PAK1 regulates spindle microtubule organization during oocyte meiotic maturation

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1. ABSTRACT

P21-activated kinase 1 (PAK1), an effector of Rho GTPase Rac 1 and Cdc42, is required for mitotic progression. However, its functions in meiosis are unclear. In the present study, we examined the expression, localization and function of PAK1 during mouse oocyte meiotic maturation and found that PAK1 was mainly associated with the meiotic spindle microtubules. Taxol treatment resulted in localization of PAK1 on spindle and aster microtubules, while nocodazole treatment induced the dispersion of PAK1 protein into the cytoplasm. Loss-of-function of PAK1 by both inhibitor treatment and morpholino oligonucleotide injection caused disorganized spindles, decreased polar body extrusion and misaligned chromosomes. In addition, inhibition of PAK1 resulted in abnormal localization of mitogen-activated protein kinase kinase (MEK). Taken together, our results suggest that PAK1 plays an important role in spindle assembly and chromosome alignment during mouse oocyte meiotic maturation.

2. INTRODUCTION

P21-activated kinases (PAKs) belong to serine/threonine kinases that are identified as downstream signaling effectors of the Rho GTPases Rac1 and Cdc42 (1). The PAK family consists of six PAK isoforms in mammals, which are divided into group A (PAK 1-3) and group B (PAK 4-6) according to sequence data and structural and biochemical properties (2). PAKs are composed of an N-terminal p21 GTPase-binding domain (PBD) and a C-terminal kinase domain. The N-terminal regulatory domain of PAK 1-3 contains a PAK auto-inhibitory domain (PID) that overlaps partially with the PBD and inhibits the kinase activation (3, 4). PAKs serve as key regulators of many cellular processes, including cytoskeletal dynamics, cell migration, focal adhesion turnover, gene transcription and angiogenesis. An increase in the PAKs protein levels is observed in several human tumors including bladder, breast, colon and ovarian cancers (5-7).

PAKs 1-3 are highly conserved in vertebrates; human PAK2 and PAK3 show 97% and 93% amino-acid identity
with PAK1, respectively (8). G2/M transition and mitosis require microtubule dynamics and actin cytoskeletal regulation. Increasing evidence indicates that PAKs, in particular PAK1, play an important role during the cell division cycle. Expression of the auto-inhibitory domain of PAK1 inhibited cyclin D1 expression and induced cell cycle arrest in mouse fibroblasts (9). Direct substrates of PAK1 involved in mitosis have been identified including polo-like kinase 1 (Plk1) and Aurora kinase which play a role in centrosome maturation, spindle assembly, anaphase onset and cytokinesis (10-15).

Rapid and dynamic cell cycle-dependent microtubule reorganizations take place at the transition from interphase to mitosis (16) when the spindle apparatus is formed with chromosomes, microtubules and centrosomes as major spindle components. Previous studies on PAK1 had mainly been focused on human mitotic cells. Recent studies on meiosis revealed that PAK1 is involved in arrest of *Xenopus* oocytes at the G2/prophase stage of the first meiotic cell cycle (17), but its function in spindle organization is unknown. Therefore, we investigated the roles of PAK1 in spindle organization during mouse meiotic maturation. Our results provide evidence that PAK1 plays an important role in meiotic spindle organization.

### 3. MATERIALS AND METHODS

#### 3.1. Antibodies

Rabbit polyclonal anti-PAK1 antibody was purchased from Signalway Antibody (Pearland, TX); mouse polyclonal anti-α-tubulin-FITC was obtained from Sigma (St Louis, MO); mouse polyclonal anti-β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit polyclonal anti-phospho-MEK1/2 antibody was purchased from Cell Signaling Technology (Beverly, MA).

#### 3.2. Oocyte collection and culture

Animal care and use were conducted in accordance with the Animal Research Committee guidelines of the Institute of Zoology, Chinese Academy of Sciences.

Immature oocytes arrested at prophase of meiosis I were collected from ovaries of 6-wk-old female CD-1 mice in M2 medium (Sigma, St. Louis, MO). Only those immature oocytes displaying a GV were cultured further in M16 medium under liquid paraffin oil at 37°C in an atmosphere of 5% CO2 in air. At different times of culture, oocytes were collected for immunostaining, microinjection or Western blot analysis.

#### 3.3. Taxol and nocodazole treatment of oocytes

Oocytes were treated at various stages with taxol or nocodazole. For taxol treatment, 5×10-3 mol l-1 taxol (Sigma) in DMSO stock was diluted in M2 medium to give a final concentration of 1×10-2 mol l-1 and oocytes were incubated for 45 min; for nocodazole treatment, 10 mg/ml nocodazole in DMSO stock (Sigma, St. Louis, MO) was diluted in M2 medium to give a final concentration of 20 µg/ml and oocytes were incubated for 10 min. After treatment, oocytes were washed thoroughly and used for immunofluorescence.

#### 3.4. IPA-3 treatment of oocytes

The PAK1 inhibitor IPA-3 (Sigma, St. Louis, MO) was dissolved in vehicle DMSO (Sigma, St. Louis, MO) to form 5×10-5 mol l-1 stock solution. The oocytes were cultured in the presence or absence of 3×10-5 mol l-1 IPA-3 for 12 h and collected for subsequent experiments as previously described (18).

#### 3.5. Morpholino oligonucleotide injection

For PAK1 knock-down in mouse oocytes, PAK1 specific morpholino oligonucleotides 5’-TGTCTACCGCGTTATTTGACATTG T-3’ (Gene Tools, LLC) was diluted in water (Sigma, St. Louis, MO) to give a concentration of 2×10-3 mol l-1. GV oocytes were microinjected with 5-10 pl of the control or PAK1-specific morpholino oligonucleotide in M2 medium containing 2.5×10-6 mol l-1 Milrinone (Sigma, St. Louis, MO) to prevent oocyte GV breakdown as previously described (19). Oocytes were incubated in M2 medium containing 2.5×10-6 mol l-1 Milrinone for 24 h, and then transferred to Milrinone-free M16 medium to resume meiosis. The oocytes cultured for 12 h were collected for subsequent experiments. The control was injected with MO standard control 5’-CCTCTTACCTCAGTTACATTTATA-3’.

#### 3.6. Immunofluorescence and Confocal Microscopy

For single staining of α-tubulin, oocytes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. After being permeabilized with 0.5% Triton X-100 at room temperature for 20 min, oocytes were blocked in 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C with 1:200 anti-α-tubulin-FITC antibody. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were co-stained with propidium iodide (PI; 10 µg/ml in PBS). Finally, the oocytes were mounted on glass slides and examined with a confocal laser scanning microscope (Zeiss LSM 510 META, Germany).

For double staining of PAK1 and α-tubulin or p-MEK, oocytes were fixed and permeabilized as above, blocked in 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C with 1:200 anti-α-tubulin-FITC and 1:100 anti-p-MEK antibody. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were co-stained with propidium iodide (PI; 10 µg/ml in PBS). The oocytes were then stained with Hoechst 33258 (10 µg/ml in PBS) for 20 min.

Each experiment was repeated at least three times, and about 100 oocytes were examined in each group. The same instrument settings were used for each replicate.

#### 3.7. Immunoblotting Analysis

A total of 200 mouse oocytes at the appropriate stage of meiotic maturation were collected in SDS sample buffer and heated for 5 min at 100°C. Immunoblotting was based on the procedures reported by us previously (20). The proteins were separated by SDS-PAGE and then electrically transferred to polyvinylidene fluoride...
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membranes. Following transfer, the membranes were blocked in TBST (TBS containing 0.1% Tween 20) containing 5% skimmed milk for 2 h, followed by incubation overnight at 4 °C with 1:1000 rabbit polyclonal anti-PAK1 antibody or 1:1000 mouse monoclonal anti-β-actin antibody. After washing three times in TBST, 10 min each, the membranes were incubated for 1 h at 37 °C with 1:1000 horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG, respectively. Finally, the membranes were processed using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

3.8. Statistics
All percentages from at least three repeated experiments were expressed as means±SEM, and the number of oocytes observed was labeled in parentheses as (n). Data were analyzed by paired-samples t-test. P<0.05 was considered statistically significant.

4. RESULTS
4.1. Expression of PAK1 during meiosis in mouse oocytes
We first examined the expression of PAK1 in mouse oocytes at different stages of meiotic maturation by Western blot. Samples were collected after oocytes had been cultured for 0 h, 4 h, 8 h, 9.5 h and 12 h, corresponding to germinal vesicle (GV), prometaphase I, metaphase I, anaphase I and metaphase II stages, respectively. As shown in Figure 1A, the expression of PAK1 was detected from prometaphase I stage to metaphase II stage. However, we did not detect any expression of PAK1 at the GV stage.

4.2. Subcellular localization of PAK1 during mouse oocyte meiotic maturation
To investigate the subcellular localization of PAK1 during meiotic maturation, mouse oocytes were analyzed with immunofluorescent microscopy at different stages of maturation. As shown in Figure 1B, no evident signal of PAK1 was found in the nucleus at GV stage. By prometaphase I, chromosomes began to move to the equator of the spindle, and PAK1 was localized at the spindle. When oocytes progressed to metaphase I, chromosomes aligned at the equatorial plate, and PAK1 became localized to the spindle and co-localized with α-tubulin. At anaphase/telophase I, PAK1 was localized in the region between the separating homologous chromosomes. At metaphase II, PAK1 was again translocated to the spindles microtubules. These data suggest that PAK1 may play a role in spindle formation.

4.3. Localization of PAK1 in mouse oocytes treated with taxol and nocodazole
To clarify the correlation between PAK1 and microtubule dynamics, the spindle-perturbing agents taxol and nocodazole were employed. Firstly, oocytes were treated with taxol to perturb microtubule dynamics and then double labeled for PAK1 and α-tubulin. After GVBD (germinal vesicle breakdown) when microtubule organization was initiated microtubules in taxol-treated oocytes became excessively polymerized and numerous asters were detected in the cytoplasm. PAK1 signals were detected on the spindle fibers and on cytoplasmic asters from prometaphase I to metaphase II stages (Figure 2A).

Next, we observed PAK1 localization when oocytes were treated with nocodazole, a potent microtubule depolymerizing agent. As shown in Figure 2B, after incubation with nocodazole, spindles were disassembled and all microtubules were depolymerized. PAK1 signals became dispersed and were detected in the cytoplasm.

4.4. Spindle organization defects and decreased polar body extrusion after treatment with IPA-3
To explore the function of PAK1 in spindle organization, the oocytes were cultured in the presence or absence of 30 μM PAK1 inhibitor IPA-3 (Sigma, St. Louis, MO) as previously described (18). In the IPA-3-treated group, the majority of spindles displayed various abnormalities and defects. The rate of abnormal spindles was 85.22±3.18% (n = 127; P<0.05, Figure 3B, left) in the IPA-3-treated group. The oocytes harboring abnormal spindles could be classified into five groups: (a) the spindle pole was disorganized; (b) the spindle had only one pole (monopolar); (c) the microtubule formed multiple spindle poles (multipolar); (d) the spindle elongated significantly. Central MII spindles were observed in IPA-3-treated oocytes (Figure 3A). However, we found that chromosomes failed to align at the equator in a significant number of oocytes. Most oocytes from the control group exhibited normal bipolar spindles, and only 16.62±1.05% (n = 120) were morphologically abnormal.

Next, we investigated the role of PAK1 in polar body (PB) extrusion and found that the rate of PB extrusion was 52.45±3.08% (n = 208) in IPA-3-treated oocytes, which was significantly lower than in the control group (81.38±2.75%, n = 211) (P<0.05, Figure 3C).

4.5. Spindle organization defects and decreased polar body extrusion after injection of PAK1 morpholino oligonucleotide
To further dissect the role of PAK1, morpholino oligonucleotide was employed to perturb the function of PAK1. We first examined PAK1 expression in oocytes injected with PAK1 morpholino oligonucleotide or control. As shown in Figure 4A, compared with the control group, the protein expression of PAK1 in morpholino oligonucleotide-injected oocytes was significantly reduced. An obvious increase in abnormal rates of spindle formation was observed in the PAK1-depleted group (69.72±1.14%, n = 82) compared to the control group (27.15±1.49%, n = 99). Similar to the observation in IPA-3-treatment experiment, knockdown of PAK1 also caused abnormal spindles, such as loss of spindle poles (8.33%), monopolar spindles (8.33%), multipolar spindles (10.42%) and elongated spindles (35.42%). Centrally localized MII spindles were seen in morpholino oligonucleotides-injected oocytes (37.5%) (Figure 4B).

Furthermore, PAK1-depletion resulted in decreased polar body extrusion. The rate of PB extrusion in
Figure 1. Expression and subcellular localization of PAK1 during mouse oocyte meiotic maturation. A) Samples were prepared after oocytes had been cultured for 0, 4, 8, 9.5 and 12 h, corresponding to GV, prometaphase I, metaphase I, anaphase I and metaphase II stages, respectively. Proteins from a total of 200 oocytes were loaded for each sample. B) Oocytes at various stages were double stained with antibodies against PAK1 and α-tubulin. Green, α-tubulin; pink, PAK1; red, chromatin; white, overlapping of green and pink; GV, oocytes at germinal vesicle; Pro-MI, oocytes at first prometaphase; MI, oocytes at first metaphase; ATI, oocytes at first anaphase and telophase; MII, oocytes at second metaphase. Each sample was counterstained with PI to visualize DNA. Bar = 20 µm.
Figure 2. Localization of PAK1 in mouse oocytes treated with spindle-perturbing agents taxol and nocodazole. A) Oocytes at different stages were incubated with 10 µM taxol in M16 medium for 45 min and then double stained with antibodies to PAK1 and α-tubulin. Green, α-tubulin; red, PAK1; blue, chromatin; yellow, overlapping of green and red; GV, oocytes at germinal vesicle; Pro-MI, oocytes at first prometaphase; MI, oocytes at first metaphase; ATI, oocytes at first anaphase and telophase; MII, oocytes at second metaphase. B) Oocytes at metaphase I and metaphase II stage were incubated in M16 medium containing 20 µg/ml nocodazole for 10 min and then double stained with antibodies against PAK1 as well as α-tubulin. Green, α-Tubulin; red, PAK1; blue, chromatin; yellow, overlapping of green and red. Each sample was counterstained with Hoechst 33258 to visualize DNA. Bar = 20 µm.
Figure 3. Effect of IPA-3 on spindle organization and polar body extrusion rate. A) Oocytes were stained with α-tubulin (green) and PI (red). The metaphase II spindle of control oocyte was anchored under the plasma membrane. However, IPA-3 had a strong effect on the spindle morphology. B) Abnormal spindle rate and polar body (PB) extrusion rates were recorded in control group and IPA-3-treated group oocytes. Data are presented as mean percentage (mean±SEM) of three independent experiments. Different letters denote statistical difference at a p < 0.05 level of significance. Bar = 20 µm.

the control group was (84.29±4.02%, n = 85), statistically higher than that in the PAK1 morpholino oligonucleotide-injected group (61.29±2.88%, n = 142).

4.6. Effect of PAK1 inhibition on proper p-MEK1/2 localization

Mitogen-activated protein kinase (MAPK) plays an important role in regulating the meiotic cell cycle progression of oocytes. MEK is an intermediate kinase that acts upstream of ERK and has a dramatic effect on microtubule organization and spindle pole tethering during mouse oocyte maturation. We observed p-MEK localization in the oocytes treated with PAK1 inhibitor or injected with morpholino oligonucleotides. As shown in Figure 5, p-MEK was localized to the two spindle poles in the control group. P-MEK was no longer accumulated at spindle poles, but detected on the spindle fibers when the oocytes were treated with PAK1 inhibitor IPA-3 or specific morpholino oligonucleotide. Therefore, inhibition of PAK1 function resulted in disruption and collapse of spindle structure that affects proper MEK localization.

5. DISCUSSION

In this study, we have shown that PAK1 plays an essential role in regulating spindle assembly during oocyte maturation. Several studies have suggested that PAK1 is localized to centrosomes; its activity peaks at entry into mitosis and is sustained during mitotic progression (4, 21). In order to dissect the function of PAK1 during meiosis in mouse oocytes, we first examined the expression of PAK1. Our data revealed that PAK1 began to translate after the GV stage and was present during meiotic progression. Furthermore, immunoﬂuorescence showed that PAK1 was localized at the spindle, suggesting that PAK1 distribution is similar to several other proteins involved in spindle formation during meiosis in mouse oocytes, such as BRCA-1 and PKC that
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Figure 4. Injection of PAK1 morpholino oligonucleotide (MO) induced spindle organization defects and decreased polar body extrusion. A) Expression of PAK1 in the morpholino oligonucleotide-injected oocytes. Germinal vesicle oocytes were microinjected with the control morpholino oligonucleotide and PAK1-specific morpholino oligonucleotide, respectively. After injection, oocytes were incubated in M16 medium containing 2.5 µM Milrinone for 24 h, and then transferred to Milrinone-free M16 for 12 h, followed by Western blotting. B) Spindle morphologies in control morpholino oligonucleotide-injected oocytes and PAK1-specific morpholino oligonucleotide-injected oocytes. After injection, oocytes were incubated in M16 medium containing 2.5 µM Milrinone for 24 h, and then transferred to Milrinone-free M16 for 12 h, followed by immunostaining with α-tubulin (green) and PI (red). In the control morpholino oligonucleotide-injected group, normal bipolar spindles were formed. In the PAK1-specific morpholino oligonucleotide-injected group, various abnormal spindles appeared. C) Abnormal spindle rate and polar body (PB) extrusion rates were recorded in control group and PAK1-specific morpholino oligonucleotide-injected group. Data are presented as mean percentage (mean±SEM) of three independent experiments. Different letters denote statistical difference at a $p < 0.05$ level of significance. Bar = 20 µm.

we previously showed to be required for proper spindle morphology in meiosis (19, 22). The colocalization of PAK1 with α-tubulin suggests that PAK1 plays a role in spindle dynamics.

Taxol is often used in studies of microtubule dynamics, as it stabilizes microtubules and promotes non-spindle microtubule formations (19). PAK1 signals were detected not only at the microtubule fibers of the abnormal meiotic spindle but also in cytoplasmic microtubule asters when oocytes were treated with taxol. We observed that PAK1 signals co-localized with α-tubulin in microtubules of abnormal spindles and asters, suggesting that PAK1 is involved in microtubule dynamics. Furthermore, PAK1
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Figure 5. Effect of PAK1 inhibition on proper p-MEK1/2 localization. Disruption of PAK1 function leads to abnormal p-MEK localization. GV oocytes that were incubated with IPA-3 or microinjected with PAK1-specific morpholino oligonucleotides were cultured in M2 medium, followed by double staining of p-MEK (pink) and α-tubulin (green). Each sample was counterstained with PI (red) to visualize DNA. Arrows indicate p-MEK abnormal localization. Bar = 20 µm.

protein became dispersed in the cytoplasm coinciding with spindle disassembly and microtubule depolymerization in nocodazole-treated oocytes. Recent studies found that PAK1 regulates microtubule dynamics by phosphorylating tubulin cofactor B which is essential for the assembly of tubulin heterodimers (23). The results from taxol- and nocodazole-treated oocytes provide further support for
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PAK1’s role in spindle and microtubule assembly in meiotic mouse oocytes.

Inhibition of endogenous PAK1 activity resulted in a higher incidence of nonfunctional spindle structure and chromosome alignment defects in HeLa cells (4). Regulatable expression of PAK1 promoted abnormal organization of mitotic spindles in human epithelial breast cancer cells (24). PAK1 is also required for the proper establishment of cell polarity and regulates microtubule dynamics in the fission yeast, Schizosaccharomyces pombe (25). Similarly, the larger proportion of abnormal spindles and decreased polar body extrusion rate in oocytes treated with PAK1 inhibitor IPA-3 compared with control oocytes suggests that PAK1 is indispensable for meiotic spindle assembly. We found that chromosomes are not aligned properly at the spindle equator. It is possible that PAK1 aids spindle microtubule assembly and governs microtubule tension, which plays an important role in correct chromosome alignment at metaphase and chromosome disjunction at anaphase (26, 27). The results from injecting the oocytes with PAK1-specific morpholino oligonucleotide further confirmed this conclusion. Therefore, inhibition of PAK1 function results in defective spindle formation from monopolar spindles to elongated spindles and decreases the first polar body extrusion rate. In addition, abnormal microtubule tension resulted in chromosome mis-segregation that is a major cause for embryonic aneuploidy in mammals and is responsible for spontaneous abortion and birth defects in human (28, 29). Taken together, these data suggest that PAK1 activity is necessary for the assembly of spindle microtubules and alignment of chromosomes.

The immature mammalian oocyte is a largely symmetrical cell arrested at prophase I and has a centrally located germinal vesicle. The resumption of meiosis is triggered after hormonal stimulation or release from the ovarian environment (30). The oocytes emit the first polar body and then are arrested at the metaphase II stage with a cortically located spindle aligned parallel to the cortex in the mouse. However, loss-of-function of PAK1 experiments showed that MII spindles detached from the cortex and located around the center of the oocyte. Previous results found that inhibition of Rac caused spindle detachment from the cortex in metaphase II-arrested oocytes (31). Therefore, it is possible that Rac regulates spindle movement through its downstream signaling effector PAK1, but the detailed mechanism needs further investigation.

Extracellular signal-Regulated Kinase (ERK) pathway controls a variety of cellular progress, including cell proliferation, adhesion and migration (32, 33). During the past decade studies have shown that the ERK pathway also plays an important role in regulating meiotic progression of oocytes in which regulation of microtubule dynamics and spindle assembly are important (34-36). MEK is an intermediate kinase of the ERK pathway and takes part in microtubule organization and spindle pole tethering (20). Cytoplasmic dynein is a microtubule minus end-directed motor protein responsible for many fundamental cellular processes. The dynein activator dynactin directly binds to both dynein and microtubules and allows the motor to traverse the microtubule lattice over long distances (37, 38). The dynein/dynactin complex is essential for the intracellular transport of centrosomal proteins including pericentrin that is needed to assemble γ-tubulin to the centrosome core structure, NuMA as well as spindle checkpoint protein Mad 2 (39, 40). Recently, we found that dynein/dynactin complex mediates poleward MEK transport. Inhibition of dynein/dynactin complex activity by microinjecting dynein and dynactin antibodies blocked poleward MEK transport so that p-MEK was localized to spindle fibers instead of spindle poles (41). Previous data have shown that PAK1 interacted with dynein light chain and regulate dynein/dynactin complex activity (42).

In the present study we found that inhibition of PAK1 function results in MEK detachment from the spindle poles and localization to the spindle fibers. Therefore, inhibition of PAK1 function led to disruption of spindle structure that may have affected dynein/dynactin complex-mediated MEK transport.

In conclusion, our data demonstrate that the spindle-associated protein PAK1 plays an important role in spindle assembly and chromosome alignment during mouse oocyte meiotic maturation.

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