Mol. Cells 2014; 37(2): 126-132
http://dx.doi.org/10.14348/molcells.2014.2259

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Chk2 Regulates Cell Cycle Progression during Mouse Oocyte Maturation and Early Embryo Development

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As a tumor suppressor homologue during mitosis, Chk2 is involved in replication checkpoints, DNA repair, and cell cycle arrest, although its functions during mouse oocyte meiosis and early embryo development remain uncertain. We investigated the functions of Chk2 during mouse oocyte maturation and early embryo development. Chk2 exhibited a dynamic localization pattern; Chk2 expression was restricted to germinal vesicles at the germinal vesicle (GV) stage, was associated with centromeres at pro-metaphase I (Pro-MI), and localized to spindle poles at metaphase I (MI). Disrupting Chk2 activity resulted in cell cycle progression defects. First, inhibitor-treated oocytes were arrested at the GV stage and failed to undergo germinal vesicle breakdown (GVBD); this could be rescued after Chk2 inhibition release. Second, Chk2 inhibition after oocyte GVBD caused MI arrest. Third, the first cleavage of early embryo development was disrupted by Chk2 inhibition. Additionally, in inhibitor-treated oocytes, checkpoint protein Bub3 expression was consistently localized at centromeres at the MI stage, which indicated that the spindle assembly checkpoint (SAC) was activated. Moreover, disrupting Chk2 activity in oocytes caused severe chromosome misalignments and spindle disruption. In inhibitor-treated oocytes, centrosome protein γ-tubulin and Polo-like kinase 1 (Plk1) were dissociated from spindle poles. These results indicated that Chk2 regulated cell cycle progression and spindle assembly during mouse oocyte maturation and early embryo development.

INTRODUCTION

In order to preserve genomic integrity, cells have evolved surveillance programs to ensure DNA replication and the accurate segregation of chromosomes. The mitotic cell cycle includes a cell growth phase (G1 phase), a DNA replication phase (S phase), a G2 phase, and M phase (Callegari and Kelly, 2007). Cell cycle checkpoints are those signal transduction pathways that control the order and timing of cell cycle transitions. These can respond to stress signals and induce cell cycle arrest during different phases (Bartek and Lukas, 2001; Masrouha et al., 2003). The cell cycle control mechanism during M phase is the spindle assembly checkpoint (SAC), which monitors chromosome segregation and reduces the probability that cell division will produce daughter cells with an abnormal number of chromosomes (Musacchio and Salmon, 2007; Sun et al., 2011).

Checkpoint kinase 2 (Chk2) is the mammalian homolog of the Saccharomy cescerevisiae Rad53 and Schizosaccharomyces pombe Cds1 protein kinases (Matsuoka et al., 1998). The amino-terminal domain of Chk2 includes a series of seven serine or threonine residues, each of which is followed by a glutamine (Matsuoka et al., 1998). Chk2 is a multi-func-tional enzyme that is involved in the regulation of replication checkpoints, DNA repair, cell cycle arrest, and apoptosis due to DNA damage (Ahn et al., 2004; Antoni et al., 2007; Bartek and Lukas, 2003; Sato et al., 2010; Stolz et al., 2011; Zhou and Elledge, 2000) Chk2 is also involved in maintaining the proper and timely assembly of the mitotic spindle apparatus, which preserves chromosome stability in human somatic cells (Joukov et al., 2006; Sato et al., 2010; Stolz et al., 2010; 2011). Chk2 has also been shown to be essential for the G2 checkpoint in response to DNA damage and unreplication (Xu and Du, 2003).

Chk2 interacts with several molecules that are involved in multiple cellular processes. Chk2 phosphorylates the related CDK2 phosphatase Cdc25A for ubiquitin-dependent degradation. At the G2/M checkpoint, Chk2 is regulated by ataxia telangiectesia mutated (ATM; Masrouha et al., 2003; Xu and Du, 2003). Activated Chk2 can phosphorylate Cdc25C, which inhibits Cdc25C from activating CDK1 at the G2/M transition in the nucleus and causes G2 arrest (Blasina et al., 1999; Stolz et al., 2011). Polo like kinase 1 (Plk1), a centrosome-associated kinase implicated in mitotic progression, can also interact with Chk2 at centrosomes and midbodies (Tsvetkov et al., 2003). Plk1 has been shown to be involved in meiotic spindle organization and cell cycle regulation *via* interactions with MAPK signaling pathways (Pahlavan et al., 2000; Tong et al., 2002).

Although the roles of Chk2 have been well studied during mitosis, its functions in oocyte meiotic maturation and subsequent

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Received 16 September, 2013; revised 13 December, 2013; accepted 13 December, 2013; published online 19 February, 2014

Keywords: cell cycle, Chk2, embryo, oocyte meiosis, spindle assembly

eISSN: 0219-1032

early embryo development remain uncertain. Mammalian oocyte meiosis involves two successive divisions: Meiosis I and Meiosis II. Chromosomes replicate once and divide twice to form haploid gametes, which is one major difference between meiosis and mitosis. Here, we investigated the roles of Chk2 during mouse oocyte maturation and embryo development. Our results demonstrate that Chk2 plays important roles in regulating cell cycle progression during female meiosis and early embryo development.

MATERIALS AND METHODS

Antibodies and chemicals

Rabbit polyclonal anti-Chk2 antibody was from Abcam (UK). Alexa Fluor 488 and 594 antibodies were from Invitrogen (USA). Mouse monoclonal anti-α-tubulin-FITC antibody was from Sigma (USA). Rabbit polyclonal anti-γ-tubulin antibody was from Santa Cruz (USA). Rabbit anti-Bub3 and mouse anti-PLK1 were gifts from Prof. Qing-Yuan Sun at the Chinese Academy of Sciences. Chk2 Inhibitor II was from Calbiochem.

Oocyte and zygote harvest and culture

ICR mice care and handling were in accordance with the policies of the Nanjing Agricultural University. Oocytes were harvested, washed thoroughly, and cultured in M16 medium covered with liquid paraffin oil at 37°C in a 5% CO_2 atmosphere. Oocytes were removed from culture at different times for immunostaining.

ICR mice (6- to 8-weeks-old) were also injected with pregnant mare serum gonadotrophin (PMSG). After 48 h they were injected with human chorionic gonadotrophin (hCG) and immediately mated with male mice. Zygotes were harvested 18 h later and cultured in K modified simplex optimized medium (KSOM; Chemicon, USA) under paraffin oil at 37°C and 5% CO_2. Embryos were removed for immunostaining after different times in culture.

Chk2 activity inhibition

Chk2 inhibitor II was prepared as a 25 mM stock solution in DMSO and stored at -20°C until used. Prior to use, it was diluted in M16 medium to final concentrations of 25 μM, 50 μM, and 100 μM, and oocytes were incubated in this medium. Controls were cultured in M16 medium only. Spindle phenotypes and chromosome alignments of oocytes treated with 50 μM inhibitor were examined using confocal microscopy after culture for 12 h. group. Polar body extrusion and germinal vesicle breakdown were observed using a microscope. Each experiment was repeated at least three times.

Immunofluorescent and confocal microscopy

Oocytes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature and permeabilized in 0.5% Triton-X-100 at room temperature for 20 min. Then, oocytes were blocked with 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C or for 4 h at room temperature with anti-Chk2 (1:100), anti-Bub3 (1:50), anti-γ-tubulin (1:50), or anti-α-tubulin (1:200) FITC-labeled antibodies. After washing three times (2 min each) in PBS that contained 1% Tween 20 and 0.01% Triton-X 100, oocytes were incubated with an appropriate secondary antibody for 1 h at room temperature. After washing three times, oocytes were stained with PI or Hoechst 33342 (10 μg/ml) for 10 min. Finally, oocytes were mounted on glass slides and viewed under a confocal laser scanning microscope (Carl Zeiss 700).

Rescue treatment

Oocytes were cultured in M16 medium for 3 h or 12 h at 37°C. Then, oocytes with inhibitor treatment were washed five times (2 min each) in M16 medium. Oocytes were transferred to fresh M16 medium and cultured for another 3 h (GVBD analysis) and another 6 h (polar body extrusion analysis) under paraffin oil at 37°C in a 5% CO_2 atmosphere.

Statistical analysis

At least three replicates were performed for each treatment. Results are given as means ± SE's. Statistical comparisons were made by analysis of variance (ANOVA) followed by Duncan's multiple comparisons test. A p-value of < 0.05 was considered significant.

RESULTS

Chk2 localization during mouse oocyte meiotic maturation

The subcellular localization of Chk2 at different stages of meiotic maturation was examined by immunofluorescent staining. As shown in Fig. 1, Chk2 was distributed at the germinal vesicles of GV stage oocytes. After GVBD, Chk2 became associated with centromeres. At MI, Chk2 was localized at spindle poles. When oocytes progressed to MII, Chk2 became localized at spindle poles. Negative control showed no specific signals.

Chk2 inhibition results in GVBD defects in mouse oocytes

The localization pattern of Chk2 prompted us to examine its roles in regulating the cell cycle of oocytes. A Chk2 specific inhibitor, Chk2 inhibitor II, was used to investigate the roles of Chk2 during mouse oocyte maturation. First, we investigated the effects of Chk2 on GVBD by inhibiting Chk2 activity during the GV stage. As shown in Fig. 2A, after 3 h in culture, most control oocytes underwent GVBD, whereas most inhibitor-treated oocytes remained at the GV stage. After releasing oocytes from inhibitor treatment, most oocytes entered GVBD. The rate of oocytes with GVBD in control oocytes was significantly higher than that of inhibitor-treated oocytes (86.9% ± 4.7%, $n = 87$, versus 0.8% ± 0.7%, $n = 90$; $p < 0.01$). The GVBD rate in rescued oocytes was significantly higher than that of treated oocytes (35.1% ± 8.8%, $n = 80$, versus 0.8% ± 0.7%, $n = 90$; $p < 0.05$).

Disrupting Chk2 activity inhibits mouse oocyte meiosis

Chk2 had a centromere localization pattern at the Pro-MI stage, which may have been related to the spindle assembly checkpoint, as most checkpoint proteins like those in the Mad and Bub families localize at centromeres. Oocytes were first cultured for 3 h, during which time most had progressed to GVBD. Then, these oocytes were treated with the Chk2 inhibitor and cultured for 12 h. The rates of first polar body (PBI) extrusion in control oocytes (77.35% ± 3.98%, $n = 79$) was remarkably higher than that in inhibitor-treated oocytes (8.18% ± 6.05%, $n = 71$; $p < 0.01$; Fig. 3B). However, after release from inhibition, PBI extrusion was rescued.

In rescued oocytes, the rate of first polar body extrusion was significantly higher than that of inhibitor-treated oocytes (28.91% ± 4.30%, $n = 66$; $p < 0.05$; Fig. 3B). The PBI extrusion rate was also inhibitor concentration dependent, as shown in Fig. 3C; the PBI extrusion rates for Control, 50 μM, and 100 μM were 77.75% ± 4.21% ($n = 120$), 47.63% ± 0.71% ($n = 101$), and 9.33% ± 5.34% ($n = 80$), respectively.

Previous studies demonstrated that Bub3 was a spindle assembly checkpoint protein that regulated cell cycle progression

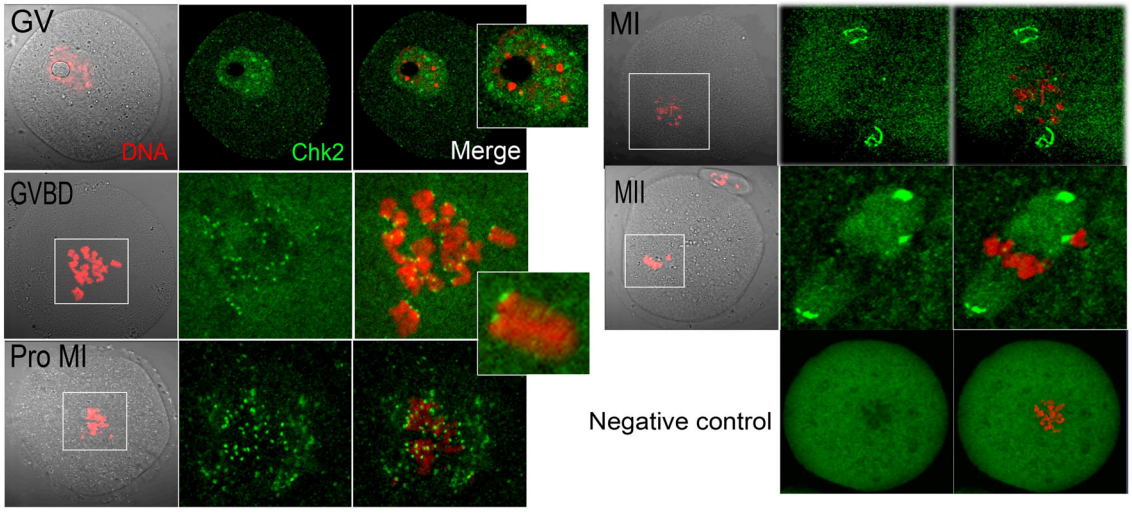

Fig. 1. Subcellular localization of Chk2 during mouse oocytes meiotic maturation. Samples were taken after culture for 0, 2, 4, 8, and 12 h, the time points at which most oocytes reached the GV, GVBD, Pro-MI, MI, and MII stages, respectively. Oocytes at these different stages were stained with an anti-Chk2 antibody. In the GV stage, Chk2 expression was restricted to germinal vesicles. After GVBD, Chk2 became located at centromeres. In the MI and MII stages, Chk2 accumulated on spindle poles. Negative control showed no specific signals. Green, Chk2; red, chromatin.

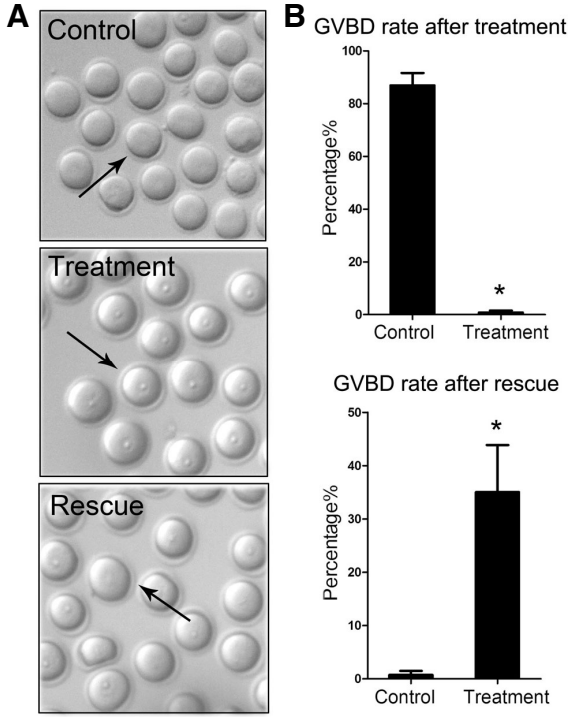

Fig. 2. Disrupting Chk2 activity inhibits mouse oocyte meiotic germinal vesicle breakdown (GVBD). (A) Oocyte germinal vesicle breakdown was inhibited after treatment with Chk2 Inhibitor II (100 μM). (B) Rates of GVBD for oocytes cultured with Chk2 Inhibitor II (100 μM) in M16 medium (n = 90), control oocytes (n = 87), and rescued oocytes (n = 80). Results are means ± SE's. *Significant difference ($p < 0.05$).

during mouse oocyte meiosis. Thus, we examined Bub3 localization after inhibitor treatment for 8 h. As shown in Fig. 3D, in control oocytes, Bub3 was observed at centromeres in the Pro-MI stage, whereas in the MI stage, Bub3 could no longer be detected at centromeres. However, in inhibitor-treated oocytes, Bub3 consistently localized at centromeres.

Disrupting Chk2 activity results in abnormal spindle morphology

Because Chk2 is required for spindle apparatus assembly during mitosis, we examined whether Chk2 had a similar role during meiosis by determining the effects of Chk2 on chromosome alignment and spindle formation in mouse oocytes. First, oocytes were incubated in M16 medium for 3 h, after which they were transferred to Chk2 inhibitor-containing M16 medium and cultured for an additional 9 h. A large proportion of oocytes exhibited severe spindle morphology disruptions and chromosome misalignments. Immunofluorescent staining results showed that chromosomes were misaligned and spindle morphology was aberrant. Chromosomes were misaligned, as elongated spindles, multipolar spindles, and nonpolar spindles were observed at both the MI and MII stages after inhibitor treatment (Fig. 4A). In control oocytes, the rates of misaligned chromosomes and aberrant spindles were 30.56% ± 5.06% and 22.98% ± 12.03% (n = 120), respectively, whereas in inhibitor-treated oocytes the rates of misaligned chromosomes and aberrant spindle were 74.71% ± 4.86% and 52.13% ± 4.34% (n = 101), respectively ($p < 0.05$; Fig. 4B).

To further characterize the roles of Chk2 for spindle assembly, we verified the localization of the spindle pole marker γ-tubulin. In control oocytes, γ-tubulin was localized at spindle poles, whereas in inhibitor-treated oocytes, γ-tubulin had disappeared from the spindle poles at metaphase I (Fig. 4C). We also examined the localization of Plk1 after Chk2 inhibition in mouse oocytes. In control oocytes, Plk1 was localized at spindle poles, but Plk1 was not associated with spindle poles in

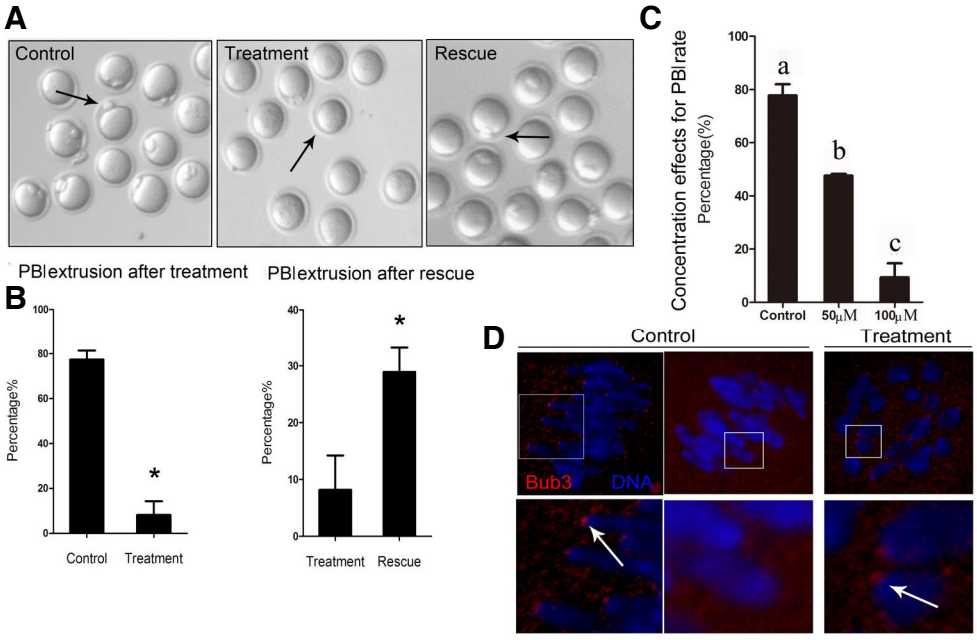

Fig. 3. Effects of Chk2 activity disruption on mouse oocyte first polar body extrusion. (A) Oocytes failed to extrude the first polar body after treatment with Chk2 Inhibitor II (50 μM). (B) Rates of first polar body extrusion for oocytes were cultured with Chk2 Inhibitor II (50 μM) in M16 medium (n = 80), control oocytes (n = 79), and rescued oocytes (n = 76). (C) Rates of first polar body extrusion after 12 h in culture with different treatments (Control, 50 μM, and 100 μM). Different letters indicate significant differences ($p < 0.05$). (D) Oocytes were fixed and single stained with antibodies against Bub3 (red) at pro-M I stage. DNA (blue) was stained with Hoechst. Arrows indicate Bub3 signals at centromeres. Results are means ± SE's. *Significant difference ($p < 0.05$).

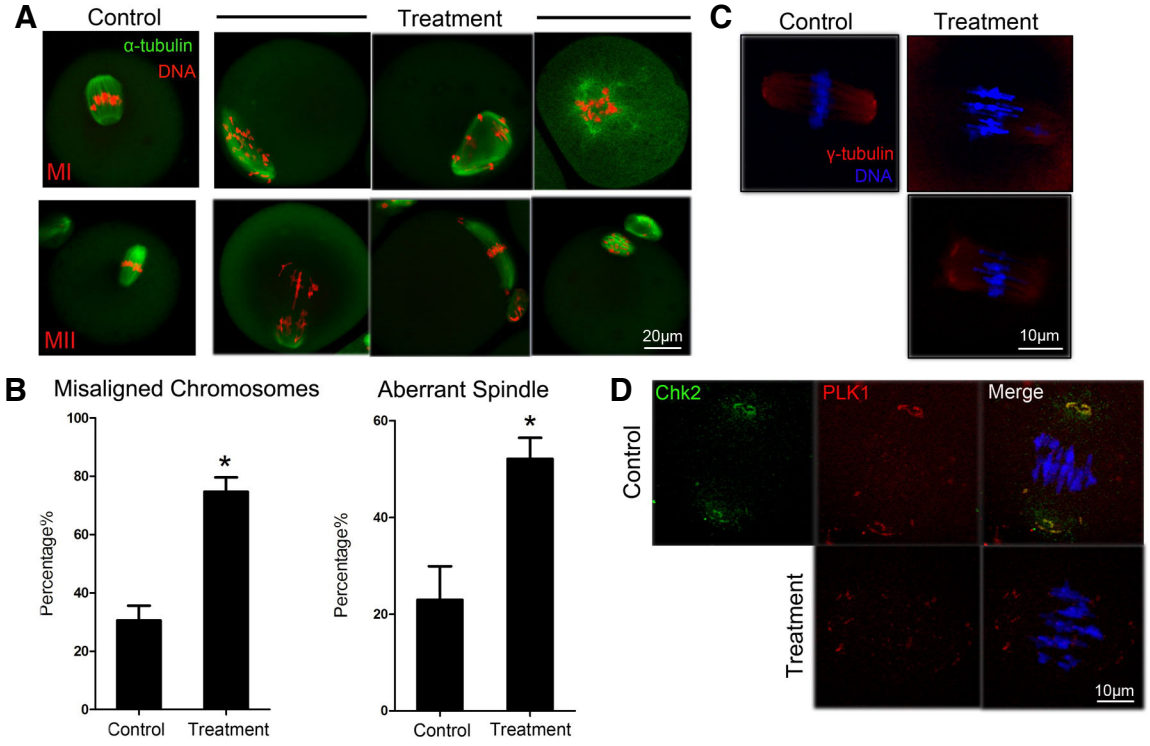

Fig. 4. Chk2 inhibition results in abnormal spindles and chromosome alignment defects. (A) Oocytes were incubated with fresh medium for 3 h and then transferred to medium with an inhibitor for another 9 h, followed by immunostaining with an anti-α-tubulin antibody (green) and PI (red). Most control oocytes exhibited normal homologous chromosome alignments and spindle formation. After treatment with Chk2 Inhibitor II (50 μM), oocytes had misaligned chromosomes (straggled or lagging) and aberrant spindles (nonpolar spindles, elongated spindles, and multipolar). (B) Rates of misaligned chromosomes and aberrant spindles for control and inhibitor treated oocytes. *Significant difference ($p < 0.05$). (C) In control oocytes, γ-tubulin was associated with spindle poles at metaphase I stage, whereas in inhibitor-treated oocytes, γ-tubulin was delocalized from abnormal spindle poles. Red, γ-tubulin; blue, chromatin. (D) Oocytes at the MI stage were double stained with antibodies against Chk2 and against Plk1. In control oocytes, Plk1 was associated with spindle poles and also distributed to centromeres at metaphase I stage, whereas in treated oocytes, Plk1 was delocalized from abnormal spindle poles. Green, Chk2; Red, Plk1; blue, chromatin.

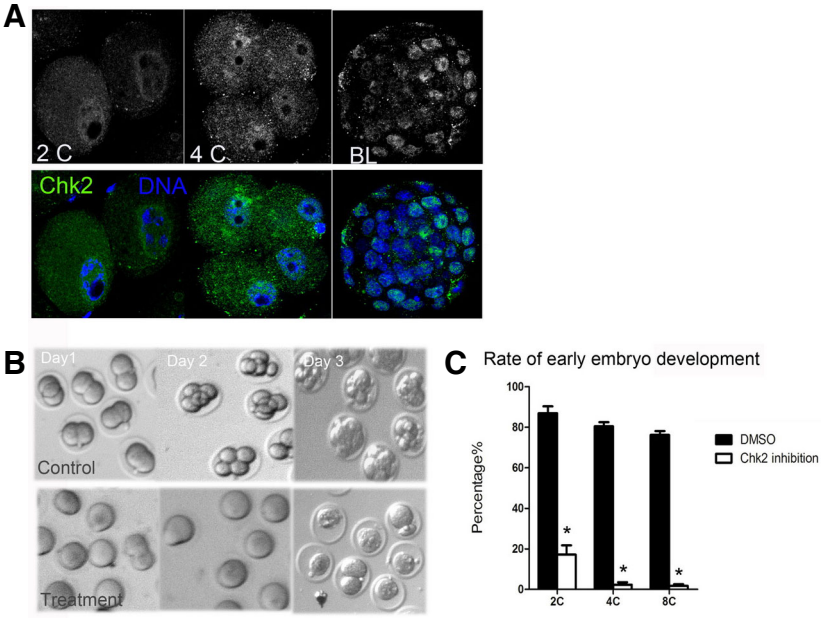

Fig. 5. Chk2 inhibition results in mouse embryo first cleavage failure. (A) Subcellular localization of Chk2 during mouse embryo development. Samples were collected from the 2-cell to the blastocyst stage. Chk2 was primarily concentrated at the nucleus. Green, Chk2; blue, chromatin. (B) Embryos failed to proceed through first cleavage after treatment with Chk2 Inhibitor II (25 μM). (C) Rates of embryo developmental stages after treatment with Chk2 Inhibitor II. *Significant difference ($p < 0.05$).

inhibitor-treated oocytes (Fig. 4D).

Chk2 inhibition results in first cleavage failure in mouse early embryo development

The localization of Chk2 was examined at different stages of mouse embryo development by immunofluorescent staining. As shown in Fig. 5A, from the 2-cell to the blastocyst stage, Chk2 was primarily localized in nuclei. We inhibited Chk2 activity starting at the zygote stage. As shown in Fig. 5B, most embryos with Chk2 inhibitor treatment did not proceed through the first cleavage, whereas most control embryos developed to the 8-cell stage. Only 17.16% ± 4.51% (n = 140) of Chk2 inhibitor-treated embryos developed to the 2-cell stage as compared with 86.97% ± 3.35% (n = 152; $p < 0.05$) of control embryos. Moreover, 2.31% ± 1.18% and 1.67% ± 0.9% of Chk2 inhibitor-treated embryos developed to the 4-cell stage and 8-cell stage as compared with 80.48% ± 2.07% and 76.27% ± 1.90% of control embryos ($p < 0.05$; Fig. 5C).

DISCUSSION

In this study, we investigated the localization and possible functions of Chk2 during mouse oocytes meiotic maturation and early embryo development. We found that Chk2 contributed to regulating spindle organization, chromosome alignment, and cell cycle progression in mouse oocytes and embryos.

We first examined the localization of Chk2 during meiosis in mouse oocytes. We found that Chk2 exhibited a novel, dynamic localization after GVBD, as Chk2 was localized at centromeres at Pro-MI in mouse oocytes, which indicated that Chk2 may be involved in centromere-related processes, such as cell cycle regulation. During the MI and MII stages when chromosomes become properly aligned and spindles form, Chk2 was localized at the spindle poles, which indicating that Chk2 may be involved in a spindle-related process. BRCA1, a downstream molecule of Chk2, has also been shown to have similar localization during mouse meiosis (Xiong et al., 2008). At the ATI stage, Chk2 staining was observed at spindle midbodies.

Thus, our results were consistent with earlier reports that showed that Chk2 localized to centrosomes and the spindle midbody during mitosis (Tsvetkov et al., 2003). Taken together, this dynamic localization pattern suggests that Chk2 has different functions during different stages of mouse oocytes meiosis.

To confirm our hypothesis, we inhibited Chk2 activity. Previous studies showed that Chk2 was necessary for proper mitosis progression (Stolz et al., 2010) and was primarily activated by ATM in response to DNA double strand breaks (Masrouha et al., 2003; Stolz et al., 2011). The Cdc25 protein, a positive regulator of cell cycle progression, was inhibited by Chk2 phosphorylation (Reinhardt and Yaffe, 2009). This caused G1/S or G2/M arrest in somatic cells.

Our results indicated that Chk2 might regulate GVBD entry during mouse oocyte meiosis, a process which is similar to G2/M in mitosis. In Chk2-inhibited oocytes, almost all oocytes failed to undergo GVBD. After the release from Chk2 inhibition, these oocytes re-entered GVBD. A previous study also showed that in *C. elegans*, Chk2 was involved in the initial establishment of pairing between homologous chromosomes during meiotic prophase (MacQueen and Villeneuve, 2001). Together with our results, Chk2 might be involved in the progression of the GV stage to GVBD. Our results also indicated that there was a G2/M checkpoint in mammalian oocytes, which will require further investigation.

Our results also indicated that Chk2 may be involved in regulating the SAC during mouse oocyte meiosis. During meiosis, chromosomes accumulate at the metaphase plate and the microtubules organized around chromosomes establish attachments with chromosome centromeres. The SAC is a surveillance mechanism that controls oocyte meiosis by blocking entry into anaphase when anomalies occur during chromosome segregation (Polanski, 2012). After GVBD, oocytes were treated with an inhibitor, and most of these oocytes failed to extrude polar bodies, which indicated that oocyte meiosis was arrested at the early stage. However, this could be rescued after release from Chk2 inhibition.

To further investigate this, we examined the expression of

the spindle assembly checkpoint protein Bub3. Bub3 supervises the meiotic metaphase-anaphase transition by preventing homologous chromosome and sister chromosome segregation during M I and II (Matsuoka et al., 1998). Bub3 expression was also shown to be consistent with centromeres after Chk2 inhibition, which indicated that inhibitor-treated oocytes were arrested at Pro-MI, as Bub3 disperses from centromeres when oocytes reach MI (Li et al., 2009).

In MI, Chk2 was localized at spindle poles, from which we hypothesized that Chk2 was responsible for spindle assembly and chromosome alignment. A spindle, which mainly consists of chromosomes, microtubules, and centrosomes, is one of the most essential elements of cellular structures. It is responsible for the accuracy of chromosome segregation, which is required for maintaining genomic integrity during both mitosis and meiosis. During mitosis, Chk2 is required for the proper and timely assembly of the mitotic spindle apparatus (Joukov et al., 2006; Sato et al., 2010; Stolz et al., 2010; 2011).

Our immunofluorescence analysis showed that the rates of chromosome misalignment and aberrant spindles in Chk2-treated oocytes were significantly higher than those for control oocytes. This was consistent with a previous report that showed that Chk2 was required for proper spindle assembly and the maintenance of chromosome stability during mitosis (Joukov et al., 2006). Moreover, cell cycle regulation by Chk2 was also conserved in mouse early embryos, as our results showed that most Chk2 inhibitor-treated embryos did not proceed through the first cleavage.

During mitosis, Chk2 co-localizes with the centrosome protein γ-tubulin (Castedo et al., 2004). To further confirm a role for Chk2 in spindle assembly, we examined the localization of γ-tubulin. During mitosis, γ-tubulin is a component of pericentriole material and is exclusively located at the centrosome throughout the cell cycle (Xiong et al., 2008). Spindle assembly is directed to a large extent by centrosomes, which are the main sites of microtubule polymerization during mitosis (Brunet and Maro, 2005; Chen et al., 2012). In this study, we found that when Chk2 activity was blocked, γ-tubulin localization was disrupted in spindle poles in mouse oocytes, which confirmed the role of Chk2 in spindle formation.

Moreover, during mitosis, Chk2 was shown to physically interact and co-localize with Plk1, a regulator of chromosome segregation, mitotic entry, and mitotic exit (Tsvetkov et al., 2003). From our results at MI, Chk2 also co-localized with Plk1 at spindle poles, and after inhibiting Chk2, Plk1 localization was disrupted. During meiosis, Plk1 accumulates at spindle poles and becomes distributed to centromeres. Plk1has been shown to be a regulator of spindle organization and the SAC (Sun et al., 2012). Our results indicated that Chk2 might regulate Plk1 localization during spindle organization and the SAC.

Taken together, our results indicate that Chk2 regulates cell cycle progression, meiotic spindle organization, and chromosome alignment during oocyte meiotic maturation and early embryo development in female mammals.

ACKNOWLEDGMENTS

This study was supported by the Scientific Research Foundation for Returned Overseas Chinese Scholars, State Education Ministry of China (2013693), China, and the Biogreen 21 Program (PJ009594 and PJ00956302), RDA, Republic of Korea.

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