Overexpression of SETβ, a protein localizing to centromeres, causes precocious separation of chromatids during the first meiosis of mouse oocytes

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Summary

Chromosome segregation in mammalian oocyte meiosis is an error-prone process, and any mistake in this process may result in aneuploidy, which is the main cause of infertility, abortion and many genetic diseases. It is now well known that shugoshin and protein phosphatase 2A (PP2A) play important roles in the protection of centromeric cohesion during the first meiosis. PP2A can antagonize the phosphorylation of rec8, a member of the cohesin complex, at the centromeres and thus prevent cleavage of rec8 and so maintain the cohesion of chromatids. SETβ is a protein that physically interacts with shugoshin and inhibits PP2A activity. We thus hypothesized that SETβ might regulate cohesion protection and chromosome segregation during oocyte meiotic maturation. Here we report for the first time the expression, subcellular localization and functions of SETβ during mouse oocyte meiosis. Immunoblotting analysis showed that the expression level of SETβ was stable from the germinal vesicle stage to the MII stage of oocyte meiosis. Immunofluorescence analysis showed SETβ accumulation in the nucleus at the germinal vesicle stage, whereas it was targeted mainly to the inner centromere area and faintly localized to the interchromatid axes from germinal vesicle breakdown to MI stages. At the MII stage, SETβ still localized to the inner centromere area, but could relocalize to kinetochores in a process perhaps dependent on the tension on the centromeres. SETβ partly colocalized with PP2A at the inner centromere area. Overexpression of SETβ in mouse oocytes caused precocious separation of sister chromatids, but depletion of SETβ by RNAi showed little effects on the meiotic maturation process. Taken together, our results suggest that SETβ, even though it localizes to centromeres, might not be essential for chromosome separation during mouse oocyte meiotic maturation, although its forced overexpression causes premature chromatid separation.

Key words: SETβ, PP2A, Mouse oocyte, Meiosis, Centromere, Cohesion, Chromosome separation

Introduction

Meiosis is a specific cell division cycle, which includes a single round of DNA replication, followed by two successive rounds of chromosome segregation (meiosis I and meiosis II). During meiosis I homologous chromosomes segregate while sister chromatids are still attached to each other; after meiosis II sister chromatids finally segregate (Lee and Orr-Weaver, 2001; Petronczki et al., 2003). Through this process, diploid parent cells generate haploid gametes. Chromosome segregation in mammalian oocyte meiosis is an error-prone process, and any mistake in this process may result in aneuploidy, which is the main cause of infertility, abortion and many genetic diseases (Cowchock et al., 1993; Hassold et al., 1996; Hassold and Hunt, 2001; Mailhes et al., 1998; Zenzes and Casper, 1992). To ensure the accuracy of chromosome segregation, a specific set of strict regulatory mechanisms has been developed in meiosis by organisms, one of which is the step-wise loss of cohesion on chromosome arms and centromeres (Yin et al., 2008b). In meiosis, the cohesion between homologous chromosomes and sister chromatids is maintained by the cohesin complex (Klein et al., 1999). The cohesin complex is a multisubunit complex containing SMC1, SMC3, rec8 and SCC3 (Gruber et al., 2003; Michaelis et al., 1997; Watanabe and Nurse, 1999), which forms a ring structure that entraps the sister chromatids to maintain the attachment of sister chromatids (Gruber et al., 2003; Nasmyth, 2002). During meiosis I, the rec8 subunits of the cohesin complex along chromosome arms are phosphorylated and then cleaved by a protease called separase (Buonomo et al., 2000; Lee et al., 2003), which leads to the segregation of homologous chromosomes. Meanwhile, the cohesin complex at the centromeres is protected from separase cleavage and maintains the attachment between sister chromatids until meiosis II (Lee et al., 2006). It is now well known that at least two protein families, shugoshin and protein phosphatase 2A (PP2A), play important roles in the protection of centromeric cohesion during first meiosis (Ishiguro et al., 2010; Kitajima et al., 2004; Kitajima et al., 2006; Lee et al., 2008; Yin et al., 2008a). Shugoshin and PP2A localize to centromeres in a bub1-dependent manner (Kitajima et al., 2005). PP2A can antagonize the phosphorylation of rec8-cohesin at the centromeres (Ishiguro et al., 2010; Kitajima et al., 2006; Lee et al., 2008; Riedel et al., 2006), in order to protect rec8 from cleavage by separase and thus keep the cohesion of chromatids.

Recently, it has been reported that a novel protein named SETβ can physically interact with shugoshin (Kitajima et al., 2006). SETβ inhibits PP2A, and it is thus also called I2PP2A (inhibitor 2 of PP2A). Its gene was originally identified as a fusion gene with the oncogene CAN in acute undifferentiated leukemia by translocation (Adachi et al., 1994). Later, some investigators found that SETβ was a histone chaperone involved in chromatin remodeling (Leung et al., 2011; Li and Damuni, 1998; Li et al., 1996). In addition, SETβ was found to be a subunit of the inhibitor of histone acetyltransferase (INHAT) (Seo et al., 2001), which can inhibit histone acetylation by acetyltransferase. It has been reported that SETβ collaborates with p21 (Cip1) to modulate cyclin-B–CDK1 activity and regulate the G2–M transition in COS and HCT116 cells (Canela et al., 2003). The most interesting feature of SET for our study is that it is a potent and specific inhibitor of PP2A (Li and Damuni, 1998; Li et al., 1996). Considering the role of shugoshin and PP2A in cohesion protection and chromosome separation, the direct interaction between shugoshin and SETβ, and the inhibitory effect of SETβ on PP2A, we hypothesized that SETβ regulates the meiotic process, especially centromeric cohesion and chromosome segregation. In this study, we explored for the first time the expression, localization and potential roles of SETβ in mouse oocyte meiosis. We found that SETβ was expressed stably and localized to the centromere during oocyte meiotic maturation. Overexpression of SETβ in mouse oocytes caused precocious segregation of sister chromatids in meiosis I, but knockdown of SETβ by siRNA had little effects on the meiotic process.

Results

Expression and localization of SETβ during mouse oocyte meiosis

To examine the expression level of SETβ during mouse oocyte meiotic maturation, oocytes were collected for western blot analysis after 0, 8 and 12 hours in culture, corresponding to the germinal vesicle (GV), first metaphase (MI) and second metaphase (MII) stages, respectively. The western blot results showed that the expression level of SETβ was stable from GV to MII stages (Fig. 1A).

Because of the lack of workable antibodies for immunofluorescent staining to investigate the subcellular localization of SETβ in whole oocytes, a low concentration of exogenous Myc–SETβ mRNA in nuclease-free water (about 0.4 mg/ml, 5–10 pl/oocyte) was injected into mouse oocytes at the GV stage. The oocytes were then cultured to different stages of meiotic maturation. The same amount of nuclease-free water was injected as a control. Anti-Myc antibody was used to detect the localization of Myc–SETβ for immunofluorescence microscopy. As shown in Fig. 1B, Myc–SETβ concentrated mainly in the germinal vesicle (nucleus) at the GV stage (Fig. 1Ba). From the germinal vesicle breakdown (GVBD) to MI stages, Myc–SETβ accumulated strongly around centromeres of chromosomes, with faint staining along the chromosome arms (Fig. 1Bb–d). At the MII stage, Myc–SETβ showed two different localization patterns: when oocytes were cultured for 12 hours (early MII), Myc–SETβ was localized only at the inner centromere area (Fig. 1Be), but when oocytes were cultured for 14 hours (late MII), Myc–SETβ was present at three points at both the inner centromere area and kinetochores (Fig. 1Bf). This

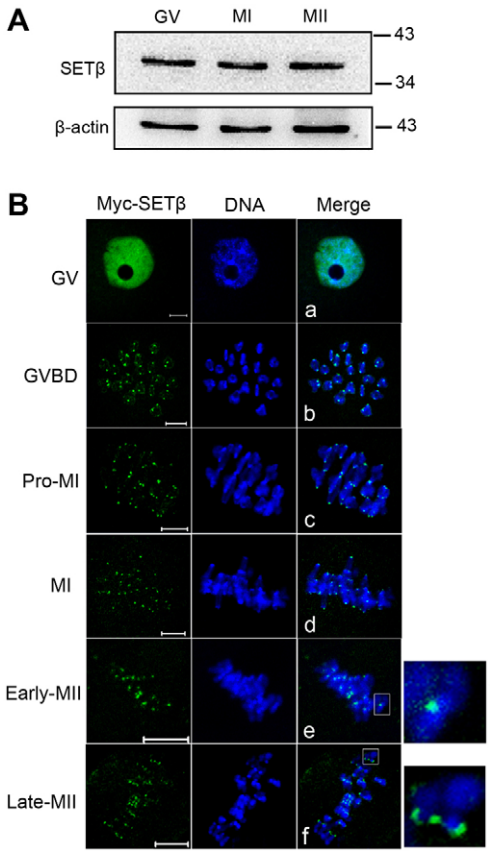

Fig. 1. (A) Expression of SETβ at GV, MI and MII stages. Samples (200 oocytes) were collected after oocytes had been cultured for 0, 8 and 12 hours, corresponding to GV, MI and MII stages respectively. Samples were immunoblotted for SETβ and β-actin. (**B**) Confocal microscopy showing the subcellular localization of Myc–SETβ (green) in mouse oocytes at GV, GVBD, Pro-MI, MI, Early-MII and Late-MII stages. DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown on the right. Scale bars: 10 μm.

may indicate that Myc–SETβ can relocate from the inner centromere area to kinetochores.

To obtain more detailed information on the SETβ localization, we employed a new method of immunofluorescence analysis after chromosome spreading (Hodges and Hunt, 2002). By using this method, we successfully detected the localization of SETβ on chromosomes with the SETβ antibody that did not work in the previous immunofluorescence analysis in intact 4% paraformaldehyde-fixed oocytes. We found that at the MI stage, when all homologous chromosomes formed bivalents, strong staining of SETβ was detected at the inner centromere and faint staining was detected along the interchromatid axes (Fig. 2A). At the MII stage, clear SETβ signal was detected at both the inner centromere area and kinetochores (Fig. 2B), as a three-point signal. Faint SETβ signals were detected along chromatid arms. We also detected localization of Myc–SETβ on both MI and MII chromosomes after Myc–SETβ mRNA injection. The localization patterns were the same as those detected with SETβ antibody (Fig. 2C,D). All the results detected by both SETβ antibody and Myc–SETβ in oocytes and chromosomes confirmed the centromeric localization of SETβ in mouse oocytes.

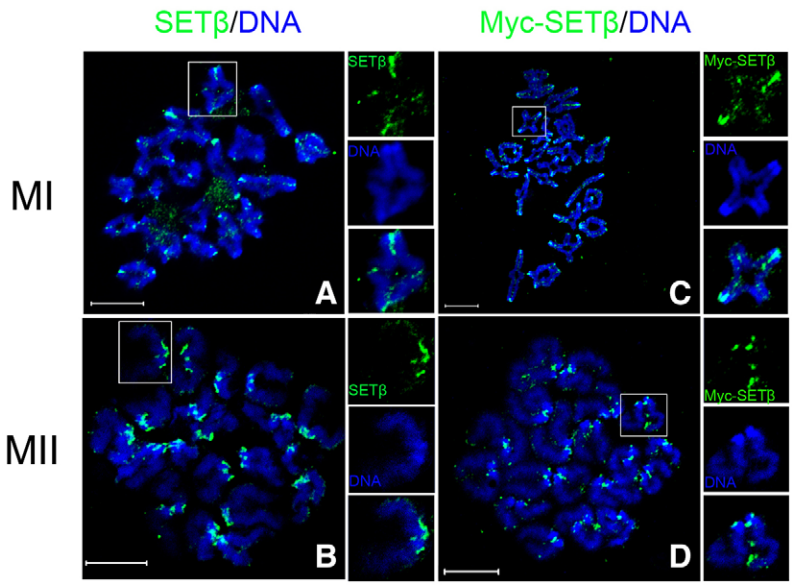

Fig. 2. The localization of SETβ and Myc–SETβ on chromosome spreads at MI and MII stages. Oocytes at MI and MII stages were used for chromosome spreading, and then stained with anti-SETβ antibody (green) and Hoechst 33342 (blue). Oocytes were injected with a low concentration of Myc–SETβ mRNA and cultured to MI and MII stages, then chromosomes were spread and stained with anti-Myc antibody (green) and Hoechst 33342 (blue). Magnifications of the boxed regions are shown on the right of each main panel. Scale bars: 10 μm.

Colocalization of SETβ with CREST, Bub3 and PP2A

To further define the centromeric localization of SETβ, Myc–SETβ mRNA was injected into oocytes that were then cultured to different stages, fixed and double stained with CREST (anti-centromere antibody) and anti-Myc antibodies. As shown in Fig. 3A, at pro-metaphase I and metaphase I stages, the Myc–SETβ signals were detected adjacent to the CREST signals (Fig. 3Aa,b). At the metaphase II stage, when Myc–SETβ was at both the inner centromere and kinetochores, we could clearly see that the Myc–SETβ signals at the inner centromere area were just between the two CREST signals of the sister chromatids, while the Myc–SETβ signals at kinetochores partly overlapped with CREST signals (Fig. 3Ac). We further investigated the colocalization of Myc–SETβ with CREST and another well-known centromeric protein, Bub3, after chromosome spreading, and the results further confirmed the localization of Myc–SETβ at the inner centromere area and kinetochores (Fig. 3B).

We then explored the relocation of Myc–SETβ from the inner centromere to kinetochores at the MII stage. We measured the distance between the sister kinetochores of chromatids with and without Myc–SETβ relocation (Fig. 4Aa,b). We found that the distance between the two sister kinetochores in those sister chromatids with Myc–SETβ relocated to the kinetochores (2.61 ± 0.06 μm, $n=63$) was significantly longer than that in sister chromatids without Myc–SETβ relocation (0.98 ± 0.05 μm, $n=56$, $P<0.05$; Fig. 4B). The distance between sister kinetochores may reflect the tension on the kinetochores. The relocation of Myc–SETβ probably results from the pulling force on the kinetochores.

As SETβ is an inhibitor of PP2A, which is also localized to centromeres, we explored the colocalization of SETβ with PP2A. We found that Myc–SETβ partly colocalized with PP2A, which may indicate functional relationships between these two important proteins (Fig. 5).

Exogenous overexpression of SETβ causes precocious segregation of sister chromatids

To investigate the functions of SETβ in meiosis, exogenous Myc–SETβ was overexpressed in mouse oocytes. The GV stage oocytes were injected with a high concentration of exogenous Myc–SETβ mRNA (about 2.5 mg/ml, 5–10 pl/oocyte), and then arrested for 12 hours by the addition of 2.5 μM milrinone for the expression of Myc–SETβ protein. The oocytes were then washed and cultured in M16 medium to resume meiosis. Immunoblotting

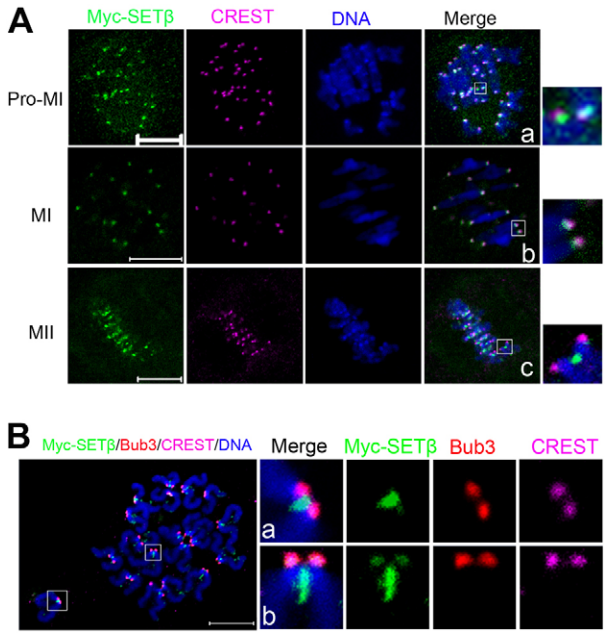

Fig. 3. Myc–SETβ localization. (**A**) Colocalization of Myc–SETβ with CREST at Pro-MI, MI and MII stages. Oocytes were injected with a low concentration of Myc–SETβ mRNA and cultured to Pro-MI, MI and MII stages, then fixed and double stained with anti-Myc antibody (green) and CREST (purple), and DNA (blue) was counterstained with Hoechst 33342. (**B**) Colocalization of Myc–SETβ, CREST and Bub3 at MII stage. Oocytes were injected with a low concentration of Myc–SETβ mRNA and cultured to MII stage, then chromosomes were spread and stained with anti-Myc antibody (green), anti-Bub3 antibody (red) and CREST (purple). DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown. Scale bars: 10 μm.

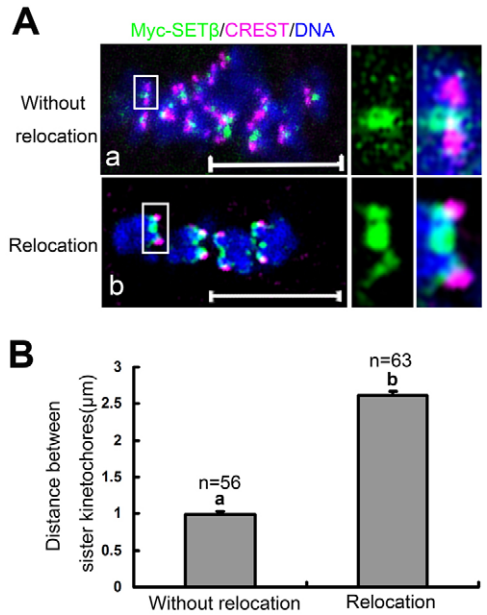

Fig. 4. Myc–SETβ relocates towards kinetochores during the MII stage. (**A**) The localization pattern of Myc–SETβ with or without relocation. Oocytes were injected with a low concentration of Myc–SETβ mRNA and cultured to the MII stage, then fixed and stained with anti-Myc antibody (green) and CREST (purple). DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown. Scale bars: 10 μm. (**B**) The sister chromatids were categorized as with or without Myc–SETβ relocation. The distance between two CREST signals (the distance between sister kinetochores) of a pair of sister kinetochores was measured in each of the categorized centromeres. The error bars represent s.e.m. Different superscript letters indicate statistical difference ($P<0.05$).

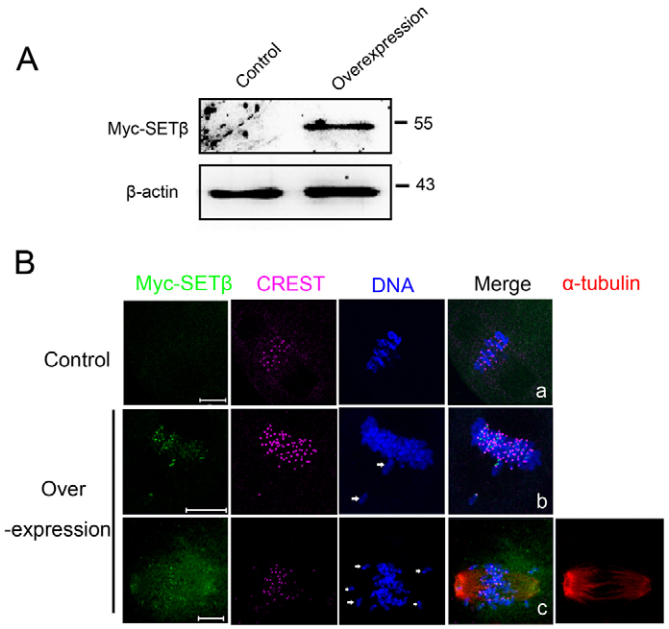

Fig. 6. SETβ overexpression leads to precocious separation of sister chromatids. (**A**) Expression of exogenous Myc–SETβ in oocytes injected with Myc–SETβmRNA: oocytes in the overexpression group were injected with a high concentration of Myc–SETβ mRNA, and oocytes of the control group were injected with nuclease-free water. The oocytes were arrested for 12 hours in 2.5 μM Milrinone for the expression of mRNA, and then collected for western blot analysis. 100 oocytes were collected per sample. The exogenous Myc–SETβ was detected with anti-Myc antibody. (**B**) Oocytes were injected with nuclease-free water or a high concentration of Myc–SETβ mRNA. The oocytes were arrested for 12 hours in 2.5 μM Milrinone for the expression of mRNA, and then released and cultured to the MII stage. The oocytes were fixed and stained with anti-Myc antibody (green), CREST (purple) and anti-α-tubulin (red). DNA (blue) was counterstained with Hoechst 33342. Arrows indicate the precociously separated chromatids. Scale bars: 10 μm.

analysis was performed to confirm the overexpression of exogenous Myc–SETβ protein (Fig. 6A). Oocytes in the overexpression group and control group were cultured for 14 hours and then collected for immunofluorescence analysis. In the SETβ-overexpression group, we observed precocious segregation of sister chromatids at the MII stage in many oocytes (Fig. 6Bb,c), whereas the chromosomes of the control oocytes were all well aligned at the equatorial plates (Fig. 6Ba). To further investigate the precocious separation of sister chromatids after SETβ overexpression, oocytes of the overexpression group and control group at the MII stage were collected for chromosome spreading analysis. As shown in Fig. 7A, the sister chromatids were closely connected at the centromeres in control oocytes (Fig. 7Aa), but in the overexpression group, the centromeric cohesion of sister chromatids were clearly loosened, and many sister chromatids were precociously segregated and became univalents (Fig. 7Ab,c). Moreover, Myc–SETβ was no longer localized at the inner centromere areas in the precociously separated chromatids. We calculated the rate of precocious separation of sister chromatids (PSSC) in the overexpression group and control group. The PSSC rate of the overexpression group was 51.58±11.68% ($n=108$), which was significantly higher than that of the control group, which was only 7.77±2.54% ($n=112$, $P<0.01$; Fig. 7B).

To investigate the causes of premature separation of sister chromatids, we then observed oocytes at anaphase I after SETβ overexpression. As shown in Fig. 7C, the precocious separation of sister chromatids had already occurred at anaphase of first

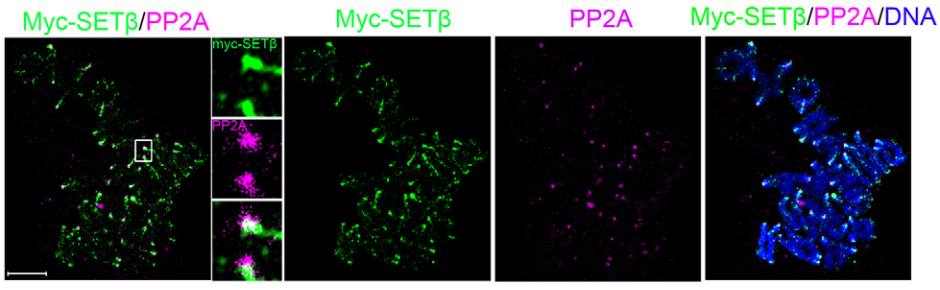

Fig. 5. Colocalization of Myc–SETβ with PP2A at the MI stage. Oocytes were injected with a low concentration of Myc–SETβ mRNA and cultured to the MI stage, then fixed and double stained with anti-Myc antibody (green) and anti-PP2A-A antibody (purple), and DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown. Scale bar: 10 μm.

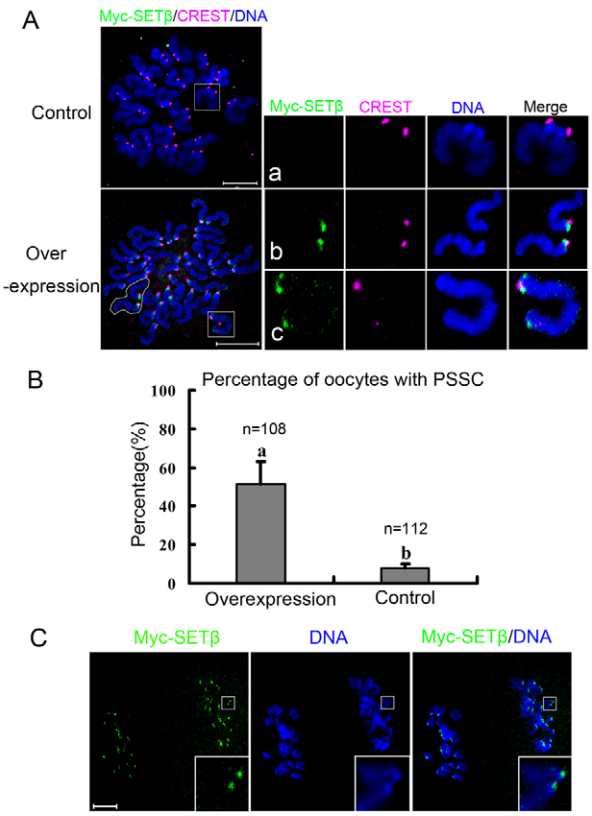

Fig. 7. SETβ overexpression leads to precocious separation of sister chromatids. (**A**) Oocytes were injected with nuclease-free water or a high concentration of Myc–SETβ mRNA. The oocytes were arrested for 12 hours in 2.5 μM Milrinone for the expression of mRNA, and then released and cultured to the MII stage. Chromosomes were spread and stained with anti-Myc antibody (green) and CREST (purple). DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown. Scale bars: 10 μm. (**B**) Percentage of oocytes with precocious separation of sister chromatids (PSSC) in the overexpression group (n=108) and control group (n=112). Data are presented as means ± s.e.m. Different superscript letters indicate statistical difference (P<0.05). (**C**) The premature segregation of sister chromatids is initiated at anaphase I. Oocytes were injected with high concentration of Myc–SETβ mRNA and cultured to the anaphase I stage. The oocytes were fixed and stained with anti-Myc antibody (green), and DNA (blue) was counterstained with Hoechst 33342. Magnifications of the boxed regions are shown. Scale bar: 10 μm.

meiosis, with the disappearance of the centromeric localization of Myc–SETβ (Fig. 7C).

Taken together, these results show that SETβ overexpression leads to significant precocious separation of sister chromatids during meiosis I.

SETβ RNAi has little effect on the meiotic process

To further investigate the roles of SETβ in mouse oocyte meiosis, siRNAs were used to knockdown the expression of SETβ. The efficiency of RNAi was measured by real-time quantitative PCR. Two siRNA oligonucleotides were used, and the real-time PCR results indicated significantly reduced mRNA levels after siRNAs injection (9.2±1% of siNRA1 and 10.7±2% of siRNA2, P<0.01; Fig. 8A). siRNA1 was selected for further experiments. Immunoblotting analysis was also performed to confirm the knockdown of SETβ protein (Fig. 8B). siRNA1 was injected into oocytes at the GV stage and the same amount of negative control siRNA was injected as a control. The oocytes were arrested in 2.5 μM Milrinone-containing M2 medium for 24 hours and then released into fresh M16 medium to resume meiosis. Immunofluorescence analysis with SETβ antibody after chromosome spreading showed a remarkable reduction of SETβ signals on kinetochores after SETβ RNAi (Fig. 8C). The rates of the first polar body extrusion (PBE) were calculated after cultured for 14 hours. No significant difference in PBE was observed between the RNAi group (78.4±4.86%, n=428) and the control group (82.14±3.06%, n=343, P>0.05; Fig. 8D). To further explore whether SETβ is involved in meiosis II exit, the SETβ and control-siRNA-injected oocytes were cultured to MII stage and then treated with 10 μM calcium ionophore A23187 plus 10 μg/ml cycloheximide to activate the meiosis-II-arrested oocytes. Three hours after the parthenogenetic activation, the oocytes were fixed and stained with α-tubulin and Hoechst33342 to show the separation of sister chromatids. We found that both the RNAi-treated group and control group oocytes entered into anaphase/telophase II normally (Fig. 8E,F). We also investigated chromosome segregation after SETβ knockdown for both divisions (meiosis I and meiosis II). MII oocytes and artificially activated MII oocytes after SETβ knockdown were collected and chromosome spreads were performed to show the pattern and number of chromosomes. However, both the RNAi-treated group and control group showed normal chromosome shape and number (Fig. 9A,B), which indicated that SETβ knockdown had little effect on chromosome separation at both meiosis I and meiosis II.

Discussion

In this study, we have investigated the expression, localization and potential functions of SETβ during mouse oocyte meiotic maturation. We demonstrated that SETβ localized around centromeres of sister chromatids and the overexpression of SETβ causes precocious segregation of sister chromatids, but SETβ RNAi has little effect on the meiotic process.

According to previous reports, SETβ is a nuclear protein (Canela et al., 2003), but it can also localize to the cytoplasm (Fan et al., 2002) and, at times, to endoplasmic reticulum (Beresford et al., 2001). In our study, we used both SETβ antibody and Myc-tagged SETβ mRNA microinjection to detect the localization of endogenous and exogenous SETβ in intact oocytes and spread chromosomes. We found SETβ mainly at the inner centromere area, with faint localization along interstitial axes of chromosomes during the first meiosis. At the MII stage, SETβ was present at the kinetochores, in addition to the inner centromere area. The localization pattern of SETβ and Myc–SETβ at the inner centromere area and along interstitial axes of chromosomes was similar to that of rec8 protein (Lee et al., 2006), a core protein of the cohesin complex, which may imply a functional relationship between SETβ and the cohesin complex. According to previous reports on cohesin localization and the high similarity of the localization pattern of the cohesin complex and SETβ, it is highly possible that SETβ participates in the regulation of cohesion and chromosome segregation.

The double staining of Myc–SETβ with CREST, a reliable centromere marker indicated that at first prometaphase (pro-MI) and MI stages, Myc–SETβ localized at the inner centromere area, just adjacent to the CREST signals (Fig. 3Aa,b), and at the MII stage, Myc–SETβ signals at the inner centromere area and

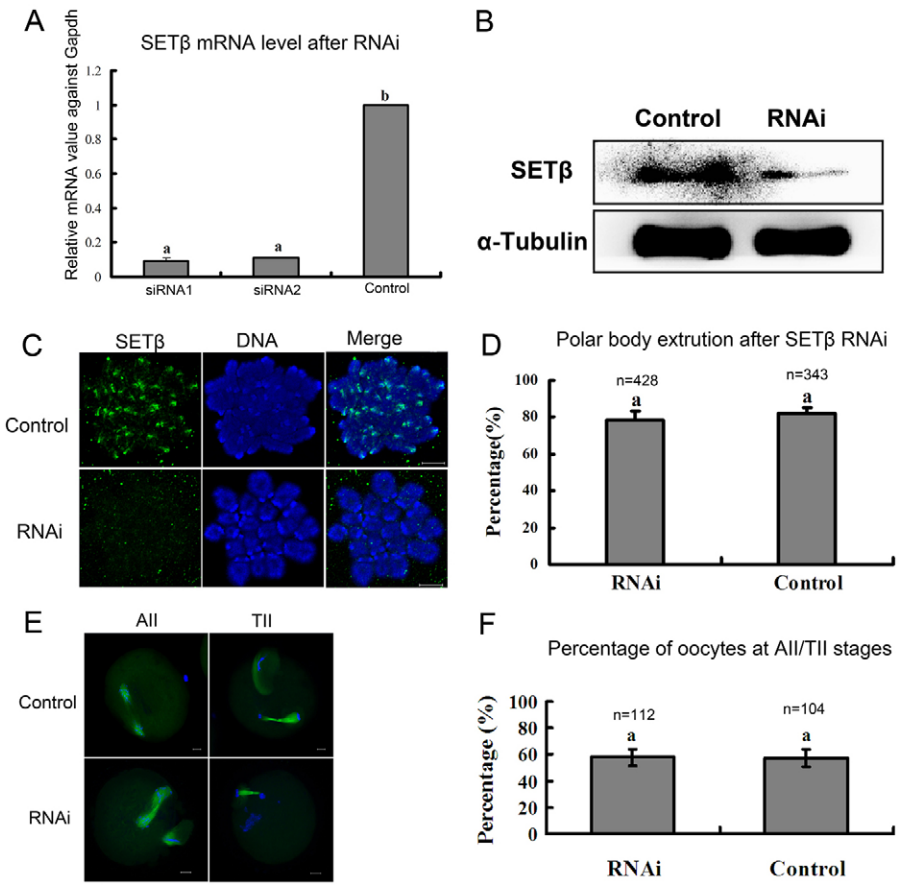

Fig. 8. SETβ depletion has little effect on the meiotic maturation process. (**A**) The knockdown efficiency of SETβ siRNA1 and siRNA2. Analysis of relative gene expression was measured using real-time quantitative PCR. Data are presented as mean ± s.e.m. Different superscript letters indicate statistical difference ($P<0.05$). (**B**) Samples from control and RNAi groups were collected to test the efficiency of SETβ RNAi. Oocytes of the RNAi group were injected with 30 μM siRNA1, and oocytes of the control group were injected with 30 μM control siRNA. The oocytes were arrested for 24 hours in 2.5 μM Milrinone for knockdown of SETβ, and then collected for western blot analysis. A total of 200 oocytes were collected per sample. (**C**) Oocytes in the RNAi group and control group were cultured to MI stage and chromosomes were spread and stained with anti-SETβ antibody (green) and Hoechst 33342 (blue). Clear SETβ signal was found on kinetochores in control groups, in contrast to the dramatic reduction in the RNAi groups. Scale bars: 10 μm. (**D**) Percentage of polar body extrusion (PBE) after SETβ depletion. SETβ siRNA1- and control siRNA-injected oocytes were cultured for 16 hours, and the rates of polar body extrusion were calculated. Data are presented as means ± s.e.m. Same superscript letters indicate no statistical difference ($P>0.05$). (**E**) Oocytes in the RNAi group and control group were cultured to MII stage and parthenogenetically activated with 10 μM calcium ionophore A23187 combined with 10 μg/ml cycloheximide. Three hours after parthenogenetic activation, oocytes were collected and stained with anti-α-tubulin (green) and Hoechst 33342 (Blue). Scale bars: 10 μm. (**F**) Percentage of oocytes entering second anaphase/second telophase (AII/TII) stages 3 hours after parthenogenetic activation in RNAi and control groups. Data were presented as means ± s.e.m. Same superscript letters indicate no statistical difference ($P>0.05$).

kinetochores were in-between and overlapped with the two CREST signals of sister chromatids, which provides direct evidence for the localization of SETβ at the inner centromere area and kinetochores. Bub3, another well-known kinetochore protein (Li et al., 2009), has also been employed to confirm the localization of SETβ.

We further explored the relocation of SETβ from the inner centromere area to kinetochores and found that the relocation of SETβ was accompanied by an increase in the distance between sister kinetochores. It is known that the distance between sister kinetochores is a reflection of tension on kinetochores, and thus we propose that the relocation of SETβ from the inner centromere area to kinetochores may be the result of pulling forces on kinetochores. Furthermore, a previous study in mouse oocytes showed a relocation pattern of both SGO1 and SGO2 from the inner centromere area to kinetochores at the MII stage (Lee et al., 2008) as a result of tension on the kinetochores, which was very similar to the situation determined for SETβ. SETβ has been reported to physically interact with both SGO1 and SGO2 (Kitajima et al., 2006), and it would be interesting to investigate the interaction between SETβ and shugoshin in meiosis. However, because shugoshin antibody was not available, it was difficult for us to pursue this investigation.

It is known that SAC (spindle assembly checkpoint) proteins, such as Mad2 and Bub3 are localized to kinetochores and play an important role in meiosis (Li et al., 2009; Sikora-Polaczek et al., 2006). Because of the kinetochore localization of SETβ, it is tempting to consider a correlation between SETβ and SAC proteins. A previous study showed that Mad2 localized to kinetochores at early MII stage but disappeared gradually from chromosomes later, which indicated that spindle assembly checkpoint was gradually switching off, and the arrest of MII

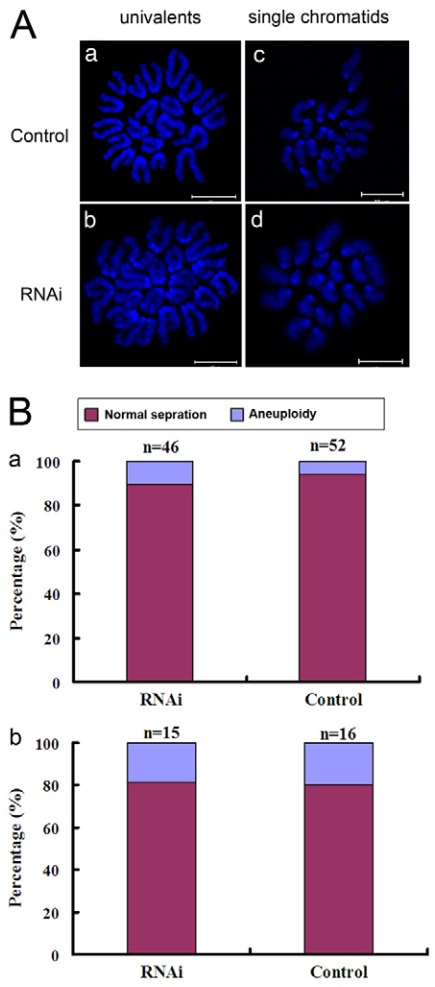

Fig. 9. SETβ knockdown has little effect on chromosome segregation for both meiosis I and meiosis II. (**A**) Chromosome spreads of oocytes in control and RNAi groups after meiosis I and meiosis II. Typical chromosomes at MII stage were univalents (a,b) and typical chromosomes after meiosis II were single chromatids (c,d). DNA was stained with Hoechst 33342 (Blue). Scale bars: 10 μm. (**B**) Percentage of oocytes with aneuploidy after meiosis I (a) and meiosis II (b) in RNAi and control groups.

was maintained by CSF alone (Sikora-Polaczek et al., 2006; Tsurumi et al., 2004). It was worthwhile, therefore, to explore the potential correlation between SETβ relocation and Mad2 disappearance from kinetochores, which may provide a link between SETβ and the spindle assembly checkpoint. However, owing to the lack of an available working Mad2 antibody, it was hard for us to investigate the correlation between SETβ and Mad2. However, we have explored the colocalization of SETβ with Bub3, another important SAC protein. We found that at early MII stage, SETβ localized between the two Bub3 spots (Fig. 3Ba), but when SETβ relocated from the inner kinetochore to the outer kinetochore, the localization of SETβ and Bub3 partly overlapped (Fig. 3Bb), which may imply the functional correlation between SETβ and SAC proteins.

It has been reported that SETβ is a potential inhibitor of PP2A. It is known that in meiosis, shugoshin localizes to centromeres and recruits PP2A to antagonize phosphorylation of the rec8 protein; we thus propose that SETβ may participate in the regulation of shugoshin–PP2A-mediated cohesion protection. We investigated the colocalization of SETβ with PP2A and found that the localization of SETβ and PP2A partly overlapped (Fig. 5), which implied a possible functional relationship between SETβ and PP2A. As centromeric PP2A protects cohesin from phosphorylation, whereas SETβ is an inhibitor of PP2A, we propose that modulation of SETβ may affect chromosome separation. Indeed, overexpression of SETβ led to precocious separation of sister chromatids (Fig. 6B; Fig. 7A,B), which provided direct evidence that SETβ can negatively regulate cohesion protection and chromosome segregation. We propose that overexpressed SETβ inhibited PP2A activity at the inner centromere area, resulting in the loss of rec8 protein protection by shugoshin–PP2A, and thus cohesin was phosphorylated and then cleaved by separase at the onset of anaphase. Moreover, the precociously separated sister chromatids lost SETβ localization at the inner centromere area, which may indicate that the localization of SETβ at the inner centromere area depends on intact cohesion.

It is surprising that knockdown of SETβ by RNAi did not produce any significant effects. However, in our study, we focused mainly on the process from the GV stage to the MII stage, which is only part of the meiotic process, so it is not clear whether SETβ participates in other meiotic events. The fact that the RNAi of SETβ does not cause obvious chromosome segregation phenotypes suggests that it may not physiologically regulate chromosome separation in mouse oocyte meiosis. The overexpression phenotype could be a non-physiological effect of inhibition of PP2A.

In conclusion, our data suggest that SETβ, a PP2A inhibitor, is a centromeric protein in oocytes and that overexpression of SETβ causes precocious chromatid segregation, but SETβ itself may not be physiologically essential for chromosome separation during mouse oocyte meiotic maturation.

Materials and Methods

All chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO) unless stated otherwise.

Antibodies

Rabbit polyclonal anti-SETβ, rabbit polyclonal anti-Bub3, mouse monoclonal anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-Myc-FITC antibody was purchased from Invitrogen (Carlsbad, CA); rabbit monoclonal anti-α-tubulin and rabbit polyclonal anti-PP2A-A antibodies were obtained from Cell Signaling Technology (Beverly, MA); CREST, human anti-centromere antibody was purchased from Fitzgerald (Acton, MA); FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-rabbit IgG were purchased from Zhongshan Golden Bridge Biotechnology Co, LTD (Beijing). Cy5-conjugated goat anti-human IgG and Cy5-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA).

Oocyte collection and culture

Animal care and handling were conducted in accordance with Animal Research Committee policies of the Institute of Zoology, Chinese Academy of Sciences. Female ICR mice 6- to 8-weeks old were sacrificed and the ovaries were isolated, then cut with a blade to release immature oocytes. Only those immature oocytes with an intact germinal vesicle (GV) were selected for further experiments. For mRNA or siRNA injection, GV oocytes were maintained at the GV stage by supplementing the M2 medium with 2.5 μM milrinone. For *in vitro* maturation, GV oocytes were cultured in M16 medium under liquid paraffin oil at 37℃ in a 5% CO_2 incubator.

SETβ plasmid construction and mRNA synthesis

Total RNA was extracted from 100 GV-stage mouse oocytes using an RNeasy micro purification kit (Qiagen), and the first strand cDNA was generated with an M-MLV first strand cDNA synthesis kit (Invitrogen), using oligo(dT) primers. The

following two pairs of nest primers were used to amplify the full-length CDS sequence of mouse SETβ by PCR. F1: 5′-TGTGGCGTGAGGGGAAGC-3′; R1: 5′-CAGAGGGACAGGGAGGAAAGG-3′; F2: 5′-GTTGGCCGGCCGACCATG-TCTGCGCCGA-3′; R2: 5′-GTTGGCGCGCCGCTAATCATCCTCGCCTTC-3′. The PCR products were purified, then digested using *Fse*I and *Asc*I (New England Biolabs, Inc.) and cloned into the pCS2+ vector, in which the SETβ sequence was linked to six Myc tags at its N-terminus.

For the synthesis of Myc-tagged SETβ mRNA, the SETβ-pCS2+ plasmids were linearized by *Sal*I and then purified. The SP6 mMESSAGE mMACHINE (Ambion) was used to produce capped mRNA and then purified by RNeasy clean up kit (Qiagen). The concentration was detected with a Beckman DU 530 Analyzer, and diluted to a concentration of 0.4 mg/ml for localization and 2.5 mg/ml for overexpression.

Micro-injection of Myc–SETβ mRNA of SETβ siRNAs

Microinjections were performed using a Narishige microinjector and completed within 30 minutes. For Myc–SETβ expression, 0.4 mg/ml (or 2.5 mg/ml for overexpression) Myc–SETβ mRNA solution was injected into cytoplasm of GV oocytes. For protein expression, oocytes were arrested at the GV stage in M2 medium supplemented with 2.5 μM milrinone for 2 hours. The same amount of RNase-free water was injected as control.

For knockdown experiments, small interfering RNAs (siRNAs) of SETβ (GenePharma) were microinjected into the cytoplasm to deplete SETβ. The subsequent siRNAs were used at 30 μM, SETβ siRNA-1: 5′-CCACCGAAA-UCAAAUGGAATT-3′; SETβ siRNA-2: 5′-GAGGAUGAUUAGCACAGAATT-3′. The same amount of negative control siRNA was used as control. After siRNA injection, oocytes were arrested at the GV stage in M16 medium supplemented with 2.5 μM milrinone for 24 hours for the depletion of SETβ.

Western blotting

A total of 200 mouse oocytes at the appropriate stage of meiotic maturation were collected and transferred to 5 μl 2× SDS sample buffer and boiled for 5 min. The samples were separated by SDS-PAGE and then transferred to PVDF membranes. After transfer, the membranes were washed briefly in TBST buffer and then blocked in TBST containing 5% skimmed milk, for 1 hour at room temperature, followed by incubation overnight at 4℃ with 1:500 rabbit polyclonal anti-SETβ antibody, 1:2000 mouse monoclonal anti-Myc antibody or 1:2000 mouse monoclonal anti-β-actin antibody. After three washes in TBST buffer, 10 minutes each, the membranes were incubated with 1:1000 HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG, for 2 hours at room temperature. Finally, the membranes were washed three times in TBST and processed using the enhanced chemiLuminescence detection system (Bio-Rad, CA).

Immunofluorescence analysis and chromosome spread

Oocytes were fixed in 4% paraformaldehyde in PBS buffer with 0.5% Triton X-100 for 30 minutes and then blocked in 1% BSA-supplemented PBS for 1 hour at room temperature. For single staining of Myc–SETβ, oocytes were incubated with FITC-conjugated anti-Myc antibody (1:100) overnight at 4℃, and after three washes in washing buffer (0.1% Tween 20 and 0.01% Triton X-100 in PBS), DNA was stained with Hoechst 33342 for 30 minutes.

For double staining of Myc–SETβ and CREST, oocytes were first stained with CREST (1:50) overnight at 4℃, and after three washes in washing buffer, oocytes were incubated with cy5-conjugated goat anti-human secondary antibody for 2 hours at room temperature, then washed three times and blocked again for 1 hour at room temperature and then incubated with FITC-conjugated monoclonal anti-Myc antibodies for 2 hours at room temperature. The oocytes were finally stained with Hoechst 333342 after three washes in washing buffer and mounted on glass slides for immunofluorescence microscopy. The photos were taken with the confocal laser-scanning microscope (Zeiss LSM 780, Germany).

Chromosome spreads were performed as described previously (Hodges and Hunt, 2002). Briefly, the oocytes were exposed in acid Tyrode's solution (Sigma) for 1 minute at 37℃ to remove the zona pellucida. After a brief recovery in M2 medium, the oocytes were transferred onto glass slides and fixed in a solution of 1% paraformaldehyde in distilled H_2O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. The slides were allowed to dry slowly in a humid chamber for several hours, and then blocked with 1% BSA in PBS for 1 hour at room temperature. The oocytes were then incubated with CREST (1:50), anti-SETβ, anti-Myc (1:50), anti-Bub3 (1:50) or anti-PP2A (1:50) primary antibodies overnight at 4℃. After brief washes with washing buffer, the slides were then incubated with the corresponding secondary antibodies for 2 hours at room temperature. DNA on the slides was stained with Hoechst 33342 and slides were mounted for observation by immunofluorescence microscopy.

Real-time quantitative PCR analysis

Total RNA was extracted from 50 oocytes using RNeasy micro purification kit (Qiagen), the first strand cDNA was generated with M-MLV first strand cDNA synthesis kit (Invitrogen), using oligo(dT) primers. The following primers were used to amplify the SETβ fragment. Forward: 5′-CCTACCTGCTCCCACCGTTA-3′; Reverse: 5′-CACTGACTGCTGACCCTGACA-3′; GAPDH was selected as a reference gene. We used a SYBR Premix Ex Tag™ kit (Takara) in an ABI prism 7500 Sequence Detection System. The steps consisted of 95℃ for 15 seconds, 40 cycles of 95℃ for 5 seconds and 60℃ for 30 seconds. Relative gene expression was calculated by the 2ΔΔCt method.

Parthenogenetic activation

Oocytes were cultured for 15 hours to develop to MII stage, and parthenogenetically activated using 10 μM calcium ionophore A23187 in KSOM at room temperature for 5 min, followed by incubation in 10 μg/ml cycloheximide in KSOM medium under liquid paraffin oil at 37℃ in a 5% CO_2 incubator.

Statistical analysis

All experiments were repeated at least three times. All percentage are expressed as means ± s.e.m. and the number of oocytes observed (*n*) given in parentheses. Data were analyzed by independent-sample *t*-tests with SPSS software (SPSS Inc., Chicago, IL). $P<0.05$ was considered statistically significant.

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Author contributions

S.-T.Q. and Q.-Y.S. conceived and designed the experiments. S.-T.Q., Z.-B.W., Y.-C.O., Q.-H.Z., M.-W.H., X.H., Z.G., L.G. and Y.-P.W. performed the experiments. S.-T.Q., Z.-B.W. and Q.-Y.S. analysed the data. Y.H. contributed reagents, materials and analysis tools. S.-T.Q., H.S. and Q.-Y.S. wrote the paper.

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