[Frontiers in Bioscience E2, 991-1000, June 1, 2010]

Borealin regulates bipolar spindle formation but may not act as chromosomal passenger during mouse oocyte meiosis

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TABLE OF CONENTS

1. ABSTRACT

In mitosis, Borealin is a novel member of the chromosomal passenger complex (CPC), which is thought to play interaction roles with INCENP and survivin in the complex. Its roles in mammalian meiosis are unknown. Here, we report the expression, localization, and function of Borealin and its relation with survivin in mouse oocyte meiosis. Borealin expression was gradually increased from the germinal vesicle (GV) stage to metaphase II (MII). Immunofluorescence microscopy revealed that Borealin was weakly expressed in the GV stage and accumulated near chromosomes after germinal vesicle breakdown (GVBD). Borealin localized mainly at the spindle poles in metaphase I and anaphase I, and at the midbody in telophase. Borealin relocalized at the spindle poles during MII. Taxol and nocodazole treatment showed that the localization of Borealin was dependent on microtubule dynamics and meiotic spindle integrity, whereas survivin distribution was independent of these factors. Disruption of Borealin function by antibody injection resulted in severe spindle assembly defects, but did not affect polar body extrusion. We also found that depletion of survivin by morpholino injection had no effect on the localization of Borealin. In conclusion, our data suggest that Borealin is required for bipolar spindle formation, but may not regulate spindle checkpoint activity as a component of the CPC during mouse oocyte meiosis.

2. INTRODUCTION

Mammalian oocytes need to undergo meiotic maturation before fertilization, and the spindle plays important roles during this process. The spindle is a subcellular structure composed of microtubules and centrosomes.(1) Microtubules emerging from spindle poles can capture chromosomes and lead them to the equatorial plate. Only when tension is detected at each chromosome on the equatorial plate can the oocyte enter the next stage. Disruption of the spindle causes chromosome missegregation and may lead to cancer if the disruption occurs during mitosis.(2, 3) In meiosis, instead of centrosomes, spindle assembly is controlled by microtubule organizing centers (MTOCs), which include centrosomal proteins.(4, 5)

The chromosomal passenger complex (CPC), which includes Aurora B, INCENP, survivin, and Borealin in the case of mitosis, plays multiple roles that are central to spindle formation and cytokinesis, such as Histone H3 Ser10 and Ser28 phosphorylation, release of arm cohesion, spindle checkpoint control, and regulation of mitotic chromosome structure, kinetochore maturation, spindle assembly, kinetochore–microtubule attachment, centromeric cohesion, chromosome alignment, and of bipolar spindle stability.(6, 7) Aurora B is the enzymatic core of the complex, its activity was conducted by

phosphorylation, and whereas survivin, INCENP and Borealin dictate the timing and localization of the kinase activity.(8)

Borealin, also named Darsa B in *X.laevis*,(7, 9) is a 31-kD protein that was first reported as a novel component of the CPC that stabilizes the bipolar mitotic spindle in human mitosis.(10) In the CPC, Borealin and INCENP associate with the helical domain of survivin to form a tight three-helical bundle(11) that promotes the binding of survivin to INCENP.(12, 13) Borealin is directly phosphorylated by Mps1 on residues that are crucial for Aurora B activity and chromosome alignment.(14) Borealin could be phosphorylated during mitosis in human cells and dephosphorylation of Borealin occurs as cells exit mitosis.(15) Depletion of Borealin leads to defective cell proliferation, p53 accumulation and early embryonic lethality(16), and depletion of *Drosophila borealin* causes polyploidy, delays apoptosis and results in abnormal tissue development.(17) Borealin could also be preferentially modified by SUMO2/3, and the modification is dynamically regulated during mitotic progression, peaking in early mitosis. (18)

Different from mitosis, meiosis includes two divisions, meiosis I and meiosis II. During meiosis I, paired maternal and paternal homologous chromosomes are segregated, oocytes developed a mechanism that maintain the cohesion of sister chromatids in first division, and during meiosis II sister chromatids are segregated in a process similar to mitosis. Although a few studies have reported the roles of Borealin in mitosis, its roles in mammalian meiosis are unknown. Here, we investigated the expression, localization and functions of Borealin during mouse oocyte meiotic maturation, and we also explored the relationship between Borealin and survivin during this process. Together, our data show that Borealin regulates bipolar spindle formation, but may not be included in the chromosomal passenger complex during mouse oocyte meiosis.

3. MATERIALS AND METHODS

3.1. Antibodies

Rabbit polyclonal anti-Borealin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit monoclonal anti-survivin antibody from Cell Signaling Technology (Beverly, MA), mouse monoclonal anti-α-tubulin antibody from Sigma (St Louis, MO), and mouse monoclonal β-actin antibody from Proteintech Group Inc. (Chicago, IL). FITC-conjugated goat anti-rabbit IgG (H+L), TRITC-conjugated goat anti-rabbit IgG (H+L) and TRITC-conjugated goat anti-mouse IgG (H+L) were purchased from Zhongshan Golden Bridge Biotechnology Co., LTD. (Beijing).

3.2. Oocyte collection and culture

All animal manipulations were conducted according to the guidelines of the Animal Research Committee of Chungbuk National University and Institute of Zoology, Chinese Academy of Sciences. Germinal vesicle-intact oocytes were collected from ovaries of six to eight week-old ICR mice. They were cultured in M2 medium (Sigma Chemical Co., St. Louis, MO) under paraffin oil at 37^{o}C, 5% CO_2 in air. Oocytes were collected at different times of culture for immunostaining, western blot analysis and microinjection.

3.3. Taxol and nocodazole treatment of oocytes

For taxol treatment, 5 mM taxol stock in DMSO was diluted in M2 medium to a final concentration of 10 μM, and MI or MII oocytes were treated with the taxol solution for 45 minutes. Oocytes were then washed three times and used for immunofluorescence microscopy. For nocodazole treatment, oocytes were incubated for 10 min in 10 μg/ml nocodazole (made from a 10 mg/ml nocodazole stock diluted in M2 medium); oocytes were incubated for 10 min, washed, and then used for immunofluorescence microscopy.

3.4. Antibody injection

About 5-10 pl anti-Borealin antibody was microinjected into the cytoplasm of a fully grown GV oocyte using a Nikon Diaphot ECLIPSE TE300 (Nikon UK Ltd., Kingston upon Thames, Surrey, UK) inverted microscope equipped with Narishige MM0-202N hydraulic three-dimensional micromanipulators (Narishige Inc., Sea Cliff, NY). After microinjection, the oocytes were cultured in fresh M2 medium under paraffin oil at 37^{o}C in an atmosphere of 5% CO_2 in air. The control oocytes were microinjected with 5-10 pl rabbit immunoglobulin G (IgG) of the same concentration as anti-Borealin antibody. Finally, spindle phenotypes and chromosomal alignment were examined by confocal microscopy.

3.5. Survivin morpholino injection

For survivin MO injection, we adopted the same method as described in a previous report(19). The survivin MO sequence is 5'-GTCACCACAACCTCCGCCAAGACGA-3'. The control group was injected with a standard MO control, 5'-CCTCTTACCTCAgTTACAATTTATA-3' (Gene Tools, LLC).

3.6.Confocal microscopy

For single staining of Borealin or α-tubulin, oocytes were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Then they were transferred to membrane permeabilization solution (0.5% Triton X-100) for 20 min. After 1h in blocking buffer (1% BSA-supplemented PBS), oocytes were incubated overnight at 4^{o}C or for 4h at room temperature with 1:50 rabbit anti-Borealin or 1:200 anti-α-tubulin-FITC antibody. After three washes in washing buffer (0.1% Tween 20 and 0.01% Triton X-100 in PBS), the oocytes were labeled with 1:100 FITC-conjugated goat-anti-rabbit IgG for 1 h at room temperature (for Borealin). They were then co-stained with PI (propidium iodide) for 5 min, followed by three more washes in washing buffer.

For double staining of Borealin and α-tubulin, after staining with anti-Borealin antibody, oocytes were labeled with second antibody (1:100 TRITC-conjugated goat-anti-rabbit IgG) for 1 h at room temperature and then

stained with 1:200 α-tubulin for 1 h after three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 for 2 min. After another three washes in washing buffer, oocytes were stained with Hoechst 33258 (10 μg/ml in PBS) for 10 min.

Finally, the oocytes were mounted on glass slides and examined with a confocal laser-scanning microscope (Zeiss LSM 510 META, Germany). At least 30 oocytes were examined for each group.

3.7. Immunoblot analysis

A total of 200 mouse oocytes were collected after being cultured for various periods of time, placed in SDS sample buffer and heated for 5 min at 100°C. The proteins were separated by SDS-PAGE and then electrically transferred to polyvinylidene fluoride membranes. Following transfer, the membranes were blocked in TBST (TBS containing 0.1% Tween 20) containing 5% non-fat milk for 2 h, followed by incubation overnight at 4°C with 1:100 rabbit polyclonal anti-Borealin antibody and 1:1000 mouse monoclonal anti-β-actin antibody. After three ten-minute washes in TBST, the membranes were incubated for 1h 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 an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

3.8. Data analysis

For each treatment, at least three replicates were performed. Statistical analyses were conducted by analysis of variance. Differences between treated groups were evaluated with the Duncan multiple comparison test. Data are expressed as mean ± SEM and $P < 0.05$ is considered significant.

4. RESULTS

4.1. Expression and localization of Borealin during mouse oocyte meiotic maturation

We cultured oocytes for 0h, 4h, 8h, 9.5h, and 12h, the time points at which most oocytes reached the GV, Pro-MI, MI, ATI, and MII stages, respectively, to examine the expression of Borealin during mouse oocyte meiotic maturation. The immunoblot analysis (Figure 1A) showed that Borealin expression gradually increased from the GV stage to MII.

We next examined the subcellular localization of Borealin at different stages of oocyte meiosis by immunofluorescence staining. As shown in Figure 1B, Borealin had very weak expression in the GV oocytes. After GVBD, Borealin accumulated near chromosomes. By metaphase I, Borealin occurred mainly at the spindle poles. When chromosomes segregated away from the equatorial plate and oocytes reached anaphase I, Borealin still localized at the spindle poles. By telophase I, Borealin accumulated mainly at the midbody. When oocytes reached metaphase II, Borealin again localized at the spindle poles.

4.2. Localization of Borealin and survivin in oocytes treated with taxol

We used taxol, a modulator of microtubule dynamics, to investigate the relationship between Borealin and microtubule dynamics. As shown in Figure 2A, microtubule fibers polymerized and formed cytoplasmic asters in the taxol-treated group. In this case, at the Pro-MI, MI, and MII stages when microtubule assembly was highly active, Borealin signal was found not only at the center of cytoplasmic asters but also near the chromosomes, which indicates that Borealin possibly colocalizes with MTOCs.

We also examined the localization of survivin after taxol treatment. In contrast to Borealin, survivin localization was not altered by taxol treatment and remained at the centromere of chromosomes (Figure 2B). There was no survivin signal at the microtubule-organizing centers (MTOCs), indicating that survivin localization may not depend on microtubule dynamics.

4.3. Localization of Borealin and survivin in oocytes treated with nocodazole

Next, we employed nocodazole, a microtubule-depolymerizing drug, to analyze localization of Borealin after microtubule disruption. In response to nocodazole treatment, microtubules disassembled, the spindle was disrupted, and Borealin localized near the chromosomes in dots at the metaphase stage (Figure 3A).

Localization of survivin following nocodazole treatment was similar to that following taxol treatment, and we still detected a survivin signal at the centromeres of chromosomes during metaphase (Figure 3B). Therefore, the polymerization and depolymerization of microtubules induced by taxol and nocodazole treatment, respectively, affect the localization of Borealin but not survivin during mouse oocyte meiosis.

4.4. Disruption of Borealin function results in spindle formation defects

Next, we sought to investigate the function of Borealin in mouse oocytes during meiosis. We evaluated the effect of Borealin antibody microinjection on microtubule assembly and spindle formation at metaphase. Using confocal microscopy, we examined the spindle morphology of oocytes after 8 h and 12 h of culture in the presence of Borealin antibody. As shown in Figure 4A, in the IgG injection group, the oocyte spindle had a normal morphology in most cases, whereas in the antibody injection group, oocyte spindles exhibited various abnormalities and defects, including disruptive spindles (Figure 4A 2-3), monopolar spindles (Figure 4A 4), and multipolar spindles (Figure 4A 5-6). The rate of abnormal spindles in the antibody injection group was significantly higher than that in the IgG group (71.5%±3.4%, n=76 vs. 32.3%±3.4%, n=95) ($P<0.05$). Similar results were observed in MII oocytes (Figure 4B) (62.5%±1.8%, n=142 vs. 26.8%±4.5%, n=83) ($P<0.05$). In addition, we also found that some oocytes exhibited larger polar body (Figure 4B 3) and small spindle-like structure in the cytoplasm (Figure 4B 5).

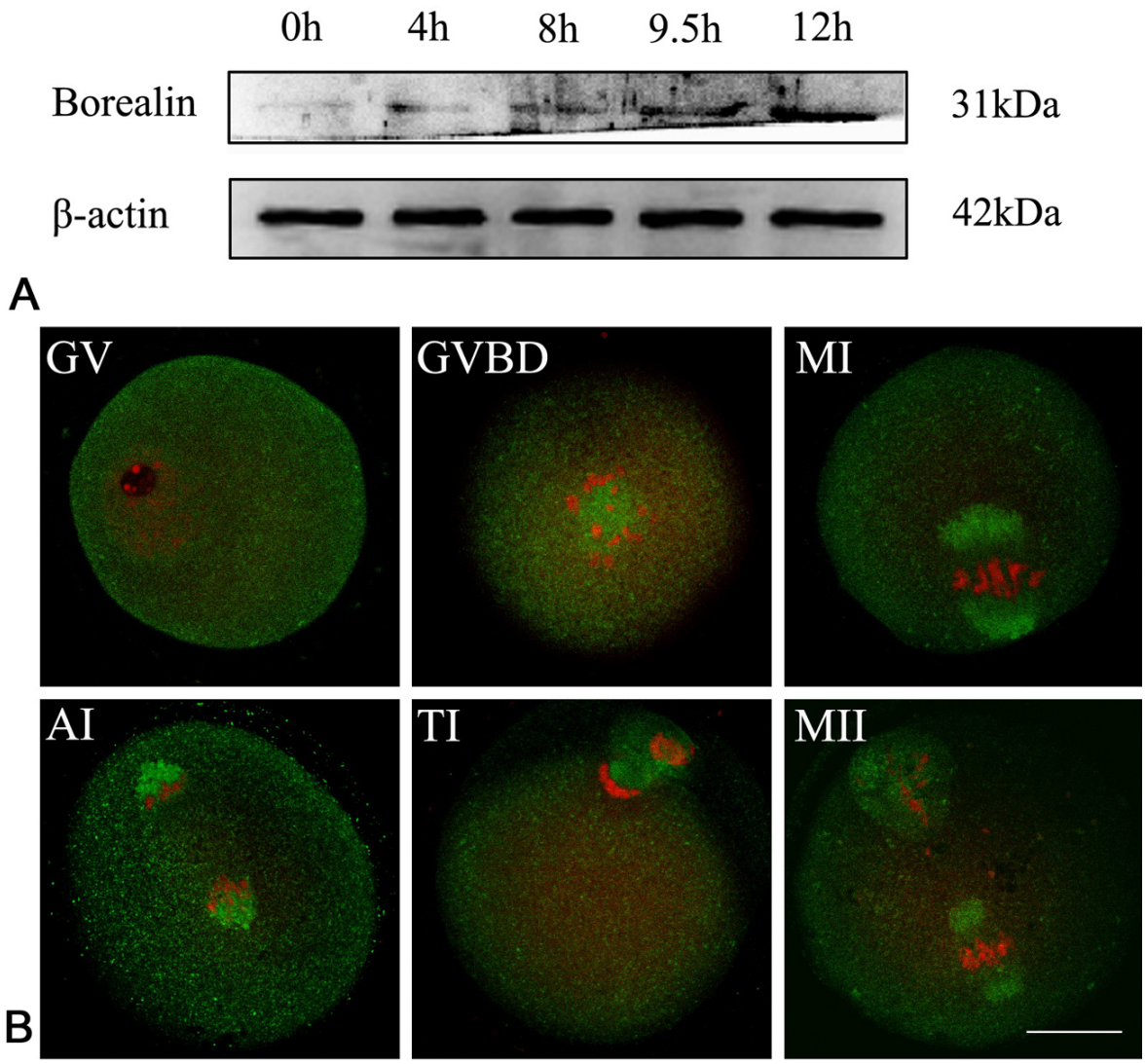

Figure 1. Expression and subcellular localization of Borealin during mouse oocyte meiotic maturation. (A) Expression of Borealin protein as revealed by western blot analysis. Samples were collected after culture for 0h, 4h, 8h, 9.5h, and 12h, when most oocytes reached the GV, Pro-MI, MI, ATI, and MII stages, respectively. The molecular mass of Borealin and β-actin are 31 kDa and 42 kDa, respectively. Each sample was collected from 200 oocytes. (B) Subcellular localization of Borealin as revealed by immunofluorescence staining. In the GV stage, little Borealin was expressed. After GVBD, Borealin accumulated near chromosomes. From MI to AI, Borealin localized at the spindle poles. Borealin localized to the midbody at telophase and to the spindle poles at metaphase II. Green, Borealin; red, chromatin. Bar=20 μm.

4.5 Disruption of Borealin function does not affect the spindle assembly checkpoint and cytokinesis

As the chromosomal passenger complex interacts with spindle checkpoint proteins in mitosis, and, as we previously demonstrated, survivin, one of the chromosomal passenger complex proteins, affects the spindle checkpoint in mouse oocyte meiosis,(19) we asked whether Borealin plays a similar role in meiosis. We examined the rates of polar body extrusion at different time points. As shown in Figure 5A, from 8h to 12h there was no significant difference between antibody injection group and IgG group (3.2%±1.63% vs. 2.3%±3.18%, 24.9%±4.45% vs. 20.2%±6.79%, 41.2%±8.56% vs. 42.1%±1.56%, 53.1%±1.27% vs. 51.8%±0.71%, 68.7%±1.84% vs. 66.3%±0.57%, n=142 vs. n=83) (P>0.1). Our results indicate that depletion of Borealin does not result in precocious anaphase and polar body extrusion, and does not affect polar body extrusion rate.

4.6. Depletion of survivin does not affect the localization of Borealin

The different localizations of Borealin and survivin in the control group and the group exposed to spindle-perturbing agents suggested that Borealin may not play the same role as the CPC. In mitosis, depletion of any component of the CPC will cause the mislocalization of other components. We then injected oocytes with survivin MO to determine the localization of Borealin after

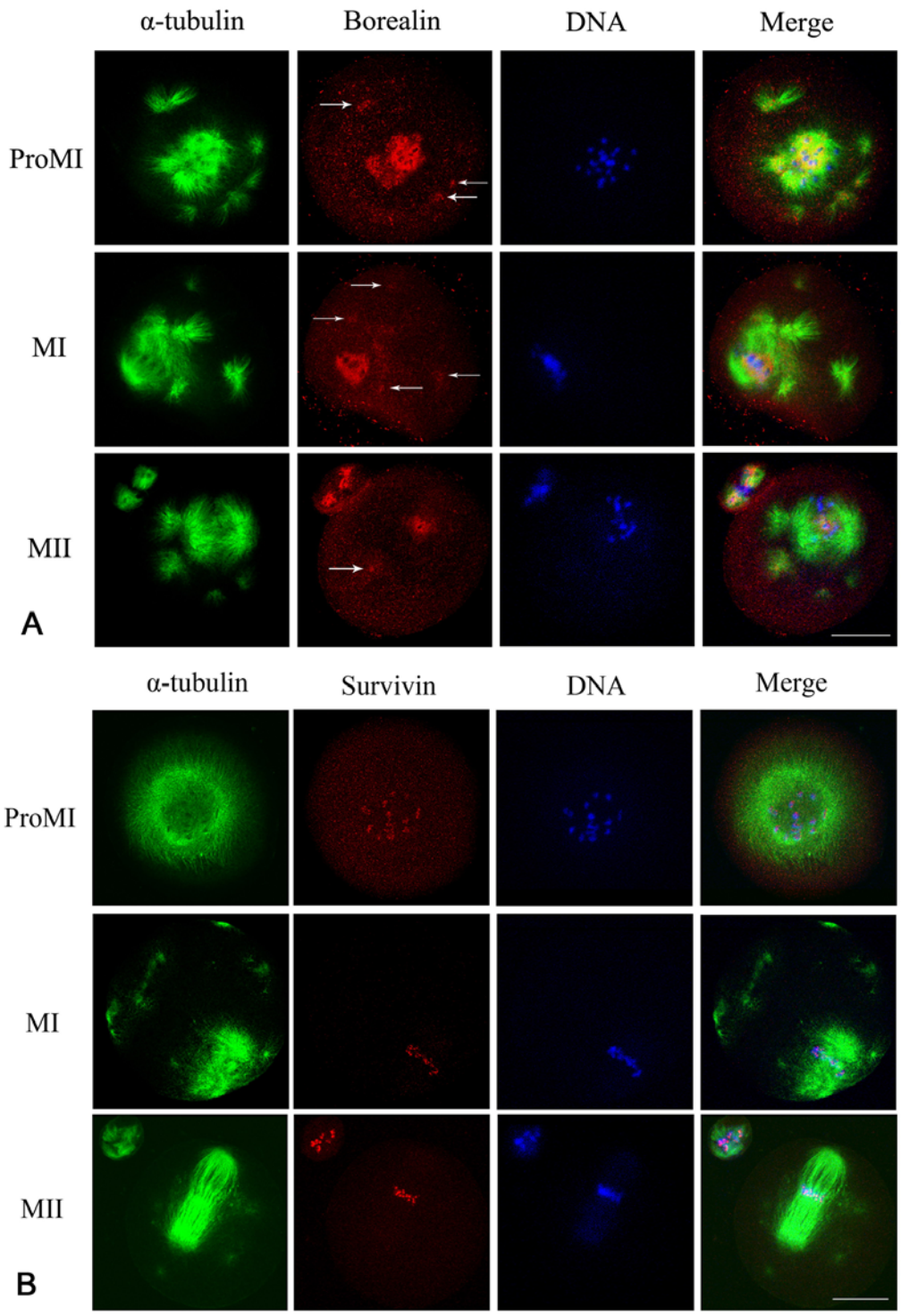

Figure 2. Localization of Borealin and survivin in mouse oocytes treated with taxol. After oocytes were incubated in M2 medium containing 10 μM taxol for 45 minutes, microtubules were polymerized and cytoplasmic asters were induced. Borealin localized at the centers of asters and near chromosomes (arrows) (A), whereas survivin still associated with centromeres (B) at the Pro-MI, MI, and MII stages. Green, α-tubulin; red, Borealin (A), survivin (B); blue, chromatin. Each sample was counterstained with Hoechst 33258 to visualize DNA. Bar=20 μm.

depletion of survivin. As shown in Figure 5B, there was no survivin expression 24h after MO microinjection, as revealed by immunoblot detection. In the group injected with survivin MO, chromosomes misaligned, but the

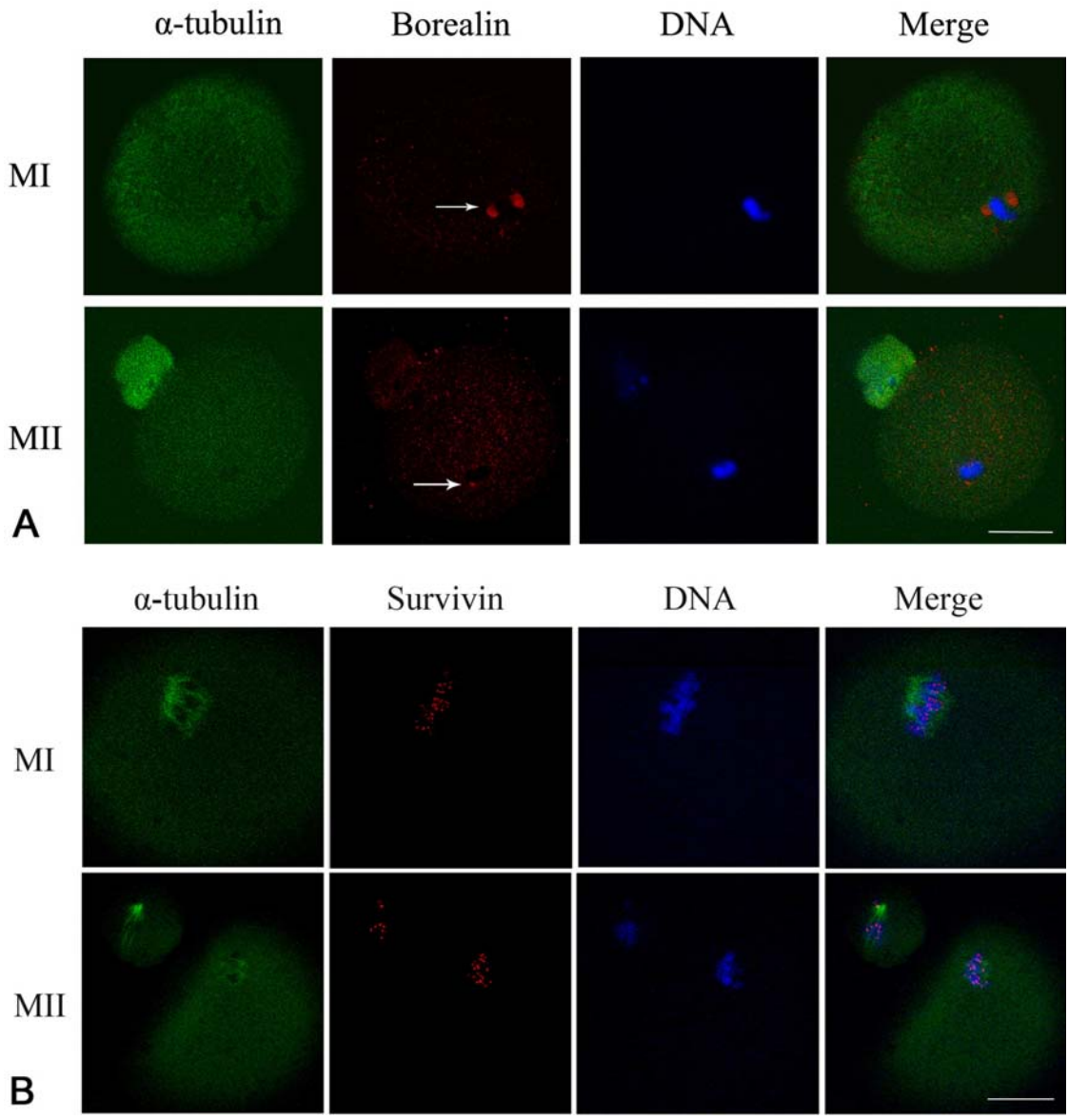

Figure 3. Localization of Borealin and survivin in mouse oocytes treated with nocodazole. After oocytes were incubated in the M2 medium containing 20 μg/ml nocodazole for 10 minutes, microtubules were depolymerized and the spindle was disrupted. Borealin localized near chromosomes (A) while survivin still occurred at the centromeres (B) during MI and MII. Green, α-tubulin; red, Borealin (A), survivin (B); blue, chromatin. Each sample was counterstained with Hoechst 33258 to visualize DNA. Bar=20 μm.

localization of Borealin did not change. As in the control group, Borealin still localized at the poles of spindle (Figure 5C).

5. DISCUSSION

In this study, we showed that the expression of Borealin gradually increased throughout meiosis in mice oocytes, that it localized to the spindle poles during metaphase, possibly colocalizing with MTOCs, that disruption of Borealin function resulted in spindle formation defects, and that Borealin localization and activity differed from that of survivin. We concluded that Borealin is required for bipolar spindle formation, but may not regulate spindle checkpoint activity as a component of the CPC during mouse oocyte meiosis.

In human mitosis, Borealin has been reported to be a centromere protein.(10) However, our immunofluorescence observations showed that Borealin was localized to spindle poles in mouse oocyte meiosis at metaphase; moreover, the expression pattern of Borealin was different from that of survivin, a component of the CPC in mouse oocyte meiosis,(19) indicating that Borealin may not serve as a centromere protein in mouse oocytes. These discrepancies may be related to species differences

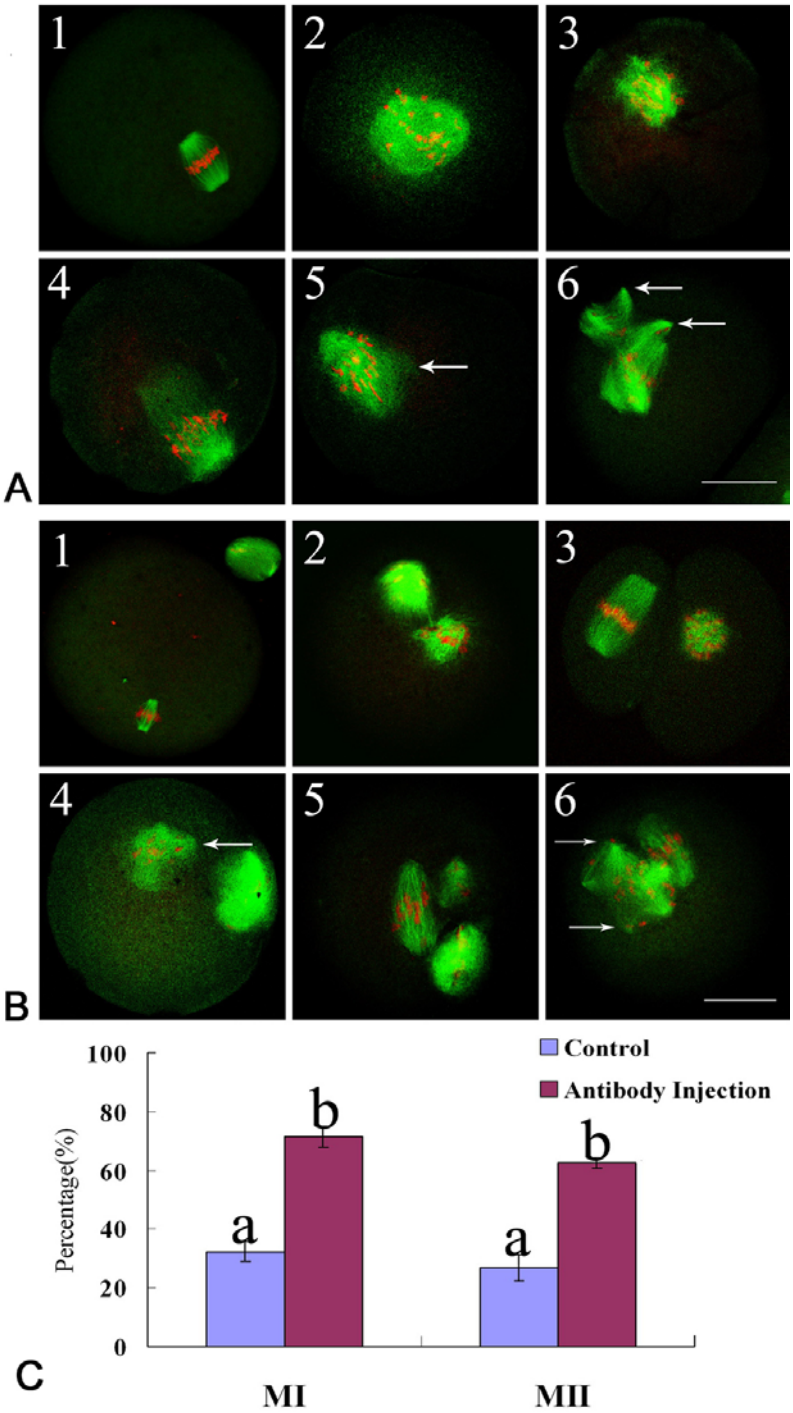

Figure 4. Effect of Borealin depletion on spindle formation in meiotic mouse oocytes. (A, B) Spindle morphology after microinjection of rabbit IgG and Borealin antibody into MI and MII mouse oocytes, respectively. In the rabbit IgG injection group, most oocytes showed normal spindle formation (A1, B1), while in the antibody injection group, most oocytes showed spindle defects (A2-6, B2-6), including spindle formation defects, monopolar spindles, multipolar spindles, larger polar body and the presence of spindle-like structure in the cytoplasm. Arrows, pole of multipolar spindle. (C) Rates of abnormal spindle formation in the IgG injection group and antibody injection group in metaphase. Green, α-tubulin; red, chromatin. Bar=20 μm.

between mice and humans. Differences in protein localization during mitosis and meiosis was also observed in previous reports.(20, 21)

To investigate the relation between microtubule dynamics and Borealin, we treated oocytes with taxol and

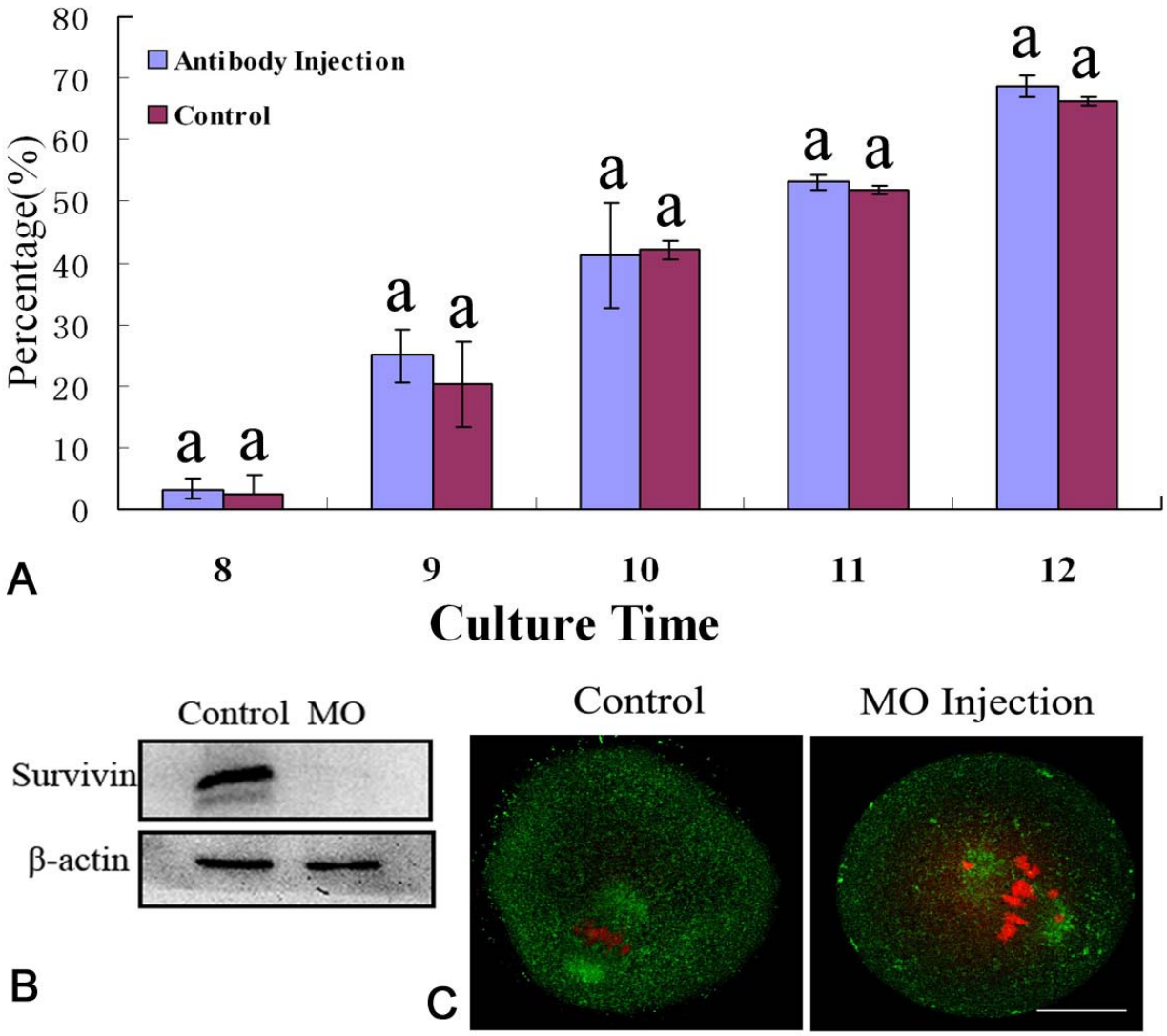

Figure 5. Effect of Borealin depletion on meiotic cell cycle progression and of survivin depletion on the localization of Borealin in mouse oocytes. (A) Rates of polar body extrusion in antibody-injected oocytes cultured for various time periods. No significant difference between the IgG injection group and antibody injection group was observed. (P>0.5) (B) Expression of survivin in the MO-microinjected oocytes. Germinal vesicle oocytes were microinjected with the MO standard control or MO and incubated for 24 h in M2 medium containing 2.5 μM Milrinone before oocytes were collected for western blot analysis. (C) Localization of Borealin after survivin MO injection. Borealin still localized at the spindle poles after depletion of survivin. Green, Borealin; red, chromatin. Bar=20 μm.

nocodazole. Our results showed that Borealin localizes at the centers of cytoplasmic asters, near the chromosomes, after taxol treatment, and only near the chromosomes after nocodazole treatment. Taxol treatment produced microtubule asters in the cytoplasm, and Borealin localized at the centers of MTOCs, which perform similar functions as centrosomes in mitosis. This localization pattern is very similar to that of MEK1/2, which we previously showed to be a microtubule assembly and spindle formation regulator,(22, 23) indicating that Borealin may play roles in regulating microtubule nucleation and bipolar spindle formation. Taxol and nocodazole treatment caused Borealin to migrate towards chromosomes, which suggests that Borealin may also be involved in connecting microtubules to centromeres. In contrast, survivin, a CPC that regulates chromosome alignment and spindle checkpoint function in mouse oocyte meiosis(19), remained associated with centromeres after taxol and nocodazole treatment. The localization of survivin following taxol and nocodazole treatment was consistent with other reports that showed that survivin is a chromosomal passenger protein in mitosis.(24) Our results indicated that survivin localization is independent of microtubule dynamics during meiosis, and that the integrity of microtubules did not affect the binding of survivin to the centromeres. Therefore, survivin may not participate in microtubule nucleation and assembly. The

observation that Borealin and survivin have different localizations following treatment with spindle-perturbing agents further suggests that Borealin may not form a complex with survivin.

We next examined the function of Borealin during meiosis by injecting Borealin antibody into mice oocytes. The observation that antibody injection resulted in a high frequency of spindle formation defects suggests that Borealin is required for meiotic microtubule assembly. Furthermore, as many oocytes also exhibited multipolar spindles and monopolar spindles, we conclude that Borealin is indispensable for the stability of meiotic bipolar spindle. The results are also consistent with previous work in human mitosis(10) and in mouse embryo.(25)

The most important roles of the CPC are the regulation of the spindle assembly checkpoint and of chromosomal alignment.(26, 27) Our previous work also showed that survivin is involved in the regulation of the spindle checkpoint in mouse oocyte meiosis(19). However, in this study, we did not find any difference in the polar body extrusion rate between the IgG injection group and the Borealin antibody injection group at all time points evaluated, indicating that there was no precocious anaphase onset, followed by precocious polar body extrusion. We conclude that disruption of Borealin may not affect spindle checkpoint activity. Moreover, the observation that there was no significant difference in polar body extrusion rate after a 12h culture period indicates that Borealin also has no effect on cytokinesis. The finding that Borealin and survivin have different effects on the spindle checkpoint provided further evidence that Borealin is not a component of the CPC, and we used survivin MO to examine the effect of survivin depletion on the localization of Borealin. We found that Borealin still localized at the spindle poles even when survivin was depleted. This was in contrast with previous work in mitosis that showed that depletion of any component of the CPC causes the mislocalization of the other components,(10, 13, 28-31) indicating that in mouse oocyte meiosis, Borealin may not be a component of the CPC.

In conclusion, the data presented here indicate that Borealin has regulatory roles in microtubule assembly and bipolar spindle formation, and that Borealin cannot be a chromosomal passenger in mouse oocyte meiosis.

6. ACKNOWLEDGEMENTS

We thank Ying-Chun OuYang for her technical assistance in micromanipulation. This work was supported by Biogreen 21 Program (2007040103403 and 20080401034062), RDA, Republic of Korea, and a research grant of the Chungbuk National University in 2007.

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Key Words: Borealin, Spindle Formation, Spindle Checkpoint, Oocyte, Meiosis

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