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Borealin is differentially expressed in ES cells and is essential for the early development of embryonic cells

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Abstract Maintaining undifferentiated state and self-renewal ability of embryonic stem cells is a process that many genes and factors participate in. Using bioinformatics analyses and suppression subtractive hybridization we cloned a novel human gene related to the proliferation of human embryonic stem (hES) cells and its mouse homologue and identified them as being borealin. Our data demonstrated that borealin was highly expressed in undifferentiated ES cells, mouse pre-implantation embryos and the brain of 8.5–9.5 day post-coitum mouse embryos. Furthermore, following Borealin depletion by microinjecting anti-Borealin antibody into the zygotes the mouse embryos were arrested at the 2 or 4-cell stage and chromosomes could not correctly localize at the equator plane of the mitotic spindle and most cells had two or more nuclei. Taken together, these results indicate that Borealin plays a crucial role in the early mouse embryonic development.

Keywords *Borealin* · Chromosomal passenger complex · ES cells · Whole-mount in situ hybridization · Mitotic spindle

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts and maintain self-renewal and pluripotency, an ability to differentiate into all types of somatic and germ cells [1]. The diverse differentiation repertoire of hES cells makes them ideal candidates for tissue regeneration, transplantation therapy and drug discovery. Previous research showed that the maintenance of undifferentiated state and pluripotency of the ES cells involves many factors including many genes and signaling pathways, such as Oct4 [2, 3], Nanog [4], Foxd3 [5], genes and LIF- [6], WNT- [7], FGF-pathway [8]. However, mechanisms of self-renewal and differentiation into specific cell types are not well elucidated. It is necessary to find more genes and more pathways to clarify the mechanism for maintaining the self-renewal and pluripotency of ES cells.

In order to further understand the mechanisms of the self-renewal and pluripotent ability of hES cells, we previously screened out some genes which were highly expressed in undifferentiated hES cells but expressed at low levels or not at all in the differentiated hES cells by suppression subtractive hybridization (SSH) [9]. We cloned a hES cell proliferation-related gene and its mouse homologue named hESPRG3 and mESPRG3, respectively (GenBank Accession No: AY508815 and AY550907, respectively, 2003). The gene was identified as the cell division associated gene 8 (cdca8, borealin, Dasra), which is a novel member of the chromosomal passenger complex [10, 11]. Proteins involved in coordination of the chromosomes and cytoskeletal events in cellular mitosis are called Chromosomal passengers. Chromosomal passenger proteins, including Aurora B [12], Survivin [13], INCENP B [14] and Borealin, are known to play crucial roles during mitosis and cell division [15]. Functions that require chromosomal passenger activity include chromatin modification (phosphorylation of histone H3), correction of kinetochore attachment errors, aspects of the spindle assembly checkpoint, assembly of a stable bipolar spindle and the completion of cytokinesis [16]. Depletion of Borealin by

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RNA interference delays mitotic progression and results in kinetochore—spindle misattachments with ectopic asters. The expression levels of Survivin and INCENP B also fell with the decrease of Borealin, but no significant changes occurred in Aurora B, Aurora A and TD-60 [17]. Loss of Drosophila Borealin causes polyploidy, delayed apoptosis and abnormal tissue development, indicating Drosophila borealin is an essential gene required for embryonic mitosis [18]. The major binding partner of Borealin within the CPC is Survivin, which contains structural features of the inhibitor of apoptosis protein family [19]. Furthermore, Borealin may be involved in targeting of the CPC to centromeres [17]. Here, we report that Borealin is differentially expressed in ES cells and that Borealin depletion results in the arrest of mouse embryo development.

Materials and methods

Culture of the ES cells

The hES cells (chES20) used in this study have previously been shown to be genuine, pluripotent ES cells in the published paper [20]. Undifferentiated hES cells were maintained on human embryo fibroblast feeder layers in DMEM/F12 supplemented with 15% Knockout-serum replacement, 2 mM l-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, and with the addition of 4 ng/ml human basic fibroblast growth factor (bFGF) (Invitrogen). Cultures were manually passaged every 7 d. Mouse R1/E ES cells (ATCC) were cultured as described previously [21].

Animal treatment and collection of mouse zygotes

ICR mice (alias Swiss hauschka, CD-1 or Ha/ICR, derived from Institute of Cancer Research, USA) and rabbits were housed in cages and maintained in the room with 35% humidity and 14 h light/10 h dark cycle at 25°C. ICR mice and rabbits received humane care as outlined in the "Research Policy Handbook" (Stanford University: Responsibilities for the Humane Care and Use of Laboratory Animals). Random-bred ICR female mice (5–8 weeks old) were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotrophin (PMSG, Sigma) and of 5 IU of human chorionic gonadotrophin (hCG, Serono) 48 h apart, after which an ICR male mouse was put into the cage for mating. At 14 h post-mating zygotes were released from the oviducts into M2 medium (Sigma) [22]. Narishige IM-200 Microinjection Systems was used for the microinjection. After injection with the anti-Borealin antibody or rabbit IgG (Santa Cruz), zygotes were brought back into the medium for culture. Cultures were maintained at 37°C in humidified 5% CO_2 for 72 h.

RT-PCR

Mouse embryos at different development stages of 1-cell, 2-cell, 4-cell, 8-cell and blastula from ICR mice were collected by controlled ovarian hyper-stimulation [22]. They were treated as follows. The embryos were washed with phosphate-buffered saline (PBS) to remove remnant granule cells followed by adding 5–10 μl acid Tyrode solution (pH 2.0–2.4) to remove zona pellucidae, centrifugation (12,000 *g*) to remove Tyrode solution, two washes with PBS and adding 3–6 μl lysate solution containing 0.8% Igepal (Sigma), 1 μl of RNase inhibitor and 5 mmol/l DTT (Gibco). Then samples were incubated at 65°C for 5 min to release RNA. The first strand cDNA was synthesized using the SMART cDNA Synthesis Kit (Clontech). Primers used for human *Borealin* open reading frame (ORF) amplification were (forward) 5′-CCATGGCT CCTAGGAAGGGCAGT-3′ and (reverse) 5′-ACGCGTC GACGCCCATTAAAAGTCCATCCTGTC-3′. Primers used for the mouse *Borealin* ORF were (forward) 5′-CATG GCTCCCAAGAAACGC-3′ and (reverse) 5′-CTGCTAC CACAGTCCCTGCT-3′. The housekeeping gene *GAPDH* was amplified with the primers 5′-TGATGATAT CGCCGCGCTCGTCGT-3′ Forward and 5′-CAGCC TGGATAGCAACGTACAT-3′ Reverse.

Whole-mount in situ hybridization of mouse embryos

Whole-mount in situ hybridization was carried out as described by Correia and Conlon [23]. Using total RNA from mouse p19 cells as the template, probes for the experiment were amplified by RT-PCR with the following primers for mouse *borealin*: Forward, 5′-TGCCTT CCATCCAAGAAGAG-3′ and Reverse, 5′-AGGTGTT GGCAGTGAAGGAC-3′. The DNA amplified by PCR was cloned into pGEM-T Easy vector (Promega) and sequenced. The digested target plasmids were used to generate digoxygenin-labeled sense and antisense riboprobes by transcription using T7 and Sp6 RNA polymerase, respectively, according to the manufacturer's protocol. ICR mice were used to collect the mouse embryos [22]. Noon of the day on which a vaginal plug was observed was considered as 0.5 dpc. The 8.5 dpc mouse embryos were collected and fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in a graded series of ethanol (25%, 50%, 75% and 100%) on ice and then stored in the 100% methanol at −20°C. Hybridization and detection were performed with a digoxygenin-labeling and detection kit (Roche).

Antibodies

An antibody to the mouse Borealin was raised in rabbit using the recombinant His-tagged full-length mouse Borealin protein expressed in bacteria. The rabbit polyclonal antibody was purified by sequential affinity chromatography on His-Boralin conjugated CNBr-activated-Sepharose 4B (Amersham Biosciences) and HiTrap protein A-Sepharose (Amersham Biosciences) [24]. The specificity of the mouse anti-Borealin antibody was confirmed by both immunoblot (mES cells) and immunhistochemistry on P19 cells. Data demonstrated that the antibody detected a single band in immunoblot and it was specific for immunhistochemistry. Diluted mouse anti-Borealin antibody was microinjected into the zygotes of ICR mice (10 pg/cell) with rabbit IgG (Santa Cruz) as the isotype control.

Western blot, immunhistochemistry and immunofluorescence staining

Protein samples were extracted from cells using RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml Aprotinin) for 30 min at 4°C. Protein extracts were separated by SDS-PAGE (12% gel) and detected by Western blotting using the Protein Detector™ LumiGLO Reserve™ Western Blot kit (KPL). Human anti-Borealin antibody (gift from Dr. Earnshaw and Dr. Chang) and peroxidase-conjugated anti-rabbit IgG (KPL) were used at 1:500 and 1:10,000 dilutions, respectively. Anti-GAPDH antibody (Santa Cruz) and peroxidase-conjugated anti-mouse IgG (Santa Cruz) were used at 1:2000 and 1:10,000 dilutions, respectively. Anti-Oct-4 antibody and anti-Survivin antibody (Santa Cruz, Chemicon) were used at 1:500 and 1:2000 dilutions, respectively. The Western blot results depicted are a representative of at least three independent experiments.

Immunhistochemistry and indirect immunofluorescence was performed as described previously [16, 24] using the antibody to Borealin and α-tubulin (Sigma). The 3,3′-diaminobenzidine (DAB) chromogen substrate was used in immunhistochemistry as indicated in the figure legend. The zygotes in the first mitotic division (16–18 h after mating) were immediately fixed in 4% formaldehyde at 4°C for 30 min after injection of anti-Borealin antibody or rabbit IgG, followed by incubation with the anti-α-tubulin antibody at 4°C overnight, two washes with PBS and incubation with fluorescein conjugated anti-mouse IgG (Sigma) for 45 min. DNA was stained with 1 × PBS containing 0.5 μg/ml DAPI (Chemicon). The embryos were then mounted onto glass coverslips and immunofluorescence data were recorded using a Nikon microscope or confocal microscope (Olympus). The results depicted are a representative of at least three independent experiments.

Results

Expression pattern of Borealin

RT-PCR was used to analyze the expression of borealin in different cells. The RT-PCR results showed that the expression level of borealin in the undifferentiated hES cells was higher than that in the differentiated hES cells and there was no expression in the human embryonic fibroblasts cells (Fig. 1a). Borealin was also highly expressed in mouse pre-implantation embryos at 1, 2, 4, 8-cell stages and blastula (Fig. 1b). To examine whether expression of borealin in ES cells is related to the undifferentiated status of the ES cells, the hES and mES cells were treated with 0.1 μM retinoic acid and the protein levels of Borealin, Survivin and Oct-4 were determined by Western blot. The results demonstrated that the protein levels of Borealin, Survivin and Oct-4 were remarkably decreased when the hES cells were induced into differentiated hES cells (Fig. 1c). The same results for Borealin were observed in the mES cells (Fig. 1d).

Localization of borealin mRNA in the 8.5–9.5 dpc mouse embryos

In order to understand the function of *borealin* in the embryonic development, whole-mount in situ hybridization was performed to detect the expression of *borealin* in the 8.5–9.5 dpc mouse embryos. The results of in situ hybridization showed that *borealin* was detected in the whole body of the embryo except the gut region, but the strongest signals were in the brain (Fig. 2b). Interestingly, there were no any signals in the extraembryonic tissues (data not shown).

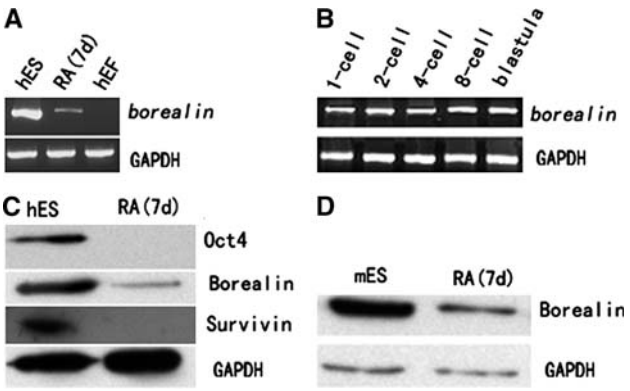

Fig. 1 RT-PCR analysis of Borealin expression showed that *Borealin* was highly expressed in the undifferentiated hES cells (**a**), pre-implantation mouse embryos (**b**). The protein levels of Borealin, Survivin and Oct-4 decline along with the hES cells differentiation induced by retinoic acid (RA), as shown by Western blot analysis (**c**). Protein level of Borealin in mES cells also declines with the cell differentiation induced by retinoic acid (RA) (**d**)

Anti-Borealin antibody arrested mouse embryo development

The specificity of the mouse anti-Borealin antibody was confirmed by both Western blot (mES cells) and immunhistochemistry on P19 cells. Western blot showed that the anti-Borealin antibody detected a single protein band about 32 kD in the lysate of mouse R1/E ES cells (Fig. 3a). Immunhistochemistry showed that the signal in brown was detected in nucleus of P19 cells (Fig. 3b). In order to determining whether *Borealin* plays a unique role during the early embryo development, the rabbit anti-Borealin antibody was microinjected into the mouse zygotes with rabbit IgG as the isotype control. Six hours later, the majority of the embryos developed into the 2-cell stage embryos without obvious difference between the embryos injected with the anti-*Borealin* antibody and IgG (Fig. 4a, e). At 48 h up to 72 h after microinjection, most embryos derived from the zygotes injected with the anti-*Borealin* antibody were arrested at the 2-cell or 4-cell stage (Fig. 4b, c), whereas, more than 75% embryos derived from the zygotes injected with IgG developed into morula at 48 h after microinjection and the embryos further developed into blastula at 72 h (Fig. 4d, f, g). There were significantly differences between the two groups except at 2-cell stage $P < 0.05$. All data were analyzed using SPSS 10.0 software. Differences of $P < 0.05$ are considered statistically significant.

In order to elucidate the mechanism underlying the development arrest of early embryos by injection of anti-Borealin antibody, the mitotic spindles of the zygotes were detected using indirect immunofluorescence. In the zygotes injected with IgG, normal spindles were observed (Fig. 5a), whereas in the zygotes injected with the antibody against Borealin the chromosome-spindle complex was disturbed and the chromosomes could not align at the equator plane of

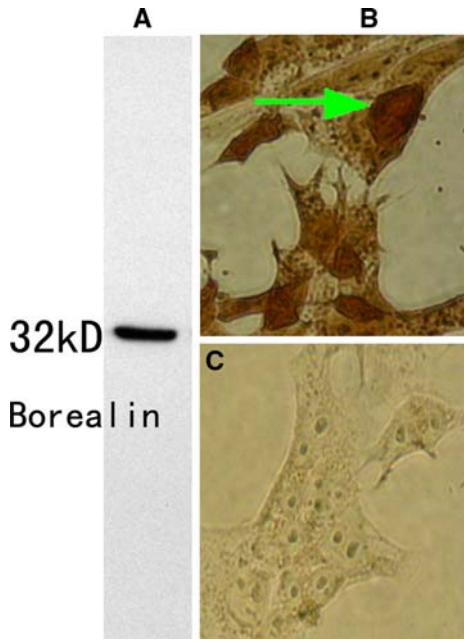

Fig. 3 The specificity of anti-Borealin antibody was tested with Western blot and immunhistochemistry. Western blot showed that the anti-Borealin antibody detected a single protein band about 32 kD in the lysate of mouse R1/E ES cells (**a**). Immunhistochemistry showed that the signal in brown was detected in nucleus of P19 cells (**b**, green arrow indicates nucleus) and no signal was detected in the control P19 cells (**c**). (**b** and **c**) 100× magnification

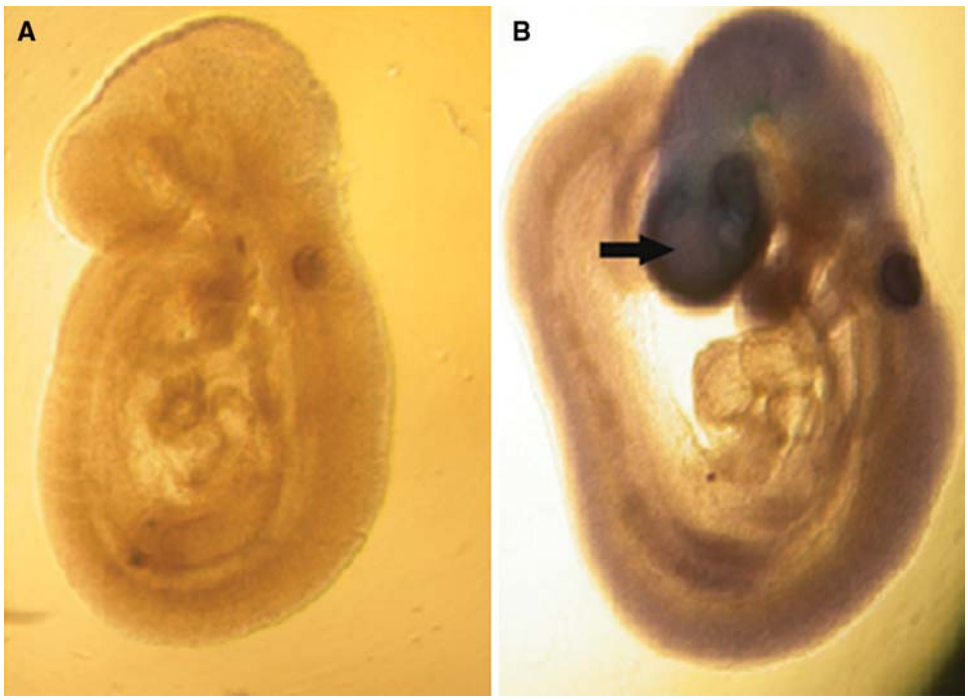

Fig. 2 Borealin mRNA was preferentially localized in the brain of whole-mount embryos. The 8.5–9.5 dpc mouse embryos were used for whole-mount in situ hybridization using digoxigenin labeled RNA sense probe for Borealin. There is no obvious signal in the control embryos (**a**) and strong signals were observed in the area of the brain using the Borealin probe (**b**)

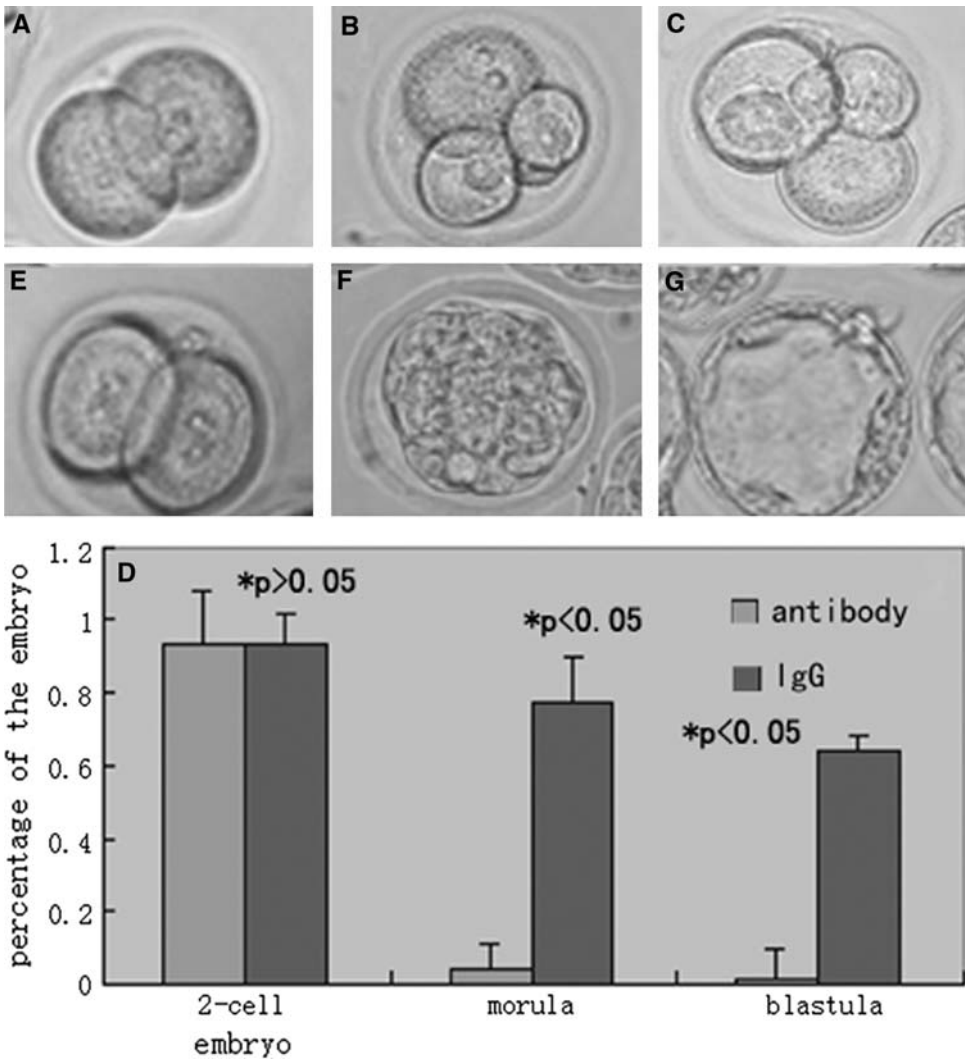

Fig. 4 The anti-Borealin antibody arrested the early development of the mouse embryos (**a**–**g** 100× magnification). Embryos injected with the anti-Borealin antibody were arrested at 2 or 4-cell stage (**a**, **b** and **c**), whereas the embryos injected with IgG could develop into 2-cell stage (**e**), morula (**f**) and blastula (**g**). The numbers of the 2-cell embryos, morulas and blastula were counted at 6–8 h, 48 h and 72 h after microinjection, respectively. The data were from three separate experiments and expressed as mean ± SD (**d**). All data were analyzed using SPSS 10.0 software. There were significant differences between the two groups except at 2-cell stage $P < 0.05$

the mitotic spindle (Fig. 5b). The zygotes injected with the IgG displayed normal mitotic division (Fig. 5c), whereas the zygotes injected with anti-Borealin antibody were arrested at 2-cell or 4-cell stages with two or more nuclei in the individual cells (Fig. 5d–f), suggesting that Borealin plays crucial roles during the mitosis and cell proliferation.

Discussion

As a key regulator of chromosome segregation and cytokinesis, the chromosomal passenger complex (CPC) first localizes to centromeres and later associates with the central spindle and midbody [25]. The chromosomal passengers are present in meiotic cells and there are growing evidences that they play a major role in meiotic chromosome segregation [26]. Borealin as a novel chromosomal passenger is a cell cycle regulator, down-regulated in response to p53/Rb-signaling, and up-regulated in many types of cancerous tissues [27]. The present study demonstrated that Borealin was highly expressed in the undifferentiated ES cells, the mouse pre-implantation embryos (Fig. 1) and the brain of 8.5–9.5 dpc mouse embryos (Fig. 2). Many genes which expressed in ES cells (Hox family, Notch, FGF) are also expressed in the early embryonic brain. Borealin may play an unknown role in the ES and embryo development. Survivin protein level is decreased in a similar pattern to Borealin in differentiated ES cells and the expression levels of Survivin decreased with the depletion of Borealin, suggesting that Borealin may regulate Survivin. Survivin is only expressed in embryonic or proliferating adult tissues and was highly over-expressed in many forms of cancer [28, 29]. It is proposed to function as a mitotic regulator and an apoptosis inhibitor during development and tumorigenesis [30].

After depletion of the Borealin by injection of anti-Borealin antibody into the zygotes, mouse embryos were arrested at 2 or 4-cell stage (Fig. 4) and their chromosomes

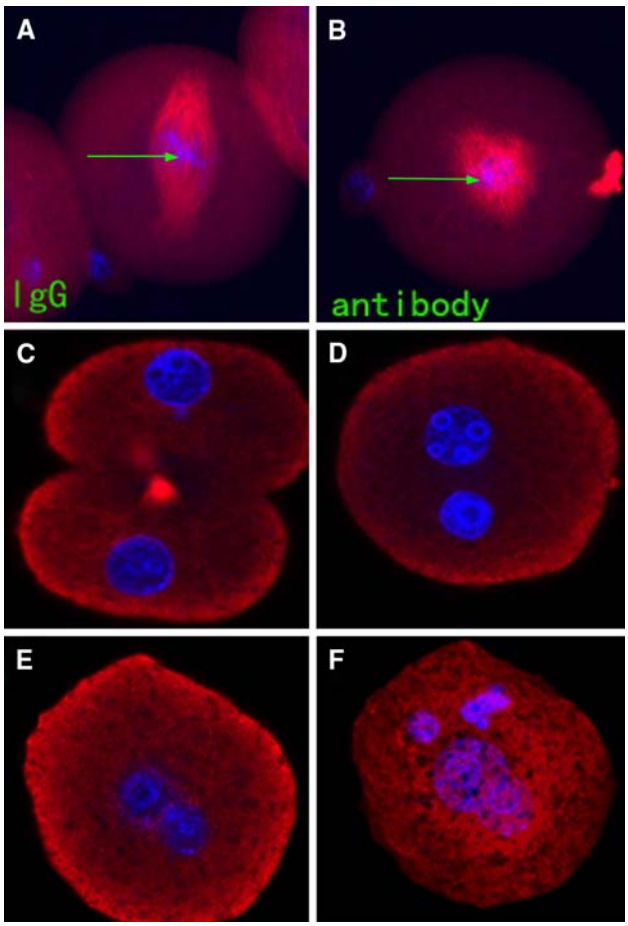

Fig. 5 The depletion of Borealin disturbed spindle formation and chromosome movement. In the mouse zygotes injected with the rabbit IgG chromosomes and spindle were normal in M phase (**a**), whereas, in zygotes microinjected with the anti-Borealin antibody the chromosomes could not attach to the spindle correctly (**b**). The nuclei of the control are normal (**c**) and the nuclei of embryos injected with mouse anti-Borealin antibody are abnormal (**d**, **e**, **f**). The picture (**f**) is a superposition of four different pictures taken at the four quarters of the unit cell and combined together to form a complete picture. (**a**–**f**) 100× magnification Red: α-tubulin Blue: DAPI

could not localize at the equator plane of the mitotic spindle completely (Fig. 5). These results showed that the depletion of Borealin is lethal to the mouse embryos, indicating that Borealin was essential for the early development of the mouse embryos. The results imply that the disturbed mitotic spindles and chromosome alignment and abnormal nuclear phenotypes caused by depletion of the Borealin are the reasons why the development was lethally arrested at the 2 or 4-cell stage. But how did the zygotes get through the first 2 cell divisions to reach the 4-cell stage following the injection with the Borealin antibody? It maybe due to the delay while the antibody works because Borealin protein already exists in zygotes before injection of the antibody. Knockout mice for Survivin are also embryonic lethal [14] and depletion of Borealin by RNAi resulted in defects in chromosome alignment, stable spindle assembly checkpoint activation and cytokinesis [26, 31]. But depletion of the CPC in somatic cells does not cause a dramatic spindle assembly phenotype [32]. Mitotic cells lacking CPC can still form spindles [32], suggesting that Borealin plays a crucial role in the early development of mouse embryos. The function as the chromosomal passenger may be only one aspect of the Borealin's role.

Identification of Borealin as an ES cell related and early embryonic development essential factor may provide a novel mechanism for ES cell self-renewal and development of the early embryo. Especially, Borealin protein level was decreased in a similar pattern to Oct-4 when ES cells were induced into differentiation with retinoic acid, implying that the protein may play a role in maintaining ES cells in undifferentiated status. However, there are more questions that need to be answered. It is very important to determine why depletion of the CPC in somatic cells does not cause a dramatic spindle assembly phenotype. Such questions need further study to be elucidated.

In conclusion, we have indicated for the first time that Borealin is differentially expressed in undifferentiated and differentiated ES cells and plays crucial roles in the early development of mouse embryos.

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