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The Distribution and Possible Role of ERK8 in Mouse Oocyte Meiotic Maturation and Early Embryo Cleavage

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Abstract: It is well known that extracellular signal-regulated kinase 8 (ERK8) plays pivotal roles in various mitotic events. But its physiological roles in oocyte meiotic maturation remain unclear. In this study, we found that although no specific ERK8 signal was detected in oocyte at the germinal vesicle stage, ERK8 began to migrate to the periphery of chromosomes shortly after germinal vesicle breakdown. At prometaphase I, metaphase I (MI), anaphase I, telophase I, and metaphase II (MII) stages, ERK8 was stably detected at the spindles. By taxol treatment, we clarified that the ERK8 signal was stained on the spindle fibers as well as microtubule asters in MI and MII oocytes. In fertilized eggs, the ERK8 signal was not observed in the two pronuclei stages. At prometaphase, metaphase, and anaphase of the first mitosis, ERK8 was detected on the mitotic spindle. ERK8 knock down by antibody microinjection and specific siRNA caused abnormal spindles, failed chromosome congression, and decreased first polar body extrusion. Taken together, our results suggest that ERK8 plays an important role in spindle organization during mouse oocyte meiotic maturation and early embryo cleavage.

Key words: ERK8, mouse oocyte meiosis, MTOC, spindle, embryo

Introduction

During mitosis, the cell accurately assigns its replicated chromosomes into two daughter cells, and this task is performed by a microtubule-based machine called the mitotic spindle. The spindle apparatus, mainly composed of chromosomes, microtubules, and centrosomes, is one of the most pivotal cellular structures (Yuan et al., 2009). In most mammalian cells, centrosomes play a dominant role in microtubule nucleation and organization (Schatten, 2008; Wadsworth et al., 2011). In stem cells, centrosomes not only play significant roles in cellular signaling but also in the differential positioning of mother and daughter centrosomes, which is important for cell differentiation (Schatten & Sun, 2011*b*). Yet in many cells lacking centrosomes, alternative mechanisms such as "self-assembly" mechanisms for spindle formation were utilized (Basto et al., 2006). Within the spindle itself, γ-tubulin complexes are recruited by factors including Nedd1/GDPWD, which contributes to both centrosome- and spindle-mediated microtubule nucleation (Haren et al., 2006). Besides the role of centrosome in spindle formation, chromosome-generated activities also make contributions to spindle self-assembly by regulating both microtubule dynamics and motor proteins, which are mediated at least in part by the small GTPase Ran (Quimby & Dasso, 2003). Importantly, both "search and capture" and "self-assembly" mechanisms appear to work in all systems and can lead to metaphase chromosome alignment (Walczak & Heald, 2008).

During oocyte meiotic maturation, centrosomes also play important roles in all stages from interphase to metaphase II. Centrosome remodeling in the meiotic oocyte is the prerequisite for accurate meiosis I and meiosis II spindle formation, specifically for accurate chromosome separation during the two successive, highly asymmetric meiotic cell divisions. Centrosomal abnormalities can bring about inaccurate microtubule organization and inaccurate chromosome alignment, with failure in chromosome segregation that leads to aneuploidy and chromosomal abnormalities (Schatten & Sun, 2011*a*). Mouse oocyte meiotic maturation differs from mitotic spindles in that it does not contain centrioles at the spindle poles (Sun et al., 2011). In mouse oocyte meiotic maturation, multiple microtubule-organizing centers (MTOCs) organize into bipolar intermediates that elongate and establish the barrel-shaped acentriolar meiotic spindle (Schuh & Ellenberg, 2007). The MTOCs contain centrosomal proteins that regulate microtubules and form the spindle poles (Compton, 2000). Many molecular components that drive acentriolar spindle pole formation in mouse egg are also present in cells containing centrosomes. These proteins include microtubule-dependent motor proteins and a variety of structural proteins that regulate microtubule orientation, anchoring, and stability (Fant et al., 2004). It is

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known that mouse oocytes contain over 80 MTOCs and rely on the self-organization of numerous acentriolar MTOCs that are functional equivalents of centrosomes in mitosis (Schuh & Ellenberg, 2007).

The mitogen-activated protein kinases (MAPKs) are a superfamily of serine/threonine protein kinases that are highly conserved and whose members have been implicated in many critical cellular processes including cell proliferation, differentiation, apoptosis, and stress responses (Waskiewicz & Cooper, 1995; Robinson & Cobb, 1997; Lewis et al., 1998; Pearson et al., 2001). Regulation of the activity of MAPK depends on the phosphorylation status of the threonine and tyrosine residues in a Thr-X-Tyr (TXY) motif, which has also been applied to classify MAPKs into three main groups. The extracellular signal regulated kinases (ERKs) is a member of MAPKs and can be classified by their TEY (Thr-Glu-Tyr) activation motif. ERK1 and ERK2 are the first identified members of the ERK family. They are most widely studied and are proven to be implicated in cellular pathways that mediate growth factor regulation of proliferation and/or differentiation in most cell types (Waskiewicz & Cooper, 1995; Robinson & Cobb, 1997). ERK8 is one of the most recently described members in ERK family that shares 69% amino acid sequence identity with ERK7. Fluorescence *in situ* hybridization localized the ERK8 gene to chromosome 8, band q24.3 (Abe et al., 2002). ERK8 contains two SH3-binding motifs in its C-terminal region and can be activated by Src-dependent signaling pathway, by serum (Abe et al., 2002), and by RET/PTC3, an activated form of the RET proto-oncogene (Iavarone et al., 2006). ERK8 is required for the stability of the proliferating cell nuclear antigen (PCNA) protein, which acts as a scaffold, coordinator, and stimulator of numerous processes required for faithful transmission of genetic information. Human double minute 2 (HDM2) mediated PCNA destruction can be prevented by ERK8 through the way of inhibiting the association of PCNA with HDM2 (Groehler & Lannigan, 2010). ERK8 also controls the cell cycle. ERK8 in *Trypanosoma brucei* strongly affected growth phenotypes and appeared to be essential for normal growth and proliferation. Silencing of ERK8 affected normal cell proliferation (Mackey et al., 2011). ERK8 is also a limiting factor in regulating the proliferation rate of MCF-10A cells and controls entry into S phase. Loss of ERK8 resulted in increase in cyclin D1, cyclin E, and p27, and decrease in cyclin A levels. Knockdown of ERK8 decreased cell proliferation approximately twofold (Groehler & Lannigan, 2010). However, ERK8 is not well studied, and information about its upstream activators, downstream effectors, and functions is scarce, and very little is known about the relationship between ERK8 and meiosis.

In this study, we investigated for the first time the expression and localization of ERK8 in the mouse oocyte and early cleavage embryos, and we also explored the functions of ERK8 in oocyte meiotic maturation by using two loss-of-function approaches: antibody injection and siRNA knockdown. Our results suggest that ERK8 plays a key role in spindle assembly, chromosome congression, and polar body (PB1) extrusion.

Materials and Methods

Antibodies and Reagents

Rabbit polyclonal anti-ERK8 antibody was purchased from Bioworld Company (St. Louis, MO, USA); mouse monoclonal anti-α-tubulin-FITC antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA); FITC or Tritc-conjugated goat anti-rabbit IgG(H+L) was produced by Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) and subpackaged by Zhongshan Golden Bridge Biotechnology Co. LTD (Beijing, China). All other reagents were purchased from Sigma-Aldrich except for those specifically mentioned.

Ethics Statement

Six- to 8-week-old ICR mice care and use were conducted in accordance with the Animal Research Committee guidelines of the Institute of Zoology, Chinese Academy of Sciences. The animal handling staffs were all trained before treating animals. The mice were killed by cervical dislocation. The only procedure performed on the dead mice was the collection of oocyte from the ovary or oviduct.

Mouse Oocyte Collection and Culture

Immature oocytes were collected from ovaries of 6–8-week-old female ICR mice in M2 medium (Sigma-Aldrich). Only those immature oocytes displaying a germinal vesicle (GV) were further cultured in M2 medium under liquid paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. After certain periods of culture, oocytes were collected for drug treatment, Western blot, microinjection, and immunofluorescence.

Zygote and 2-Cell Embryo Collection

ICR females were induced to superovulate by an intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin at 12:00 AM, 48 h ahead of 10 IU of human chorionic gonadotropin injection (hCG) on the day of breeding. ICR stud males were introduced to the female mice 5 h post hCG. Females that had been mated with the same strain of males were selected for retrieval of zygotes 18–20 h after hCG injection. Oocyte-cumulus complex were first dispersed by 1 mg/mL of hyaluronidase for 1 min and removed by gently pipetting, then the fertilized eggs were washed and cultured in 30 μL droplets of potassium simplex optimized medium (KSOM) under liquid paraffin oil at 37°C in humidified air containing 5% CO_2. The formation of pronuclei was examined at 24 h after hCG, and only those eggs that extruded second polar bodies and formed pronuclei were used for further study. Starting from 27 h after hCG, zygotes were checked every 10–20 min to determine the time for nuclear envelope breakdown (NEBD) and then collected for further research at different times after NEBD. Embryos were further cultured in KSOM for certain periods to reach NEBD (0 min), pro-metaphase (60 min),

metaphase (90 min), anaphase (110 min), and 2-cell stages (140 min). The zygotes and 2-cell embryos at the above phases were used for immunofluorescence.

Taxol and Nocodazole Treatment of Oocytes

For taxol treatment experiments, 5 mM taxol (Sigma-Aldrich) in DMSO stock was diluted in M2 medium to get a final concentration of 10 μM and oocytes at metaphase I (MI) and metaphase II (MII) stages were cultured for 45 min; for nocodazole treatment experiments, 10 mg/mL nocodazole in DMSO stock (Sigma-Aldrich) was diluted in M2 medium to get a final concentration of 20 μg/mL and oocytes at MI and MII stages were incubated for 10 min. After treatment, oocytes were washed thoroughly and used for immunofluorescent staining.

Immunofluorescence and Confocal Microscopy

Oocytes were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) (pH 7.4) for 30 min. After being permeabilized with 0.5% Triton X-100 at room temperature for 20 min, oocytes were blocked in 1% bovine serum albumin supplemented PBS for 1 h and then incubated with rabbit polyclonal anti-ERK8 antibody (Bioworld; 1:50), or mouse polyclonal anti-α-tubulin-FITC (Sigma-Aldrich; 1:100) overnight at 4°C. After three washes in washing buffer (0.1% Tween 20 and 0.01% Triton X-100 in PBS) for 5 min each, the oocytes were labeled with FITC conjugated goat-anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology Co.; 1:100) for 1 h at room temperature and then washed three times with washing buffer. The oocytes were co-stained with propidium iodide (PI) for 15 min or Hoechst 33342 for 30 min.

For double staining of ERK8 and α-tubulin, after staining of ERK8 (the secondary antibody was TRITC conjugated goat-anti-rabbit IgG, 1:100), and three washes in washing buffer for 5 min each, oocytes were again blocked in 5% normal mouse serum for 1 h at room temperature, then stained with mouse polyclonal anti-α-tubulin-FITC (Sigma-Aldrich; 1:100) for 2 h at room temperature, then after three washes in washing buffer for 5 min each, they were co-stained with Hoechst 33342 for 30 min.

Immunofluorescent staining of zygotes or 2-cell embryos for ERK8 was performed identically with the same procedure that was used in oocyte staining. Finally, the oocytes, zygotes, or 2-cell embryos were mounted on glass slides and examined with a confocal laser scanning microscope (Zeiss LSM 780 META, Carl Zeiss, Oberkochen, Germany).

Immunoblotting Analysis

A total of 250 mouse oocytes were collected in SDS sample buffer and heated for 5 min at 100°C. The proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 2.5 h at 120 V and then transferred by electrophoresis on to a poly-vinylidene difluoride membrane (Amersham, Piscataway, NJ, USA) for 2.5 h at 200 mA at 4°C. After transfer, the membrane was blocked in TBST (TBS with 0.1% Tween20) containing 5% skimmed milk for 2 h, followed by incubation overnight at 4°C with 1:1,000 rabbit anti-ERK8 antibody and 1:1,000 mouse monoclonal anti-β-actin antibody, respectively. Then the membranes were washed three times with TBST, 10 min each, and incubated for 1 h at 37°C with an appropriate secondary antibody coupled to horseradish peroxidase. Finally, the membranes were processed using the enhanced chemiluminescence detection system (Amersham).

RNA Interference and Antibody Injection

Microinjections were performed using an Eppendorf microinjector (Hamburg, Germany) and completed within 30 min. 5–10 pL of the negative control siRNA (UUCUCCGAACGU GUCACGUTT.genepharma) or ERK8 siRNA (CGAAGAAUG ACAGGGAUAUTT.genepharma) was microinjected into the GV-intact oocytes in M2 medium containing 2.5 μM Milrinone.The final concentration of the control or ERK8 siRNA was 30 μM. Microinjected oocytes were incubated in M2 medium containing 2.5 μM Milrinone for 24 h to knock down ERK8 and then was washed six times and transferred to Milrinone-free M2 medium to resume meiosis. Oocytes were cultured for certain periods and collected for the subsequent experiments. For ERK8 antibody injection, the same method was adopted as above, except that after injection, the oocytes were cultured in M2 medium directly. The control oocytes were microinjected with 5–10 pL rabbit immunoglobulin G.

Data Analysis

For each treatment, at least three replicates were performed. 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 =). Statistical analyses were conducted by ANOVA. Differences between treated groups were analyzed by Independent Samples T-test (ANOVA) using SPSS17.0 software (SPSS Inc., Chicago, IL, USA) followed by Student-Newman-Keuls test and $P < 0.05$ was considered significant.

Results

Expression and Subcellular Localization of ERK8 during Mouse Oocyte Meiotic Maturation

We cultured oocytes for 0, 4, 8, and 12 h, the time points when most oocytes reached the GV, prometaphase I (ProI), MI, and MII stages, respectively, to examine the expression of ERK8 during mouse oocyte meiotic maturation. The immunoblotting results showed that ERK8 was expressed at all stages and no detectable difference was found from GV to MII stage (Fig. 1A). To investigate the subcellular localization of ERK8 during meiotic maturation, mouse oocytes were processed for immunofluorescent staining at different stages of maturation. We did not observe any specific staining of ERK8 in GV oocytes. Shortly after germinal versicle breakdown (GVBD; 2 h of culture), ERK8 began to migrate to the periphery of chromosomes. At ProI, MI, anaphase I

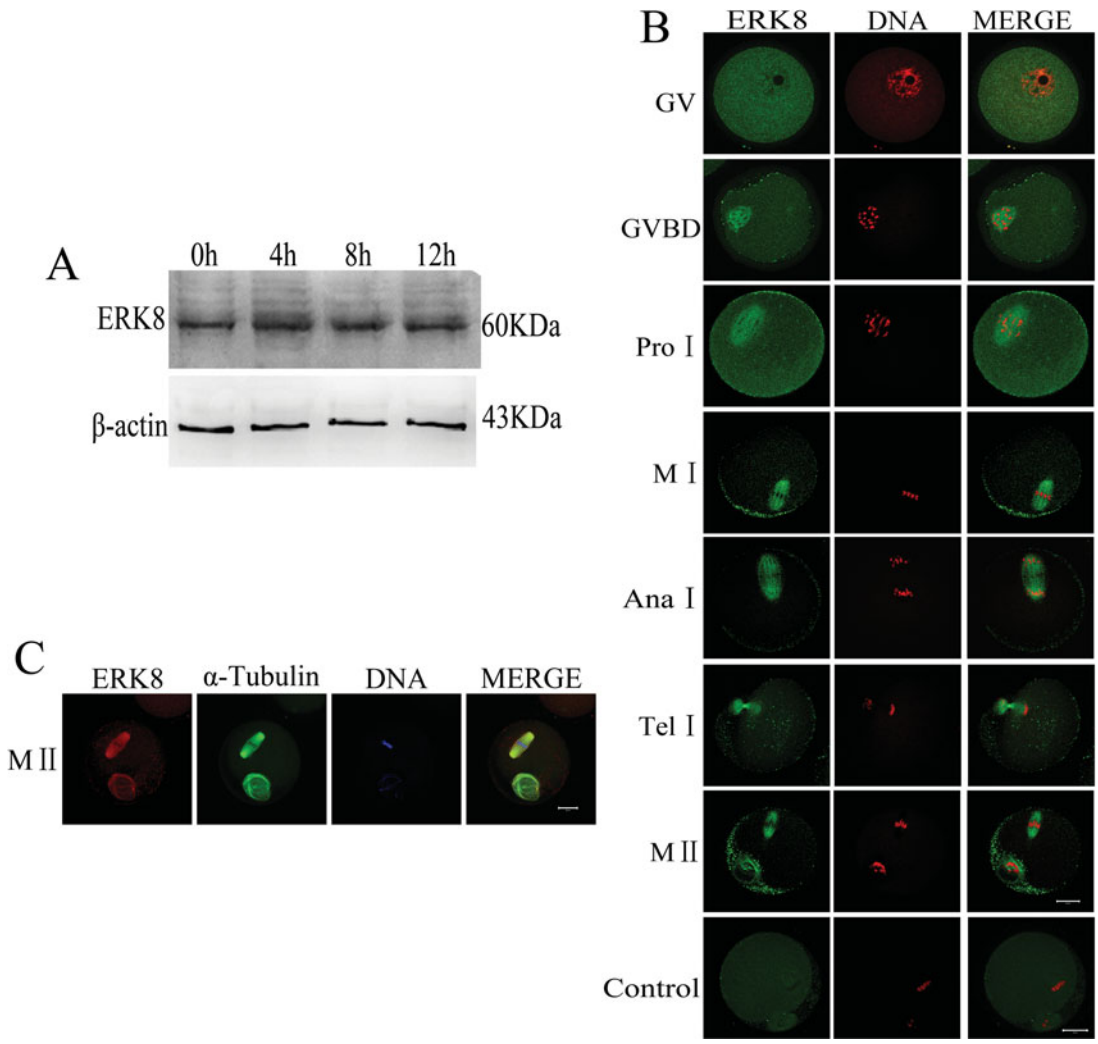

Figure 1. Expression and subcellular localization of ERK8 during mouse oocyte meiotic maturation. **A:** Expression of ERK8 was detected by Western blotting. Samples were collected after culture for 0, 4, 8, and 12 h, the time points when most oocytes reached to the GV, ProI, MI, and MII stages, respectively. The molecular mass of ERK8 and β-actin were about 60 kDa and 43 kDa, respectively. Each sample was collected from 250 oocytes. **B:** Subcellular localization of ERK8 as shown by immunofluorescent staining. Oocytes at various stages were stained with an antibody against ERK8 (green); each sample was counterstained with PI to visualize DNA (red). GV, oocytes at germinal vesicle stage; GVBD, oocytes at germinal vesicle breakdown stage; ProI, oocytes at first meiotic prometaphase; MI, oocytes at first meiotic metaphase; AnaI, oocytes at first meiotic anaphase; TelI, oocyte at first meiotic telophase; MII, oocytes at second meiotic metaphase. An MII oocyte was used as negative control for ERK8 confocal microscopy, in which a rabbit IgG instead of rabbit polyclonal anti-ERK8 antibody was used and the fluorescent second antibody was used just as the experimental group. Bar = 20 μm. **C:** Double staining of ERK8 and α-tubulin at the MII stage. Bar = 20 μm.

(AnaI), telophase I (TelI), and MII stages, ERK8 was stably distributed on the entire length of the meiotic spindle (Fig. 1B). The localization of ERK8 at the spindle in meiotic process was confirmed by the double staining of ERK8 and α-tubulin (Fig. 1C).

Subcellular Localization of ERK8 in Mouse Zygote and 2-Cell Embryo

At two pronuclei zygote stage, no specific ERK8 signal was observed. After NEBD, ERK8 began to aggregate to the periphery of chromosomes and then on the mitotic spindle. When the zygote progressed into anaphase, ERK8 still showed the same location with α-tubulin. By the completion of the first mitosis, two blastomeres formed and both of them entered interphase. At this stage, again no specific ERK8 signal was observed, similar to the stage of two pronuclei (Fig. 2).

Subcellular Localization of ERK8 in Mouse Oocytes Treated with Taxol or Nocodazole

After observing that ERK8 was apparently localized at the spindle at the MI and MII stages, we investigated the correlation between ERK8 and microtubules. Taxol, a microtubule-stabilizing agent, was first used to treat oocytes. As shown in Figure 3A, the microtubule fibers in taxol-treated oocytes were excessively polymerized, leading to significantly numer-

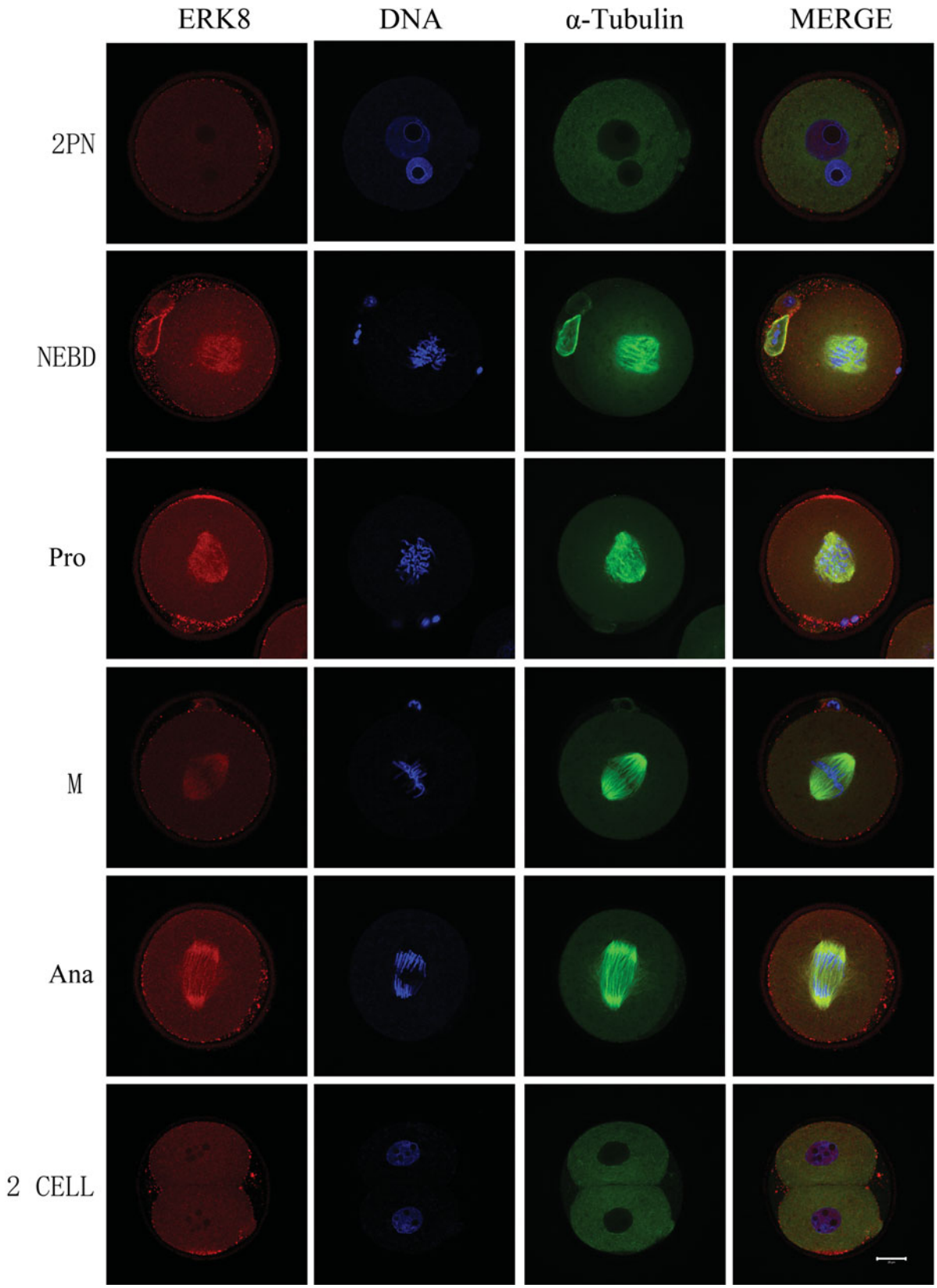

Figure 2. Localization of ERK8 in mouse early embryo cleavage. Subcellular localization of ERK8 in mouse early embryo cleavage as shown by immunofluorescent staining. Embryos at various stages were stained with an antibody against ERK8 (red), α-tubulin (green), and DNA (blue). 2PN, zygotes at two pronuclei stage; NEBD, zygotes at nuclear envelope breakdown; Pro, zygotes at prometaphase of the first mitosis; M, zygotes at metaphase of the first mitosis; Ana, zygotes at anaphase of the first mitosis; 2-cell embryo, embryo at 2-cell interphase stage. Bar = 20 μm.

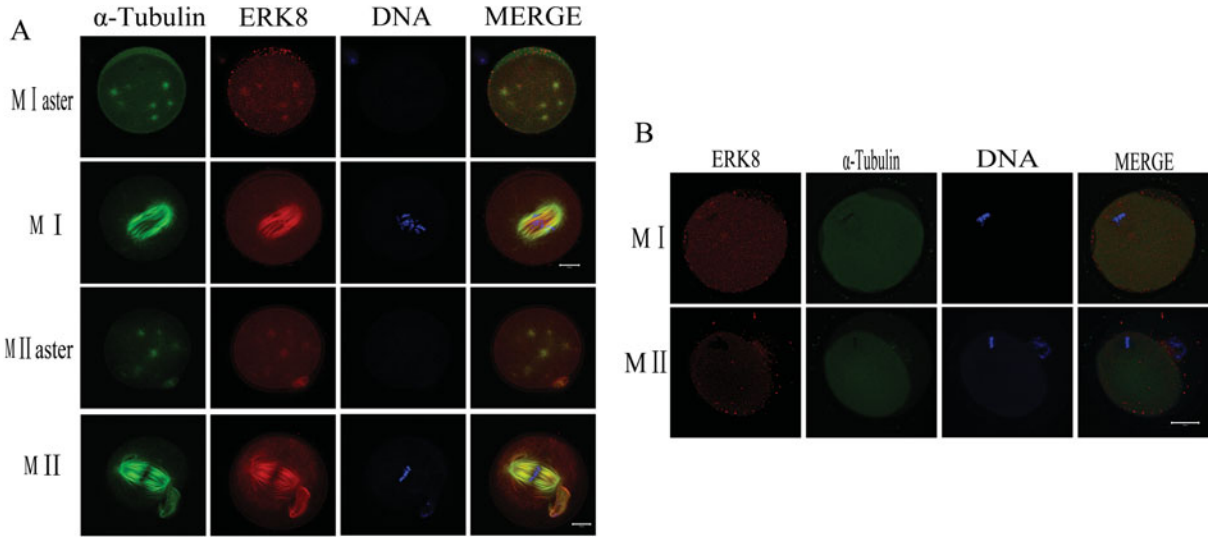

Figure 3. Localization of ERK8 in mouse oocytes treated with spindle perturbing agents during meiotic maturation. **A:** Oocytes at the MI and MII stage were incubated in M2 medium containing 10 μM taxol for 45 min and then double stained with antibodies against ERK8 and anti-α-tubulin-FITC. Both ERK8 and α-tubulin signals were localized at the cytoplasmic asters and spindles of the same oocytes. α-tubulin (green); ERK8 (red); DNA (blue). Bar = 20 μm; **B:** Double staining of ERK8 and α-tubulin in oocytes whose microtubules were completely disassembled with nocodazole treatment for 10 min. α-tubulin (green), ERK8 (red), and DNA (blue). Bar = 20 μm.

ous asters in the cytoplasm. In taxol-treated oocytes, as shown in Figure 3A, ERK8 signal was detected on the abnormal spindles as well as cytoplasmic asters (Fig. 3A).

Next, we briefly exposed oocytes at the MI and MII stages to nocodazole to induce depolymerization of microtubules. After treatment with nocodazole for 10 min to disassemble microtubules completely, ERK8 became distributed into the cytoplasm (Fig. 3B).

ERK8 Antibody Injection Caused Abnormal Spindles and Chromosome Congression Defect at the MI Stage

To explore the roles of ERK8 in mouse oocyte meiosis, we first disrupted the ERK8 protein activity by antibody injection. Oocytes with intact GV were injected with ERK8 antibody or immunoglobulin G, respectively. After injection, the oocytes were cultured in M2 medium directly for 8 h and then collected and immunostained with α-tubulin and Hoechst 33342. In the ERK8 antibody injection group, the oocytes exhibited various morphologically defective spindles and chromosome misalignment phenotypes. The major defect was no poles, and other defects include spindles with one pole, multipoles, small spindles, long spindles, and unformed spindles with astral microtubules (Fig. 4A). A severely impaired spindle in the ERK8 antibody injection group also accompanied chromosome misalignment phenotypes, which include minor and severe misalignment. Compared to the control group (23.2% $\pm$ 2.7%, n = 217), an obvious increase in abnormal spindle formation was observed in the ERK8 antibody injection group (57.1% $\pm$ 4.9%, n = 185, $p < 0.05$) (Fig. 4B). The ERK8 antibody injection group (60.4% $\pm$ 3.8%, n = 185) also showed higher chromosome misalignment than that of the control group (20.6% $\pm$ 1.8%, n = 217, $p < 0.05$) (Fig. 4B).

ERK8 siRNA Knockdown Caused Abnormal Spindles and Chromosome Congression Defect at the MI Stage

Oocytes injected with ERK8 siRNA were held in prophase for 24 h before meiotic maturation was allowed to proceed and then released into milrinone-free medium for an 8 h culture. Western blotting analysis showed that the expression level of ERK8 was significantly reduced by siRNA injection (Fig. 5A). After an 8 h culture, the oocytes in the control and ERK8-depleted group were collected and immunostained with anti-α–tubulin and Hoechst 33342. Abnormal spindle and misaligned chromosomes phenotypes were also found in the ERK8-siRNA depleted group (Fig. 5B). As shown in Figure 5C, the rate of abnormal spindles in the ERK8-siRNA injection group (59.3 $\pm$ 3.5%, n = 159) was significantly higher than that of the control group (25.3 $\pm$ 1.5%, n = 155, $p < 0.05$) (Fig. 5C). The ERK8 siRNA knockdown group (60.5 $\pm$ 3.8%, n = 159) also showed higher chromosome misalignment than that of the scrambled siRNA-injected group (30.6 $\pm$ 3.4%, n = 155, $p < 0.05$) at the MI stage.

ERK8 siRNA and Antibody Injection Caused MI/AnaI Transition Failure and Decreased PB1 Extrusion

After the ERK8 siRNA injection, oocytes were held in prophase for 24 h before meiotic maturation was allowed to proceed and then released into milrinone-free medium for 12 h for maturation. We found that the majority of oocyes arrested at the Pro-MI/MI stage in the ERK8 siRNA group; however, the oocytes entered MII stage in the control-siRNA group (Fig. 6A). The PB1 extrusion rate in the ERK8 siRNA group (31.1 $\pm$ 2.8%, n = 144) was significantly lower

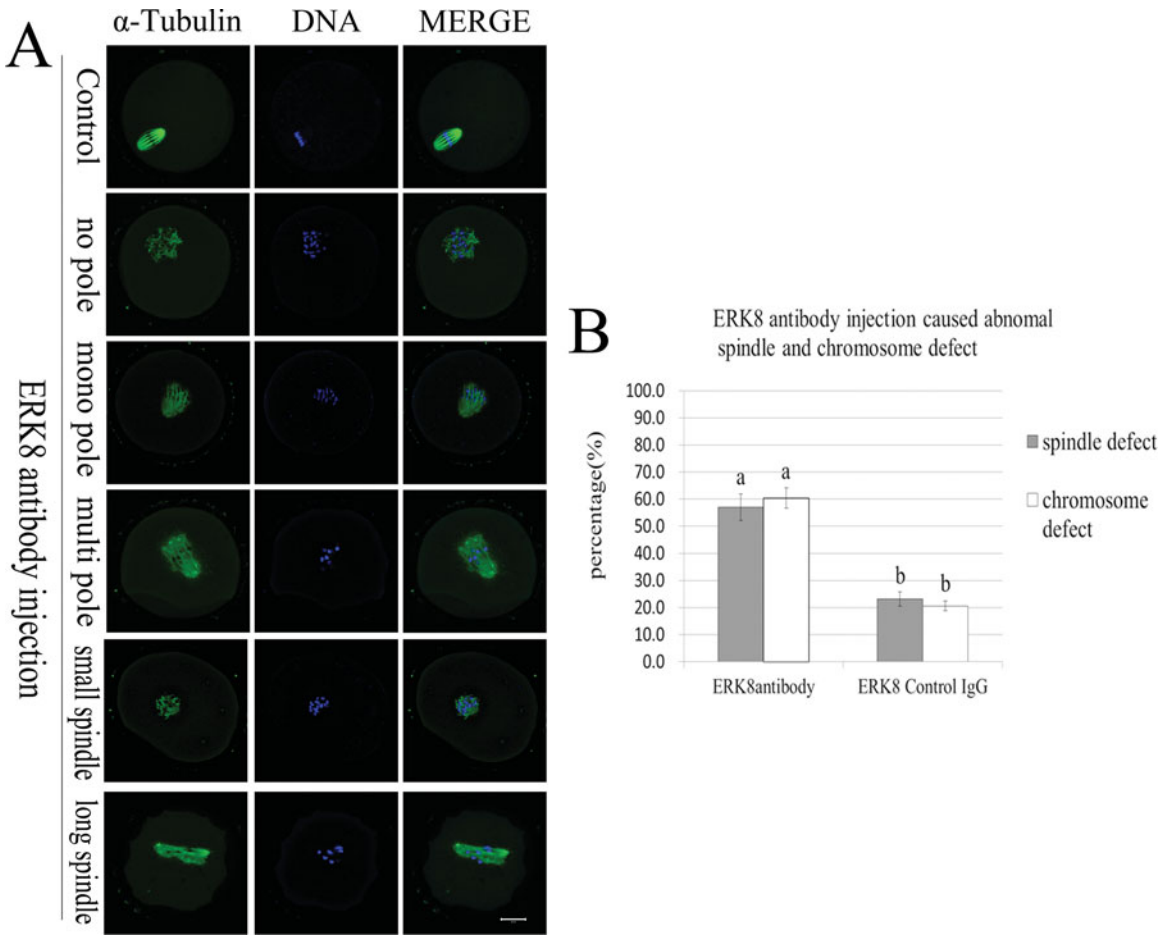

Figure 4. ERK8 antibody injection caused abnormal spindles and chromosome congression defect at the MI stage. **A:** ERK8 antibody injected oocytes exhibited chromosome misalignment and abnormal spindle including minispindles, spindle with no poles, monopoles, and multipoles. Injected oocytes were stained with α-tubulin (green) and Hoechst 33342 (blue). Bar = 20 μm. **B:** The rate of oocytes with abnormal spindles or chromosome misalignment in the ERK8 antibody injection group and control group. Data were presented as means ± SEM of three independent experiments. Different letters indicated statistical difference ($p < 0.05$).

than that of the control siRNA group (63.4 ± 4.2%, n = 157, $p < 0.05$) (Fig. 6B). Similar to the ERK8 siRNA group, the rate of PB1 extrusion (35.3 ± 3.1%, n = 114) in the ERK8 antibody injection group was also significantly lower than that of the control IgG injection group (72.7 ± 2.8%, n = 125, $p < 0.05$) (Fig. 6B).

Discussion

In the present study, we investigated the expression, localization of ERK8 in the mouse oocytes and early cleavage embryos, and explored its role in mouse oocyte meiotic maturation. The results demonstrated that ERK8 was expressed in mouse oocytes and early embryos, and it was localized on spindle microtubules. Especially, the inhibition of ERK8 activity by siRNA and antibody injection affected the assembly of spindle, chromosome alignment, and PB1 extrusion during oocyte meiotic maturation. The study for the first time provides direct evidence of the involvement of ERK8 in oocyte meiotic maturation and early embryo cleavage.

ERK8 is a newly discovered protein by using ERK7 cDNA as a screening tool. The ERK8 sequence of 544 amino acids produces a 60-kDa protein with a characteristic T–E–Y motif in its activation domain (Bogoyevitch & Court, 2004). The C-terminal tail of ERK8, like that of ERK7, contains SH3 domain binding motifs and acts with the SH3 domain of the tyrosine kinase c-Src. But ERK8 is not activated by MEK1, MEK2, or MEK5 (Abe et al., 2002). To date, the possible physiological roles of ERK8 remain the least studied. In our study, Western blot analysis showed that the quantity of ERK8 protein remained stable during mouse oocyte maturation. Shortly after GVBD, similar to ERK1/2 (Verlhac et al., 1993; Wang et al., 2007), ERK8 was spatially correlated with the spindle organization during meiotic maturation in mouse oocyte. Then we explored the relation of ERK8 and the α-tubulin. ERK8 followed the same localization pattern as α-tubulin at MII stage. These results indicate that the distribution of ERK8 may be associated with the organization of microtubules.

The subcellular localization of ERK8 during fertilization and first mitosis is similar to that in meiosis. After

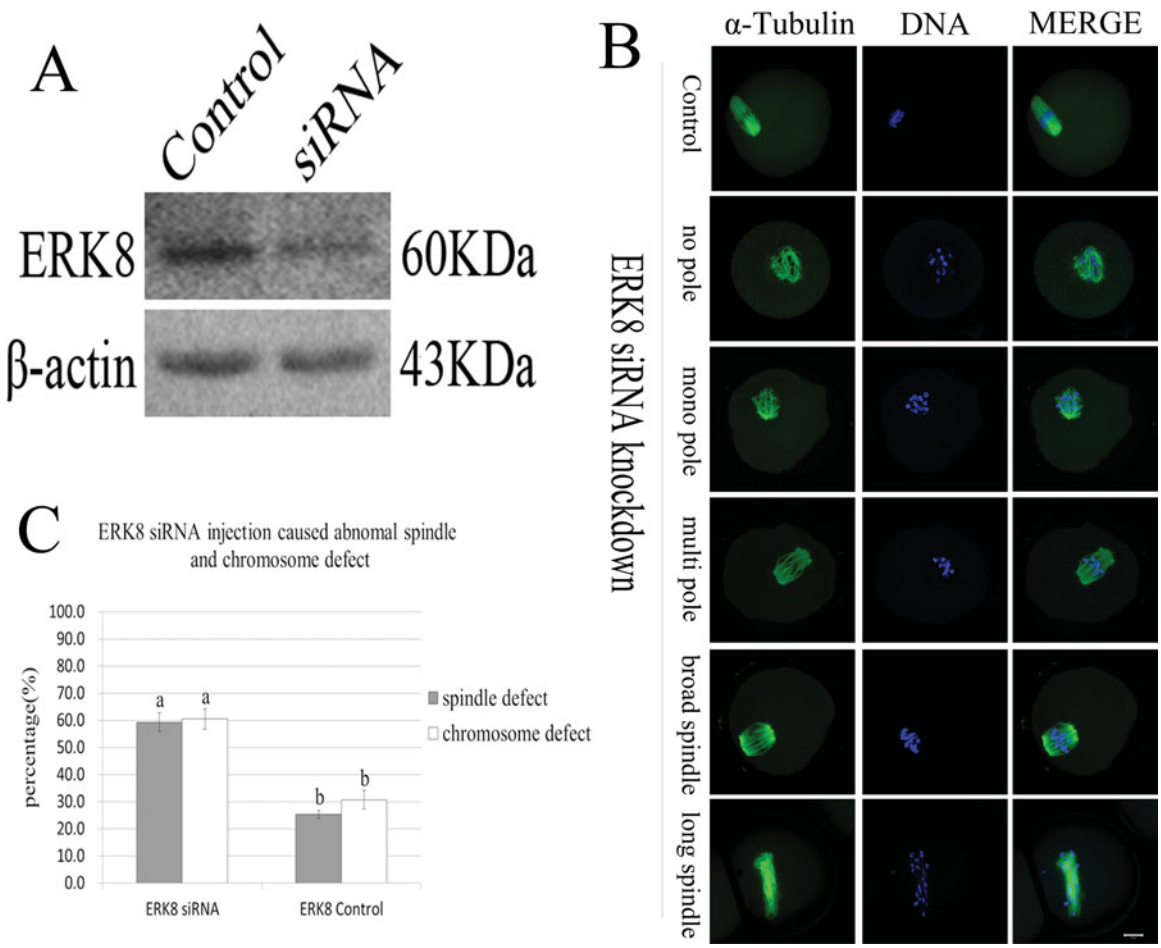

Figure 5. ERK8 siRNA knockdown caused abnormal spindles and affected chromosome congression at the MI stage. **A:** Western blot of oocytes in the ERK8 siRNA group and in the scrambled siRNA control group. The molecular mass of ERK8 is 60 kDa and that of β-actin is 43 kDa. **B:** ERK8-siRNA depleted oocytes exhibited chromosome misalignment and abnormal spindle including minispindles, spindle with no poles, monopoles, and multipoles, similar to the phenotypes of ERK8 antibody injected oocytes. Injected oocytes were stained with α-tubulin (green) and Hoechst 33342 (blue). Bar = 20 μm. **C:** The rate of oocytes with abnormal spindles or abnormal spindles and in the ERK8 siRNA injection group and control group. Data were presented as means ± SEM of three independent experiments. Different superscripts indicate statistical difference at $P < 0.05$ level of significance.

fertilization, no specific ERK8 signal was found at the pronuclear stage just as in the GV stage. After NEBD, ERK8 followed the same subcellular location as α-tubulin during the first embryo cleavage. By the completion of the first mitosis, two blastomeres entered interphase, and at this time ERK8 again showed no specific localization.

This subcellular localization pattern may indicate that ERK8 regulates spindle assembly and chromosome dynamics. Next, we further explored the relationship between microtubules and ERK8 with spindle-perturbing agents. Taxol treatment stabilized microtubule fibers and led to significantly enlarged spindles, together with numerous asters in the cytoplasm. ERK8 signals were detected to co-localize with α-tubulin of the spindle and asters. Localization of ERK8 was similar to those of many other proteins involved in spindle formation that have been studied previously, e.g., Septin1 (Zhu et al., 2011), Chk1 (Chen et al., 2012), BRCA1 (Xiong et al., 2008), and PLK1 (Tong et al., 2002), which are present at the center of MTOCs or asters after taxol treatment. These results further support the involvement of ERK8 in microtubule organization. In addition, when we briefly exposed oocytes to nocodazole, ERK8 disappeared from the spindle upon entire spindle collapse.

To further explore the function of ERK8, we used ERK8 antibody microinjection and ERK8 specific siRNA microinjection to deplete ERK8 expression or function. Our previous research showed that either Aurora-A antibody or BRCA1 antibody microinjection can distort MI spindle organization, indicating that Aurora-A and BRCA1 are critical regulators of cell cycle progression and microtubule organization during mouse oocyte meiotic maturation (Yao et al., 2004; Xiong et al., 2008). The siRNA approach is another frequently used method to down-regulate a specific gene expression and explore its function in oocyte. For example, depletion of MK2 by siRNA causes spindle assembly and chromosome alignment defects showing its role in meiotic maturation (Yuan et al., 2010).

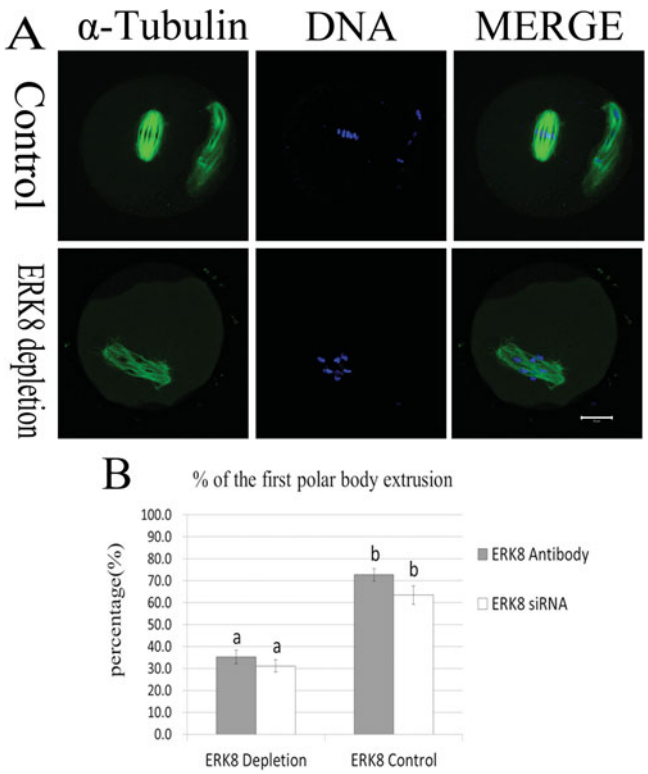

Figure 6. ERK8 depletion caused a decrease in PBE. **A:** Oocytes of control and ERK8 depletion groups were cultured for 12 h followed by immunostaining with α-tubulin antibody (green) and Hoechst 33342 (blue). Control oocytes extruded PB1 and reached MII stage. In the ERK8 depleted group, many oocyes arrested at the MI stage. **B:** Frequencies of polar body extrusion of oocytes cultured for 12 h in the ERK8 antibody injection group, ERK8 siRNA group, and their control counterparts. Data are presented as means ± SEM of three independent experiments. Different superscripts indicate statistical difference ($p < 0.05$).

Knockdown of Nedd1 transcripts using specific siRNAs resulted in a high incidence (65–70%) of MI arrest. The arrested oocytes were characterized by disrupted meiotic spindle structure, reduced microtubule density, and evident chromosome misalignment (Ma et al., 2010). In the present study, high incidence of severely abnormal spindles and misaligned chromosomes was observed in ERK8-antibody injected oocytes compared to control oocytes, indicating that ERK8 is important for the meiotic spindle assembly. This conclusion is further supported by the results of injecting the oocytes with ERK8-specific siRNA. Consistent with the results of ERK8 antibody injection experiments, most ERK8-specific siRNA injected oocytes showed higher abnormal spindle and chromosome defect. We conclude that ERK8 is involved in meiotic spindle assembly.

Critical steps in mammalian oocytes meiotic maturation are the progression through meiosis I and the asymmetric meiotic division that result in extrusion of the first polar body. Accurate chromosome alignment is important for correct spindle formation and special ring cap structure formation at the cortex of the oocyte, both of which contribute to polar body extrusion (Wang et al., 2011). Correct spindle assembly is also needed for spindle movement during the first meiosis, which is essential for asymmetric meiotic divisions and the first polar body extrusion (Ai et al., 2008*b*). Depolymerization of microtubules can inhibit spindle migration and rotation (Ai et al., 2008*a*). Previous studies show that many proteins such as DYNLT3 and Dicer are involved in spindle assembly, and they also play roles in the first polar body extrusion (Liu et al., 2010; Huang et al., 2011). The higher abnormal spindle chromosome defect in ERK8 depleted oocytes prompted us to explore the role of ERK8 in polar body extrusion. We analyzed the oocyte maturation process after ERK8 depletion. Compared to the control oocytes, most ERK8 antibody injected and siRNA knockdown oocytes were arrested at the Pro-MI/MI stage, and they failed to extrude the first polar body (PB1). These results suggest that ERK8, via its effects on meiotic spindles, is required for the mouse oocyte maturation process.

Why and how ERK8 regulates microtubule assembly is still unclear. Proteins such as c-Jun and Src are potential candidates in the ERK8 regulation pathway (Abe et al., 2002; Iavarone et al., 2006; Xu et al., 2010). In porcine oocytes, Src acts as an essential upstream regulator of MAPK activity and mediates reinitiation of meiosis promoted by testosterone and androgen receptors (Li et al., 2008). The Src family kinase (SFK) is also needed for meiotic maturation in mouse oocyte. Depletion of SFK activity can cause the oocyte failure to initiate GVBD, to go into MII, and to extrude PB1 (Zheng et al., 2007). ERK8 contains two SH3-binding motifs in its C-terminal region and can co-immunoprecipitate with c-Src *in vivo*. ERK8 can be activated downstream of c-Src and v-Src, suggesting its participation in a Src-dependent signaling pathway (Abe et al., 2002). The relationship between ERK8 and Src could be the next step for ERK8 function studies in mouse oocyte meiosis. Another possible candidate in ERK8 pathway in meiosis is c-Jun N-terminal kinase (JNK). In previous studies, JNK has been shown to be a player in spindle assembly and maintenance. JNK2 antibody microinjection and the specific inhibitor SP600125 treatment can cause abnormal spindle formation and decrease PB1 extrusion (Huang et al., 2011). It was reported that in HCT15 colorectal cancer cells, overexpression of ERK8 caused an increased phosphorylation of c-Jun at Ser63 and Ser73, and knockdown of ERK8 in HCT15 colorectal cancer cells blocked c-Jun phosphorylation suggesting c-Jun's role in the ERK8 signal pathway (Xu et al., 2010). We can infer that ERK8 might affect spindle assembly in oocyte meiosis through c-Jun phosphorylation, which needs further investigation.

Taken together, our results suggest that ERK8 may play important roles in the microtubule organization and meiotic cell cycle progression in mouse oocytes, fertilized eggs, and early embryos. Further studies are necessary to identify the upstream kinases and the downstream substrates of the ERK8 family in mammalian meiosis.

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