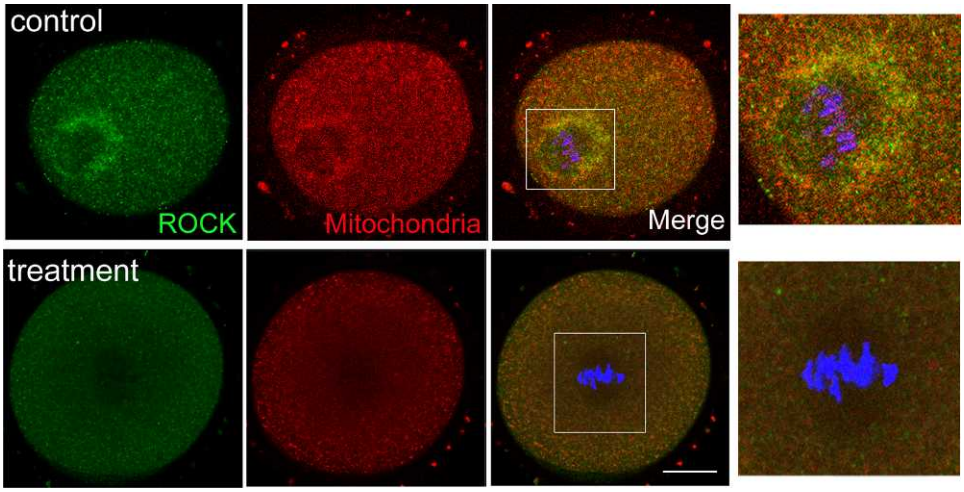

FIG. 5. Effects of disrupting ROCK activity on mitochondrial distributions. In control oocytes, mitochondria were distributed around spindles, similar to that of ROCK. In treated oocytes, mitochondria disappeared from the spindle areas. Red, mitochondria; green, ROCK; blue, chromatin. Bar = 20 μm.

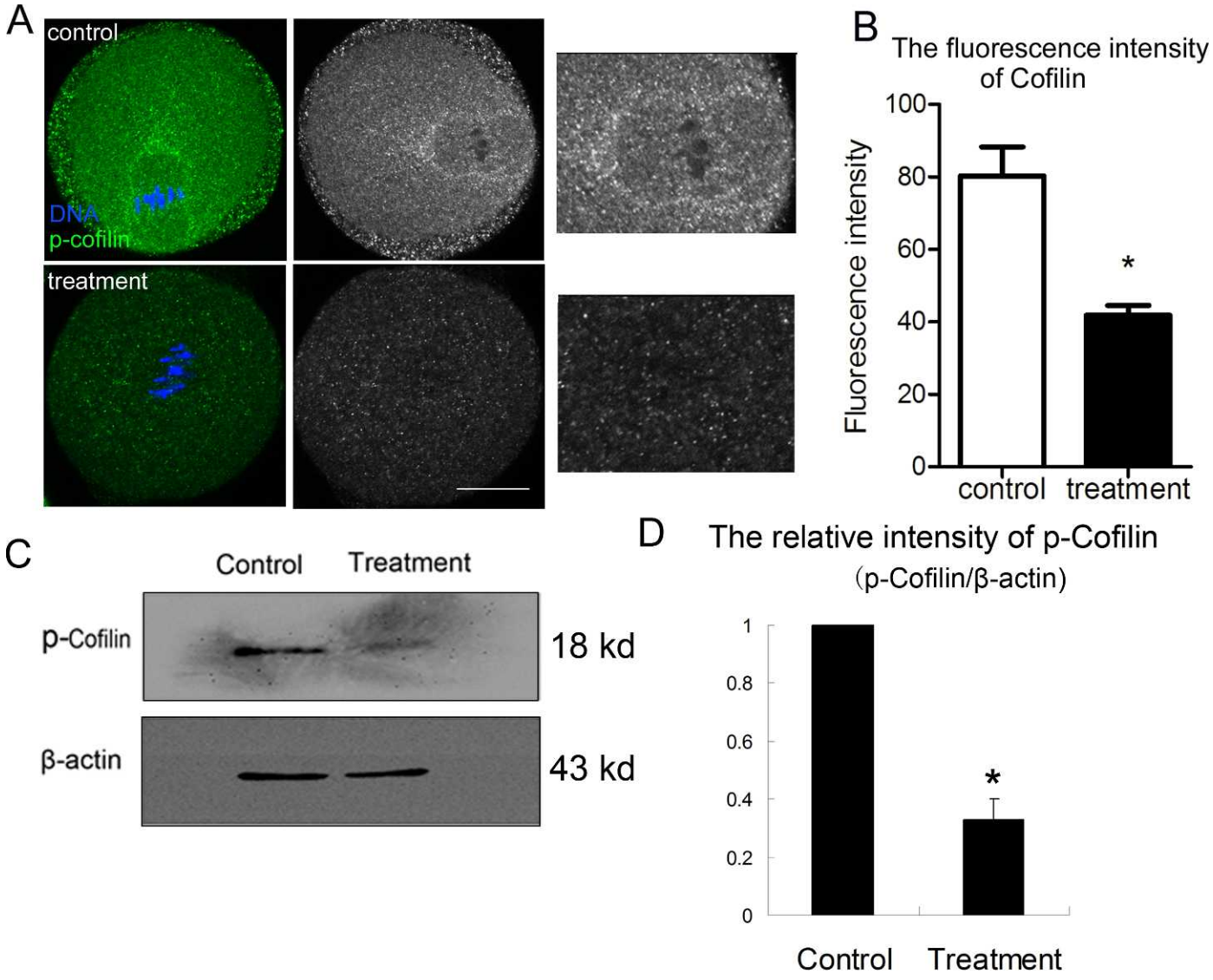

FIG. 6. Effects of disrupting ROCK activity on Cofilin phosphorylation levels. **A**) Cofilin phosphorylation levels after culture for 9 h. In the control group, p-Cofilin was expressed around spindles, whereas there was no specific localization of p-Cofilin around the spindles in treated oocytes. Bar = 20 μm. **B**) p-Cofilin fluorescence intensity in mouse oocytes. *Significantly different ($P < 0.05$). Green, Cofilin; blue, chromatin. **C**) p-Cofilin expression examined by Western blot. **D**) Relative p-Cofilin protein intensity after treatment. The expression of p-Cofilin was significantly decreased (p-Cofilin:β-actin, 1.0 vs. 0.329 ± 0.071; *$P < 0.05$).

treatment group, this distance was 24.50 ± 6.78 μm (n = 15); this distance was significantly greater than that in the control group ($P < 0.05$). These results indicated that disrupting ROCK activity resulted in a failure of spindle migration and further disrupted polar body extrusion.

Formation of CGFDs was examined as a feature of oocyte polarization, the process that results from spindle migration. As shown in Figure 4B, in the control group, CGs were absent near the regions of the cortex close to chromosomes during the MI stage. However, in the ROCK disrupted group, CGs were distributed uniformly over the entire cortex, and no CGFDs had formed. This indicated that oocyte polarization had been disrupted after this treatment and further demonstrated spindle migration disruption.

Mitochondrial Distribution Disruption after ROCK Inhibition

Mitochondria play a unique role in cellular processes by producing ATP, which is essential for actin assembly [19]. Thus, we examined the function and distribution of mitochondria after ROCK inhibition. As shown in Figure 5, in the control group, there was one phenotype with a polarized mitochondrial distribution around spindles, which was similar to that of ROCK. However, in the treatment group, mitochondria were minimally distributed around MI spindles and were diffusely distributed throughout the cytoplasm in the MI stage. This indicated that the localization pattern of mitochondria was related to ROCK in mouse oocytes.

Correlation Between ROCK and p-Cofilin Expression in Mouse Oocytes

Cofilin is an actin-depolymerizing factor that regulates actin dynamics. Its activity is inhibited by phosphorylation. We next explored the relationship between ROCK and Cofilin during oocyte meiosis by examining the phosphorylation status of Cofilin after disrupting ROCK activity. As shown in Figure 6A, in the treatment group, the Cofilin phosphorylation level was significantly lower than that in the control group. By using immunofluorescence staining, the subcellular localization of phosphorylated-Cofilin (p-Cofilin) was around MI spindles in the control group, whereas in the treatment group, there was no specific p-Cofilin expression around MI spindles.

We also determined the average fluorescence intensity of p-Cofilin in the cytoplasm. In the control group, the average p-Cofilin fluorescence intensity was significantly higher (80.27 $\pm$ 31.15; n = 15) than that in the treatment group (41.83 $\pm$ 10.33; n = 15; Fig. 6B). To confirm this, we assessed p-Cofilin protein expression by Western blot. The p-Cofilin protein expression in the treatment group was significantly reduced compared with that in the control group (Fig. 6C). Relative protein intensity analysis also confirmed this (p-Cofilin:β-actin, 1.0 vs. 0.329 $\pm$ 0.071; $P < 0.05$; Fig. 6D). These results revealed that ROCK was essential for Cofilin phosphorylation during mouse oocyte meiosis.

DISCUSSION

In this study, we investigated the expression, localization, and possible functions of ROCK during mouse oocyte meiosis. We found that ROCK played important roles in regulating cytokinesis and polar body extrusion in mouse oocytes. These results provided direct evidence for ROCK involvement in oocyte polarization.

ROCK expression was restricted to the nucleus during the GV stage and then accumulated around spindles during the entirety of meiosis. In the cytoplasm, ROCK colocalized with actin, which was consistent with a previous study and showed that ROCK played a crucial role in actin dynamics [20]. There is an ''actin cloud'' around the meiotic spindles in mouse oocytes that drives spindle migration [21]. This indicates that ROCK may play an important role in mouse oocyte meiosis and is related to actin dynamics.

To confirm our hypothesis, we disrupted ROCK activity by RNAi and by inhibitor treatment. We found that this affected polar body extrusion during oocyte meiosis. Time-lapse microscopy showed that spindles were arrested in the center of cytoplasm and that polar bodies were not extruded in treated oocytes. These results were similar to those in previous studies that showed that, in mouse oocytes, disrupting the actin nucleation factors Spire1/2 [22], Formin-2 [23], and Arp2/3 complex [24] affected polar body extrusion.

We next examined actin expression during oocyte meiosis. Live cell imaging by time-lapse microscopy and immunofluorescent microscopy showed that in treated oocytes, actin expression at the membrane and in the cytoplasm had decreased, and no clear actin caps were formed. These results suggested that ROCK might be related to actin assembly and might affect polar body extrusion. Similar results were reported in which it was shown that Formin-2 (Fmn2), concentrated around chromosomes, mediated actin to drive chromosomes to the cortex and promoted cytokinesis and polar body extrusion [25]. Our results indicated that ROCK regulation of oocytes polar body extrusion was related to actin.

To further investigate the mechanisms of ROCK's involvement in oocyte polar body extrusion, we examined spindle migration during oocyte maturation. A previous study showed that an actin flow drove the migration of spindles and moved to the cortex, resulting in oocyte asymmetric division [26]. Thus, we examined whether ROCK regulated the spindle migration that was involved during oocyte cytokinesis. The average distance of a spindle pole to the cortex was significantly greater in most treated oocytes, and most spindles were arrested in the central cytoplasm. CGFDs, a feature of oocyte polarization resulting from spindle migration [27], did not form in the treated group. All of these results indicated that spindle migration failed after disrupting ROCK activity.

The effects of ROCK on spindle migration were similar to those of Rho GTPase RhoA, Rac, and Cdc42, which were shown to regulate spindle positioning and polar body extrusion during mouse oocyte meiosis [28, 29]. Similar results were observed with the actin nucleation factor Arp2/3 complex [30] and its NPFs JMY [27], N-WASP [31], WAVE2 [32], and Formin-2 [33] in mouse oocytes. These molecules are downstream regulators of small GTPases and have been shown to regulate polar body extrusion and cytokinesis through their effects on spindle migration during mouse oocyte meiosis. Thus, our results suggest that ROCK participates in oocyte asymmetric division through its regulation of spindle migration.

Previous studies showed that mitochondria accumulated around developing meiotic spindles [34, 35] and further associated with spindles during their migration toward the cortex [36]. The asymmetrical inheritance of mitochondria during mouse oocyte maturation is dependent on an intact actin cytoskeleton [36], while the polarization of mitochondria-rich myoplasm depends on the actin cytoskeleton in ascidian oocytes [37]. ROCK was shown to regulate mitochondrial dynamics in podocytes and endothelial cells of mice [38]. Moreover, ROCK inhibition reduced mitochondria-associated processes during transplantation of embryonic stem cell-derived neural precursors [39].

Our current results showed that the mitochondrial distribution was similar to that of ROCK around the spindles in MI oocytes and that the mitochondrial distribution around spindles was decreased after disrupting ROCK activity. Mitochondrial generation of ATP is essential for actin assembly and plays an important role during oocyte maturation. Thus, ROCK may be associated with mitochondrial function in oocytes.

Cofilin, a downstream molecule of ROCK, is a key regulator of actin filament dynamics and reorganization by promoting depolymerization and severing actin filaments [40, 41]. This led us to hypothesize that Cofilin may participate in actin-mediated oocyte cytokinesis in conjunction with ROCK. The decreased p-Cofilin expression in treated oocytes confirmed that ROCK might regulate actin assembly through its phosphorylation of Cofilin and further affect oocyte meiotic maturation.

In conclusion, our results indicated that a ROCK-Cofilin-actin pathway regulated meiotic spindle migration and cytokinesis during mouse oocyte maturation.

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